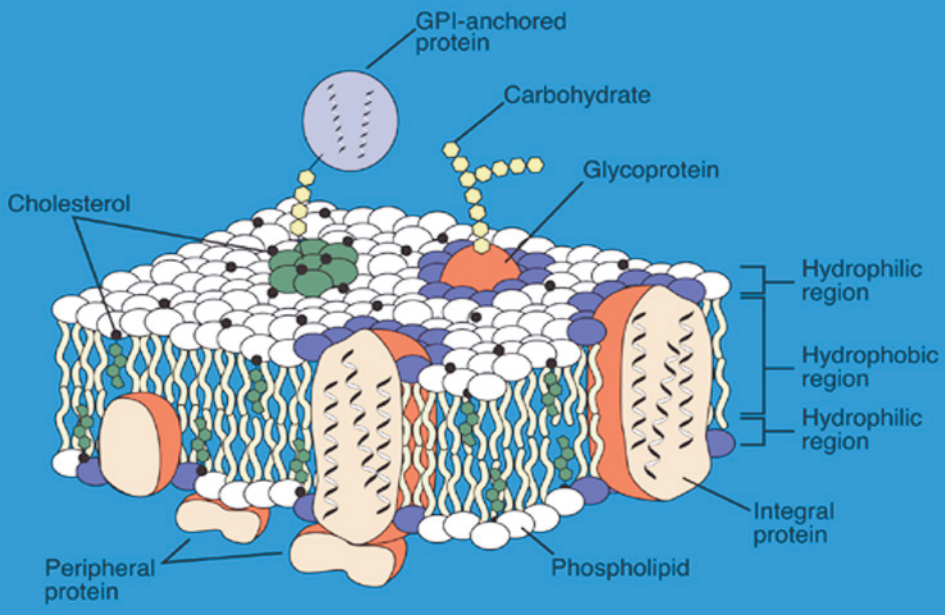
Biochemistry of Lipids, Lipoproteins and Membranes, 5th edition



edited by
D.E. Vance and J.E. Vance

BIOCHEMISTRY OF LIPIDS, LIPOPROTEINS AND MEMBRANES (5TH EDN.)

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Biochemistry of Lipids, Lipoproteins and Membranes (5th Edn.)

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Preface

The first edition of this textbook was published in 1985. Since that time, studies on the biochemistry and molecular biology of lipids and lipoproteins have underscored the physiological importance of lipids. Important new roles of genes involved in lipid metabolism, and their relationship to diseases such as heart disease, diabetes, obesity, stroke, cancer, and neurological disorders, have been revealed. The 5th edition of this textbook, therefore, takes into account the major advances in these fields and, in addition, provides basic knowledge of the genes and proteins involved in lipid metabolic processes. This edition has been written with two major objectives in mind. The first is to provide students and teachers with an advanced up-to-date textbook covering the major areas in the biochemistry and molecular biology of lipids, lipoproteins, and membranes. As in previous editions of this book, the chapters are written at a level that is accessible to students who have already taken an introductory course in biochemistry and are therefore familiar with the basic concepts and principles of biochemistry and lipid metabolism. Thus, we hope that this volume will provide the basis for an advanced course for the undergraduate and graduate students with an interest in the lipid field. The second objective of this book is to satisfy the need for a general reference book for scientists and researchers who are presently working in, or are about to enter, the lipid and related fields. This book is unique in that it is not a collection of exhaustive reviews on the topics covered in each chapter, but rather is a current, readable, and critical summary of the field. Our goal was to present a clear summary of each research area. This book should allow scientists to become familiar with recent discoveries related to their own research interests, and should also help clinical researchers and medical students keep abreast of developments in basic science that are important for clinical advances in the future.

All of the chapters have been extensively revised since the 4th edition appeared in 2002. We have not attempted to describe in detail the structure and function of biological membranes or the mechanism by which proteins are assembled into membranes as these topics are covered elsewhere in a number of excellent books. The first chapter, however, contains an up-to-date summary of the principles of membrane structure as a basis for the material covered in the subsequent chapters.

As the chapters do not constitute comprehensive reviews of the various topics, we have limited the number of references cited at the end of each chapter and have emphasized recent review articles. Additional up-to-date reviews are available on all the topics included in this book. In addition, some of the primary literature is cited within the body of the text by providing the name of one of the authors and the year in which the article was published. Using this system, readers should be able to find the original citations by searching an on-line database.

The editors and contributors assume full responsibility for the content of the chapters. We shall be pleased to receive any comments and suggestions for future editions of this book.

Dennis E. Vance and Jean E. Vance
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Contents

D.E. Vance and J.E. Vance (Eds.) *Biochemistry of Lipids, Lipoproteins and Membranes (5th Edn.)*

CHAPTER 1

Functional roles of lipids in membranes

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Contents

1. Introduction and overview

Lipids as a class of molecules display a wide diversity in structure and biological function. A primary role of lipids in cellular function is to form the lipid bilayer permeability barrier of cells and organelles (Fig. 1). Glycerophospholipids (termed phospholipids hereafter) are the primary building blocks of membranes but other lipids are important components. Table 1 shows the major lipids found in the membranes of various cells and organelles but does not take into account the minor lipids, many of which are functionally important. Sterols are present in all eukaryotic cells and in a few bacterial membranes. The ceramide-based sphingolipids are also present in the membranes of all eukaryotes. Neutral diacylglycerol glycans are major membrane-forming components in many gram-positive bacteria and in the membranes of plants, while gram-negative bacteria utilize a saccharolipid (Lipid A) as a major structural component of the outer membrane. The variety of hydrophobic domains of lipids results in additional diversity. In eukaryotes and eubacteria, these domains are saturated and

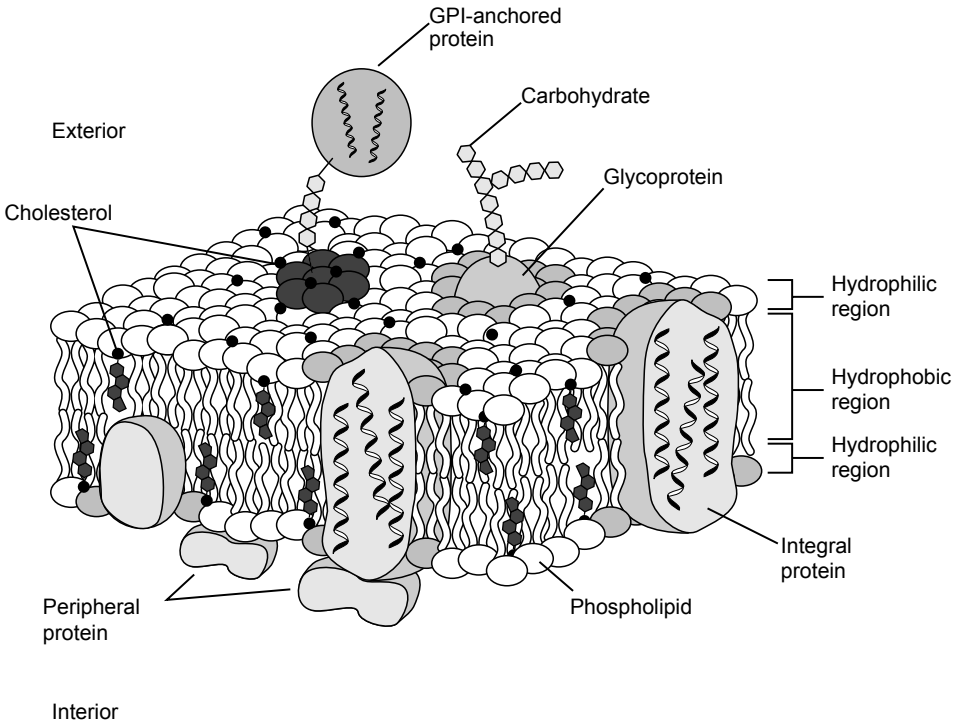

Fig. 1. Model for membrane structure. This model of the plasma membrane of a eukaryotic cell is an adaptation of the original model proposed by Singer and Nicholson (S.J. Singer, 1972). The phospholipid bilayer is shown with integral membrane proteins largely containing α-helical transmembrane domains. Peripheral membrane proteins associate either with the lipid surface or with other membrane proteins. Lipid rafts (dark gray head groups) are enriched in cholesterol and contain a phosphatidylinositol glycan-linked (GPI) protein. The light gray head groups depict lipids in close association with protein. The irregular surface and wavy acyl chains denote the fluid nature of the bilayer.

Table 1
Lipid composition of various biological membranes

Lipid	Erythrocyte[a]	CHO cells[b]	Mitochondria[c]		Endoplasmic reticulum[d]	*E. coli*[e]
			Outer	Inner		
Cholesterol	25	–	N.D.	N.D.	20	N.D.
PE	18	21	33	24	21	75
PC	19	51	46	38	46	N.D.
Sphingomyelin	18	9	–	–	9	N.D.
PS	9	7	1	4	2	<1
PG	0	1	N.D.	N.D.	–	20
CL	0	2.3	6	16	–	5
PI	1	8	10	16	2	N.D.
Glycosphingolipid	10	–	–	–	–	N.D.
PA	–	1	4	2	–	<2

The data are expressed as mole% of total lipid. N.D. indicates not detected and blank indicates not analyzed.
[a]Human [5].
[b]Chinese hampster cells (T. Ohtsuka, 1993).
[c]*S. cerevisiae* inner and outer mitochondrial membrane (E. Zinser, 1991).
[d]Murine L cells (E.J. Murphy, 2000).
[e]Inner and outer membrane excluding Lipid A (C.R. Raetz, 1990).

unsaturated fatty acids or alkyl alcohols. Archaebacteria contain long-chain reduced polyisoprene moieties in ether linkage to glycerol instead of fatty acids. If one considers a simple organism such as *Escherichia coli* with three major phospholipids and several different fatty acids along with many minor precursors and modified products, the number of individual phospholipid species ranges in hundreds. In more complex eukaryotic organisms with greater diversity in both the phospholipids and fatty acids, the number of individual species is in thousands. Sphingolipids also show a similar degree of diversity and when added to the steroids, the size of the eukaryotic lipodome approaches the size of the proteome.

In this chapter, the diversity in structure, chemical properties, and physical properties of lipids will be outlined. The various genetic approaches available to study lipid function in vivo will be summarized. Finally, how the physical and chemical properties of lipids relate to their multiple functions in living systems will be reviewed to provide a molecular basis for the diversity of lipid structures in natural membranes [1].

2. Diversity in lipid structure

Lipids are defined as the biological molecules readily soluble in organic solvents such as chloroform, ether, or toluene. However, many peptides and some very hydrophobic proteins are soluble in chloroform, and lipids with large hydrophilic domains such as saccharolipids are not soluble in these solvents. Here we will consider only those lipids that contribute significantly to membrane structure or have a role in determining protein structure or function. The broad area of lipids as second messengers is covered in Chapters 12–14. The LIPID Metabolites and Pathways Strategy (LIPID MAPS) consortium is identifying, characterizing, and classifying the components of the lipidome and developing a

web-based systematic nomenclature for lipids and repository for structural information on lipids. The website of this consortium [2] provides a comprehensive and evolving picture of the lipodome.

2.1. Glycerolipids

The diacylglycerol backbone in eubacteria and eukaryotes is *sn*-3-glycerol esterified at positions 1 and 2 with long-chain fatty acids (Fig. 2). In archaebacteria (Fig. 3), the opposite isomer *sn*-1-glycerol forms the lipid backbone and the hydrophobic domain is composed of phytanyl (saturated isoprenyl) groups in ether linkage at positions 2 and 3 (an archaeol) [3]. In addition, two *sn*-1-glycerol groups are connected in ether linkage by two biphytanyl groups (dibiphytanyldiglycerophosphatetetraether) to form a covalently linked bilayer. Some eubacteria (mainly hyperthermophiles) have dialkyl (long-chain alcohols in ether

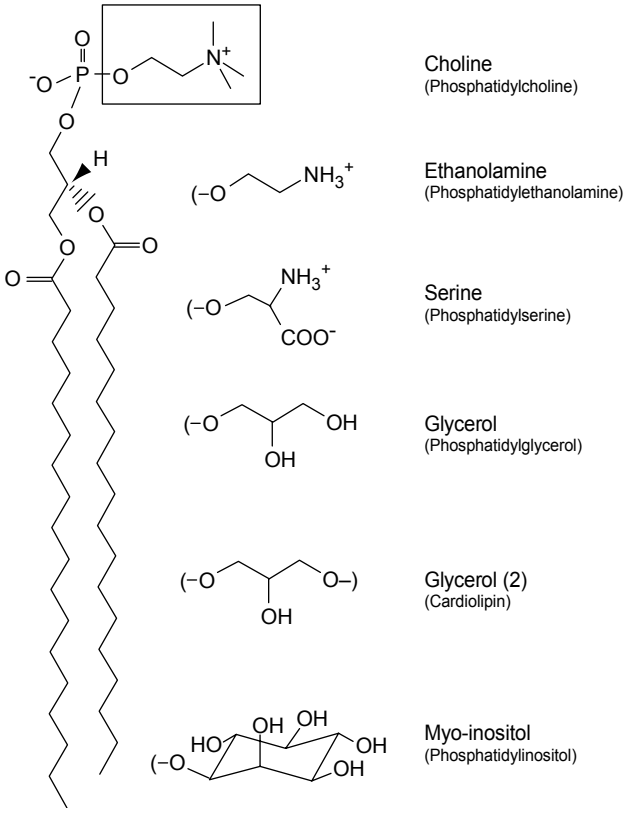

Fig. 2. Structure of glycerophosphate-based lipids. The complete lipid structure shown is 1,2-distearoyl-*sn*-glycerol-3-phosphocholine or phosphatidylcholine (PC). Substitution of choline in the box with the head groups listed below results in the other phospholipid structures. CDP–diacylglycerol has a CMP and phosphatidic acid (PA) has a hydroxyl group in place of choline (not shown). Cardiolipin (CL) is also referred to as diphosphatidylglycerol since it contains two PAs joined by a glycerol.

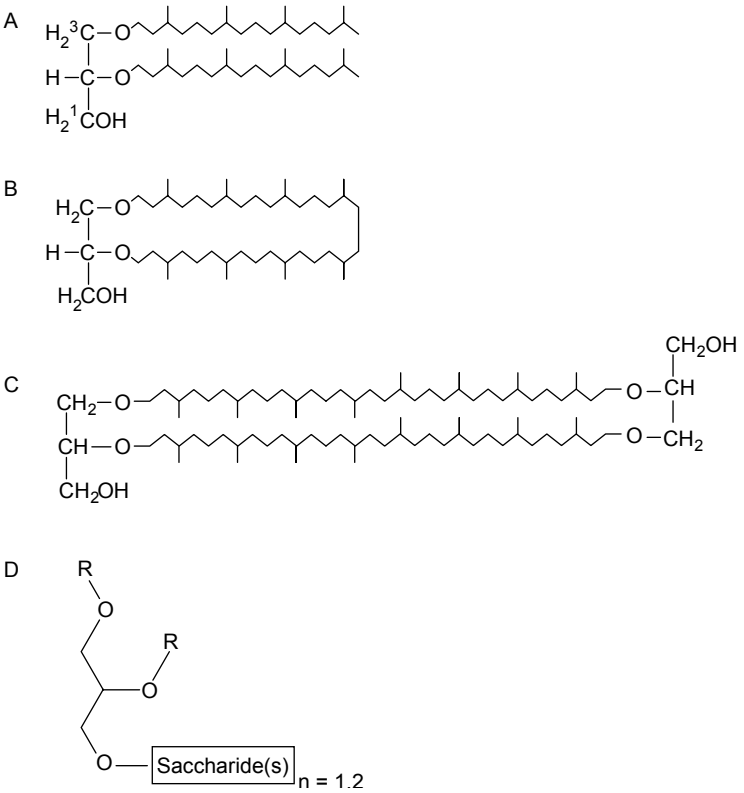

Fig. 3. Structure of dialkylglycerols in archaebacteria. Archaebacteria have phytanyl chains in ether linkage to positions 2 and 3 of *sn*-1-glycerol (archaeol). The position 1 can be derivatized with phosphodiesters. (A) Diphytanylglycerol (C20–C20 diether) with the stereochemistry of glycerol indicated. (B) Cyclic biphytanyl (C40) diether. (C) Biphytanyl diglycerol diether. (D) A glyceroglycan with either a mono or disaccharide (glucose or galactose) at position 1 of *sn*-1-glycerol. The R groups are ether-linked phytanyl chains. Similar glyceroglycans are found in eubacteria and plants with a *sn*-3-glycerol backbone and ester-linked fatty acid chains at positions 1 and 2.

linkage) phospholipids and similar ether linkages are found in the plasmalogens of eukaryotes. The head groups of the phospholipids (boxed area of Fig. 2) extend the diversity of lipids defining phosphatidic acid (PA, with OH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin (CL). Archaebacteria analogs exist with head groups of glycerol and glyceromethylphosphate as well as all of the above except PC and CL. Archaebacteria, also have neutral glycan lipid derivatives in which mono- and disaccharides (glucose or galactose) are directly linked to *sn*-1-archaeol (Fig. 3). Plants (mainly in the thylakoid membrane) and many gram-positive bacteria also have high levels of neutral diacylglycerol glycans with mono- or disaccharides linked to the 3-carbon of *sn*-3-glycerol (Chapter 4). Added to head group diversity is the range of alkyl chains of the lipids. Fatty acid chain lengths can vary from 12 to 18 in eubacteria and contain double bonds. Some gram-positive bacteria contain odd-numbered

branched-chain fatty acids rather than unsaturated fatty acids. Eukaryotic lipids contain fatty acid chains up to 26 carbons in length with multiple or no double bonds. Therefore, the diversity of glycerol-based lipids in a single organism is significant, but the diversity throughout nature is enormous.

The majority of information on the chemical and physical properties of lipids comes from studies on the major phospholipid classes of eubacteria and eukaryotes with only limited information on the lipids from archaebacteria. The biosynthetic pathways and the genetics of lipid metabolism have also been extensively studied in eubacteria (Chapter 3) and eukaryotes (Chapter 8). Clearly, the archaeol lipids confer some advantage with respect to the environment of archaebacteria. Interestingly, the pathways for phospholipid biosynthesis in eubacteria and archaebacteria are very similar even though their lipids differ in chirality of the glycerol backbone. Many of these organisms exist in harsh environments that call for more chemically stable lipid bilayers that are afforded by the above lipids. How the physical properties of the more commonly studied lipids change with environment will be discussed later.

2.2. Saccharolipids

The outer membrane of gram-negative bacteria (Fig. 4) contains lipopolysaccharide or endotoxin, which is a lipid made up of a head group derived from glucosamine phosphate [4]. The core lipid (Lipid A, see Fig. 5 and Chapter 3) in *E. coli* is a phospholipid containing two glucosamine groups in β 1–6 linkage that are decorated at positions 2, 3, 2′, and 3′ with *R*-3-hydroxymyristic acid (C14) and at positions 1 and 4′ with phosphates. Further modification at position 6′ with a KDO disaccharide (two 3-deoxy-D-*manno*-octulosonic acids in a 1–3 linkage) results in KDO_2–Lipid A that is further modified by an inner core, an outer core, and the O-antigen. Laboratory strains of *E. coli* such as K-12 and *Salmonella typhimurium* lack the O-antigen found in the wild-type and clinically important strains.

The core Lipid A forms the outer monolayer of the outer membrane bilayer of gram-negative bacteria with the inner monolayer (Fig. 4) being made up of phospholipids. This structure is modified post-assembly in response to environment including host fluids, temperature, ionic properties, and antimicrobial agents [27] and displays additional diversity among enteric and non-enteric gram-negative bacteria. Studies on Lipid A are of clinical importance because it is the primary antigen responsible for toxic shock syndrome.

2.3. Sphingolipids

All eukaryotic cells contain sphingolipids derived from the condensation of palmitoyl-CoA and serine followed by slightly different species-specific conversion to the core ceramide molecule (Chapter 14). In plants and higher eukaryotes, there is additional diversity of the long-chain base derived from palmitate with additional double bonds and hydroxyl groups as well as considerable diversity in the fatty acid in amide linkage. Yeast contains mainly derivatives of phytoceramide (4-hydroxy ceramide) and C26 fatty acid chains. The major classes of sphingolipids are grouped according to what is esterified at the primary hydroxyl β to the amide carbon of ceramide. Sphingomyelin has choline phosphate at this position, while the glycosphingolipids have various lengths of oligosaccharides at this position.

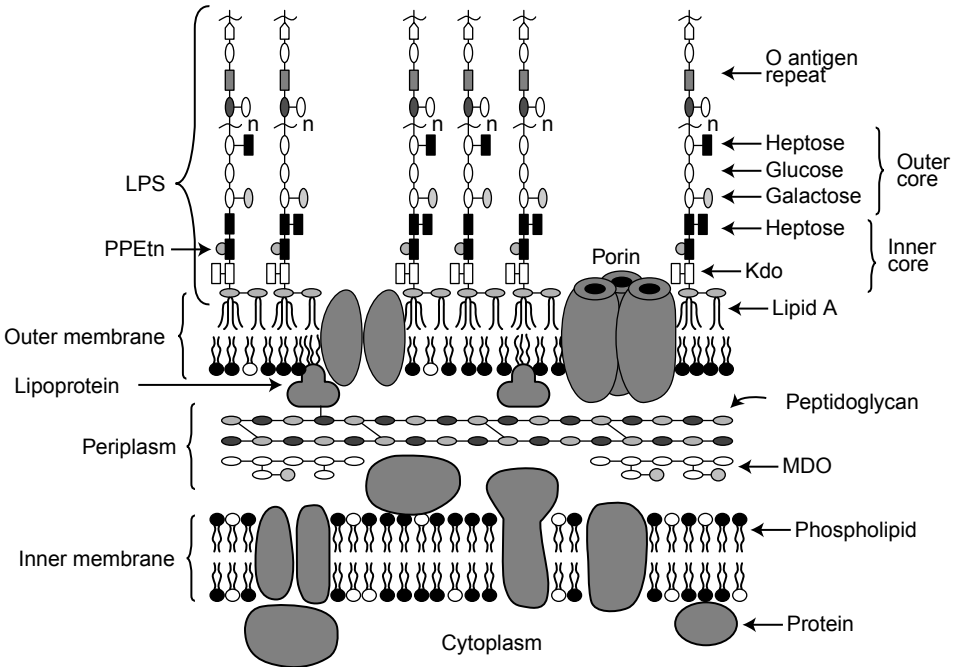

Fig. 4. *E. coli* cell envelope. The complete cell envelope of gram-negative bacteria contains an inner membrane that is a typical phospholipid bilayer and is the permeability barrier of the cell. The outer membrane is composed of an inner monolayer of phospholipid and an outer monolayer of the Lipid A portion of lipopolysaccharide (LPS). The structure of KDO_2–Lipid A is shown in Fig. 5 and is connected to a polysaccharide to build up the inner core, outer core, and the O-antigen repeat. PPEtn is ethanolamine pyrophosphate. The outer membrane is a permeability barrier for molecules larger than 750–1000 Da that cannot pass through various pores in the outer membrane. The periplasmic space contains many proteins and the membrane-derived oligosaccharide (MDO) that is one component of the osmolarity regulatory system. MDO is decorated with *sn*-glycerol-1-phosphate and ethanolamine phosphate derived from PG and PE, respectively. The amino acid–sugar cross-linked peptidoglycan gives structural rigidity to the cell envelope. One-third of the lipoproteins (*lpp* gene product) are covalently linked via its carboxyl terminus to the peptidoglycan and, in complex with the remaining lipoproteins as trimers, associates with the outer membrane via covalently linked fatty acids at the amino terminus. The amino terminal cysteine is blocked with a fatty acid, derived from membrane phospholipids, in amide linkage and is derivatized with diacylglycerol, derived from PG, in thioether linkage. Figure is courtesy of C.R.H. Raetz.

The acidic glycosphingolipids, found primarily in mammalian cells, contain either sulfated sugars (sulfatide) or sialic acid (gangliosides) in the terminal sugar position. Yeast sphingolipids contain inositol phosphate and mannose inositol phosphates linked at this hydroxyl. Although the synthesis of sphingolipids occurs in the endoplasmic reticulum and the Golgi, they are primarily found in the outer leaflet of the plasma membrane.

3. Properties of lipids in solution

The matrix that defines a biological membrane is a lipid bilayer composed of a hydrophobic core excluded from water and an ionic surface that interacts with water and defines the

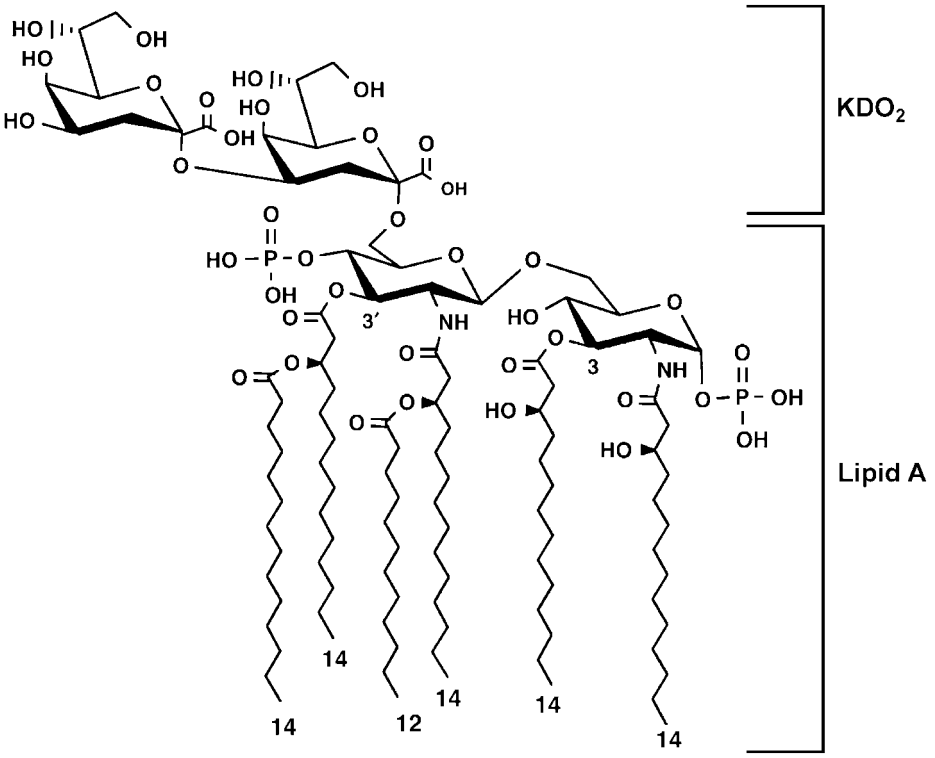

Fig. 5. Structure of KDO_2–Lipid A. Lipid A is a disaccharide of glucosamine phosphate that is multiply acylated with both amide and ester linkages to fatty acids of the chain lengths indicated (12 and 14). As illustrated in Fig. 4, Lipid A is attached to KDO_2 that is then elongated with the remainder of lipopolysaccharide structure. Figure is courtesy of C.R.H. Raetz.

hydrophobic–hydrophilic interface (Fig. 1). Much of our understanding of the physical properties of lipids in solution and the driving force for the formation of lipid bilayers comes from the concept of the 'hydrophobic bond' as described by Walter Kauzmann (1959) in the context of the forces driving protein folding and later extended as the 'hydrophobic effect' by Tanford [5] to explain self-association of lipids within biological systems. The 'fluid mosaic' model for membrane structure further popularized these concepts (S.J. Singer, 1972). This model envisioned membrane proteins as undefined globular structures freely moving in a homogeneous sea of lipids. Although this model stimulated research in the area of membrane proteins, it relegated lipids to a monolithic role as a fluid matrix within which membrane proteins reside and function. As will be apparent, our current understanding of the role of lipids in cell function is as specific and dynamic as that of proteins.

3.1. Why do polar lipids self-associate?

Polar lipids are amphipathic in nature containing both hydrophobic domains, which do not interact with water, and hydrophilic domains, which readily interact with water. The basic

premise of the hydrophobic effect is that the hydrocarbon domains of polar lipids distort the stable hydrogen bonded structure of water by inducing ordered cage-like structures around the apolar domains. Self-association of the hydrophobic domains minimizes the total surface area in contact with water resulting in an entropy-driven relaxation of water structure and an energy minimum for the final self-associated molecular organization. The polar domains of lipids interact either through hydrogen bonding or ionic interaction with water or other lipid head groups and therefore are energetically stable in an aqueous environment. The structural organization that a polar lipid assumes in water is determined by its concentration and the law of opposing forces, i.e., hydrophobic forces driving self-association of hydrophobic domains versus steric and ionic repulsive forces of the closely associated polar domains opposing self-association. At low concentrations, amphipathic molecules exist as monomers in solution. As the concentration of the molecule increases, its stability in solution as a monomer decreases until the unfavorable repulsive forces of closely packed polar domains are outweighed by the favorable self-association of the hydrophobic domains. At this point, a further increase in concentration results in the formation of increasing amounts of self-associated monomers in equilibrium with a constant amount of free monomer. This point of self-association and the remaining constant free monomer concentration is the critical micelle concentration [6]. Due to the increased hydrophobic effect, a larger hydrophobic domain results in a lower critical micelle concentration. However, the larger the polar domain, either because of the size of neutral domains or charge repulsion for like-charged ionic domains, the higher becomes the critical micelle concentration due to the unfavorable steric hindrance or charge repulsion in bringing these domains into close proximity. The critical micelle concentration of amphipathic molecules with a net charge is lowered by increasing ionic strength of the medium due to dampening of the charge repulsion effect. Addition of chaotropic agents such as urea that disrupts water structure or organic solvents that lower the dielectric constant of water raises the critical micelle concentration by stabilizing the hydrophobic domain in an aqueous environment. Therefore, the critical micelle concentration of the detergent sodium dodecyl sulfate is reduced 10-fold when the NaCl concentration is raised from 0 to 0.5 M but is increased upon addition of urea or ethanol.

These physical properties and the shape of amphipathic molecules define three supermolecular structural organizations of polar lipids and detergents in solution (Fig. 6). Detergents, lysophospholipids (containing only one alkyl chain), and phospholipids with short alkyl chains (eight or fewer carbons) have an inverted-cone shape (large head group relative to a small hydrophobic domain) and self-associate above the critical micelle concentration with a small radius of curvature to form micellar structures with a hydrophobic core excluding water. The micelle surface, rather than being a smooth spherical or elliptical structure with the hydrophobic domains completely sequestered inside a shell of polar residues that interact with water, is a very rough surface with many of the hydrophobic domains exposed to water. The overall structure reflects the optimal packing of amphipathic molecules at an energy minimum by balancing the attractive force of the hydrophobic effect and the repulsive force of close head group association. The critical micelle concentration for most detergents ranges from micromolar to millimolar. Lysophospholipids also form micelles with critical micelle concentrations in the micromolar range. However, phospholipids with chain lengths of 16 and above self-associate at a concentration around

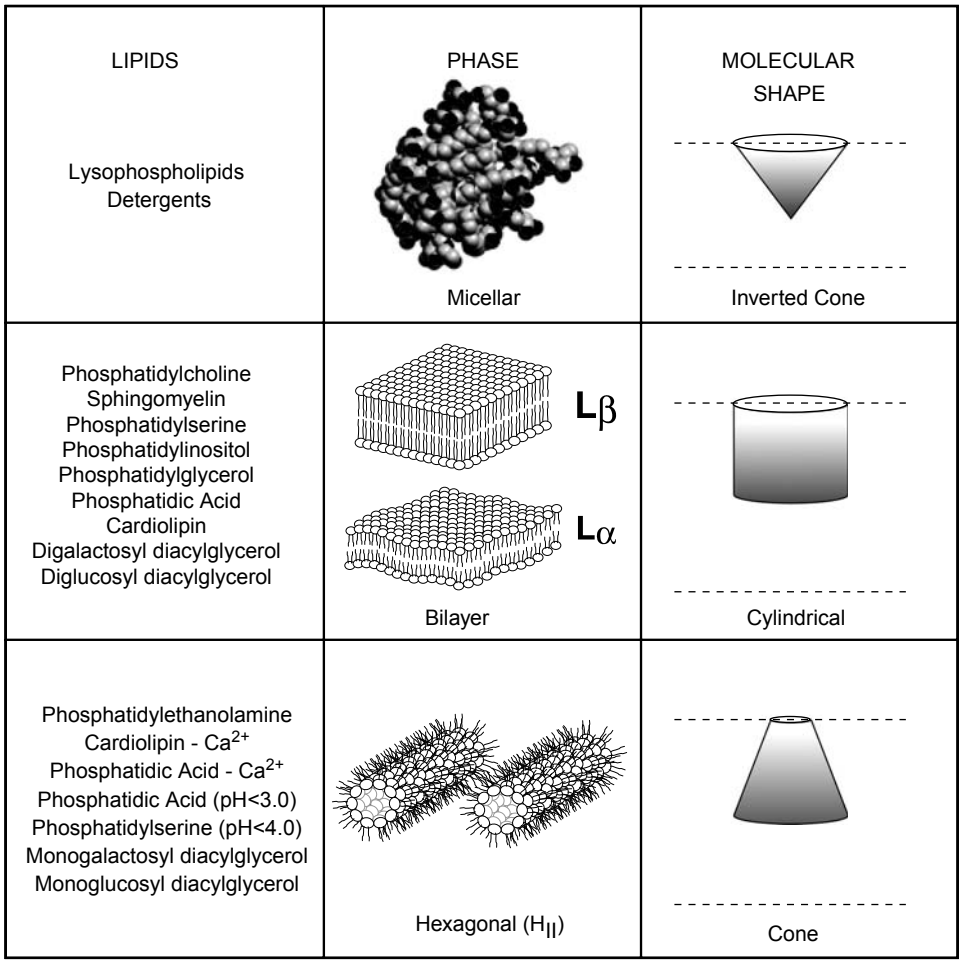

Fig. 6. Polymorphic phases and molecular shapes exhibited by lipids. The space-filling model for the micellar phase is of the β-D-octyl glucoside micelle (50 monomers). The polar portions of the detergent molecules (oxygen atoms are black) do not completely cover the micelle surface (hydrocarbons in gray) leaving substantial portions of the core exposed to bulk solvent. Inverted-cone-shaped molecules form micelles. Model adapted from Garavito and Ferguson-Miller [6]. Copyright 2001 The American Society for Biochemistry and Molecular Biology. Polar lipids with two long alkyl chains adopt a bilayer or non-bilayer (H_{II}) structure depending on the geometry of the molecule (cylinder- or cone-shaped, respectively) and environmental conditions. The L_β (ordered gel) and L_α (liquid crystalline) bilayer phases differ in the order within the hydrophobic domain and in mobility of the individual molecules.

10^{-10} M due to the hydrophobic driving force contributed by two alkyl chains. Phospholipids with long alkyl chains do not form micelles but organize into bilayer structures, which allow tight packing of adjacent side chains with the maximum exclusion of water from the hydrophobic domain. In living cells, phospholipids are not found free as monomers in solution but are organized into either membrane bilayers or protein complexes. When long-chain phospholipids are first dried to a solid from organic solvent and then hydrated,

they spontaneously form large multilamellar bilayer sheets separated by water. Sonication disperses these sheets into smaller unilamellar bilayer structures that satisfy the hydrophobic nature of the ends of the bilayer by closing into sealed vesicles (also termed liposomes) defined by a continuous single bilayer and an aqueous core much like the membrane surrounding cells. Liposomes can also be made by physical extrusion of multilamellar structures through a small orifice, by dilution of a detergent–lipid mixture below the critical micelle concentration of the detergent, or by sonication (Z. Zawada, 2004).

Cylinder-shaped lipids (head group and hydrophobic domains of similar diameter) such as PC form lipid bilayers. Cone-shaped lipids (small head groups relative to a large hydrophobic domain) such as PE (unsaturated fatty acids) favor an inverted micellar structure where the head groups sequester an internal aqueous core and the hydrophobic domains are oriented outward and self-associate in non-bilayer structures. These are denoted as the hexagonal II (H_{II}) and cubic phases (a more complex organization similar to the H_{II} phase). The ability of lipids to form multiple structural associations is referred to as lipid polymorphism. Lipids such as PE, PA, CL, and monosaccharide derivatives of diacylglycerol can exist in either a bilayer or non-bilayer phase depending on solvent conditions, alkyl chain composition, and temperature. These phases are governed by the packing geometry of the hydrophilic and hydrophobic domains upon self-association as discussed below.

Both cone-shaped and inverted-cone-shaped lipids are considered non-bilayer-forming lipids and when mixed with the bilayer-forming lipids change the physical properties of the bilayer and introduce stress or strain in the bilayer structure. When bilayer-forming lipids are spread as a monolayer at an aqueous–air interface, they orient with the hydrophobic domain facing air, and they have no tendency to bend away from or toward the aqueous phase due to their cylindrical symmetry. Monolayers of the asymmetric cone-shaped lipids (H_{II}-forming) tend to bend toward the aqueous interface (negative radius of curvature), while monolayers of asymmetric inverted-cone-shaped lipids (micelle-forming) tend to bend away from the aqueous phase (positive radius of curvature). The significance of shape mismatch in lipid mixtures will be covered below.

3.2. Physical properties of membrane bilayers

The organization of diacylglycerol-containing polar lipids in solution (Fig. 6) is dependent on the nature of the alkyl chains, the head groups, and the solvent conditions (i.e., ion content, pH, and temperature). The transition between these phases for pure lipids in solution can be measured by various physical techniques such as ^{31}P NMR and microcalorimetry. The difference between the ordered gel (L_β) and liquid crystalline (L_α) phases is the viscosity or fluidity of the hydrophobic domains of the lipids, which is a function of temperature and the alkyl chain structure. At any given temperature, the 'fluidity' (the inverse of the viscosity) of the hydrocarbon core of the bilayer increases with increasing content of unsaturated or branched alkyl chain or with decreasing alkyl chain length. Due to the increased mobility of the fatty acid chains with increasing temperature, the fluidity and space occupied by the hydrophobic domain of lipids increase. A bilayer-forming lipid such as PC assumes a cylindrical shape over a broad temperature range and with different alkyl chain compositions. When analyzed in pure form, PC exists in either the L_β or L_α phase mainly

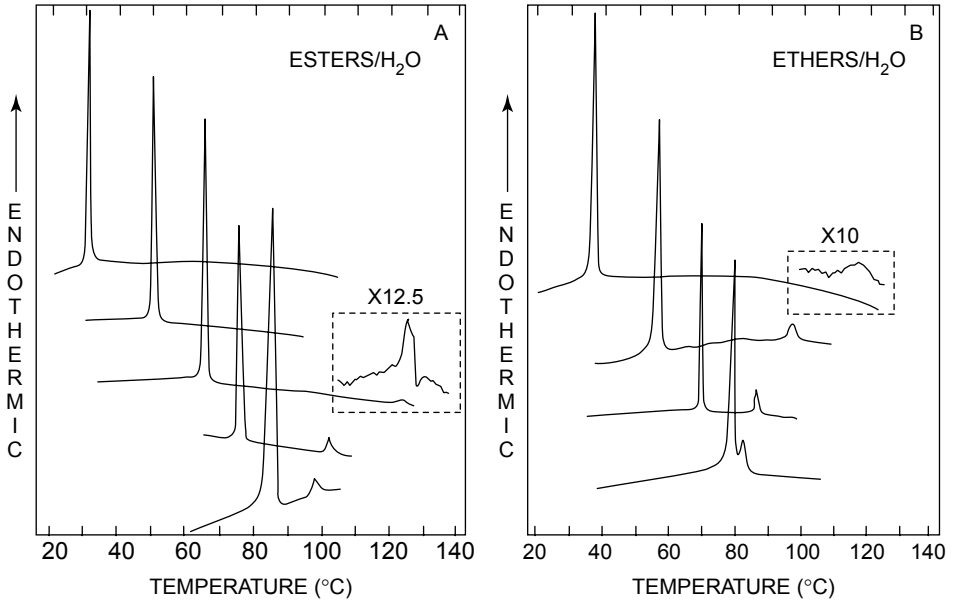

Fig. 7. Phase behavior of PE as a function of temperature and chain length. As hydrated lipids pass through a phase transition, heat is absorbed as indicated by the peaks. The large peaks at the lower temperatures are due to the L_β to L_α transition and the smaller peaks at higher temperatures are due to the L_α to H_{II} transition. (A) Even-numbered diacyl-PEs ranging from C12 to C20 (top to bottom). (B) Even-numbered dialkyl-PEs in ether linkage ranging from C12 to C18 (top to bottom). The inserts indicate an expanded scale for the transition to H_{II}. Adapted from Seddon et al. [7]. Copyright 1983 American Chemical Society.

dependent on the alkyl chain composition and the temperature. Non-bilayer-forming lipids such as PE exist at low temperatures in the L_β phase, at intermediate temperatures in the L_α phase, and at elevated temperatures in the H_{II} or cubic phase (Fig. 7). The last transition is not only temperature dependent but also depends on the shape of the lipid. The supermolecular organization of lipids with relatively small head groups can change from cylindrical to conical (H_{II} phase) with increasing unsaturation or length of the alkyl chains or with increasing temperature. As can be seen from Fig. 7, the midpoint temperature (T_m) of the transition from the L_β to the L_α phase increases with an increase in the length of the fatty acids, but the midpoint of the transition temperature (T_{LH}) between the L_α and H_{II} phases decreases with increasing chain length (or increasing unsaturation; not shown).

Similar transition plots as well as complex phase diagrams have been generated with mixtures of lipids. The physical property of a lipid mixture is a collective property determined by each of the component lipids. A large number of studies indicate that the L_α state of the membrane bilayer is required for cell viability and cells adjust their lipid composition in response to many environmental factors so that the collective property of the membrane exhibits the L_α state. Addition of non-bilayer-forming lipids to bilayer-forming lipids can result in non-bilayer formation but at a higher temperature than for the pure non-bilayer-forming lipid. Addition of non-bilayer-forming lipids also adds another parameter of

tension between the two monolayers. These lipids in each half of the bilayer tend to reduce the radius of curvature of each monolayer that results in a tendency to pull the bilayer apart by curving the monolayers away from each other (Section 3.1). This process results in potential energy residing in the bilayer that is a function of the presence of non-bilayer lipids. Forcing non-bilayer-forming lipids into a bilayer structure also exposes the hydrophobic core of the non-bilayer-forming lipids to the aqueous phase, which when relieved by insertion of proteins into the bilayer results in a release of free energy. Mixtures of lipids with dissimilar phase properties can also generate phase separations with local domain formation (Section 5.5). Such discontinuities in the bilayer structure may be required for many structural organizations and cellular processes such as accommodation of proteins into the bilayer, movement of macromolecules across the bilayer, cell division, and membrane fusion and fission events. The need for bilayer discontinuity may be the reason that all natural membranes contain a significant proportion of non-bilayer-forming lipids even though the membrane under physiological conditions is in the L_α phase.

Addition of cholesterol to lipid mixtures has a profound effect on the physical properties of a bilayer. Increasing amounts of cholesterol inhibit the organization of lipids into the L_β phase and favor a less fluid but more ordered structure than the L_α phase, resulting in the lack of a phase transition normally observed in the absence of cholesterol. The solvent surrounding the lipid bilayer also influences these transitions primarily by affecting the size of the head group relative to the hydrophobic domain. Ca^{2+} and other divalent cations (Mg^{2+}, Sr^{2+} but not Ba^{2+}) reduce the effective size of the negatively charged head groups of CL and PA allowing organization into the H_{II} phase. Low pH has a similar effect on the head group of PS. Since Ca^{2+} is an important signaling molecule that elicits many cellular responses, it is possible that part of its effect is transmitted through changes in the physical properties of membranes. In eukaryotes, CL is found almost exclusively in the inner membrane of the mitochondria where Ca^{2+} fluxes play important regulatory roles.

3.3. Special properties of CL

CL has the unique property of being either a bilayer or non-bilayer lipid depending on the absence or presence of divalent cations. CL is found almost exclusively in the energy-transducing membranes of mitochondria and bacteria (Table 1). A largely unrecognized property of CL is the ionization constants of its two phosphate diesters. Rather than displaying two pK values in the range of 2–4, pK_2 of CL is $>$8.5 [8] indicating that CL is protonated at physiological pH (Fig. 8). This property results in CL being a proton sink or a conduit for protons in transfer processes. Although PG appears to substitute for CL in many processes in both bacteria and yeast, lack of CL in yeast results in a reduction in cell growth dependent on oxidative processes [9]. CL also serves as the binding site for cytochrome *c* on the outer surface of the inner mitochondrial membrane. Reduced levels of mitochondrial CL in mammalian cells lead to significant release of cytochrome *c* to the cytoplasm that initiates an apoptotic cascade (J.B. McMillin, 2002). In mammalian cells, unlike yeast, PG may not be able to substitute for CL because of a requirement for CL in preventing continuous induction of apoptosis.

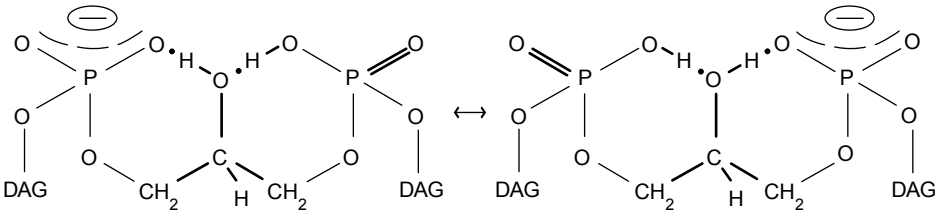

Fig. 8. Ionization state of CL at physiological pH. CL is only partially ionized under these conditions ($pK_2 > 8.5$) and therefore can trap a proton by hydrogen bonding with the *sn*-2 hydroxyl of glycerol that joins the two PAs in the CL structure. Adapted from Haines and Dencher [8]. Copyright 2002 Elsevier Science Ltd.

3.4. What does the membrane bilayer look like?

The functional properties of natural fluid bilayers are not only influenced by the hydrophobic core and the hydrophilic surface but by the interface region containing bound water and ions. Fig. 9A shows the distribution of the component parts of dioleoyl PC across the bilayer [10] and illustrates the dynamic rather than static nature of the membrane. The length of the fatty acid chains defines the bilayer thickness of 30 Å for the above phospholipid. However, the thickness is not a static number as indicated by the probability of finding CH_2 residues outside of this limit. Bilayer thickness can vary over the surface of a membrane if microdomains of lipids are formed with different alkyl chain lengths. The width (15 Å on either side of the bilayer) of the interface region between the hydrocarbon core and the free water phase is generally not appreciated. This region contains a complex mixture of chemical species defined by the ester-linked glycerophosphate moiety, the variable head groups, bound water and ions. Many biological processes occur within this interface region and are dependent on its unique properties including the steep polarity gradient (Fig. 9B) within which surface-bound cellular processes occur.

4. Engineering of membrane lipid composition

Given the diversity in both lipid structure and function, how can the role of a given lipid be defined at the molecular level? Unlike proteins, lipids have neither inherent catalytic activity nor obvious functions in isolation (except for their physical organization). Many functions of lipids have been uncovered serendipitously based on their effect on catalytic processes or biological functions studied in vitro. Although considerable information has accumulated with this approach, such studies are highly prone to artifacts. The physical properties of lipids are as important as their chemical properties in determining function. Yet there is little understanding of how the physical properties of lipids measured in vitro relate to their in vivo function. In addition, the physical properties of lipids have been ignored in many in vitro studies. Genetic approaches are generally the most useful in studying in vivo function, but this approach has considerable limitations when applied to

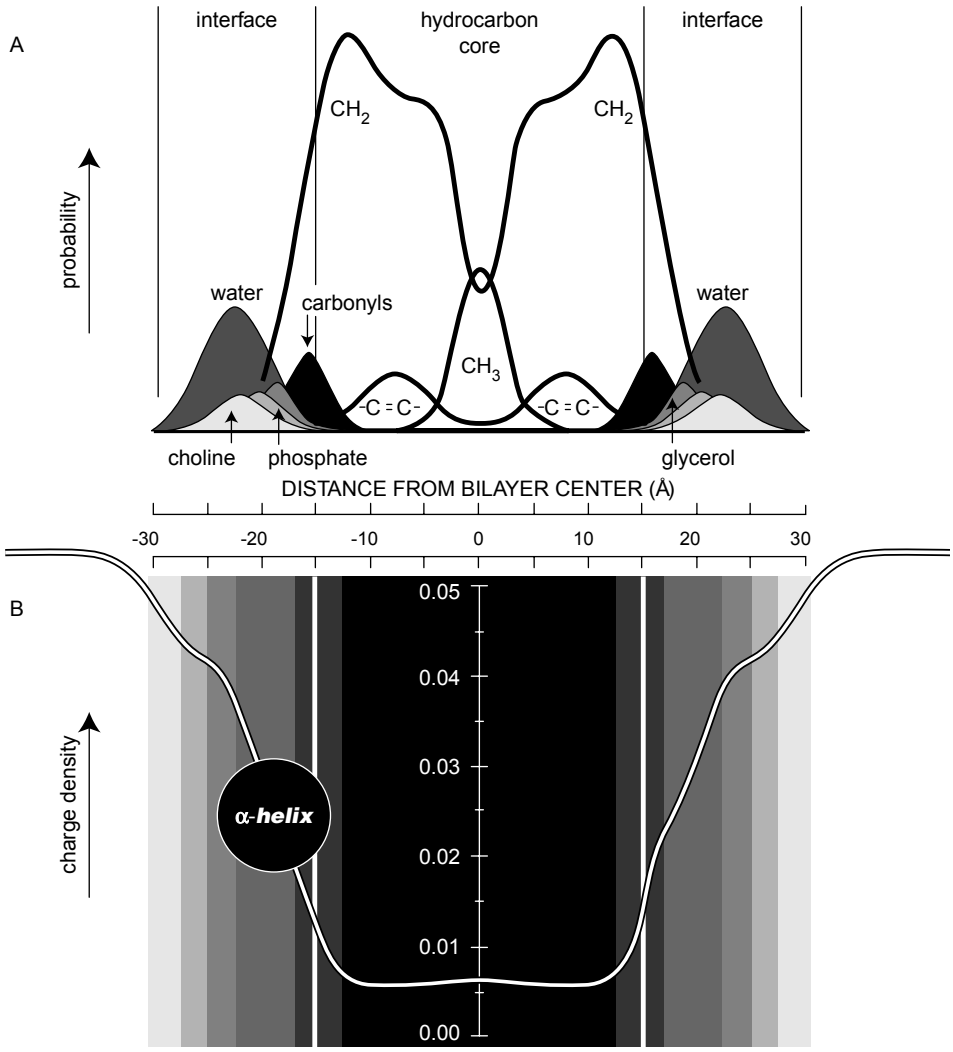

Fig. 9. The probability distribution for chemical constituents across a bilayer of PC. (A) The diagram was generated from X-ray and neutron diffraction data. The interface region between the hydrocarbon core and the free solvent region extends for approximately 15 Å on either side of the 30-Å-thick bilayer. The width of each peak defines the mobility of each constituent of PC. (B) As an α-helical peptide moves from either side of the bilayer towards the center, the charge density of the environment steeply declines as indicated by the line. Adapted from White et al. [10]. Copyright 2001 The American Society for Biochemistry and Molecular Biology.

lipids. First, genes do not encode lipids, and in order to make mutants with altered lipid composition, the genes encoding enzymes along a biosynthetic pathway must be targeted. Therefore, the results of genetic mutation are indirect and many times far removed from the primary lesion. Second, a primary function of most membrane lipids is to provide the permeability barrier of the cell. Therefore, alterations in lipid composition may compromise

cell permeability before other functions of a particular lipid are uncovered. One may learn from genetics that a lipid is essential for cell viability but one might never learn the molecular bases for other requirements. The challenge is to use this genetic information to manipulate the lipid composition of cells without severely compromising cell viability. In cases where this has been possible, the combination of the genetic approach to uncover phenotypes of cells with altered lipid composition, and the unraveling in vitro of the molecular basis for the phenotype, has proven to be a powerful approach for defining lipid function. The most useful information to date has come from genetic manipulation of prokaryotic and eukaryotic microorganisms. However, the basic molecular principles underlying lipid function will be generally applicable to more complex mammalian systems.

4.1. Alteration of lipid composition in bacteria

The pathways for formation of the major phospholipids (PE, PG, and CL) of *E. coli* were biochemically established mainly by Eugene Kennedy and coworkers and subsequently verified using genetic approaches, as described in Chapter 3. The design of strains in which lipid composition can be genetically altered in a systematic manner has been very important in defining new roles for lipids in cell function [1].

Surprisingly, *E. coli* mutants completely lacking either PE and PS or PG and CL are viable. Null mutants in the *pgsA* gene (encodes PG phosphate synthase) that cannot synthesize PG and CL are lethal, but suppressors of this mutation have been identified [11]. In such mutants, the major outer membrane lipoprotein precursor (Fig. 4), which depends on PG for its lipid modification, accumulates in the inner membrane and apparently kills the cell. Cells unable to make this lipoprotein are viable but are temperature sensitive for growth at 42°C indicating that PG and CL are not absolutely required for viability, only for optimal growth. However, the anionic nature of these lipids (apparently substituted by increased levels of PA) is necessary for the proper membrane association and function of peripheral membrane proteins, as discussed in Sections 5.2.5 and 5.5.2.

Mutants of *E. coli* lacking the amine-containing lipids PE (*psdA* null) or PS and PE (*pssA* null) are viable when grown in the presence of millimolar concentrations of Ca^{2+}, Mg^{2+}, and Sr^{2+} but have a complex mixture of defects in cell division, growth rate, outer membrane barrier function, energy metabolism, assembly of membrane proteins, and sugar and amino acid transport [1].

4.2. Alteration of lipid composition in yeast

The pathways of phospholipid synthesis and the genetics of lipid metabolism in yeast *Saccharomyces cerevisiae* [12] are as well understood as in *E. coli*. Yeasts have pathways (Chapters 3 and 8) similar to the *E. coli* CDP–diacylglycerol-dependent synthesis of PE and PG. However, CL synthesis in all eukaryotes involves transfer of a phosphatidyl moiety from CDP–diacylglycerol to PG rather than from one PG to another PG as in bacteria. In addition, yeasts utilize the mammalian pathways for synthesis of PI, PE, and PC including the methylation of PE to form PC. All gene products necessary for the synthesis of diacylglycerol, CDP–diacylglycerol, and PI in yeast are essential for viability. PS synthesis is not essential if growth medium is supplemented with ethanolamine in

order to make PE and PC. However, PE is definitely required whereas apparently PC is not (J.Y. Choi, 2004).

No gene products involved in lipid metabolism are encoded by mitochondrial DNA, which in *S. cerevisiae* encodes eight proteins primarily required for oxidative phosphorylation. Mitochondrial PS is imported from its site of synthesis in the endoplasmic reticulum and converted to PE by the mitochondrial PS decarboxylase (*PSD1* gene product). PG and CL are synthesized from CDP–diacylglycerol via nuclear gene products imported into mitochondria [1]. Null mutants of *crd1* (encodes CL synthase) lack CL but accumulate the immediate precursor PG (normally 10-fold lower than CL) to levels approaching those of CL (S.-C. Chang, 1998). These mutants grow normally on glucose for which mitochondrial function is not required. However, on non-fermentable carbon sources such as glycerol or lactate they grow slower. Therefore, CL appears to be required for optimal mitochondrial function but is not essential for viability. However, lack of PG and CL synthesis due to a null mutation in the *PGS1* gene (encodes PG phosphate synthase) results in the inability to utilize non-fermentable carbon sources for growth (S.-C. Chang, 1998). Similar effects are seen in mammalian cells with a mutation in the homologous *PGS1* gene (T. Ohtsuka, 1993). The surprising consequence of the lack of PG and CL in yeast is the lack of translation of mRNAs of four mitochondria-encoded proteins (cytochrome *b* and cytochrome *c* oxidase subunits I–III) as well as cytochrome *c* oxidase subunit IV [13] that is nuclear encoded. These results indicate that some aspects of translation of a subset of mitochondrial proteins (those associated with electron transport complexes in the inner membrane but not ATP metabolism) require PG and/or CL.

5. Role of lipids in cell function

There are at least two ways by which lipids can affect protein structure and function and thereby cell function. Protein function is influenced by specific protein–lipid interactions that depend on the chemical and structural anatomy of lipids (head group, backbone, alkyl chain length, degree of unsaturation, chirality, ionization, and chelating properties). However, protein function is also influenced by the unique self-association properties of lipids that result from the collective properties (fluidity, bilayer thickness, shape, and packing properties) of the lipids organized into membrane structures.

5.1. The bilayer as a supermolecular lipid matrix

Biophysical studies on membrane lipids coupled with biochemical and genetic manipulation of membrane lipid composition have established that the L_α state of the membrane bilayer is essential for cell viability. However, membranes are made up of a vast array of lipids that have different physical properties, can assume individually different physical arrangements, and contribute collectively to the final physical properties of the membrane. Animal cell membranes are exposed to a rather constant temperature, pressure, and solvent environment and therefore do not change their lipid makeup dramatically. The complex membrane lipid composition that includes cholesterol stabilizes mammalian cell membranes in the L_α phase over the variation in conditions they encounter. Microorganisms are

exposed to a broad range of environmental conditions so have developed systems for changing membrane lipid composition in order to maintain the L_α phase. Yet all biological membranes contain significant amounts of non-bilayer-forming lipids.

5.1.1. Physical organization of the bilayer

As the growth temperature of *E. coli* is lowered, the content of unsaturated fatty acids in phospholipids increases. Genetic manipulation of phospholipid fatty acid composition in *E. coli* is possible by introducing mutations in genes required for the synthesis of unsaturated fatty acids (Chapter 3). The mutants require supplementation with unsaturated fatty acids in the growth medium and incorporate these fatty acids to adjust membrane fluidity in response to growth temperature. When mutants with membranes containing a high content of unsaturated fatty acids are grown at low temperature, they lyse if the temperature is rapidly raised probably due to the increased membrane permeability of fluid membranes and a transition from the L_α to H_{II} phase of the lipid bilayer. Conversely, when mutants with membranes containing a high content of saturated fatty acids are grown at high temperatures, growth arrest occurs after a shift to low temperature due to the reduced fluidity of the membrane. Wild-type cells, which do not normally contain such extremes in fatty acid content as can be generated with mutants, arrest growth after a temperature shift until fatty acid composition is adjusted to provide favorable membrane fluidity.

Bacterial cells also regulate the ratio of bilayer- to non-bilayer-forming lipids in response to growth conditions [14]. Bacterial non-bilayer-forming lipids are PE with unsaturated alkyl chains, CL in the presence of divalent cations, and monoglucosyl diacylglycerol (MGlcDG). Extensive studies of lipid polymorphism have been carried out on *Acholeplasma laidlawii* because this organism alters its ratio of MGlcDG (capable of assuming the H_{II} phase) to DGlcDG (diglucosyl diacylglycerol, which only assumes the L_α or L_β phase) in response to growth conditions (Å. Wieslander, 1981). High temperature and unsaturation in the fatty acids favor the H_{II} phase for MGlcDG. At a given growth temperature, the MGlcDG to DGlcDG ratio is inversely proportional to the unsaturated fatty acid content of MGlcDG. As growth temperature is lowered, *A. laidlawii* either increases the incorporation of unsaturated fatty acids from the medium into MGlcDG or increases the ratio of MGlcDG to DGlcDG to adjust the H_{II} phase potential of its lipids to remain just below the transition from bilayer to non-bilayer. Therefore, the cell maintains the physical properties of the membrane well within that of the L_α phase but with a constant potential to undergo transition to the H_{II} phase. Plants contain the galactose homolog of these lipids, which have similar properties to the bacterial lipids.

In contrast to *A. laidlawii*, *E. coli* maintains its non-bilayer lipids, CL (in the presence of divalent cations) and PE, within a narrow range and in wild-type cells adjusts the fatty acid content of PE to increase or decrease its non-bilayer potential (S. Morein, 1996). The unsaturated fatty acid content of inner membrane PE is higher than that of the PE on the inner leaflet of the outer membrane (which is 90% PE). The result is that the L_α to H_{II} transition for the inner membrane pool is only 10–15°C above the normal growth temperature of 37°C, while this transition for the outer membrane phospholipids is 10°C higher than the inner membrane phospholipids. This increased potential for the inner membrane lipids to form non-bilayer structures is believed to be biologically significant for the function of the inner membrane. In mutants completely lacking PE, the role of non-bilayer lipid appears

to be filled by CL. The growth defect of mutants lacking PE is suppressed by divalent cations in the same order of effectiveness ($Ca^{2+} < Mg^{2+} < Sr^{2+}$) for these ions to induce the formation of non-bilayer phases of CL. The CL content of these mutants varies with the divalent cation used during growth. However, the L_α to H_{II} transition for the extracted lipids (in the presence of the divalent cation) is always the same as that of lipids from wild-type cells (containing PE) grown in the absence of divalent cations. Therefore, even though *E. coli* normally does not alter its PE or CL content to adjust the physical properties of the membrane, these mutants are able to adjust CL levels to maintain the optimal physical properties of the membrane bilayer.

5.1.2. Biological importance of non-bilayer lipids

It is obvious why prevention of formation of large amounts of non-bilayer phase would be important to maintaining cell integrity. However, why is there a need for non-bilayer-forming lipids? Numerous biological processes can be envisioned as requiring discontinuity in the membrane bilayer. Integration of proteins into the bilayer might require 'annular lipids' (those in close proximity to the protein) to interface between the more regular structure of the bilayer and the irregular surface of proteins. Movement of proteins or other macromolecules through the bilayer might also require such discontinuity. The process of membrane vesicle fusion and fission requires a transition state that is not bilayer in nature. Finally, the tension resulting from the pulling apart of the two halves of the bilayer induced by either one or both monolayers containing non-bilayer lipids may be of biological importance (Section 3.1).

Since cells homeostatically adjust the mixture of bilayer- and non-bilayer-forming lipids, some proteins must be sensitive to the intrinsic curvature of the composite membrane lipids. There is a correlation between the spontaneous curvature of the membrane and the performance of embedded proteins (S.M. Bezrukov, 2000). Protein kinase C is a membrane protein that reversibly binds to the membrane surface (an amphitropic protein) and is activated by a complex of PS (probably at least six molecules), one molecule of diacylglycerol, and one molecule of Ca^{2+}. In the presence of diacylglycerol (non-bilayer lipid), protein kinase C is highly specific for PS, but in the absence of diacylglycerol the kinase will bind to any anionic lipid. Stereoselectivity for the 1,2-diacyl-*sn*-glycerol is not absolute, but protein kinase C is stereospecific for the natural L-serine isomer of PS independent of whether other non-bilayer-forming lipids are present. However, this stereoselectivity appears to be related to the fact that in the presence of Ca^{2+}, the natural isomer of PS undergoes the L_α to H_{II} transition at a lower temperature than D-PS (R.M. Epand, 1998). Diacylglycerol is highly non-bilayer promoting and might selectively partition to a non-bilayer domain formed by the natural isomer of PS. The specific interaction of these two lipids might provide the unique allosteric switch that regulates protein kinase C activity.

Phospholipase C activity is not directly influenced by the formation of non-bilayer structures. However, the presence of lipids (e.g., PE) with a tendency to form such structures stimulates the enzyme even under conditions at which purely bilayer phases exist. Conversely, sphingomyelin, a stabilizer of the bilayer phase, inhibits the enzyme. Thus, phospholipase C appears to be regulated by the overall geometry and composition of the bilayer (M.B. Ruiz-Arguello, 1998) supporting the hypothesis that the collective physical properties of the lipid bilayer can modulate the activities of membrane-associated proteins.

Therefore, it is not always clear which property of lipids, i.e., chemical or physical, is required for optimum physiological function of a protein or process. The complex interplay between chemical and physical properties of lipids exemplifies the difficulty in understanding how lipids affect biological processes at the molecular level.

5.2. Selectivity of protein–lipid interactions

A specific phospholipid requirement has been determined for optimum in vitro reconstitution of function for more than 50 membrane proteins. If one considers specific lipid requirements for membrane association and activation of amphitropic proteins, the number is in hundreds. Integral membrane proteins fold and exist in a very complex environment and have three modes of interaction with their environment. The extramembrane domains are exposed to the water milieu, where they interact with water, solutes, ions, and water-soluble proteins. Part of the protein is exposed to the hydrophobic–aqueous interface region (Fig. 9). The remainder of the protein is buried within the approximately 30-Å thick hydrophobic interior of the membrane. Amphitropic proteins may spend part of their time completely in the cytosol and are recruited to the membrane surface, or even partially inserted into the membrane, in response to various signals.

Much of what is known about these protein–lipid interactions has come from protein purification and reconstitution of function dependent on lipids. Genetic approaches coupled with in vitro verification of function have uncovered new roles for lipids. Most exciting have been results from X-ray crystallographic analysis of membrane proteins, which have revealed lipids in specific and tight association with proteins. The predominant structural motif for the membrane-spanning domain of membrane proteins is an α-helix of 20–25 amino acids, which is sufficient to span the 30-Å core of the bilayer. A β-barrel motif is also found to a lesser extent.

5.2.1. Lipid association with α-helical proteins

CL is found aligned with a high degree of structural complementarity within a high-resolution structure of the light-harvesting photosynthetic reaction center from *Rhodobacter sphaeroides*. The head group of CL is located on the surface of the reaction center, is in close contact with residues from all three of the reaction center subunits, and is engaged in hydrogen bond interactions with polar residues in the membrane interface region (at the cytoplasmic side of the membrane) (Fig. 10A). A striking observation was that the acyl chains of CL lie along grooves in the α-helices that form the hydrophobic surface of the protein and are restricted in movement by van der Waals interactions. A PE molecule was resolved in the X-ray structure of the photosynthetic reaction center from *Thermochromatium tepidum*. The phosphate group of PE is bound to Arg and Lys by electrostatic interaction and to Tyr and Gly by hydrogen bonds. PE acyl chains fit into the hydrophobic clefts formed between α-helices of three different subunits of the complex. The fatty acid chains of these phospholipids are unsaturated to allow bending of the chains to fit the grooves on the surface of these proteins.

Bacteriorhodopsin is a light-driven ion pump that is found in the purple membrane of the archaebacterium *Halobacterium salinarum*. Bacteriorhodopsin monomers consist of a bundle of seven transmembrane α-helices that are connected by short interhelical loops and enclose a molecule of retinal that is buried in the protein interior, approximately half way

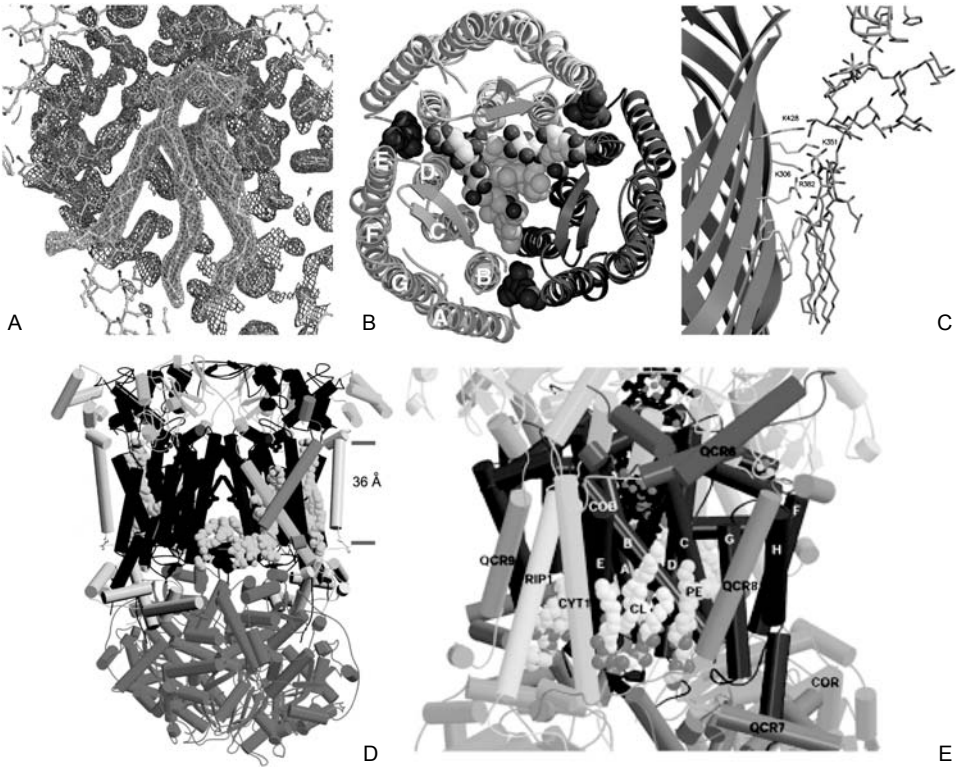

Fig. 10. Atomic structure of protein-bound lipids. (A) Model of CL (green) tightly bound to the surface of the photosynthetic reaction center (blue) from *R. sphaeroides*. The space-filling model was derived from X-ray crystallographic data that resolved between 9 and 15 carbons of the acyl chains of CL. Figure adapted from McAuley et al. [15]. Copyright 1999 National Academy of Sciences, USA. (B) Lipid packing in crystals of bacteriorhodopsin. Top view of the trimer in three different colors (domains A–E noted in one of the trimers) in complex with lipid (space-filling models) viewed from the extracellular side. Three phytanyl chains of lipid (gray) lie in the crevices formed between the A–B domain of one monomer and the D–E domain of the adjacent monomer. The central core of the trimer is filled with a sulfated triglycoside attached to archaeol. Red denotes the oxygen atoms of the sugars in white. Figure adapted from Essen et al. [16]. Copyright 1998 National Academy of Sciences, USA. (C) Crystal structure of FhuA complexed with lipopolysaccharide. The ribbon structure (blue) represents the outside surface of the β barrel of FhuA with extended chains (yellow) of amino acids. The amino acids of the aromatic belt interact with the acyl chains (gray) and the basic amino acids interact with the phosphate (green and red) groups of Lipid A. The remainder of the lipopolysaccharide structure extends upward into the periplasm. Adapted from Ferguson et al. [17]. Copyright 2000 Elsevier Science, Ltd. (D) Crystal structure of yeast Complex III dimer with the interface between monomers in the center and the putative interface with Complex IV (shown in (E)) that lies within the transmembrane region between red lines on either side. The cyan CL on the right of (D) is the white CL in the center of (E). Bottom faces the mitochondrial matrix. (D) is adapted from Pfeiffer et al. [18]. Copyright 2003 The American Society for Biochemistry and Molecular Biology. (E) is adapted from Hunte [19]. Copyright 2005 Biochemical Society, London. (See color plate section, plate no. 1.)

across the membrane [16]. Proton pumping by bacteriorhodopsin is linked to photoisomerization of the retinal and conformational changes in the protein, in a series of changes called a photochemical cycle. Specific lipids can influence the steps in this cycle. A combination of squalene (an isoprenoid) and the methyl ester of PG phosphate is required to maintain normal photochemical cycle behavior. In a high-resolution structure of

bacteriorhodopsin, 18 full or partial lipid alkyl chains per monomer were resolved (Fig. 10B), four pairs of which are linked with a glycerol backbone to form diether lipids identified as native archaeol-based lipids. One of the lipid alkyl chains buried in the center of the membrane appears to be squalene. Lipid chains were also observed in the hydrophobic crevices between the ends of the monomers in the trimeric structure and probably hold the complex together. This organization explains the requirement for the natural archaeol lipids to maintain structure and function of the protein. The two glycolipid molecules are positioned at the extracellular side (top) of the molecule leaving a central hole in the bacteriorhodopsin trimer facing the cytoplasm resulting in a 5 Å 'membrane thinning' relative to the surrounding bilayer. This may cause a steeper electric gradient across the central core than in the bulk lipid phase.

5.2.2. Lipid association with β-barrel proteins

The pore-forming proteins of the outer membrane of *E. coli* are organized as antiparallel β chains forming a barrel structure with an internal aqueous pore and an exterior hydrophobic interface with the membrane bilayer (Fig. 10C). The X-ray crystal structure of *E. coli* outer membrane ferric hydroxamate uptake receptor (FhuA) contains bound lipopolysaccharide in 1:1 stoichiometric amounts [17]. The acyl chains of the lipopolysaccharide are ordered on the protein surface approximately parallel to the axis of the β-barrel along the half of the hydrophobic belt oriented toward the extracellular surface of the outer membrane. Numerous van der Waals interactions with surface-exposed hydrophobic residues are observed. The large polar head group of lipopolysaccharide makes extensive interactions with a cluster of eight positively charged residues on the surface of the barrel. In the interface region of the membrane, there are clusters of aromatic amino acid residues positioned as belts around the protein. Similar organization of aromatic amino acids of α-helical proteins near the membrane interface region has been observed in other membrane proteins and may be involved in π-bonding interactions with the head groups of lipids.

5.2.3. Organization of protein complexes

Rather than being associated with the exterior surface of membrane proteins, many phospholipids are found wedged between the subunits of oligomeric complexes. Anionic phospholipids have a particularly important function in energy-transducing membranes such as the bacterial cytoplasmic membrane and the inner mitochondrial membrane. In particular, CL has been shown to be a key factor in the maintenance of the optimal activity of the major integral proteins of the inner mitochondrial membrane, including NADH dehydrogenase, the cytochrome bc_1 complex, ATP synthase, cytochrome *c* oxidase, and the ATP/ADP translocase [9]. CL is an integral part of the structure of *E. coli* succinate dehydrogenase and formate dehydrogenase-N.

The yeast ubiquinol:cytochrome *c* oxidoreductase is a membrane protein complex (Complex III) of the inner mitochondrial membrane. The catalytic core is composed of the *b* subunit, which is encoded by mitochondrial DNA, and the c_1 and Rieske iron–sulfur protein subunits, which are encoded by nuclear DNA. The complex also contains an additional seven non-identical and non-catalytic nuclear-encoded subunits, three heme groups, and two quinones. Fourteen phospholipid molecules have been identified in the 2.3-Å-crystal

structure (Fig. 10D and E) of the dimer from yeast (H. Palsdottir, 2004): four are CL, two are PI, six are PE, and two are PC. Six of the phospholipids per dimer are integral to the structure. Two PE molecules (one per monomer, Fig. 10D, lime green lipid at the center) contact the *b* subunit from both monomers of the dimer and lie at the interface between the two monomers. Two CL molecules (one per monomer, cyan lipid near the center) are located near the PEs. Each PI is intercalated between the three catalytic subunits of each monomer. The acyl chains fit tightly in grooves between the helices and are fixed by hydrophobic interactions with residues of all catalytic subunits. Importantly, PI is wrapped around the transmembrane helix of the Rieske protein, which may stabilize the helix packing between the transmembrane anchor of the Rieske protein and the core of the complex. Since PI binds close to the point of movement of the extrinsic domain of the Rieske protein, it could dissipate torsion forces generated by the fast movement of this domain in the process of catalysis. The remaining phospholipids are immobilized annular lipids on the surface of the complex that define the transmembrane domain of Complex III (red bars). Among these annular lipids are the CL (cyan) and PE (lime green) shown on the right front side and left rear side of Fig. 10D. These faces, shown in Fig. 10E, form a cleft containing CL (white) and PE (yellow) that has been postulated to be the interface with Complex IV in formation of a supermolecular complex (Section 5.2.4) [18]. This CL molecule lies at the entrance to one of the proton uptake sites associated with quinone reduction.

In the above cases, specific lipids mediate protein–protein contacts within a multimeric complex and are very important for structural and functional integrity of complex membrane proteins. The advantage of using lipid molecules to form a significant part of the contact surface between adjacent protein subunits is that they have a high degree of conformational flexibility, and are usually available in a range of molecular shapes and sizes. The use of lipids as interface material reduces the need for highly complementary protein–protein interactions and provides flexible interactions between subunits.

5.2.4. Supermolecular complex formation

Kinetic and structural analysis of the mammalian mitochondrial respiratory chain suggested that its individual Complexes I–IV (NADH dehydrogenase, succinate dehydrogenase, bc_1 complex, and cytochrome *c* oxidase, respectively) physiologically exist in equilibrium with supermolecular complexes or 'respirasomes' composed of the individual complexes [20]. Thus, electron transfer in the respiratory chain would be through either substrate channeling or random collision mechanisms depending on metabolic conditions. In *S. cerevisiae* mitochondria, this equilibrium is shifted to supermolecular complex organization. Recent evidence strongly suggests that phospholipids, in particular CL, play an important role in 'gluing' these complexes together in a functional manner (M. Zhang, 2002, 2005). In intact yeast mitochondria or in mild detergent extracts of mitochondria, Complexes III and IV behave kinetically or on gel electrophoresis, respectively, as an associated supermolecular complex. However, in mutants of yeast lacking the ability to make CL, Complexes III and IV behave kinetically or are displayed on gel electrophoresis as individual complexes. A cavity in the Complex III formed by membrane-embedded transmembrane helices of cytochromes c_1 and *b* with a lid on top of this cavity formed by subunits Qcr8 and Qcr6p has been suggested as a possible site of interaction between Complexes III and IV (Fig. 10D and E). CL together with PE fills this cavity and might provide a flexible

amphipathic linkage between the complexes [19]. Complexes III and IV are dissociated by detergents at low ionic strength indicating that they primarily associate through hydrophobic interactions.

Mutations in the human *TAZ* gene encoding Tafazzin, a phospholipid acyltransferase that is involved in remodeling CL to its mature highly unsaturated fatty acid composition (Y. Xu, 2006) (Chapter 8), are associated with Barth syndrome, an X-linked genetic disorder characterized by cardiomyopathy, skeletal myopathy, neutropenia, and growth retardation. It was demonstrated that CL deficiency in lymphoblasts from patients with Barth syndrome is accompanied by decreased stability of the $I/III_2/IV$ supermolecular complex suggesting that loss of mature CL species in Barth syndrome results in an altered organization of the respiratory chain (K. Brandner, 2005; M. McKenzie, 2006).

The high levels of CL in the inner membrane of mitochondria may be important for efficient utilization of the proton electrochemical potential generated by oxidative processes. The ability of CL to trap protons (Section 3.3), and consequently restrict the amount of pumped protons escaping to the bulk water phase, might increase the efficiency of protonic coupling between respiratory and ATP synthase supermolecular complexes in the cristae membrane microdomains [8]. This feature would prevent significant formation of a delocalized proton electrochemical gradient. A delocalized proton electrochemical gradient promotes superoxide formation by the respiratory chain with further generation of other free radicals, which in higher eukaryotes induces the mitochondrial pathway of apoptosis (S. Papa, 2006).

5.2.5. Binding sites for peripheral membrane proteins

A common mechanism of cellular regulation is to organize functional complexes at the membrane on demand from existing components. Peripheral membrane proteins are proteins that interact with the membrane surface or are partially embedded in the membrane surface and are easily released from the membrane by chelating agents, high salt, high pH, or chaotropic agents that do not disrupt the membrane bilayer. Integral membrane proteins with at least one domain that spans the bilayer are generally not released by such treatments and require detergents or other agents that disrupt the bilayer in order to be rendered in a 'soluble' form. Amphitropic proteins are a subclass of peripheral membrane proteins that transiently associate with the membrane usually in response to a metabolic signal. Peripheral membrane proteins can interact with the membrane lipids in at least four modes [21]: coulombic interactions between a positively charged domain on the protein surface with a membrane domain of anionic phospholipids (PA, PG, PI, CL, or PS), aromatic residues exposed on the protein surface interacting through π-bonding with the positive head group of phospholipids (PC or PE), interaction of specific binding sites on the protein with lipid second messengers (polyphosphorylated PI or diacylglycerol), or partial insertion of hydrophobic domains of the protein into the membrane bilayer (insertion of an amphipathic α-helix). In many cases, the hydrophobic face of an amphipathic helix inserts parallel to the bilayer with the cationic face residing in the interface region and interacting with anionic lipid head groups.

Three structure-specific domains, mostly found in eukaryotic cells, have been identified for membrane association and activation of amphitropic proteins [21]. The C1 lipid clamp is a conserved cysteine-rich protein domain that binds lipids and is found in protein kinases

C and other enzymes regulated by the second messenger diacylglycerol. This receptor domain interacts with one molecule of diacylglycerol and recruits protein kinase C to specific membrane sites. The C1 domain adopts a β-sheet structure with an open cavity. The C2 domain generally binds anionic phospholipids such as PS in a Ca^{2+} dependent manner and is conserved among phospholipases C, phospholipases A_2, PI-3-phosphate kinases, and calcium-dependent protein kinases C. The crystal structure of the C2 domain of protein kinase Cα in complex with PS reveals that the recognition of PS involves a direct interaction with two Ca^{2+} ions. The pleckstrin homology (PH) domain is shared by protein kinase Cβ and some phospholipases C. This domain is responsible for association of peripheral membrane proteins with the membrane via the phosphoinositide head group of polyphosphorylated PIs in an enantiomer-specific manner. PH domains consist of seven-stranded β-sheets with positively charged pockets that attract the negatively charged PI head group.

In prokaryotic cells, the protein structural features defining lipid-binding domains are less well conserved than in eukaryotes, and the membrane ligand appears to be an anionic lipid-rich domain with little selectivity for the chemical species of lipid. DnaA (K. Boeneman, 2005), MinD [20], and SecA (Section 5.3) are amphitropic proteins in *E. coli* that perform different functions but become membrane associated and activated by similar mechanisms. The involvement of anionic lipids in the function of these proteins was discovered through the use of *E. coli* mutants in which the anionic lipid content could be controlled [1]. DnaA is required for initiation of DNA replication and is active in its ATP-bound but not ADP-bound form. In vitro the exchange of ADP for ATP in the complex is greatly stimulated by almost any anionic phospholipid including non-*E. coli* lipids like PI. An anionic phospholipid-specific positively charged amphipathic helix has been identified in DnaA that appears to direct initial membrane association followed by partial insertion of the protein into the bilayer. The resulting conformational changes alter the ATP/ADP binding properties.

MinD is also an ATP/ADP binding protein containing a similar highly conserved carboxyl terminal amphipathic motif responsible for binding to anionic phospholipids. This motif is not structured in crystals of MinD but is predicted to be an amphipathic α-helix, with one side of the helix containing mainly hydrophobic amino acids and the other side containing mainly positively charged amino acids. ATP binding to MinD induces a conformational change in the protein that results in exposure of the C-terminal hydrophobic motif followed by binding to phospholipid bilayers with the induction of α-helix formation. The ATP-bound form of the protein has high affinity for anionic phospholipids and is localized to the cell poles in co-localization with anionic phospholipid-enriched domains (Section 5.5.2). Interaction of MinD with MinE results in ATP hydrolysis and release of MinD from the membrane. ATP binding and hydrolysis causes the rapid movement of MinD from one cell pole to the opposite cell pole, by alternately forming membrane-associated zones of coiled oligomeric MinD structures extending from each pole. This cycle, combined with the polar localization of anionic phospholipids, restricts MinD and its tightly associated partner MinC to the membrane surface at the poles. MinC strongly inhibits formation of the Z-ring that initiates cell septum formation in preparation for cell division. This mechanism appears to be critical for positioning of the Z-ring at the center of cell and not at the poles.

What was once thought to be a specific interaction of DnaA, MinD, and SecA proteins with either PG or CL is actually an interaction with an anionic surface charge on the membrane. Mutants completely lacking PG and CL but with elevated levels of PA (Y. Shiba, 2004) still initiate DNA replication (DnaA), divide properly (MinD), and export proteins (SecA). These proteins can be activated in in vitro reconstituted systems with a wide range of anionic lipids including those not found in *E. coli*. It appears that these proteins recognize, via positively charged amphipathic helices, clusters or domains of negative charge rather than specific lipids on the membrane surface.

CTP:phosphocholine cytidylyltransferase is responsible for the synthesis of CDP–choline, a precursor of PC synthesis in mammalian cells (Chapter 8). The enzyme has affinity for membranes depleted of PC that leads to activation of the enzyme and increased synthesis of PC. A complex mixture of factors including anionic lipids and non-bilayer-forming lipids stimulates membrane association [22]. When binding occurs via two positively charged amphipathic helices, a large structural change occurs leading to enzyme activation. Affinity for an anionic membrane surface is understandable, but the role of other lipids such as PE and diacylglycerol in activation and the negative effect of fatty acids have only been recently clarified [22]. The former two non-bilayer-forming lipids induce a negative curvature of the two halves of the bilayer toward the aqueous domain and away from the hydrophobic domain. Incorporation of fatty acids (micelle-forming) has the opposite effect. Surface association of the amphipathic helices would be favored by the decrease in hydration of the interface region due to the induced negative curvature, and penetration into the surface of the bilayer would reduce the stress imposed by negative curvature, thus favoring membrane association.

5.3. Translocation of proteins across membranes

Movement of proteins across membranes is often required to transfer a protein from its site of synthesis to its site of function. The process involves the transfer of hydrophobic and hydrophilic segments of proteins through the hydrophobic core of the membrane. The in vivo evidence for the participation of anionic phospholipids in protein translocation was obtained from experiments with *E. coli* mutant strains defective in the biosynthesis of PG and CL [1]. The in vivo translocation of the outer membrane precursor proteins, prePhoE and proOmpA, is severely hampered in these mutants. The molecular basis for this anionic lipid requirement involves the function of SecA, which moves secreted proteins through the membrane translocation pore composed of SecY and two other membrane proteins, SecE and SecG. SecA requires both anionic phospholipids and the pore component SecY for high-affinity binding to the membrane. Functional reconstitution of the purified and delipidated SecYEG complex from *E. coli* and *Bacillus subtilis* into liposomes of defined lipid composition revealed an absolute requirement for PG (C. van der Does, 2000). Translocation activity was proportional to the amount of PG in reconstituted proteoliposomes and optimum activity was obtained only with the specific lipid composition of each organism. In chloroplasts, SecA-coupled ATPase activity is optimally stimulated in the presence of the neutral lipid DGalDG and the anionic phospholipid PG at a molar ratio of 8:2, consistent with the content of neutral and anionic lipids in the thylakoid membranes of higher plants (C. Sun, 2007). Moreover, the presence of neutral DGalDG is favored over the zwitterionic PE as a partner with PG.

Non-bilayer-forming lipids are also required for protein translocation across the membrane of *E. coli*. The only non-bilayer-forming lipid in *E. coli* mutants lacking PE is CL. Protein translocation into inverted membrane vesicles prepared from PE-lacking cells (now enriched in CL) is reduced with divalent cation-depletion but can be enhanced by inclusion of Mg^{2+} or Ca^{2+} [1]. Protein translocation in the absence of divalent cations was restored by incorporation of non-bilayer PE (18:1 acyl chains) but not by bilayer-prone PE (14:0 acyl chains). These results indicate that lipids with a tendency to form non-bilayer structures provide a necessary environment for translocation of proteins across the membrane.

5.4. Assembly of integral membrane proteins

Much less is known about the role of phospholipids in insertion and organization of integral membrane proteins than about the protein machinery required for protein insertion. Most membrane proteins are organized with several α-helical transmembrane domains spanning the membrane bilayer. These helices are connected by extramembrane loops alternately exposed on either side of the membrane. How do lipids act in specific ways to guide and determine final membrane protein structure and organization?

5.4.1. Lipid-assisted folding of membrane proteins

The membrane clearly serves as the solvent within which integral membrane proteins fold and function [10]. Recent evidence supports a role for lipids in the folding and topological organization of transmembrane domains with respect to the plane of the bilayer. The major evidence for lipid-assisted folding and topological organization of membrane proteins comes from studies on the requirement for PE in the assembly and function of three secondary transport proteins of *E. coli*, lactose permease (LacY), phenylalanine permease (PheP), and γ-aminobutyrate permease (GapP) ([23] and references within). LacY is a polytopic membrane protein with 12 transmembrane-spanning domains (Fig. 11). LacY transports lactose either in an energy independent mode to equilibrate lactose across the membrane (downhill transport) or by coupling uphill movement of lactose against a concentration gradient with downhill movement of a proton coupled to the proton electrochemical gradient across the membrane (uphill transport). Uncovering a role for PE in the assembly of LacY came about by the fortuitous availability of reagents and techniques. The availability of viable *E. coli* strains in which the level of PE can be regulated provided a reagent to study the requirement for PE in the assembly of LacY in vivo and in isolated membranes (M. Bogdanov, 2002). The development of a blotting technique termed 'Eastern–Western' made possible the screening for lipids affecting the refolding of LacY in vitro or the conformation of LacY made in vivo (M. Bogdanov, 1999).

In the Eastern–Western procedure, lipids are first applied to a solid support such as nitrocellulose. Next, proteins subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis are transferred by standard Western blotting techniques to the solid support in such a manner that the protein of interest is transferred to the lipid patch. During electrotransfer of protein to the solid support, protein, lipid, and sodium dodecyl sulfate mix and as transfer continues the sodium dodecyl sulfate is removed leaving behind the protein to refold in the presence of lipid. Attachment of the refolded protein to a solid support allows one to probe protein structure using conformation-sensitive antibodies or protein function

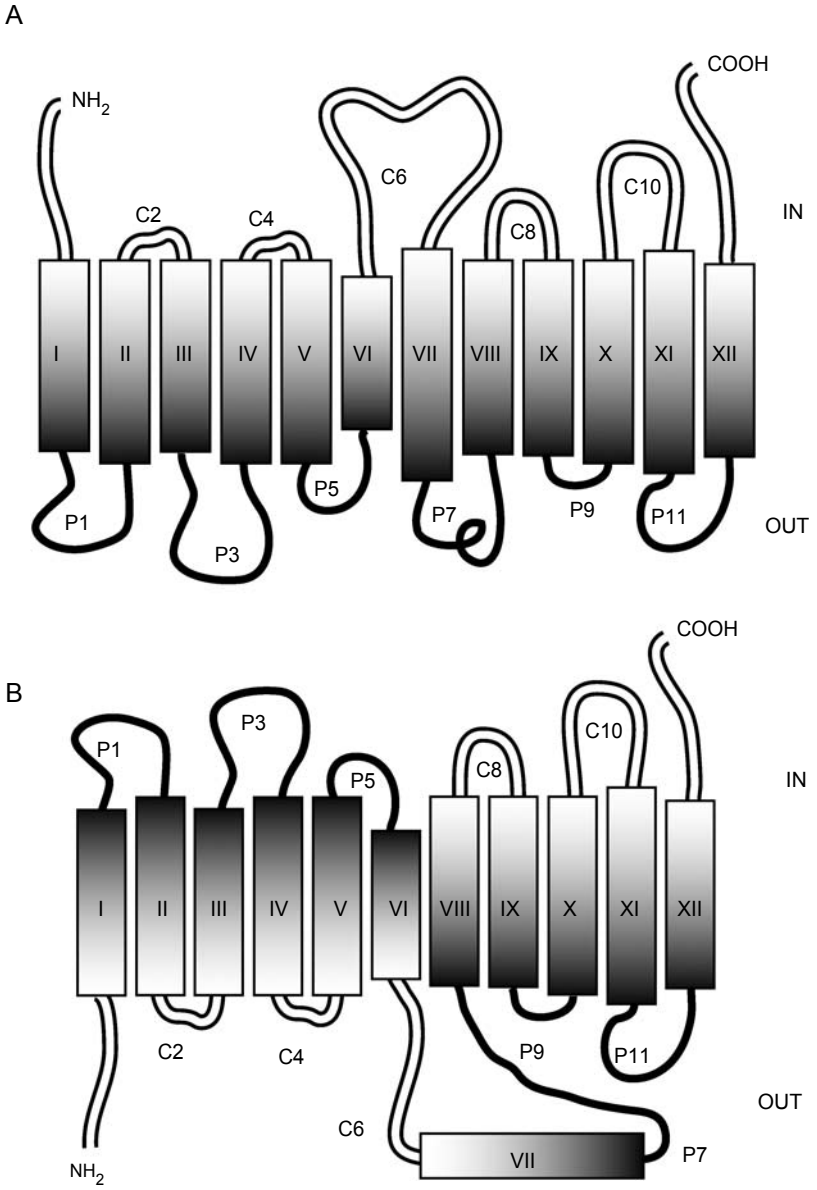

Fig. 11. Topological organization of LacY in the *E. coli* inner membrane. (A) The topological organization of LacY when assembled in membranes containing PE (normal configuration). The 12 hydrophobic membrane spanning α-helices are numbered in Roman numerals from the amino (NH_2) to the carboxyl (COOH) terminus. The even-numbered hydrophilic loops ('C') connect the transmembrane domains on the cytoplasmic side (IN) of the membrane. The odd-numbered hydrophilic loops ('P') connect the transmembrane domains on the periplasmic side (OUT) of the membrane. (B) The topological organization of LacY when assembled in the absence of PE. Note the first six transmembrane domains along with their connecting hydrophilic loops are inverted with respect to the plane of the membrane when compared to (A). Transmembrane domain VII is very hydrophilic and resides outside of the membrane in the altered structure.

by direct assay. This combined blotting technique makes possible the detection of membrane protein conformational changes as influenced by individual lipids during refolding.

The initial observation that PE was required for LacY function was concluded from studies of reconstitution of transport function in sealed vesicles made of purified LacY and lipid. When reconstituted in lipid vesicles containing PE, both active and facilitated transport was observed. In vesicles lacking PE and containing PG, or CL or PC, only facilitated transport occurred. The physiological importance of PE for LacY function was established using mutants lacking PE. LacY expressed in PE-containing cells displayed uphill transport of substrate, but cells lacking PE only displayed downhill transport (M. Bogdanov, 1995). Using Western and Eastern–Western blotting techniques and a conformation-sensitive antibody, it was established that LacY assembled in the presence, but not in the absence, of PE displays 'native' structure. LacY maintains its native structure even when PE is completely removed, and LacY originally assembled in the absence of PE is restored to native structure by partial denaturation in sodium dodecyl sulfate followed by renaturation in the presence of PE specifically. LacY assembled (either in vivo or in vitro) in membranes lacking PE is restored to native structure by post-assembly addition of PE to the membranes (M. Bogdanov, 1998). Taken together these data strongly suggest that PE assists in the folding of LacY by a transient non-covalent interaction with a late folding and non-native intermediate thereby fulfilling the minimum requirements of a lipid-assisted folding mechanism analogous to that propagated by protein molecular chaperones (M. Bogdanov, 1996).

Can lipids assist the folding of soluble proteins that transiently associate with membranes? The refolding of the denatured soluble enzyme horseradish peroxidase was followed in the presence and absence of liposomes of different lipid composition (D. Debnath, 2003). Remarkably, dimyristoyl PE, a bilayer-forming lipid, was able to significantly increase the yield of renatured enzyme relative to refolding in the absence of liposomes. However, dioleoyl PE, which does not favor bilayer organization, did not support proper refolding. Therefore, LacY and the peroxidase share common requirements for proper refolding dependent on lipids.

The molecular basis for the loss of native structure and function of LacY assembled in the absence of PE is a topological mis-assembly of the protein. In the absence of PE, the first six transmembrane helical bundle and the connecting loops are inverted with respect to the plane of the membrane bilayer (Fig. 11). Cytoplasmic loops become periplasmic, and periplasmic loops become cytoplasmic. Transmembrane domain VII, a helical domain of low hydrophobicity normally stabilized in the membrane bilayer by intramolecular salt bridges, stably resides outside of the membrane (M. Bogdanov, 2007). The ability of LacY to adopt alternative conformations in different lipid environments is consistent with the existence of two separately folded subdomains (N-terminal and C-terminal halves) that can be co-expressed independently within the cell and associate into a functional protein (M. Sahin-Toth, 1996). Therefore, each domain can display considerable conformational flexibility and respond independently to the lipid environment. However, in order for these domains to respond to the lipid environment independently of each other, either during initial assembly or during a change in lipid environment, the interface between these domains must possess considerable flexibility so that the intrinsic preferred orientation in the lipid bilayer is governed by their respective thermodynamic minima. Indeed the hydrophobicity

of transmembrane domain VII influences the operation of the molecular hinge within LacY; mutation of the Asp 240 to Ile within this domain, which increases its stability within the bilayer, prevents inversion of the first helix bundle in cells lacking PE [28]. Therefore, transmembrane domains on either side of a flexible hinge region can organize independently of each other in response to lipid environment, while proteins without such a hinge region either cannot assume different topologies or cannot fold and are degraded.

If LacY is first assembled in the absence of PE and then post-assembly PE synthesis is initiated, there is correction of topology, i.e., a re-orientation of transmembrane domains with respect to the bilayer, and uphill transport function is regained (Bogdanov, 2002). Thus, membrane protein topology can be rearranged after membrane insertion challenging the dogma that once transmembrane orientation is established during assembly it is static and not subject to post-insertional topological editing or alteration. The ability of LacY to adopt different topologies dependent on the presence or absence of PE raises the question of whether protein topogenesis is determined primarily by protein–lipid interactions or depends on the lipid requirements of the components of the assembly machinery. To address this question, mis-assembled LacY or properly assembled LacY was purified from PE-lacking or PE-containing cells, respectively, and then reconstituted into liposomes of various lipid compositions (X. Wang, 2002). Irrespective of the source of LacY, the final lipid composition of proteoliposomes determined both topology and function. Therefore, both the topological organization and transport function of LacY are determined primarily by the phospholipid composition independent of cellular protein assembly machinery.

Several secondary transport proteins with high overall structural homology to LacY are also dependent on PE for assembly and function. In the case of PheP (W. Zhang, 2003) and GapP [23], only the N-terminal helical hairpin (two transmembrane domains) are inverted in cells lacking PE. Therefore, the role of PE in determining topological orientation of transmembrane domains may be a general requirement for proper assembly of this family of secondary transport proteins. These results dramatically illustrate the specific effects of membrane lipid composition on structure, function, and dynamics of membrane proteins. The ability of changes in lipid composition to effect such large changes in protein structure has important implications for regulatory roles of lipids in cell processes. For example, as eukaryotic proteins move through the secretory pathway, they encounter different membrane lipid compositions that might affect protein structure in dramatic ways to turn on or turn off function. Local changes in lipid composition may also result in similar changes in structure and function. Incompatibility of lipids involved in correct topogenesis of membrane proteins from different sources might indicate a general problem for heterologous expression of foreign membrane proteins.

5.4.2. Molecular determinants of protein topology

Based on statistical analysis and experimental determination, the cytoplasmic extramembrane domains connecting transmembrane domains predominantly carry a positive charge in contrast to the remaining extramembrane domains that are either neutral or negative (positive inside rule) [24]. Although amino acid sequence determines membrane protein topology, the sequence is encoded for a specific membrane lipid environment as has been demonstrated for the secondary transport proteins of *E. coli*. Reducing the net positive

charge for cytoplasmic extramembrane domains increases their potential for translocation to the opposite side of the membrane. However, genetically reducing the anionic phospholipid content of the membrane also results in a higher potential for translocation of weakly positive extramembrane domains [1]. Similarly, increasing the net negative charge density on the membrane surface by eliminating PE, as described above, also results in translocation of some but not all extramembrane domains. The role of lipid head group charge density as a topological determinant is further supported by expression of foreign lipids in *E. coli*. In *E. coli* lacking PE but genetically engineered to synthesize the neutral glycan lipid MGlcDG, LacY assumes its native topological organization and functions in uphill transport of substrate (J. Xie, 2006). The extramembrane domains of LacY, which are sensitive to lipid composition (those of the N-terminal six transmembrane helical bundle), carry a net positive charge and also each contains one or more acidic amino acids that lie within the interface region of the membrane. Substitution of any one of these acid residues with a neutral amino acid prevents mis-orientation of the whole helical bundle in cells lacking PE [29]. Thus, the final topology of membrane proteins appears to result from a finely tuned interaction between topogenic signals on the protein and topological determinants within the membrane that are influenced by the net charge of protein extramembrane domains and lipid head groups and direct lipid–protein interaction on either side of the membrane.

5.5. Lipid domains

Compartmentalization of many biological processes such as biosynthesis, degradation, energy production, and metabolic signaling plays an important role in cell function. Subcellular organelles, multiple membrane structures, cytosol versus membrane localization are all utilized to compartmentalize functions. The original fluid mosaic model envisioned the membrane bilayer as a homogenous sea of lipids into which proteins are dispersed (Fig. 1). The current view of biological membranes is that they contain microdomains of different lipid and protein composition and that these domains serve to further compartmentalize cellular processes.

Defined lipid mixtures undergo phase separations due to lipid polymorphism, differences in steric packing of the acyl chains, and length of acyl chains. Mixtures of bilayer and non-bilayer lipids undergo multiple phase transitions as a function of temperature supporting the existence of segregated domains within the bilayer. In model systems, amphipathic polar lipid analogs self-associate into domains if their hydrophobic domains are the same even if their polar domains carry the same net charge (T. Mizushima, 1996). Therefore, head group repulsive forces can be overcome by orderly packing of the hydrophobic domains. There is considerable acyl chain mismatch between phospholipids and sphingolipids, i.e., phospholipids tend to have shorter acyl chains (16–18) with higher degrees of unsaturation compared to the longer (20–24 for the acyl group) saturated chains of sphingolipids. Naturally occurring sphingolipids undergo the L_β to L_α transition near the physiological temperature of 37°C, while this transition for naturally occurring phospholipids is near or below 0°C. Therefore, the more laterally compact hydrophobic domains of sphingolipids can readily segregate from the more disordered domains of unsaturated acyl chains of phospholipids. Lipid segregation can also be facilitated by

specific polar head group interactions, particularly intermolecular hydrogen bonding to other lipids and to protein networks involving hydroxyls, phosphates, amines, carbohydrates, and alcohols. The hydrogen bonding properties of CL due to its high pK_2, as noted earlier (Fig. 8), may be the basis for the formation of clusters of CL in natural and artificial membranes [25].

5.5.1. Lipid rafts

Lipid rafts are liquid-ordered phases of lipids and proteins that exist as microdomains within the more dispersed L_α bilayer. Lipid rafts are operationally defined as the membrane fraction of eukaryotic cells that is resistant to solubilization by the detergent Triton X-100 (detergent-resistant membrane fraction) near 4°C. This fraction is greatly enriched relative to the total membrane in cholesterol, glycosphingolipids, sphingomyelin, and a subset of membrane proteins. The liquid-ordered- to liquid-disordered-phase transition temperature of rafts is up to 15°C above the transition temperature of the surrounding lipid bilayer because of the high cholesterol and sphingomyelin content (C. Leidy, 2004). Many cell surface receptors co-localize to lipid rafts by virtue of the apparent specificity of their transmembrane domains for the raft environment (J. Bock, 2003). Also co-clustered with lipid rafts are soluble globular protein domains tethered to the raft lipids via covalent linkage to fatty acids, cholesterol, isoprenoid compounds, or PI (Chapter 2). The PI-linked proteins are attached via their carboxyl terminus directly to the amino group of ethanolamine phosphate, which in turn is linked to a trisaccharide and then to the inositol of PI (Fig. 12). The sphingolipids and PI glycan-linked proteins occupy the outer surface monolayer of the plasma membrane bilayer, and the acyl chains of these lipids are generally more saturated and longer than those of the plasma membrane phospholipids. The similarity in the structure of the more ordered hydrophobic domains of the raft lipids and their dissimilarity with the surrounding more fluid phospholipids favor a self-association of the raft lipids and the PI glycan-linked proteins. The hydrogen bonding properties of the glycosphingolipids with themselves and with the constituents of the PI glycan-linked proteins stabilize the complexes. Finally, the planar shape of cholesterol favors its intercalation parallel to the ordered acyl chains of the raft lipids with its single hydroxyl group facing the surface. The stability of this structure appears to explain why it is not dissipated by detergent extraction.

Lipid rafts appear to be a mechanism to compartmentalize processes on the cell surface by bringing together various receptor-mediated and signal transduction processes. A general phenomenon is that when PI glycan-linked proteins aggregate on the membrane surface, they also become enriched in the detergent-resistant membrane fraction and are phosphorylated by kinases believed to localize to lipid rafts from the cytosolic side of the membrane via covalently attached fatty acids that insert into the membrane (Fig. 12). How phosphorylation of the substrate on the trans side of the membrane occurs is not clear. A class of lipid domains related to rafts is caveolae, which are invaginations on the surface of the plasma membrane of eukaryotic cells thought to be involved in cholesterol transport (Chapter 20).

The existence of lipid rafts and their function is still an evolving area of research (D.A. Brown, 2006). Isolation and characterization of detergent-resistant membrane fractions, studies in model systems, and studies with whole cells all support the concept of lipid rafts. However, they have never been observed in eukaryotic cells in the native state, presumably due to their dynamic properties.

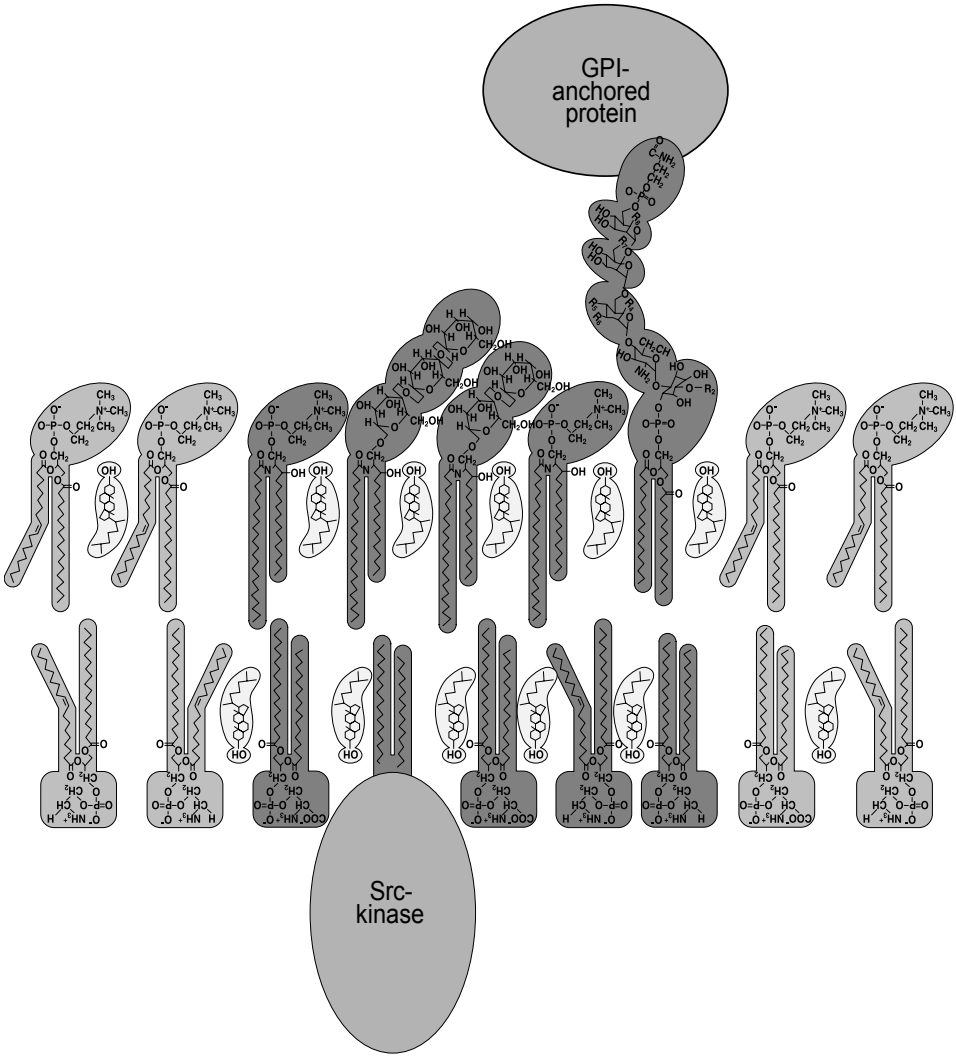

Fig. 12. Model of lipid raft. A phosphatidylinositol glycan (GPI)-linked protein is attached to the exterior monolayer of the membrane and Src-kinase to the interior monolayer of the membrane by their respective covalently attached lipids. The mechanism for clustering and coupling Src-kinase with a PI glycan-linked protein is hypothetical. Clustered (dark gray) around the PI glycan are ordered (straight alkyl chains) glycosphingolipids, sphingomyelin, and PC with intercalated cholesterol. The phospholipids with kinked (unsaturated) chains indicate the more disordered L_α state of the surrounding bilayer. Reprinted (abstracted/excerpted) with permission from Simons and Ikonen [26]. Copyright 2000 American Association for the Advancement of Science.

5.5.2. Lipid domains in bacteria

One of the few examples of lipid domains observed in living cells is the detection of CL in membranes of *E. coli* by the fluorescent dye 10-*N*-nonyl acridine orange [1]. This reagent has a higher affinity for CL than other anionic lipids and association with CL induces a shift from green to red in the emission spectrum of the compound. Spectral shift upon

10-*N*-nonyl acridine orange binding to CL is due to π–π bond stacking induced by neighboring CL molecules, analogous to acridine orange binding to DNA. The interaction and associated red shift are not pH dependent or due to ionic interactions. The red shift in the 10-*N*-nonyl acridine orange emission spectra is indicative of CL microdomains in the membrane. In wild-type *E. coli*, the fluorescence is localized at the cell pole, and in cells following duplication of the nucleus, fluorescence is observed between the nuclei. In filamentous cells of *E. coli* with multiple genomes distributed along their length, the fluorescence is localized between the genomes. The 10-*N*-nonyl acridine orange-stained CL domains are also found in septal regions and on the cell poles in *B. subtilis* but not in mutant cells lacking measurable levels of CL (K. Matsumoto, 2006). Visualization of CL in *B. subtilis* cells in different phases of sporulation revealed specific targeting of CL into the engulfment and forespore membranes.

PE can also be visualized in the bacterial membrane by means of a cyclic peptide probe, which binds specifically to the head group of PE (K. Matsumoto, 2006). Treatment of *B. subtilis* with biotinylated peptide probe followed by detection with tetramethylrhodamine-conjugated streptavidin revealed that PE is localized in the septal membranes of exponential-growth-phase cells and in sporulating cells in the polar, septal, engulfment, and forespore membranes. In *E. coli* cells, the distribution of the fluorescence signal of the PE-specific probe is consistent with a uniform distribution of PE along the cell membrane. This finding might imply that PE plays different physiological roles in the two bacterial types.

5.6. Cytokinesis

The function of cytokinesis is to divide one cell into two by building a membrane barrier between the two daughter cells. In eukaryotic cells, the interaction of actin filaments with myosin filaments applies tension to the membrane to form a cleavage furrow, which gradually deepens until it encounters the narrow remains of the mitotic spindle between the two nuclei. Phospholipids play an essential role in the division processes in eukaryotic cells.

In the plasma membrane of eukaryotic cells, phospholipids are distributed asymmetrically with PE and PS localized to the inner leaflet and PC and sphingolipids enriched in the outer leaflet (H. Sprong, 2001). Using the above probe highly specific for binding to PE, it was demonstrated that PE is exposed on the cell surface of the cleavage furrow of eukaryotic cells at the final stage of cytokinesis (K. Emoto, 2005). Immobilization of cell surface PE by the probe inhibited disassembly of the contractile ring resulting in formation of a long cytoplasmic bridge between the daughter cells. Removal of the probe from the surface of arrested cells allowed cell division to proceed with disappearance of exposed PE.

In *E. coli,* cell division is initiated after genome duplication by oligomerization of FtsZ, a prokaryotic analog of tubulin, in a ring structure midway between the poles of the cell. This protein ring is the scaffold that recruits a series of proteins to the division site that brings about constriction and eventually cell division. An *E. coli* mutant completely lacking PE propagates as long multinucleated filamentous cells. FtsZ and other early cell division proteins are still recruited to the division sites between nucleoids, however they often assemble in aberrant spiral rather than ring structures whose ability to undergo constriction is strongly inhibited [20]. Discontinuity between nucleoid segregation and cell division

in the absence of PE might result from aberrant organization of FtsZ polymers and also from a possible requirement as cells proceed through the cell cycle for dynamic movement of PE into and out of CL-enriched lipid domains located at the cell division site (Section 5.5.2). On the other hand, the neutral non-bilayer-forming lipid MGlcDG can substitute for PE in cell division (M. Wikström, 2004), suggesting that common properties of these two lipids, such as the ability to form non-bilayer structures and dilution of negative charge, are important for cell division [1].

The heterogeneous distribution of phospholipids in bacterial membranes is an essential factor in the bacterial cell cycle. First, it is important for initiation of DNA replication and division site selection, since it involves amphitropic proteins DnaA and MinD, whose functions are directly controlled by membrane anionic phospholipids through the interaction with amphipathic helices (Section 5.2.5). The next step, namely division site recognition, is also (at least partially) controlled by the amphitropic protein FtsA (containing a C-terminal amphipathic motif, which is functionally interchangeable with that of MinD) that tethers the cytoskeletal protein FtsZ to the division site. Thus, the mid-cell domain provides optimal phospholipid composition first for initiation of DNA replication and then for Z-ring positioning. Concentration of non-bilayer phospholipids such as CL (*E. coli*) and CL and PE (*B. subtilis*) at the division site is consistent with this requirement.

6. Summary and future directions

Biological membranes due to their lipid component are flexible self-sealing boundaries that form the permeability barrier for cells and organelles and provide the means to compartmentalize functions, but at the same time they perform many other duties. As a support for both integral and peripheral membrane processes, the physical properties of the lipid component directly affect these processes in ways that are often difficult to assess. Each specialized membrane has a unique structure, composition, and function. Also, within each membrane exist microdomains such as lipid rafts, lipid domains, and organizations of membrane-associated complexes with their own unique lipid composition. Lipids provide the complex hydrophobic–hydrophilic solvent within which membrane proteins fold and function, and they can also act in a more specific manner in determining final membrane protein organization and orientation in the membrane. These diverse functions of lipids are made possible by a family of low-molecular-weight molecules that are physically fluid and deformable to enable interaction in a flexible and specific manner with other macromolecules. At the same time, they can organize into the very stable but highly dynamic supermolecular structures we know as membranes.

Defining lipid function is a challenging undertaking because of the diversity of chemical and physical properties of lipids and the fact that each lipid type potentially is involved at various levels of cellular function. The challenge for the future will be to determine the function, at the molecular level, of the many lipid species already discovered and yet to be discovered. The LIPID MAPS project is an ambitious undertaking to fully define and characterize the lipodome and will increase the complexity of known lipid structures and potential functions. Coupling genetic and biochemical approaches has been historically a very powerful approach to defining structure–function relationships of physiological

importance. Using this approach in microorganisms has proven to be very fruitful. As the sophistication of mammalian cell and whole animal genetics evolves, genetic manipulation coupled with biochemical characterization will begin to yield new and useful information on the function of lipids in more complex organisms. The interest in understanding biodiversity through the detailed characterization of the vast number of microorganisms will yield additional novel lipids that must be characterized structurally and functionally. Finally, as we discover more about the role of lipids in normal cell function, the role lipids play in disease will become more evident.

Abbreviations

CL	cardiolipin
DGalDG	digalactosyl diacylglycerol
DGlcDG	diglucosyl diacylglycerol
MGlcDG	monoglucosyl diacylglycerol
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine

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CHAPTER 2

Lipid modifications of proteins

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Contents

1. Introduction

Lipid modifications of proteins (Fig. 1) are widespread and functionally important in eukaryotic cells. Intracellular proteins such as the signal-transducing heterotrimeric GTP-binding proteins (G proteins) and the Ras superfamily of G proteins are modified by 14- or 16-carbon fatty acids and/or 15- or 20-carbon isoprenoids. Numerous cell surface glycoproteins such as acetylcholinesterase and the folate receptor are modified at their C-terminus by a structurally complex glycosylphosphatidylinositol (GPI) anchor. Even some secreted protein ligands are lipid modified: the morphogen Hedgehog has a covalently attached cholesterol moiety at its C-terminus and an amide-linked C16 fatty acid at its N-terminus. In most cases, the lipid moiety is crucial to protein function as it can regulate the interaction of an otherwise water-soluble protein with membranes. In some instances, the covalent lipid acts as a functional switch resulting in membrane association of certain protein conformations but not of others. The lipid moiety may also aid in the sorting of the protein to membrane domains that promote lateral and transbilayer protein–protein interactions that are critical for cell function.

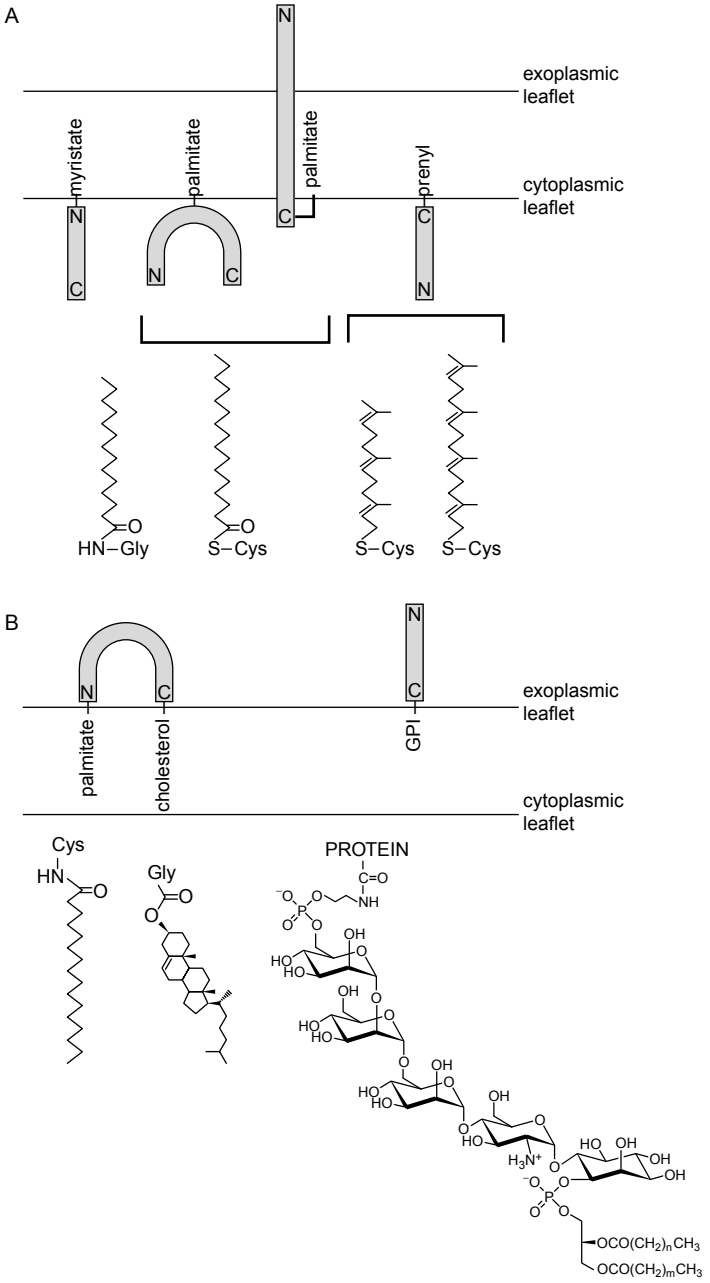

Fig. 1. Structures of lipids covalently attached to proteins. Panel A shows proteins that are lipidated on cytoplasmically exposed amino acids, whereas panel B shows lipidated proteins in the extracellular leaflet. (A) *N*-myristoyl glycine, palmitate thioester-linked to cysteine, farnesyl, or geranylgeranyl (prenyl) thioether-linked to cysteine. (B) *N*-palmitoyl cysteine, cholesterol ester-linked to glycine, and a minimal GPI anchor linked to the ω amino acid in a GPI-anchored protein. The GPI structure is shown with a diacylglycerol moiety containing two ester-linked fatty acids. Other GPI anchors are based on ceramide, while yet others have monoacylglycerol, a fatty acid ether-linked to glycerol, and/or a fatty acid ester-linked to inositol.

The covalent attachment of lipid to protein was first described in a study of myelin protein in 1951, but only clearly documented as important for protein biosynthesis and function in a study of the outer membrane murein lipoprotein of *Escherichia coli* by Braun and Rehn in 1969. These early discoveries were followed by the identification, in the 1970s, of fatty acids linked to viral glycoproteins and, in the 1980s, of isoprenoids covalently attached to fungal mating factors and to GTP-binding proteins. The 1980s also saw the identification and characterization of *N*-myristoylated proteins and GPI-anchored proteins, and work on tissue patterning factors in the 1990s revealed a new class of autoprocessed proteins modified by cholesterol as well as secreted proteins modified at their N-terminus by amide-linked palmitate. This chapter surveys eukaryotic protein lipidation (for a description of bacterial diacylglycerol-modified proteins the reader is directed to articles by Wu and colleagues (H.C. Wu, 1993)) by illustrating the structure of the known lipid modifications, describing their biosynthesis, and considering their functional significance. A number of excellent review articles treat these topics in depth [1–16].

2. Protein prenylation

Prenylated proteins constitute approximately 0.5–2% of all proteins in mammalian cells. They contain a farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoid attached via a thioether linkage to a cysteine residue at or near the carboxy-terminus of the protein. Protein prenylation was originally discovered when certain fungal peptide mating factors were shown to contain a carboxy-terminal cysteine modified by a thioether-linked farnesyl group. However, it was not until Glomset and colleagues (R.A. Schmidt, 1984) subsequently showed that animal cell proteins could be metabolically radiolabeled with radioactive mevalonate, an isoprenyl group precursor, that protein prenylation was more widely appreciated. Glomset and colleagues initially observed that the growth arrest of mammalian cells induced by compactin, an inhibitor of isoprenoid biosynthesis, could not be reversed by exogenously added sterols including cholesterol, the major product of the isoprenoid pathway (M.S. Brown, 1980). The compactin-induced growth arrest could, however, be alleviated by small amounts of mevalonate, suggesting that mevalonate itself or a non-sterol metabolite of mevalonate played an important role in the growth cycle of cells. This result prompted studies in which cells were metabolically labeled with radioactive mevalonate and led to the discovery that almost 50% of the cell-associated radioactive mevalonate could not be extracted into lipid solvents as a result of post-translational (cycloheximide-insensitive) covalent association with proteins.

Protein prenylation is catalyzed by one of three different multi-subunit prenyltransferases located in the cytoplasm of cells [1]. A majority of prenylated proteins, including most members of the Ras family of G proteins [2], contain a carboxy-terminal CaaX motif (CaaX box) composed of a conserved cysteine residue, two aliphatic amino acids (a), and a variable carboxy-terminal residue (X). The CaaX box is recognized by CaaX prenyltransferases that catalyze the attachment of a farnesyl or geranylgeranyl group from the corresponding isoprenyl pyrophosphate to the cysteine residue (Fig. 2). The CaaX prenyltransferases involved in these reactions are protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase-I). FTase recognizes CaaX boxes where X = M,

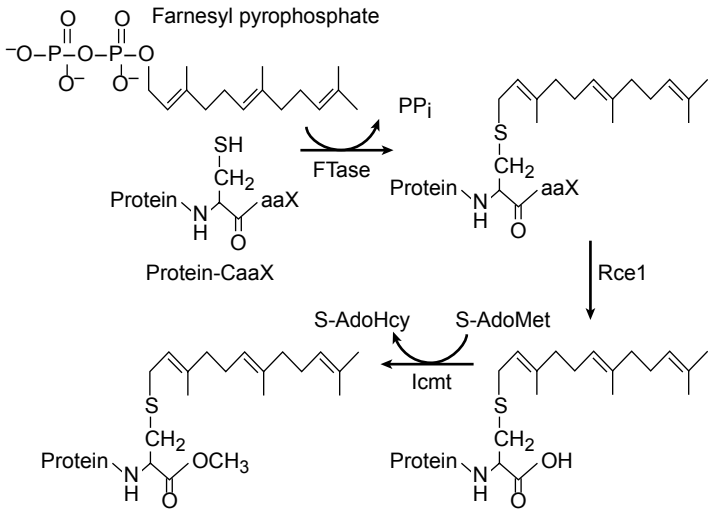

Fig. 2. Farnesylation, proteolysis, and carboxymethylation of a CaaX protein. The farnesyl donor is farnesyl pyrophosphate. FTase, farnesyltransferase; Rce, CaaX protease; Icmt, methyltransferase.

S, Q, A, or C, whereas GGTase-I recognizes CaaX boxes with X = L or F. Other prenylated proteins, such as the Rab proteins involved in vesicular transport, terminate in a CC, CXC, or $CC(X)_{n\,=\,1–3}$ motif; these proteins are substrates for protein geranylgeranyltransferase type II (GGTase-II) [3]. In order to be prenylated, Rab proteins must associate with Rab escort protein; after geranylgeranylation, Rab escort protein delivers the prenylated Rab to membranes.

Subsequent to prenyl modification, Ras and most other CaaX proteins are further processed by two endoplasmic reticulum (ER)-localized, membrane-bound enzymes. The first prenyl-dependent processing step is the proteolytic removal of the –aaX tripeptide by the CaaX protease Rce1; this is followed by carboxymethylation of the now C-terminal prenylcysteine residue by the methyltransferase Icmt (Fig. 2). The result of these modifications is to produce a protein that exhibits some affinity for cellular membranes and also to impart a unique structure at the C-terminus that can serve as a specific recognition motif in certain protein–protein interactions.

The importance of prenylation in CaaX protein function, most notably as a regulator of the oncogenic potential of the Ras proteins [2], has led to considerable efforts to identify inhibitors of the prenyltransferases involved in evaluation as therapeutic agents [4]. A majority of these studies have focused on FTase, since this enzyme modifies Ras proteins, and early pre-clinical studies indicated significant anticancer potential for FTase inhibitors. A wide variety of FTase inhibitors have been developed, including some very promising ones that possess antitumor activity in animal models and are now in clinical development [4]. In addition, the success of FTase inhibitors in pre-clinical models of tumorigenesis, the increasing realization that proteins modified by GGTase-I play important roles in oncogenesis, and the finding that post-prenylation processing by Rce1 is

important in the function of Ras and other CaaX proteins have led to the current situation in which all of the enzymes involved in CaaX protein processing are viewed as potential therapeutic targets [5]. New developments extend the use of these approaches to other diseases that involve prenylated proteins. Progeroid (prematurely old) syndromes result from aberrant processing of farnesylated lamin A, an intermediate filament protein that is associated with the inner nuclear membrane [17]. Administration of an FTase inhibitor to mouse models of progeria relieves the disease phenotype (L.G. Fong, 2006), raising hope for a similar treatment for afflicted children.

2.1. The CaaX prenyltransferases FTase and GGTase-I

FTase is a heterodimer consisting of 48 kDa (α) and 46 kDa (β) subunit polypeptides. GGTase-I also consists of two subunits, a 48 kDa α subunit shared with FTase, and a 43 kDa β subunit that shares 25% identity with the β subunit of FTase. The isoprenoid substrates for the two enzymes are farnesyl pyrophosphate and geranylgeranyl pyrophosphate. Protein substrates for FTase in mammalian cells include Ras GTPases, lamins, several proteins involved in visual signal transduction, and at least three protein kinases and phosphatases. Known targets of GGTase-I include most γ subunits of heterotrimeric G proteins and Ras-related GTPases such as members of the Ras and Rac/Rho families. Both FTase and GGTase-I recognize short peptides containing appropriate CaaX motifs, and tetrapeptide substrates were instrumental in purifying the enzymes to homogeneity.

Both FTase and GGTase-I are zinc metalloenzymes in which the single bound zinc atom participates directly in catalysis. FTase additionally requires high concentrations (>1 mM) of magnesium for catalysis. FTase proceeds via a functionally ordered kinetic mechanism, with farnesyl pyrophosphate binding first to create an FTase-farnesyl pyrophosphate binary complex that then reacts rapidly with a CaaX substrate to form a prenylated product. In the absence of excess substrate, the dissociation rate is so slow that FTase-product complexes can be isolated. A wealth of structural information has emerged for FTase in the past several years beginning with the first X-ray crystal structure of unliganded FTase solved at 2.2 Å resolution (H.-W. Park, 1997), greatly enhancing the ability of investigators to conduct structure–function analyses on the enzyme and investigate the roles of specific residues in substrate binding and catalysis. The structural biology of the prenyltransferases is the subject of recent reviews [1,3].

3. Fatty acylation of proteins: N-myristoylation

Myristate, a relatively rare 14-carbon, saturated fatty acid, is amide-linked to the N-terminal glycine residue of *N*-myristoylated proteins [6] (Fig. 1A). Protein *N*-myristoylation is catalyzed by the enzyme myristoyl CoA:protein *N*-myristoyltransferase (NMT) which recognizes and modifies N-terminal glycine residues that occur in the general sequence context (M)GXXX(S/T)K. A web-based program (http://mendel.imp.ac.at/myristate/) can be used to predict whether a particular protein will be *N*-myristoylated; a collection of data from such predictions may be found at MYRbase (http://mendel.imp.ac.at/myristate/ myrbase).

Approximately 1% of the *S. cerevisiae* genome encodes proteins that are known to be — or predicted to be — *N*-myristoylated. Although eukaryotic proteins form the bulk of this class of lipid-modified proteins, many viral proteins and bacterial proteins become *N*-myristoylated on entering the cytoplasm of the host cell where they encounter NMT (S. Maurer-Stroh, 2004).

Myristoylation is typically considered to be an early co-translational event that occurs in the cytoplasm as soon as ~60 amino acids of the nascent peptide emerge from the ribosomal tunnel (I. Deichaite, 1988), and after the N-terminal glycine residue is made available by cellular methionyl-aminopeptidases that remove the initiator methionine residue. However, myristate can be attached post-translationally to N-terminal glycine in synthetic peptides of the appropriate sequence, and also to N-terminal glycines that are newly generated after caspase-mediated cleavage of proteins during apoptotic cell death. The latter process is termed morbid myristoylation (J. Zha, 2000; M. Mishkind, 2001).

NMT was first purified from *S. cerevisiae* and characterized as an ~55 kDa monomeric protein with no apparent cofactor requirements (D.A. Towler, 1987). The crystal structures of the *S. cerevisiae* and *C. albicans* NMTs have been determined, the former as a co-crystal with a non-hydrolyzable myristoyl-CoA derivative and a dipeptide inhibitor of the enzyme (R.S. Bhatnagar, 1999). NMT uses an ordered Bi–Bi reaction mechanism: myristoyl CoA binds first, followed by the peptide substrate (D.A. Rudnick, 1991). After catalysis, CoA is discharged first, followed by the *N*-myristoyl peptide. The enzyme is highly selective for myristoyl CoA. Fungi express a single NMT that is essential for cell viability (R.J. Duronio, 1989); vertebrates express two NMT proteins with partially overlapping functions. Since *E. coli* cells do not have NMT activity, expression of recombinant *N*-myristoylated proteins requires the simultaneous expression of NMT and the protein of interest (R.J. Duronio, 1990). In vitro analyses of human and fungal NMTs indicate that while their reaction mechanism and acyl-CoA substrate specificities are the same, their peptide specificities are different; this difference has been exploited to develop species-selective NMT inhibitors that act as fungicidal agents. Because of the importance of myristoylation of viral proteins for entry, assembly, and budding of certain viruses, NMT inhibitors may also have antiviral effects (S. Maurer-Stroh, 2004).

4. Fatty acylation of proteins: S-acylation

Thioacylated proteins contain fatty acids in thioester linkage to cysteine residues [7–9] (Fig. 1A). Protein thioacylation is frequently referred to as palmitoylation, although fatty acids other than palmitate are found on thioacylated proteins. Membrane proteins as well as hydrophilic proteins are thioacylated, the latter, in many cases, acquiring the modification when they become associated with a membrane compartment as a result of initial *N*-myristoylation or prenylation. Examples include G-protein coupled receptors, the transferrin receptor, the cation-dependent mannose-6-phosphate receptor, and hydrophilic proteins such as members of the Src family of protein tyrosine kinases (e.g., $p59^{fyn}$ and $p56^{lck}$) as well as H-Ras, N-Ras, and the synaptic vesicle protein SNAP-25. The yeast palmitoyl proteome, i.e., the collection of all *S*-acylated proteins in yeast, was recently defined via a comprehensive proteomics approach (A.F. Roth, 2006). It consists of 50 proteins including

polytopic amino acid permeases with 12 predicted transmembrane spans, monotopic membrane proteins such as the SNARE proteins involved in vesicle fusion and Golgi localized mannosyltransferases, and various cytoplasmic proteins, many of which are known to also be myristoylated or prenylated. Thioacylated cysteine residues in cytoplasmic proteins are found in a variety of sequence contexts; for membrane proteins such cysteines are close to a transmembrane domain, near the membrane–cytoplasm interface. Unlike the other known lipid modifications of proteins, thioacylation is reversible: the protein undergoes cycles of acylation and deacylation, and so the half-life of the acyl group is much shorter than that of the polypeptide (~20 min for the acyl group versus ~1 day for the polypeptide in the case of N-Ras (A.I. Magee, 1987)).

For intrinsically hydrophilic proteins such as $p59^{fyn}$ and SNAP-25, thioacylation functions together with a second signal to provide membrane anchoring in the cytoplasmic leaflet of the membrane bilayer for an otherwise soluble protein and to promote partitioning of the protein into sterol/sphingolipid-rich membrane domains (lipid rafts). Thioacylation also plays a role in protein trafficking as discussed below. In the case of huntingtin protein, whose intracellular aggregation is a hallmark of Huntington's disease, palmitoylation plays a protective role by inhibiting protein aggregation (A. Yanai, 2006); palmitoylation-defective huntingtin is more prone to aggregate and more toxic to cells. The functional significance of thioacylation of polytopic and monotopic membrane proteins is less clear, but new data (J. Valdez-Taubas, 2005; L. Abrami, 2006) suggest that the acyl modification may dictate protein stability by blocking ubiquitination, a modification that marks proteins for degradation in the proteasome or in multi-vesicular bodies.

4.1. Protein acyltransferases (PATs) and thioacyl protein thioesterases (APTs)

A family of protein acyltransferases (PATs) is responsible for *S*-acylation of proteins in cells (S. Lobo, 2002; A. Roth, 2002) [8]. Members of this family are characterized by the presence of a cysteine-rich domain containing a DHHC (Asp-His-His-Cys) motif. PATs are polytopic membrane proteins with the putative catalytic DHHC motif localized to a cytoplasmic loop between transmembrane spans. Some PATs function alone whereas others, such as the yeast Ras PAT Erf2, require a cytoplasmic protein, Erf4, for activity. It is likely that particular classes of substrate have a dedicated PAT that accounts for most, if not all, of their *S*-acylation. For example, Swf1p modifies SNARE proteins and other monotopic membrane proteins with a juxtamembrane cysteine residue. The yeast vacuolar protein Vac8 is mainly *S*-acylated by the vacuolar DHHC protein Pfa3 (J.E. Smotrys, 2005) but is also modified by Ykt6 (L.E. Dietrich, 2004).

Two thioacyl protein thioesterases (APTs) have been identified. Unlike PATs, both are soluble proteins. Acylprotein thioesterase-1 (APT1) was purified from rat liver cytosol using palmitoylated G-protein α-subunit as a substrate (J.A. Duncan, 1998, 2002). This thioesterase, a 29-kDa monomeric protein, is likely to be the one involved in turnover of cytoplasmically disposed thioacyl groups of proteins. It displays both acylprotein thioesterase activity as well as lysophospholipase activity, but thioacylproteins are by far the preferred substrates. The second acylprotein thioesterase, protein palmitoylthioesterase-1 (L.A. Camp, 1994), was purified from bovine brain on the basis of its ability to depalmitoylate H-Ras. It is localized to lysosomes where it is involved in the catabolism of thioacylated proteins,

an essential process in humans and mice [10]. Thioacylated molecules are presumed to gain access to the lysosomal lumen by an autophagic pathway in which membrane fragments are captured into a vacuole that subsequently fuses with lysosomes. A defect in protein palmitoylthioesterase-1 leads to a severe neurodegenerative disorder termed infantile neuronal ceroid lipofuscinosis characterized by the accumulation of autofluorescent material (including lipid thioesters) in all tissues. Structural analyses of protein palmitoylthioesterase-1 have provided insight into the molecular basis of this disease (J.J. Bellizzi, 2000).

5. Membrane anchoring of fatty acylated and prenylated proteins: the two-signal hypothesis, lipid switches, and dynamic acylation

When they are first synthesized, *N*-myristoylated proteins and prenylated proteins possess only a single lipid moiety that is not sufficiently hydrophobic to confer stable membrane anchorage. For example, *N*-myristoylated peptides bind membranes with a K_d of only $\sim 10^{-4}$ M (R.M. Peitzch, 1993). Similarly a hydrophilic protein or peptide modified by a single thioacyl chain does not bind strongly to membranes. Thus, for stable membrane association, lipid-modified proteins require a second membrane attachment signal ('two-signal hypothesis') [11,12]. This could be another lipid chain or a motif recognized by a membrane-bound receptor or a polybasic stretch of amino acids that interacts electrostatically with negatively charged phospholipids in the membrane. For *N*-myristoylated proteins such as MARCKS (myristoylated alanine-rich C-kinase substrate) and the tyrosine kinase Src, this second interaction is provided by the electrostatic affinity between a polybasic region of the protein and the negatively charged head groups of phospholipids in the cytoplasmic leaflets of cell membranes (D. Murray, 1998). Neither myristate nor the polybasic domain alone is sufficient, but together they provide strong membrane binding.

The *N*-myristoylated Src family protein tyrosine kinases are frequently thioacylated at one or more cysteine residues near the myristoylated glycine, and these doubly or triply lipid-modified proteins are found associated with the cytoplasmic face of the plasma membrane (the N-terminal sequences of $p59^{fyn}$ and $p56^{lck}$ are ^{1}M**G**C̲VC̲---- and ^{1}M**G**C̲VQC̲----, respectively, where the *N*-myristoylated glycine is shown in bold and the thioacylated cysteine residues are underlined). A similar situation is seen for the prenylated Ras proteins that must be thioacylated before they associate firmly with membranes (the C-terminal sequences of H-Ras and N-Ras are ---GC̲MSCK**C**VLS–COOH and ---GC̲MGLP**C**VVM–COOH, respectively (the farnesylated cysteine is in bold and the thioacylated cysteines are underlined). A third example is provided by proteins such as SNAP-25 which are exclusively thioacylated, but display at least four thioacyl chains through which they become stably associated with the synaptic vesicle membrane.

The membrane association of fatty acylated and prenylated proteins is reversible and can be regulated in a number of ways, using switches that obscure the primary lipid modification or affect the strength of the 'second signal' (Fig. 3). For example, phosphorylation of the polybasic domain of *N*-myristoylated MARCKS reduces the strength of the second signal and causes MARCKS to dissociate from membranes (S. McLaughlin, 1995).

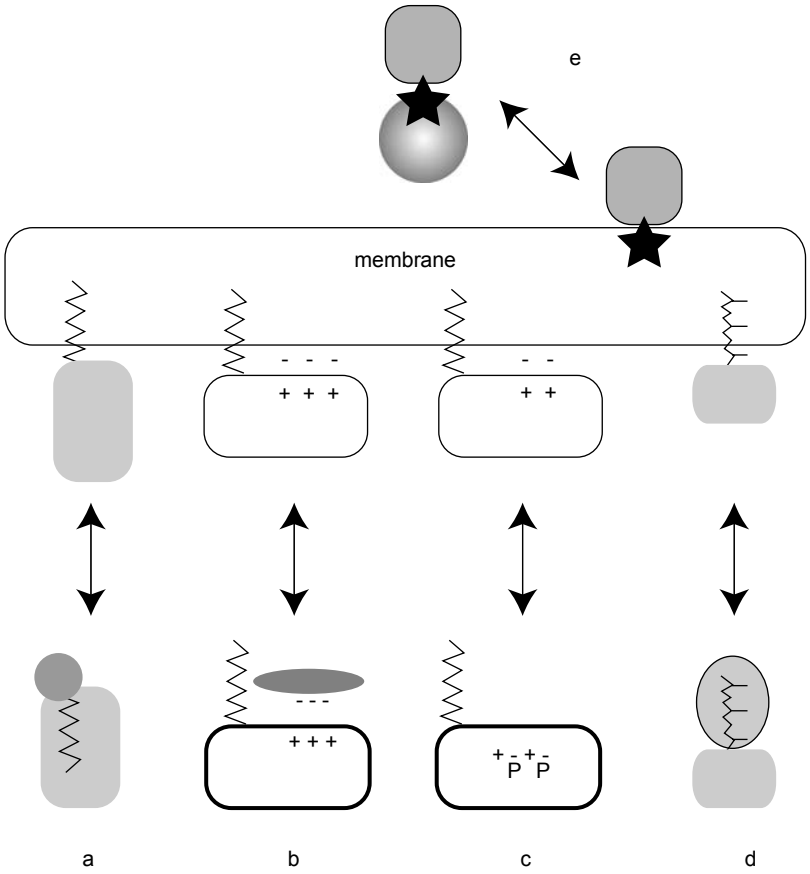

Fig. 3. Reversible membrane association of lipidated proteins (redrawn from Ref. [11]). (a) Binding of a ligand (shaded circle) to an *N*-myristoylated protein triggers a myristoyl switch. (b) Binding of a ligand (shaded oval) to the polybasic motif of a singly lipidated protein reduces the 'second signal' allowing the protein to desorb from membranes. (c) Phosphorylation within the polybasic motif lowers its affinity for anionic phospholipids (electrostatic switch). (d) A prenyl group is sequestered by a binding partner. (e) Lipidated secreted proteins (the star represents a lipid modification: cholesterol and/or fatty acid) spread from their source by binding to lipoprotein carriers.

Similarly, APT1-mediated removal of the *S*-acyl chain from farnesylated, palmitoylated H-Ras results in reduced membrane association. Some *N*-myristoylated proteins such as ADP ribosylation factor and recoverin exist in alternate conformations in which the myristoyl group is exposed and available for membrane binding, or sequestered within a hydrophobic pocket in the protein. On ligand binding (GTP for ADP ribosylation factor, and Ca^{2+} for recoverin), the myristoyl group is exposed and becomes available to promote interactions with target membranes and protein partners (J.B. Ames, 1996; J. Goldberg, 1998). Prenyl switch mechanisms also exist. Here interaction of the prenylated protein with a binding partner obscures the prenyl group and allows the protein to dissociate from the membrane.

In this way, membrane association of prenylated Rho- and Rab-GTPases is modulated by their cognate GDP dissociation inhibitor proteins (Y. An, 2003; A. Rak, 2003).

6. Membrane targeting and intracellular trafficking of fatty acylated and prenylated proteins

Proteins modified with myristate or farnesyl alone undergo transient interactions with a variety of intracellular membranes whereas tandem modifications promote stable membrane association. Thus, a protein with a single lipid modification, such as the cytoplasmically synthesized *N*-myristoylated $p59^{fyn}$, becomes stably associated with the cytoplasmic face of the plasma membrane only when it becomes thioacylated by a plasma membrane-localized PAT. In this scenario, thioacylation would not only provide for stable membrane association of a protein with a single lipid modification, but also ensure targeting of that protein to the membrane where thioacylation occurs. Thus, within 5 min of the completion of peptide synthesis, $p59^{fyn}$ becomes *N*-myristoylated, thioacylated, and located to the cytoplasmic face of the plasma membrane (W. van't Hof, 1997). Removal of the palmitoylation sites slows the kinetics of membrane association, and reduces the proportion of $p59^{fyn}$ that is membrane associated. The dynamic nature of thioacylation suggests that the duration of association of $p59^{fyn}$ with the plasma membrane is dictated by the half-life of the thioacyl chain (kinetic trapping). However, since $p59^{fyn}$ is doubly thioacylated, it is unlikely that it would significantly revert to its solely *N*-myristoylated state and re-enter the cytoplasm.

Thioacylation frequently dictates plasma membrane targeting of proteins lacking transmembrane spans. In the case of $p59^{fyn}$, targeting occurs directly, with the *N*-myristoylated protein becoming thioacylated and plasma membrane associated rapidly upon completion of synthesis. In contrast, $p56^{lck}$ appears to be thioacylated on intracellular membranes and arrive at the plasma membrane via vesicular transport (bound to the cytoplasmic face of secretory vesicles) (M. Bijlmakers, 1999). In yet another targeting variation, newly synthesized *N*-myristoylated $G_{z\alpha}$, a dually acylated trimeric G protein α-subunit, associates with all cellular membranes but accumulates eventually at the plasma membrane: the plasma membrane form is the only one that is both *N*-myristoylated and thioacylated.

The trafficking of mammalian Ras proteins provides a further illustration of these principles (Fig. 4) [13]. Farnesylated and carboxymethylated Ras proteins are generated at the cytoplasmic face of the ER; N-Ras and H-Ras are subsequently *S*-acylated whereas K-Ras has a polybasic second signal for membrane association. Dual-lipid-modified H/N-Ras proteins are kinetically trapped on the ER and use the secretory pathway to traffic to the plasma membrane whereas K-Ras moves to the plasma membrane via a non-vesicular mechanism. At the plasma membrane, a putative APT removes the *S*-acyl group allowing farnesylated H/N-Ras to exchange with other intracellular membranes, including the Golgi, by non-vesicular means (J.S. Goodwin, 2005).

The examples narrated here predict defined subcellular locations for PATs and APTs in relationship to their substrates. DHHC PAT proteins have been found at the ER, Golgi, and plasma membrane (Y. Ohno, 2006); there is no information on the predicted thioesterases.

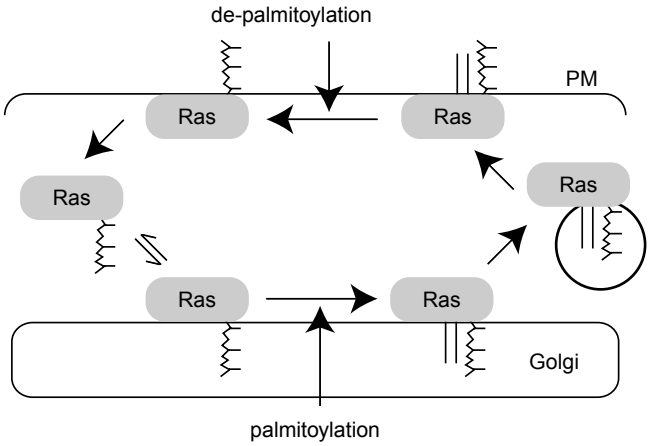

Fig. 4. Palmitoylation-dependent trafficking of Ras (redrawn from Ref. [13]). Farnesylated Ras weakly associates with Golgi membranes but is kinetically trapped once it is *S*-acylated (straight lines). *S*-acylated, farnesylated Ras is transported to the plasma membrane (PM) via secretory vesicles. At the PM, a thioesterase depalmitoylates Ras, releasing the protein and allowing it to reassociate with the Golgi.

7. Lipid modifications of secreted proteins: Hedgehog, Wingless/Wnt, and Spitz

Hedgehog (Hh) proteins are secreted signaling molecules involved in the patterning of diverse tissues during development of multicellular organisms. These proteins — morphogens — function in a graded manner, emanating from cells in which the protein is produced and acting upon target cells several cell diameters away, thus specifying different cell fates by altering programs of gene expression. Hh is dually lipidated [14] — the mature protein is modified by cholesterol at its C-terminus (J.A. Porter, 1996) and by palmitate at its N-terminus (R.B. Pepinsky, 1998) (Fig. 1B).

Hh is synthesized as a 45-kDa precursor protein containing an N-terminal signal sequence (which targets the protein to the ER and is subsequently trimmed), followed by an N-terminal signaling domain (Hh-N; ~19 kDa), an absolutely conserved Gly–Cys–Phe tripeptide motif, and a C-terminal processing domain (Hh-C) which is removed to yield the active signaling molecule (Fig. 5). Addition of cholesterol to Hh proteins proceeds via an autoproteolytic internal cleavage reaction at the Gly–Cys–Phe tripeptide sequence. Cleavage is initiated through a nucleophilic attack by the thiol side chain of Cys on the Gly carbonyl replacing the Gly–Cys peptide bond with a thioester linkage. A second nucleophilic attack on the same carbonyl by the hydroxyl group of cholesterol results in removal of the C-terminal processing domain and produces the active Hh signaling molecule containing a C-terminal cholesterol modification. Although other sterols can substitute for cholesterol in in vitro Hh processing assays, the free 3β-hydroxyl group is essential for processing. Hh-C mediates the auto-processing reaction. The first 145 amino-terminal residues of this domain are sufficient for thioester formation and cleavage in the presence of dithiothreitol. At least some part of the 63 carboxy-terminal residues is required for

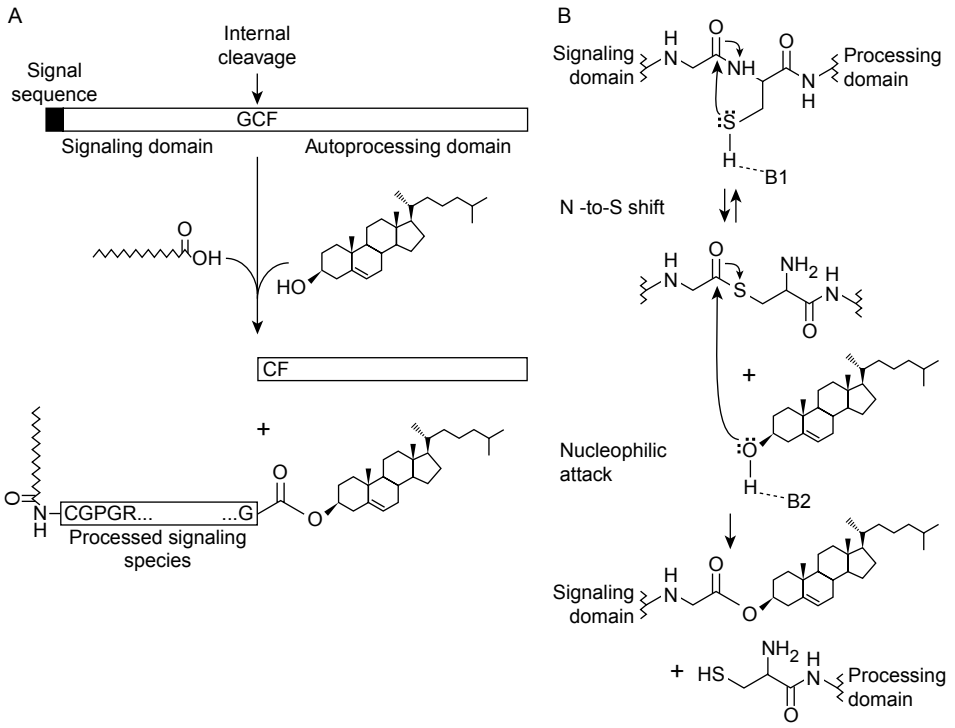

Fig. 5. Modification of hedgehog with ester-linked cholesterol and an N-terminal amide-linked fatty acid (redrawn from Ref. [14]). (A) Overall processing steps leading to lipid-modified Hedgehog (Hh). (B) Mechanism of cholesterol addition. Residues indicated as B1 and B2 in panel B are catalytic bases presumably contained in the carboxy-terminal domain of the Hh precursor.

cholesterol addition and contains a sterol recognition region (T.M. Hall, 1997). The chemistry involved in cholesterol modification of Hh can also be seen in self-splicing proteins where an internal sequence (termed intein) in the protein is excised and the flanking sequences (exteins) are ligated. The intein, such as Hh-C, mediates the protein-splicing reaction (the 145 amino acid active portion of Hh-C is accordingly referred to as the Hh intein module or Hint module). The same chemistry is mimicked in synthetic approaches used for chemical ligation of peptides (P.E. Dawson, 1994).

After cholesterol modification, Hh proteins are palmitoylated at their N-terminus; the N-terminal residue is a Cys that forms part of a CGPGR motif. Unlike the *S*-acylation of proteins discussed above, Hh palmitoylation occurs in the lumen of the secretory pathway. Also, although the N-terminal cysteine in Hh is initially conventionally *S*-acylated, a spontaneous S-to-N shift occurs that results in the palmitate residue being amide-linked to the amino terminus of the protein. Hh palmitoylation is catalyzed by Rasp [also called Ski (skinny hedgehog), Sit (sightless), or Cmn (central missing)], a member of the MBOAT family of enzymes (membrane-bound *O*-acyltransferase) (K. Hofmann, 2000). Rasp is also implicated in the *N*-palmitoylation of Spitz, a membrane-bound epidermal growth factor ligand that is cleaved intracellularly and then secreted (G.I. Miura, 2006).

Wnt proteins are also acylated. The Wnt3a protein is modified by thioester-linked palmitate at a conserved cysteine residue and also by an unsaturated fatty acid, palmitoleic acid, which is oxyester-linked to a conserved serine residue (R. Takada, 2006). Porcupine (porc), a member of the MBOAT family, is required for the *O*-acylation of Wnt3a. It is not clear whether porc or another acyltransferase carries out the *S*-acyl modification.

Lipid modification of Hh and Wingless is required for their proper activity. Hh proteins truncated at the cleavage site are not modified by cholesterol, but nevertheless produce active signaling molecules; however, studies in *Drosophila* have shown that these proteins are not targeted properly and cause mispatterning and lethality in embryos. Since morphogens have both short-range and long-range activity, it can be readily envisaged that lipidation would reduce their diffusibility and enhance short-range signaling activity. It has been suggested that the long-range signaling activity depends on association of the morphogens with lipoprotein particles (Fig. 3). Reversible insertion of the lipid anchors of Hh or Wingless into the phospholipid monolayer of lipoproteins would allow these morphogens to spread through tissues (S. Eaton, 2006; I. Guerrero, 2006; F. Wendler, 2006).

8. GPI anchoring of proteins

Roughly 1% of all proteins encoded by eukaryotic genomes are post-translationally modified at their C-terminus by GPI, a complex glycophospholipid that anchors proteins to the cell surface [15,16]. The core structure of a GPI anchor is ethanolamine-*P*-6Manα1-2Manα1-6Manα1-4GlcNα1-6-*myo*-inositol-P-lipid; the amino group of ethanolamine is amide-linked to the carboxy terminus of the mature GPI-anchored protein (Fig. 1B). Proteins destined to receive a GPI anchor are synthesized on membrane-bound ribosomes. The primary translation product has an ER-targeting N-terminal signal sequence and a C-terminal signal that directs the attachment of a GPI anchor. The GPI anchoring enzyme, GPI transamidase (GPIT), replaces the GPI signal with a preassembled GPI on the lumenal face of the ER. Web-based programs are available that can predict whether or not a particular protein is likely to be GPI-anchored (http://mendel.imp.univie.ac.at/sat/gpi/gpi_prediction.html).

In contrast to most other forms of protein lipidation where a second signal is needed to ensure stable membrane association, GPI represents the sole means of membrane anchorage of a GPI-protein. Like *N*-myristoylation and prenylation, GPI attachment is irreversible. However, unlike myristoyl, palmitoyl, and prenyl groups, GPIs can be modified by the addition of side-branching groups to their glycan core, and their lipid moieties can be remodeled as well. These modifications can be species-specific. The fatty acyl chains of the GPI lipid portion are typically saturated. Examples of GPI-proteins include hydrolases (e.g., 5′-nucleotidase, acetylcholinesterase, alkaline phosphatase), cell surface receptors (e.g., folate receptor), cell adhesion molecules (e.g., isoforms of the neural cell adhesion molecule), complement regulatory proteins (e.g., decay accelerating factor, CD55), the scrapie prion, and protozoal coat proteins (e.g., *Trypanosoma brucei* variant surface glycoprotein).

8.1. Biosynthesis of GPI-anchored proteins: assembly of GPIs

The discussion here focuses on the GPI assembly pathway in mammalian cells (Fig. 6); however, the pathway is highly conserved in all eukaryotes and variations of the pathway as described here are found in all eukaryotic organisms studied thus far.

GPI assembly occurs in the ER via a multi-step pathway involving at least 20 proteins, most of which are polytopic membrane proteins. Synthesis is initiated on the cytoplasmic leaflet of the ER by adding *N*-acetylglucosamine (GlcNAc) from UDP–GlcNAc to phosphatidylinositol (PI) to generate GlcNAc–PI. The GlcNAc transferase that mediates this first step is a complex consisting of at least six proteins in mammalian cells: PIG-A, PIG-C, PIG-H, PIG-, PIG-Q, PIG-Y (a seventh non-essential subunit, DPM2, has also been proposed). PIG-A is the catalytic component, but other subunits are required for enzyme activity. In the second step of the pathway, GlcNAc–PI is de-*N*-acetylated by the GlcNAc–PI de-*N*-acetylase, PIG-L, to generate GlcN–PI. In the third step the inositol residue of GlcN–PI is palmitoylated at the 2-position by PIG-W to generate GlcN–acyl–PI. This acylation is required for eventual phosphoethanolamine addition to the third mannose residue but the acyl chain is typically removed once the complete GPI anchor is attached

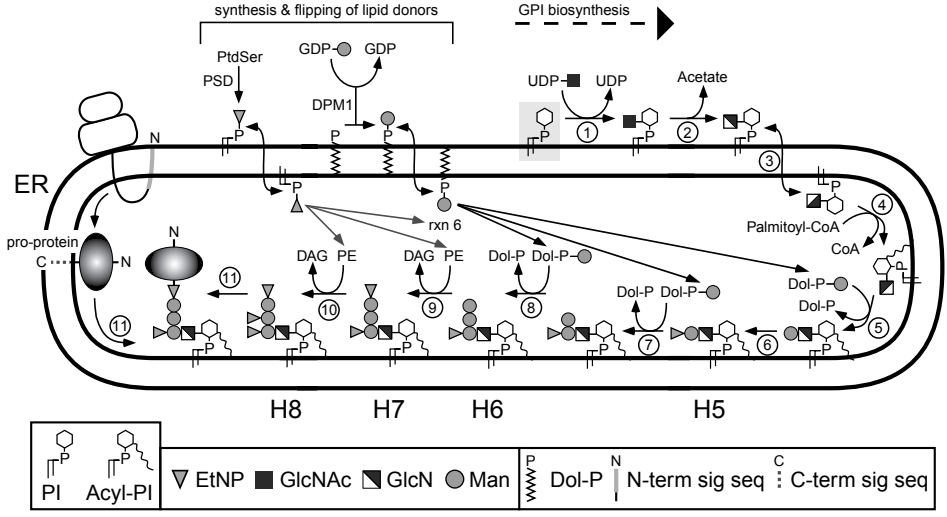

Fig. 6. GPI biosynthesis in the ER (reproduced from Ref. [16]). The ER is depicted as a topologically defined compartment. Biosynthesis begins at the top of the figure with a PI acceptor (gray box). PI is glycosylated to generate GlcNAc-PI on the cytoplasmic face of the ER (1). GlcNAc-PI is de-*N*-acetylated (2) and flipped across the ER membrane (3). GlcN–PI is inositol acylated (4) to yield GlcN–acyl–PI. This lipid is mannosylated and modified by phosphoethanolamine (Etn-P) (6–10). Mannose (Man) is derived from Dol (dolichol)-P-Man (synthesized from Dol-P and GDP-Man on the cytoplasmic face of the ER by the enzyme Dpm1) and Etn-P is derived from phosphatidylethanolamine (typically synthesized by decarboxylation of phosphatidylserine via the action of Psd (PS decarboxylase)). Both Dol-P-Man and PE must be flipped into the lumenal leaflet of the ER to participate in GPI biosynthesis. The EtnP-capped GPIs that are synthesized by this pathway (H7 and H8 in mammals) are attached to ER translocated proteins (shown counterclockwise at left, beginning with a ribosome-associated nascent chain) displaying a C-terminal GPI signal sequence (11). Step 11 is catalyzed by GPI transamidase.

to protein. GlcN–acyl–PI is elaborated by a series of mannosylation and phosphoethanolamine additions to yield a mature GPI suitable for attachment to protein. In contrast to the first two steps of synthesis, which occur on the cytoplasmic leaflet of the ER, the inositol acylation, mannosylation, and EtNP addition reactions occur lumenally. This indicates that GlcN–PI must be flipped across the ER membrane bilayer (R.A. Vishwakarma, 2005). The flipping step is the only step of the GPI assembly pathway for which a protein catalyst remains to be assigned.

Dolichol-P-mannose is the mannose donor for all three mannosylation steps, whereas phosphatidylethanolamine contributes the phosphoethanolamine residues. Both these precursors are synthesized on the cytoplasmic face of the ER and must be flipped into the ER lumen to participate in GPI biosynthesis. The mannosyltransferases required for the assembly of the first, second, and third mannoses are PIG-M/PIG-X, PIG-V, and PIG-B (a fourth mannose residue that is essential for GPI assembly in yeast is transferred by the protein SMP3). Phosphoethanolamine side chains attached to the first, second, and third mannose residues are transferred by PIG-N, hGPI7/PIG-F, and PIG-O/PIG-F. Fully assembled GPI structures, or the GPI moiety in GPI-anchored proteins, are frequently subject to lipid re-modeling reactions in which fatty acids or the entire lipid structure is replaced with different fatty acids or lipids.

8.2. Biosynthesis of GPI-anchored proteins: attachment of GPIs to proteins

The C-terminal GPI-signal sequence has a conserved general structure consisting of three small amino acids (termed ω, ω + 1, ω + 2; ω is the GPI attachment site), followed by a hydrophilic spacer of ~10 moderately polar amino acids, and a hydrophobic tail. The ~10 amino acids immediately N-terminal to ω form a solvent-accessible region that links the signal sequence to the protein. GPIT activates the carbonyl group of the ω amino acid in the GPI-protein precursor by displacing the GPI signal sequence. Nucleophilic attack on the activated carbonyl by the amino group of a phosphoethanolamine-capped GPI yields a GPI-anchored protein and regenerates GPIT. GPI is amide-linked to the ω residue. Small nucleophiles like hydrazine can replace GPI in microsomal assays, providing a simple test of whether carbonyl activation has occurred (Fig. 7).

Human GPIT is a complex of five membrane proteins: GPI8, GAA1, PIG-S, PIG-T, and PIG-U (K. Ohishi, 2001; P. Fraering, 2001; Y. Hong, 2003). GPITs from *S. cerevisiae*, *D. melanogaster*, *C. elegans,* and *A. thaliana* are similar. In contrast, GPITs from trypanosomatids such as the African sleeping sickness parasite *Trypanosoma brucei* share three subunits with human GPIT (homologs of GPI8, GAA1, and PIG-T termed *Tb*GPI8, *Tb*GAA1, and *Tb*GPI16/PIG-T, respectively) but have two novel subunits (TTA1 and TTA2) in lieu of PIG-S and PIG-U (K. Nagamune, 2003). All five subunits are essential in both human and trypanosomatid-type GPIT, and all are needed for the nucleophilic attack on the ω residue that produces the activated carbonyl intermediate to initiate transamidation.

GPI8 is presumed to be the catalytic center of the enzyme since it shares sequence homology with a family of cysteine proteases, one member of which has transamidase activity in vitro. Mutagenesis of a Cys–His catalytic dyad in human GPI8 inactivates GPIT. The functions of the remaining subunits are unknown. It seems likely that they recruit substrates, or regulate substrate access to the catalytic site (S. Vainauskas, 2004, 2006).

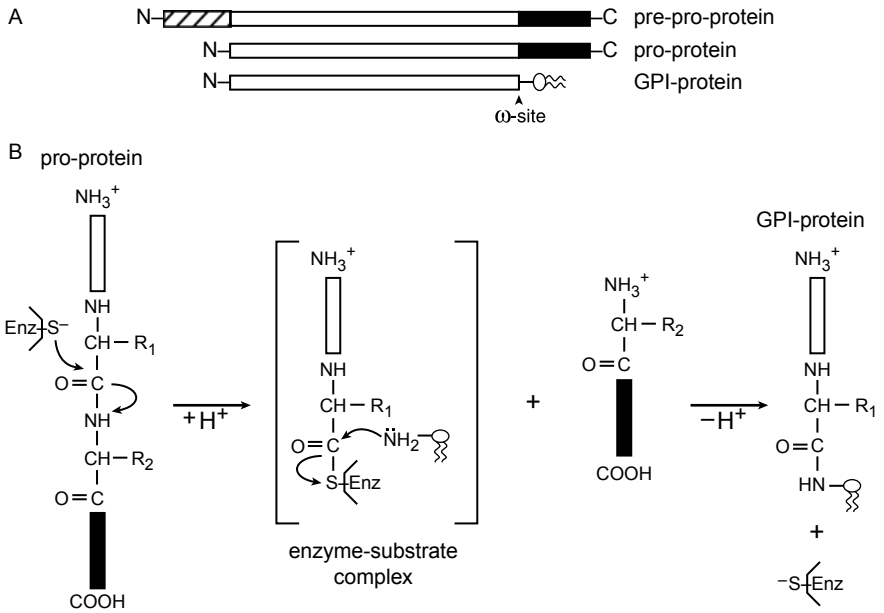

Fig. 7. GPI anchoring of proteins. Panel A outlines the processing steps involved in the conversion of a pre-pro-protein to a GPI-anchored protein. Panel B shows the transamidation reaction in which a GPI anchor is attached to a pro-protein. The transamidase enzyme (Enz), which contains an enzymatically critical cysteine residue, is depicted as Enz-S$^-$. The ω amino acid is depicted with a side chain labeled R_1; the ω + 1 amino acid has a side chain labeled R_2.

8.3. GPI-anchoring in mammals, parasitic protozoa, and yeast

Macromolecules that are GPI-anchored rely on the anchor for cell surface expression. If GPI transfer to protein is blocked, either through a GPI biosynthetic defect or a defect in GPIT, the protein is not expressed at the cell surface and is accumulated in the ER or degraded.

GPI-deficient mammalian cells are viable in tissue culture and many GPI-deficient mutant cell lines have been established. However, GPI deficiency has major consequences at the level of tissues and the whole body. This was revealed in transgenic mouse models in which the *PIG-A* gene (required for the first step of GPI biosynthesis) was knocked out in specific tissues or in the whole animal. For example, keratinocyte-specific disruption of *PIG-A* caused abnormal development of skin leading to death of the mutant mice a few days after birth (M. Tarutani, 1997), and disruption of *PIG-A* in the whole animal resulted in embryos that did not develop beyond day 9 of gestation (M. Nozaki, 1999). A somatic mutation of *PIG-A* in multipotent hematopoietic human stem cells causes paroxysmal nocturnal hemoglobinuria, an acquired hemolytic disease in humans characterized by abnormal activation of complement on erythrocytes due to a deficiency of GPI-anchored complement regulatory proteins such as decay accelerating factor (N. Inoue, 2003). This disease is characterized by intravascular hemolysis and anemia.

GPI anchoring is the most prominent mode of attachment for cell surface proteins and glycans in parasitic protozoa (M.A.J. Ferguson, 1999). Pathogenic protozoa, including species of the genera *Trypanosoma, Leishmania,* and *Plasmodium,* display abundant GPI-anchored cell surface macromolecules that play crucial roles in parasite infectivity and survival. An example is the GPI-anchored variant surface glycoprotein of bloodstream forms of *Trypanosoma brucei*, the causative agent of African sleeping sickness. A recent work indicates that GPI biosynthesis is essential for the growth of bloodstream stages of *T. brucei* and that insect stages of the parasite, although viable in the absence of GPI synthesis, are impaired in their ability to infect the tsetse fly vector (M.A.J. Ferguson, 2000). Thus the GPI pathway is an attractive drug target for African sleeping sickness and possibly other diseases, such as Chagas disease, leishmaniasis, and malaria, which are caused by GPI-rich protozoan parasites.

GPIs serve a unique function in yeast (*S. cerevisiae*). In addition to anchoring secretory proteins to the cell surface, GPIs play an additional role in yeast cell wall biosynthesis (F.M. Klis, 2006). The yeast cell wall consists of a fibrous lattice of mannoproteins, β1,3-glucan, β1,6-glucan, and chitin. During cell wall biosynthesis, GPI-anchored mannoproteins are transported through the secretory pathway to the cell surface. After arrival at the plasma membrane a transglycosylation reaction (catalyzed by an as-yet-unknown enzyme) results in cleavage of the GPI moiety between GlcN and the first mannose residue and formation of a glycosidic linkage between the mannoprotein-GPI-remnant and β1,6 glucan. Mutations in GPI biosynthesis are lethal in yeast (S.D. Leidich, 1994, 1995) and decreased levels of GPI biosynthesis cause growth defects and aberrant cell wall biogenesis. However, not all GPI-anchored proteins become crosslinked to β1,6 glucan; the amino acids within four or five residues upstream of the ω-site determine whether the protein becomes incorporated into the cell wall or remains anchored to the plasma membrane. A unique characteristic of yeast GPIs is the addition of a fourth mannose residue to the core GPI structure (via an α1-2 linkage to the third mannose). Most GPI-anchored proteins in yeast also undergo lipid remodeling replacing the glycerolipid backbone with ceramide. The remodeling occurs after the attachment of protein to the GPI and can occur in both the ER and the Golgi.

8.4. Functions of GPI anchors

GPIs provide targeting signals that affect the intracellular trafficking of GPI-anchored proteins (J.B. Helms, 2004; S. Schuck, 2006). In yeast (but not in mammals or trypanosomes), GPI-proteins are packaged into unique transport vesicles for export from the ER — these vesicles do not carry transmembrane protein cargo and their delivery to the Golgi apparatus depends on ongoing ceramide synthesis (M. Muñiz, 2001; S. Sutterwala, 2007). In many instances, GPI-linked proteins are sorted into unique transport vesicles at the *trans*-Golgi network and are delivered to the apical surface of polarized epithelial cells (C. Zurzolo, 1994). A similar situation occurs in neurons, where axons and dendrites constitute the polarized domains (C.G. Dotti, 1991). GPI-anchored proteins are endocytosed slowly compared to transmembrane proteins bearing internalization motifs; they are also slower to exit the endosomal recycling compartment (S. Chatterjee, 2001). The retention of GPI proteins in endosomes, and the opportunity for the protein to persist in an acidic endosomal

compartment, has important implications for the biology associated with GPI-anchored proteins such as the folate receptor and the cellular scrapie protein. The release of folate and its delivery to the cytosol are enhanced under these conditions, as is the conversion of the scrapie protein to the infectious prion form (A. Taraboulos, 1995).

A GPI-anchor may also allow a protein to be selectively released from the cell surface upon hydrolysis by a GPI-specific phospholipase (e.g., PI-phospholipase C or GPI-phospholipase D). This has been shown to occur for certain GPI-anchored proteins in mammalian cell culture. One example is GPI-anchored membrane dipeptidase which is released from the adipocyte cell surface by a phospholipase C in response to insulin (S. Movahedi, 2000). Interestingly, other GPI-anchored proteins are not released, indicating a level of regulation in insulin-stimulated hydrolysis of GPI-anchored proteins. GPI-anchored molecules have also been shown to transfer between cells and stably insert in the external leaflet of the acceptor cell's plasma membrane (M.G. Low, 1998). The biological significance of this event is unclear; however, the ability of GPI-anchored proteins to transfer between cells has implications for the expression of foreign proteins on the cell surface.

9. Future directions

This chapter describes post-translational lipid modifications of proteins that represent functionally critical elaborations of protein structure. These modifications, with the possible exception of thioacylation, can be anticipated at the level of primary protein sequence, enabling predictions about the localization and likely behavior of the modified proteins within cells. The availability of web-based tools enhances the ability to make such predictions. The functional diversity of lipid-modified proteins makes it interesting to generalize the evolutionary impetus for lipid anchoring compared to the use of 'conventional' protein transmembrane domains. It is now recognized that lipid modifications do not simply provide hydrophobic anchors for the otherwise hydrophilic proteins; rather, they provide specific information on regulation of the targeting and trafficking of proteins that is crucial to their function. Many lipid-modified proteins cluster in the plane of the membrane in a way that depends on the membrane's cholesterol and sphingolipid content, as well as its associated actin cytoskeleton (S.J. Plowman, 2005). This clustering — which requires the protein to be lipid-modified — regulates signal transduction cascades in which the lipid-modified proteins play a part. Also of note is the ability to regulate the membrane association of lipid-modified proteins by titrating the strength of the second signal for membrane anchoring, or by using the bound lipid as a switch, regulated by protein conformation or protein–protein interactions. Analyses of the biophysical characteristics of membrane association of lipid-modified proteins and the regulation of protein function by lipid modification remain critical areas of investigation.

Enzymes involved in most protein lipidation pathways have been identified, with the exception of members of the acyltransferase and thioesterase family involved in *S*-acylation and its turnover. Although many PATs and a single cytoplasmic APT have been identified, many more remain to be discovered. For example, new APTs will have to be identified in order to understand the acylation cycle-dependent trafficking of *S*-acylated proteins like Ras. Also, proteins/enzymes have been assigned to all steps of the GPI biosynthetic pathway

except the step where a lipid precursor flips from the cytoplasmic face of the ER to the lumenal leaflet. The identification of this catalyst remains a challenge for the future. Structural biological analyses and enzymological studies of many of the lipidation pathways are at an advanced stage, with the exception of the GPI pathway where most of the enzymes are membrane-bound proteins and the substrates remain challenging targets for chemical synthesis. These aspects are open to new investigation.

Since many lipidated proteins function in crucial aspects of cell signaling, many of the enzymes involved in lipid modification are attractive targets for drug development. This has long been recognized for prenyltransferases and NMT, but the newly identified PAT and APT enzymes are also the likely foci for new therapeutic strategies.

Abbreviations

APT	thioacyl protein thioesterase
DHHC-CRD	Asp-His-His-Cys cysteine-rich domain-containing protein acyltransferases
ER	endoplasmic reticulum
FTase	protein farnesyltransferase
GGTase	protein geranylgeranyltransferase
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol
GPIT	GPI transamidase
Hh	hedgehog protein
MBOAT	Membrane-bound *O*-acyltransferase
NMT	*N*-myristoyltransferase
PAT	protein acyltransferase
PI	phosphatidylinositol
PIG	phosphatidylinositol glycan

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CHAPTER 3

Fatty acid and phospholipid metabolism in prokaryotes

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Contents

1. Bacterial lipid metabolism

Bacteria are a versatile tool for the study of metabolic pathways. This is especially true for the gram-negative *Escherichia coli*. These bacteria are easy to grow, and the growth conditions can be controlled and manipulated by the investigator. Most importantly, they are suitable for genetic manipulation and their genome sequence is available [1]. *E. coli* is the most extensively studied bacterium, and the genes and enzymes of fatty acid and phospholipid metabolism were first delineated using this organism [2,3]. Although the *E. coli* paradigm provides a solid core of information that is shared by all bacteria, the biochemical details of the lipid metabolic pathways in other bacteria cannot be inferred from the *E. coli* model. The explosion of genomic information in the last several years has allowed detailed comparisons of the gene complements of bacteria. Some key differences between the model organism and important human pathogens are highlighted in the relevant sections.

Phospholipid synthesis requires significant energy investment by the cell, and the advantages of maintaining fine control over the pathway are obvious. The pathway in most bacteria is catalyzed by a series of discrete proteins: the enzymes of fatty acid synthesis are cytosolic, while those of membrane phospholipid synthesis are mainly integral inner membrane proteins. The differences between the bacterial and mammalian enzymes offer attractive targets for novel anti-microbial drugs, and this has been a driving force behind much of the recent research.

The study of phospholipid enzymology in *E. coli* dates back to the early 1960s, when work in the laboratory of Vagelos discovered that the intermediates in fatty acid synthesis are bound to a heat-stable cofactor termed acyl carrier protein (ACP) [2,3]. The enzymes of fatty acid synthesis are soluble proteins whose individual activities can be assayed in crude cell extracts or purified preparations. This is markedly different from the mammalian fatty acid synthase, a large multi-functional polypeptide with intermediates covalently attached (Chapter 6). Thus, the bacterial enzymes became a focus of intensive study, especially in the laboratories of Vagelos, Bloch, and Wakil. Soon, the structures of all of

the intermediates were known, and the basic chemical reactions required were described [4]. During the late 1960s, work on the enzymes of phospholipid synthesis in bacteria flourished, and, mainly through classical identification experiments in the Kennedy Lab, the intermediates in that pathway were established [5].

A second phase of bacterial phospholipid research, during the 1970s and early 1980s, was the identification of mutants in the pathway [6]. The genetic manipulation of *E. coli* became relatively facile. Mutants in many specific enzymes were generated by employing mutagens in combination with a battery of clever selection and screening techniques [4,6]. Such mutations generally fall into one of the two classes. First, they may confer an auxotrophy on a strain, such as a requirement for unsaturated fatty acids or glycerol phosphate. Such mutants have generally lost the ability to produce a key biosynthetic enzyme (e.g., *fabA* mutants, which require supplementation with unsaturated fatty acids), or they may be more complex (e.g., *plsB* mutants require high glycerol phosphate concentrations due to a K_m defect in the enzyme and a second site mutation). Second, they may be conditionally defective, usually at elevated temperatures. For example, strains with the *fabI*(Ts) mutation grow at 30°C, but are not viable at 42°C. This type of defect is usually ascribed to an amino acid change in an essential enzyme that renders it unstable at higher temperatures. Techniques using the bacteriophage P1 are available for the movement of these alleles into other host strains, thus allowing for the mapping of the genes to specific regions of the chromosome, or the generation of strains with particular combinations of mutations. Regulatory mutants were identified, affecting multiple enzymes with a single mutation that allowed for regulatory networks to be investigated. The membrane-bound enzymes of phospholipid synthesis were not amenable to analysis using standard biochemical approaches, and the genetic approach allowed these enzymes to be identified.

Next came the cloning and detailed study of the enzymes of lipid metabolism during the late 1980s and 1990s [3]. Plasmid-based expression systems were used to examine overexpression of enzymes on pathway regulation, and purified enzymes could be more easily obtained for biochemical analysis. With the availability of the sequence data generated by these clones, more precise methods for the construction of specific mutations also became available. Specific genes, or portions of genes, could be 'knocked out' by targeted replacement based on sequence information, as opposed to random insertion of phage DNA. In the late 1990s, the genomic sequences of a broad spectrum of bacteria started to become available on the web complemented by user-friendly software tools. The explosion of bacterial genomic sequences continues in the 21st century and there are now over 400 complete sequences available that provide a solid bioinfomatic framework to compare lipid metabolism in different strains. This infrastructure fuels the current phase of bacterial lipid metabolic research and comparative enzymology that is focused primarily on understanding the nature and importance of these pathways in the important human pathogens.

With the advent of genome information, many of the genes and gene products first identified and characterized in *E. coli* (Table 1) were identified in other bacteria, thus these genes can easily be cloned and their properties compared to those of *E. coli*. The pathways, especially fatty acid biosynthesis, are generally highly conserved among bacteria and can be reconstructed based on the bioinformatic database. Coupled with recent findings about the efficacy of certain inhibitors of the pathway, considerable attention is now being focused on

Table 1
Abbreviated list of genes in bacterial lipid metabolism

Gene	Protein
aas	2-Acyl-GPE acyltransferase
accA	Carboxyltransferase subunit
accB	Biotin carboxy carrier protein
accC	Biotin carboxylase
accD	Carboxyl transferase subunit
acpP	Acyl carrier protein
acpH	Acyl carrier protein hydrolase
acpS	Acyl carrier protein synthase
cdh	CDP–diacylglycerol hydrolase
cdsA	CDP–diacylglycerol synthase
cdsS	Stabilizes mutant CDP–diacylglycerol synthase
cfa	Cyclopropane fatty acid synthase
cls	Cardiolipin synthase
desA	Phospholipid desaturase
desB	Acyl-CoA desaturase
desT	Transcriptional regulator
dgkA	Diacylglycerol kinase
fabA	β-Hydroxydecanoyl-ACP dehydrase I
fabB	β-Ketoacyl-ACP synthase I
fabD	Malonyl-CoA:ACP transacylase
fabF	β-Ketoacyl-ACP synthase II
fabG	β-Ketoacyl-ACP reductase
fabH	β-Ketoacyl-ACP synthase III
fabI	Enoyl-ACP reductase I
fabK	Enoyl-ACP reductase II
fabL	Enoyl-ACP reductase III
fabM	*trans*-2-enoyl-ACP isomerase
fabN	β-Hydroxydecanoyl-ACP dehydrase/isomerase II
fabR	Transcriptional regulator
fabT	Transcriptional regulator
fabZ	β-Hydroxyacyl-ACP dehydrase
fadA	β-Ketoacyl-CoA thiolase
fadB	4-function enzyme of β-oxidation: β-hydroxyacyl-CoA dehydrogenase and epimerase; *cis*-β-*trans*-2-enoyl-CoA isomerase and enoyl-CoA hydratase
fadD	Acyl-CoA synthetase
fadE	Electron-transferring flavoprotein
fadF	Acyl-CoA dehydrogenase
fadG	Acyl-CoA dehydrogenase?
fadH	2,4-dienoyl-CoA reductase
fadL	Long-chain fatty acid transport protein precursor
fadR	Transcriptional regulator
fapR	Transcriptional regulator
fatA	Unknown, possible transcription factor
gpsA	Glycerol phosphate synthase
htrB	KDO_2–lipid IV_A acyloxy lauroyltransferase
kdtA	KDO transferase
lplT	Lysophospholipid flippase
lpxA	UDP–GlcNAc β-hydroxymyristoyl-ACP acyltransferase

Table 1 (continued)

Gene	Protein
lpxB	Disaccharide-1-phosphate synthase
lpxC	UDP–β-*O*-hydroxymyristoyl-GlcNAc deacetylase
lpxD	UDP–β-*O*-hydroxymyristoyl-GlcN *N*-acyltransferase
lpxK	Disaccharide-1-phosphate 4′-kinase
lpxP	KDO_2–lipid IV_A acyloxy palmitoyltransferase
mdoB	Phosphatidylglycerol–membrane-oligosaccharide glycerophosphotransferase
msbA	Lipid flippase
msbB	KDO_2–lipid IV_A acyloxy myristoyltransferase
pgpA	PtdGroP phosphatase
pgpB	PtdGroP phosphatase
pgsA	PtdGroP synthase
pldA	Detergent-resistant phospholipase A
pldB	Inner membrane lysophospholipase
plsB	Glycerol phosphate acyltransferase
plsC	1-Acylglycerol phosphate acyltransferase
plsX	Acyl-ACP phosphotransferase
plsY	Acylphosphate:glycerol phosphate acyltransferase
psd	PS decarboxylase
pss	PS synthase
tesA	Thioesterase I
tesB	Thioesterase II

fatty acid synthesis as a target for novel drug design [7]. The availability of pure enzymes has also stimulated progress in structural biology, and many new three-dimensional structures of enzymes related to lipid metabolism have been recently solved, including the entire set of fatty acid biosynthetic proteins found in the *E. coli* model [8].

2. Membrane systems of bacteria

Phospholipids in *E. coli* and other gram-negative bacteria are used in the construction of the inner and outer membranes (Fig. 4 in Chapter 1). The inner membrane is impermeable to solutes unless specific transport systems are present. The outer membrane contains pores that allow the passage of molecules having a molecular weight less than 600, and is rich in structural lipoproteins and proteins involved in the transport of high-molecular-weight compounds. The outer layer of the outer membrane is composed primarily of lipopolysaccharides (LPSs) rather than phospholipids. Between the inner and outer membranes is an osmotically active compartment called the periplasmic space. Membrane-derived oligosaccharides (MDOs), peptidoglycan, and binding proteins involved with metabolite transport are found in this compartment. Gram-positive bacteria do not possess an outer membrane. Instead, they have a membrane bilayer surrounded by a thick layer of peptidoglycan decorated with proteins, carbohydrates, and often, teichoic and lipoteichoic acid.

3. Bacterial fatty acid biosynthesis

3.1. Acyl carrier protein

A unique feature of fatty acid synthesis in bacteria is the presence of the small (8.86 kDa), acidic and highly soluble ACP, the product of the *acpP* gene. ACP is one of the most abundant proteins in *E. coli*, constituting about 0.25% of the total soluble protein ($\sim 6 \times 10^4$ molecules/cell). The acyl intermediates of fatty acid biosynthesis are bound to the protein through a thioester linkage to the terminal sulfhydryl of the 4′-phosphopantetheine prosthetic group. The prosthetic group sulfhydryl is the only thiol group of ACP and is attached to the protein via a phosphodiester linkage to Ser-36. ACP must interact specifically and transiently with all of the enzymes of fatty acid biosynthesis (except acetyl-CoA carboxylase), and does so through interactions with exposed negative residues on ACP with a patch of positive residues on the surfaces of the *fab* enzymes.

The ACP pool in normally growing cells is approximately one-eighth of the coenzyme A (CoA) pool, the other acyl group carrier in cells. The prosthetic group of ACP is produced from CoA, and a common feature of both is the pantetheine arm for thioester formation. Virtually all of the ACP is maintained in the active, holo-form in vivo, indicating that the supply of prosthetic group does not limit fatty acid biosynthesis. During logarithmic growth, a significant pool of ACP is unacylated. The ACP pool must be severely depleted before an effect on fatty acid and phospholipid synthesis can be detected. Overproduction of ACP generally yields high levels of apo-ACP, which is toxic to the cell by inhibition of the glycerol phosphate acyltransferase (PlsB). The 4′-phosphopantetheine prosthetic group is transferred from CoA to apo-ACP by the 14-kDa monomeric [ACP]synthase. The [ACP]synthase from *Bacillus subtilis* has been crystallized in complex with ACP to give the first detailed look at ACP–protein interactions (K.D. Parris, 2000). ACP plays other roles in cell physiology, donating acyl chains to MDOs, lipoic acid, and quorum signals and secondary metabolites.

ACP is one of the most interactive proteins in bacterial physiology [9] and the physiological significance of its recognition by all its binding partners will be the subject of much future research. The ACPs from one bacterial species readily substitutes for the other ACPs in fatty acid synthesis due to the conservation of residues along helix-2, termed the recognition helix [10]. This conserved sequence of negatively charged and hydrophobic amino acid side chains interacts with a complementary constellation of basic and hydrophobic residues on the target protein that are found adjacent to the active site tunnel. Whereas the enzymes of fatty acid biosynthesis do not have a conserved primary sequence that can be recognized as an ACP recognition motif, the three-dimensional structures of all the enzymes illustrate the existence of the basic-hydrophobic recognition region at the active site entrance [8,10]. Site-directed mutagenesis confirms the importance of these residues to ACP binding.

3.2. Acetyl-CoA carboxylase

Acetyl-CoA carboxylase (ACC) catalyzes the first committed step of fatty acid synthesis, the conversion of acetyl-CoA to malonyl-CoA. Acetyl-CoA is a key intermediate in many

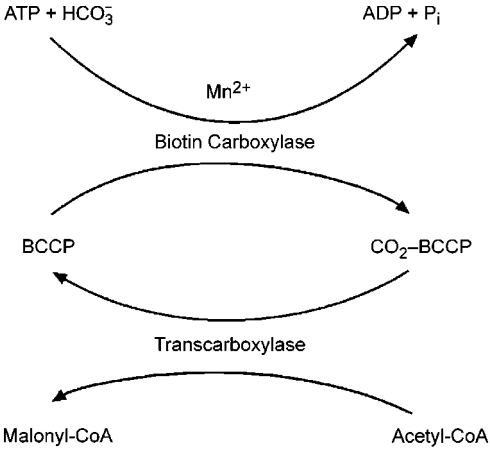

Fig. 1. The acetyl-CoA carboxylase reaction is performed in two steps. Biotin, covalently attached to BCCP (biotin carboxyl carrier protein, *accB*), is carboxylated by the carboxylase subunit (*accC*). The heterodimeric transcarboxylase (*accA* and *accD*) then transfers the CO_2 to acetyl-CoA, forming malonyl-CoA.

pathways, and constitutes the majority of the acyl-CoA species within the cell at concentrations of about 0.5–1.0 mM during logarithmic growth on glucose. Malonyl-CoA is normally present at 0.5% of this level, and is used exclusively for fatty acid biosynthesis. The overall carboxylation reaction is composed of two distinct half reactions: the ATP-dependent carboxylation of biotin with bicarbonate to form carboxybiotin; and transfer of the carboxyl group from carboxybiotin to acetyl-CoA, forming malonyl-CoA (Fig. 1) [11].

Each ACC half-reaction is catalyzed by a different protein sub-complex. The vitamin biotin is covalently coupled through an amide bond to a lysine residue on biotin carboxyl carrier protein (BCCP, a homodimer of 16.7-kDa monomers encoded by *accB*) by a specific enzyme, biotin–apoprotein ligase (encoded by *birA*), and is essential to activity. The crystal and solution structures of the biotinyl domain of BCCP have been determined, and reveal a unique 'thumb' required for activity (J. Cronan, 2001). Carboxylation of biotin is catalyzed by biotin carboxylase (encoded by *accC*), a homodimeric enzyme composed of 55-kDa subunits that is copurified complexed with BCCP. The *accB* and *accC* genes form an operon. The three-dimensional structure of the biotin carboxylase subunit has been solved by X-ray diffraction revealing an 'ATP-grasp' motif for nucleotide binding. The mechanism of biotin carboxylation involves the reaction of ATP and CO_2 to form the short-lived carboxyphosphate, which then interacts with biotin on BCCP for CO_2 transfer to the 1′-nitrogen.

The carboxyltransferase enzyme that transfers the carboxy group from the biotin moiety of BCCP to acetyl-CoA is a heterotetramer composed of two copies of two dissimilar subunits, α (35 kDa) and β (33 kDa) (encoded by *accA* and *accD*, respectively). Sequence analysis suggests that the acetyl-CoA binding site lies within the AccA subunit. Strains with mutations in *accB* and *accD* have been obtained that are temperature sensitive for growth, indicating that this reaction is essential. It is thought that the ACC complex present in vivo is composed of one copy of each sub-complex, with a combined molecular weight of 280 kDa.

3.3. Initiation of fatty acid biosynthesis

For the malonate group to be used for fatty acid synthesis, it must first be transferred from malonyl-CoA to malonyl-ACP by the 32.4-kDa monomeric malonyl-CoA:ACP transacylase, the product of the *fabD* gene (Fig. 2). A stable malonyl-serine enzyme intermediate is formed during the course of the FabD reaction, and subsequent nucleophilic attack on this ester by the sulfhydryl of ACP yields malonyl-ACP. The high reactivity of the serine in malonyl-ACP transacylase is due to the active site being composed of a nucleophilic elbow as observed in alpha/beta hydrolases. The serine is hydrogen bonded to His-201 in a fashion similar to serine hydrolases.

The last two carbons of the fatty acid chain (i.e., those most distal from the carboxylate group) are the first introduced into the nascent chain, and acetyl-CoA can be thought of as the 'primer' molecule of fatty acid synthesis in *E. coli*. The initial condensation reaction, catalyzed by β-ketoacyl-ACP synthase III (FabH), utilizes acetyl-CoA and malonyl-ACP to form the four-carbon acetoacetyl-ACP with concomitant loss of CO_2 (Fig. 2). FabH also possesses acetyl-CoA:ACP transacylase activity, and for many years it was thought that acetyl-ACP was the actual primer. However, acetyl-ACP appears to be a product of a side reaction, and the role, if any, played by this intermediate in the pathway is unknown.

The FabH proteins play a major role in specifying product diversity. *E. coli* FabH is specific for acetyl-CoA as the primer and this organism makes only straight-chain, even-numbered fatty acids. The FabH from gram-positive bacteria that produce branched-chain fatty acids are selective for five- and seven-carbon branched-chain precursors derived from amino acids. In *Mycobacterium tuberculosis*, the FabH prefers long-chain fatty acids and this organism is characterized by the presence of very long-chain mycolic acids in the membrane.

3.4. Elongation of acyl chains

Four enzymes participate in each iterative cycle of chain elongation (Fig. 3). The acetoacyl-ACP formed from the initiating FabH condensation is reduced by an NADPH-dependent β-ketoacyl-ACP reductase (*fabG*), and a water molecule is then removed by a β-hydroxyacyl-ACP dehydrase (*fabA* or *fabZ*). The last step is catalyzed by enoyl-ACP reductase (*fabI* or *fabK*) to form a saturated acyl-ACP, which serves as the substrate for another condensation reaction or when the chain length reaches 16–18 carbons is utilized for membrane phospholipid synthesis. β-Ketoacyl-ACP synthase I or II (*fabB* or *fabF*) initiates additional

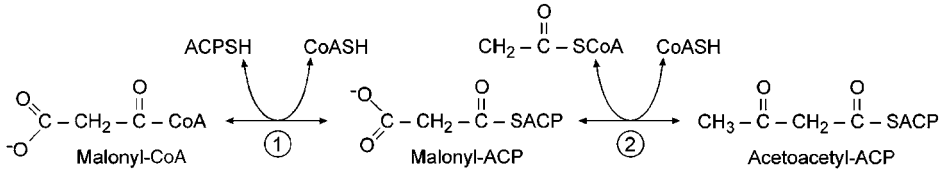

Fig. 2. Initiation of fatty acid synthesis. (1) Malonyl-CoA:ACP transacylase (FabD) transfers the malonyl group from CoA to ACP and then (2) β-ketoacyl-ACP synthase III (FabH) catalyzes the initial irreversible condensation of malonyl-ACP with acetyl-CoA to form acetoacetyl-ACP.

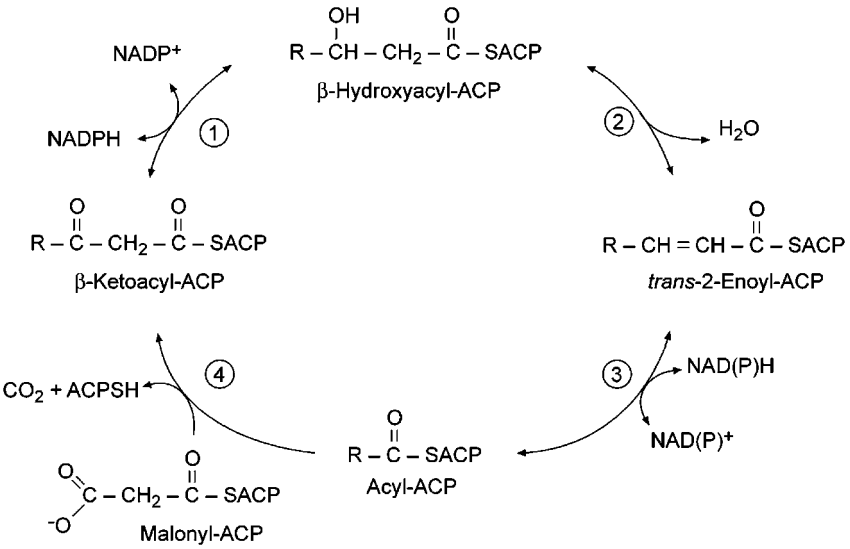

Fig. 3. Cycles of fatty acyl chain elongation. All intermediates in fatty acid synthesis are shuttled through the cytosol as thioesters of the acyl carrier protein (ACP). (1) β-Ketoacyl-ACP reductase (FabG), (2) β-hydroxyacyl-ACP dehyrase (FabA or FabZ), (3) *trans*-2-enoyl-ACP reductase I (FabI), (4) β-ketoacyl-ACP synthase I or II (FabB or FabF).

rounds of elongation by adding two-carbon units from malonyl-ACP to the growing acyl-ACP.

3.4.1. β-Ketoacyl-ACP reductase

The β-ketoacyl-ACP reductase gene (*fabG*) is located within the *fab* gene cluster between the *fabD* and *acpP* genes and is co-transcribed with *acpP*. Insertional mutants that prevent *fabG* transcription while allowing ACP to be produced were generated in Cronan's laboratory and suggest that *fabG*-encoded reductase activity is essential in *E. coli*. The *fabG*-encoded NADPH-specific β-ketoacyl-ACP reductase is a homotetrameric protein of 25.6-kDa monomers. The protein functions with all chain lengths in vitro and exhibits cooperative binding of NADPH. A dramatic conformational change occurs on cofactor binding, as evidenced by the crystal structures of the free and NADPH-bound protein (A.C. Price, 2004). FabG is the only protein in the elongation cycle where a single isoform is known and is found universally distributed in bacteria.

3.4.2. β-Hydroxyacyl-ACP dehydrase

E. coli possesses two β-hydroxyacyl-ACP dehydratases. One is encoded by *fabZ*, and is active on all chain lengths of saturated and unsaturated intermediates. This enzyme is distinct from the dual-function β-hydroxydecanoyl-ACP dehydratase/isomerase (encoded by *fabA*) first described by Bloch and coworkers. The FabA enzyme dehydrates saturated, but not unsaturated, fatty acid intermediates and catalyzes a key isomerization reaction at the point where the biosynthesis of unsaturated fatty acids diverges from saturated fatty

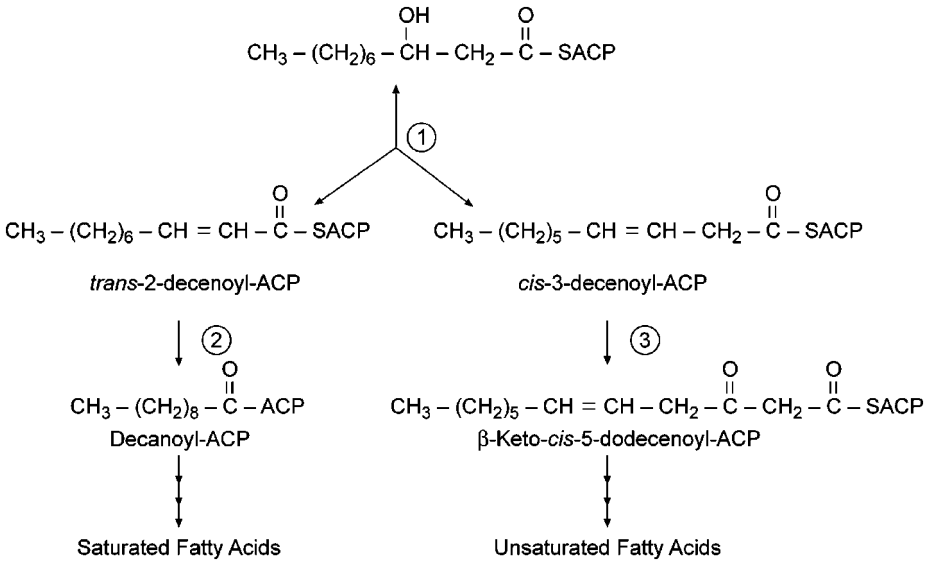

Fig. 4. Branch point in unsaturated fatty acid synthesis. (1) FabA catalyzes the inter-conversion of β-hydroxydecanoyl-ACP, *trans*-2-decenoyl-ACP, and *cis*-3-decenoyl-ACP. (2) *trans*-2-Decenoyl-ACP is a substrate for enoyl-ACP reductase (FabI), while (3) *cis*-3-decenoyl-ACP is elongated by β-ketoacyl-ACP synthase I (FabB). Competition between FabI and FabB is partly responsible for the ratio of saturated to unsaturated fatty acids.

acids (Fig. 4). FabA and FabB share weak overall homology (28% identity and 50% similarity at the amino acid level). The mono-functional FabZ protein (17 kDa) is somewhat smaller than the *fabA*-encoded bifunctional enzyme (19 kDa). The FabA dehydrase/isomerase has been crystallized, and its structure solved (M. Leeson, 1996). The active site His is located in a long tunnel which acts as a molecular ruler to ensure that only 10-carbon intermediates are isomerized. Interestingly, some gram-positive bacteria that make unsaturated fatty acids express a FabZ-like protein that, like FabA, possesses dehydratase or isomerase activity. Thus, the differences in the active site residues do not account for the catalytic properties of the protein. Instead, domain-swapping experiments suggest that it is the shape of the active site tunnel that is important. Some dehydratases can accommodate the kink that arises through introduction of the *cis*-3 double bond, while others cannot.

3.4.3. Enoyl-ACP reductase

The final step in each round of fatty acyl elongation in *E. coli* is the NADH-dependent reduction of the *trans* double bond, catalyzed by the homotetrameric (subunit mass of 29 kDa) NADH-dependent enoyl-ACP reductase I (encoded by *fabI*). The FabI amino acid sequence is similar (34% identical) to the product of a gene (called *inhA*) from mycobacteria. InhA is involved in mycolic acid biosynthesis (A. Banerjee, 1994). The synthesis of these unusual 70–80 carbon mycobacterial acids requires a pathway composed of enzymes essentially identical to those of fatty acid synthesis. Missense mutations within the *inhA* gene result in resistance to the anti-tuberculosis drugs, isoniazid and ethionamide. The crystal structures of FabI and InhA have been solved, and are virtually superimposable for most

of the protein. FabI has a flexible substrate-binding loop that becomes ordered on binding of the specific inhibitors, diazaborine or triclosan (and presumably substrate), while InhA has two short helices in this region that move 4 Å on drug or substrate binding. A novel enoyl-ACP reductase II (FabK) is found in gram-positive bacteria. FabK is an NADH-dependent enzyme, but also has a flavin mononucleotide cofactor.

3.4.4. β-Ketoacyl-ACP synthases

Three *E. coli* enzymes catalyze the Claisen condensation that is the β-ketoacyl-ACP synthase reaction. These enzymes are the products of the *fabB*, *fabF*, and *fabH* genes. β-Ketoacyl-ACP synthase I, or FabB, is composed of two identical 42.6-kDa subunits, and has both malonyl-ACP and fatty acyl-ACP binding sites. In the condensation reaction, the acyl group is covalently linked to the active site cysteine. The acyl-enzyme undergoes condensation with malonyl-ACP to form β-ketoacyl-ACP, HCO_3^-, and free enzyme. Overproduction of FabB has two effects: an increased amount of *cis*-vaccenic acid (18:1d11) in phospholipids, and resistance to the antibiotics thiolactomycin and cerulenin (Section 12). β-Ketoacyl-ACP synthase II (FabF) is very similar to FabB (38% identical at the amino acid level). Like FabB, FabF has a dimeric structure of 43-kDa subunits, and is inhibited by cerulenin and thiolactomycin. FabF is not essential to growth in *E. coli*, but is essential for the regulation of fatty acid composition in response to temperature fluctuations. Mutants lacking FabF activity, unlike wild-type *E. coli*, do not produce increased amounts of the long, unsaturated fatty acid *cis*-vaccenate at lower temperatures. However, FabF is by far the most common condensing enzyme in bacteria, and most organisms contain only a single condensing enzyme and it is the FabF class. β-Ketoacyl-ACP synthase III (FabH) is a dimeric protein of identical 33.5-kDa subunits first detected as a condensation activity resistant to cerulenin. The FabH reaction is characterized by the preference for a CoA-linked primer, rather than acyl-ACP.

The crystal structures of all three synthases have been determined, and all share a common thiolase fold [8]. Structures of FabB and FabF are virtually identical. Both utilize a conserved catalytic triad of Cys–His–His. FabH has a much more closed active site, reflecting its use of less hydrophobic, shorter chain, substrates than for FabB and FabF. FabH also differs in that it contains a catalytic triad composed of Cys–His–Asn. The exact reason for this difference is not clear at this time, since the key nitrogens of the His or Asn residues of the respective synthases occupy equivalent space in the respective structures.

3.5. Synthesis of unsaturated fatty acids

The pathway described above suffices to produce the straight-chain saturated fatty acids found in the membrane phospholipids, mainly palmitic acid (16:0) in *E. coli*. Gram-negative bacteria also contain unsaturated fatty acids, and the ratio of saturated to unsaturated fatty acids in the membrane phospholipids is a key determinant in membrane fluidity, and changes according to temperature [12]. The *fabA*-encoded dehydrase or isomerase (Section 3.4.3) catalyzes a vital reaction at the branch point of the two pathways: the isomerization of *trans*-2-decenoyl-ACP to *cis*-β-decenoyl-ACP (Fig. 4). The *cis*-3 compound is not a substrate for the enoyl-ACP reductase, but instead it is rapidly condensed in a reaction requiring FabB, but not FabF. Strains with mutations in either *fabA* or *fabB* require supplementation

with unsaturated fatty acids for growth, showing the specific requirement for both enzymes. As their names suggest, these were the first two mutants in fatty acid biosynthesis identified by classical genetic techniques [13,14].

Both FabB and FabF are capable of participating in saturated and unsaturated fatty acid synthesis, and the enzymes have been shown, in vitro, to function similarly with all long-chain acyl-ACPs except palmitoleoyl-ACP. Palmitoleoyl-ACP is an excellent substrate for FabF, but not for FabB. In vivo, the reactivity of FabF toward this substrate increases at lower temperatures, leading to increased amounts of the more fluid *cis*-vaccenate (18:1Δ11) in membrane phospholipids. Factors that control unsaturated fatty acid composition in bacteria that lack the FabA and FabB systems, such as *Streptococcus pneumoniae*, are less well understood. Transcription factors exist that modulate the membrane fatty acid composition, but the ligands that control the DNA binding of these factors are unknown.

3.6. Dissociable or dissociated enzymes?

Historically, scientists in the field have debated whether the enzymes of bacterial fatty acid synthase system are 'dissociable' or 'dissociated'. This seemingly minor semantic distinction has larger ramifications for the in vivo physiology of the cell. The implication of the use of the word *dissociable* is that the enzymes form a complex in vivo, and only became separated (or dissociated) on cell disruption; whereas in a *dissociated* system, the enzymes do not form a complex in vivo. The concept of a large complex mimicking the multifunctional type I enzyme would support the notion of substrate channeling between the active sites, increasing the catalytic efficiency of the pathway as a whole. However, there are no data to support the existence of either a large complex or substrate channeling. A minimal fatty acid synthetic unit must consist of at least six separate activities encoded by the *acpP*, *fabD*, *fabH*, *fabG*, *fabI*, *fabZ*, and *fabB* genes. In vitro, these enzymes appear as monomers (ACP, FadD), dimers (FabH, FabZ, and FabB), or tetramers (FabG and FabI). Thus, a complex of these proteins in vivo would possess 16 subunits (assuming only one of the each individual complex was present) with a combined mass of over 440 kDa. With our current knowledge of the three-dimensional structures of these proteins [8], it is hard to envision how the pieces of such a puzzle would fit together. The enzymes have active sites located at the bottom of narrow tunnels, leaving no opportunity for the prosthetic group to swing between them. The acyl-ACP intermediate must instead completely dissociate from the enzyme to interact with the next enzyme in the pathway. Studies with the yeast two-hybrid system have failed to detect interactions between different enzymes. Thus, most type II fatty acid synthases should be considered dissociated and not dissociable. Nonetheless, the debate continues as recent data with *M. tuberculosis* suggested that several of the enzymes form a complex (R. Veyron-Churlet, 2004). More work will be needed to validate these results and determine if *M. tuberculosis* is an oddball type II system or reveals a fundamental level of organization that has been missed in other systems.

There is one very important feature of the type II system that should not be overlooked. Although a 'hard-wired' type I mechanism may seem more efficient, it only produces a single product. In contrast, the type II system is not only responsible for producing all the diversity of fatty acids in membranes, the intermediates of the pathway are diverted for the synthesis of other key molecules. These include biotin, lipoic acid, and the quorum-sensing

acylhomoserine lactones. These molecules are produced in relatively low abundance, but in the case of *Pseudomonas aeruginosa*, the intracellular storage lipids (hydroxyalkonoates) and the extracellular rhamnolipids are produced in quantities that exceed the number of acyl groups in the membrane. The existence of a dissociated pathway allows these ancillary metabolic pathways to compete with the enzymes of fatty acid synthesis for the acyl-ACP intermediates that are liberated at each step in the pathway.

4. Transfer to the membrane

Fatty acid biosynthesis in *E. coli* is normally complete when the acyl chain is 16 or 18 carbons in length. These acyl-ACPs are now substrates for the acyltransferases that transfer the fatty acyl chain into the membrane phospholipids (Fig. 5). Alternatively, *E. coli* can incorporate exogenous fatty acids, following esterification to CoA. The first enzyme (the *plsB* gene product) transfers fatty acids from either the soluble acyl-ACP or acyl-CoA to the 1-position of glycerol phosphate. The product of the reaction, 1-acylglycerol phosphate, partitions into the membrane. The PlsB protein is an integral inner membrane protein of 91 kDa, and has a preference for saturated fatty acids. The second acyltransferase (the *plsC* gene product), a membrane protein of 27 kDa, esterifies the 2-position of the glycerol backbone and prefers unsaturated acyl chains. Thus, bacterial phospholipids have an asymmetric distribution of fatty acids between the 1-position and 2-position of the glycerol phosphate backbone. The PlsB system influences the chain length of the fatty acids incorporated

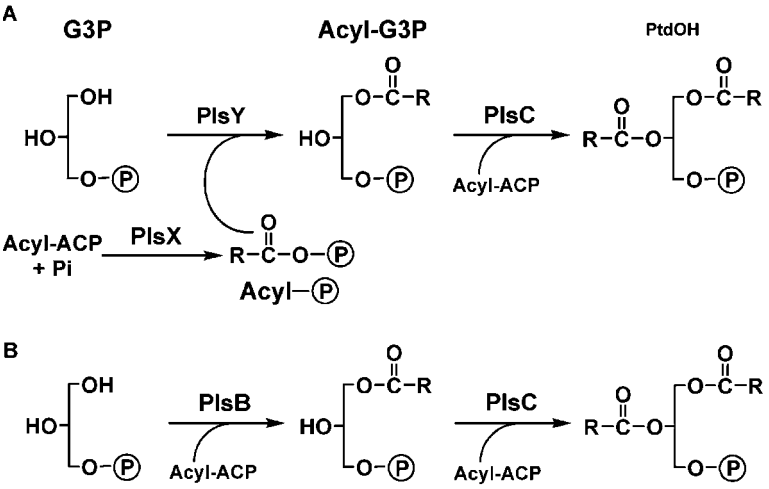

Fig. 5. Transfer of fatty acyl groups to the membrane. Panel A, PlsX catalyzes the transfer of the fatty acid from acyl-ACP to phosphate to form the activated acylphosphate. PlsY acylates the 1-position of the glycerol backbone using acylphosphate as the acyl donor. Panel B, a fatty acid is transferred from acyl-ACP or acyl-CoA to the 1-position of *sn*-glycerol-3-phosphate (G3P) by glycerol phosphate acyltransferase (PlsB). A second fatty acid is transferred to the 2-position by the 1-acylglycerol phosphate acyltransferase (PlsC). PlsC is common to both pathways.

into the phospholipids by competition with the elongation condensing enzymes for acyl-ACP, and the rate of fatty acid biosynthesis via modulation of its activity by ppGpp (Section 10.5).

The isolation of *E. coli* mutants with defective acyltransferase activity (*plsB*) by Bell's laboratory heralded a major advance in the study of the acyltransferases (R.M. Bell, 1974). These mutants were glycerol phosphate auxotrophs and exhibited an increased Michaelis constant for glycerol phosphate in in vitro acyltransferase assays. The increased K_m was subsequently shown to arise from a single missense mutation in the open reading frame. Therefore, *plsB* mutants require an artificially high intracellular concentration of glycerol phosphate for activity. Complementation of these mutants facilitated the cloning of the PlsB. Plasmids that suppressed the glycerol phosphate requirement of *plsB* strains overexpressed PlsB activity 10-fold. PlsB possesses a catalytic His–Asp dyad commonly associated with all glycerolipid acyltransferases utilizing acyl thioester substrates (bacterial or mammalian). The PlsB is specifically activated by acidic phospholipids, phosphatidylglycerol (PtdGro) and cardiolipin (Ptd_2Gro), as shown by micelle assays containing detergents and phospholipid, and is active as a monomer. PlsB also exhibits negative cooperativity with respect to glycerol phosphate binding, a property that may account in part for the finding that dramatic increases in the intracellular glycerol phosphate concentration do not increase the amount of phospholipid in *E. coli*.

However, PlsB is not widely distributed in bacteria, and most organisms use the PlsX–PlsY system to initiate membrane phospholipid synthesis [15]. This pathway begins by the conversion of acyl-ACP end product to acylphosphate by the *plsX* gene product. Next, an acylphosphate-specific PlsB, encoded by *plsY*, transfers the acylphosphate to the 1-position of the glycerol backbone. The *plsX* gene is located in the *fab* cluster next to *fabH* in *E. coli*, but is not associated with the *fab* genes in most bacteria. This discovery clarifies the finding that the *plsB* growth phenotype depends on mutations in two unlinked genes. One mutation is in the *plsB* gene discussed above, and the second is in a gene called *plsX*. Both mutations are required for a strain to exhibit a requirement for glycerol phosphate since strains harboring either the *plsB* or the *plsX* lesion do not have a defective growth phenotype.

The next step in phospholipid biosynthesis is catalyzed by 1-acylglycerol phosphate acyltransferase (the *plsC* gene product) which acylates the product of the PlsB step to form phosphatidic acid (Fig. 5). Phosphatidic acid comprises only about 0.1% of the total phospholipid in *E. coli* and turns over rapidly, a property consistent with its role as an intermediate in phospholipid synthesis. The 1-acylglycerol phosphate acyltransferase is thought to transfer unsaturated fatty acids selectively to the 2-position. The *plsC* gene is universally expressed in bacteria.

5. Phospholipid biosynthesis

E. coli possesses only three major phospholipid species in its membranes, making it one of the simplest organisms to study with regard to phospholipid biosynthesis. Phosphatidylethanolamine (PE) comprises the bulk of the phospholipids (75%), with PtdGro and Ptd_2Gro forming the remainder (15–20% and 5–10%, respectively). The scheme for the synthesis of membrane phospholipids follows the classic Kennedy pathway (Fig. 6).

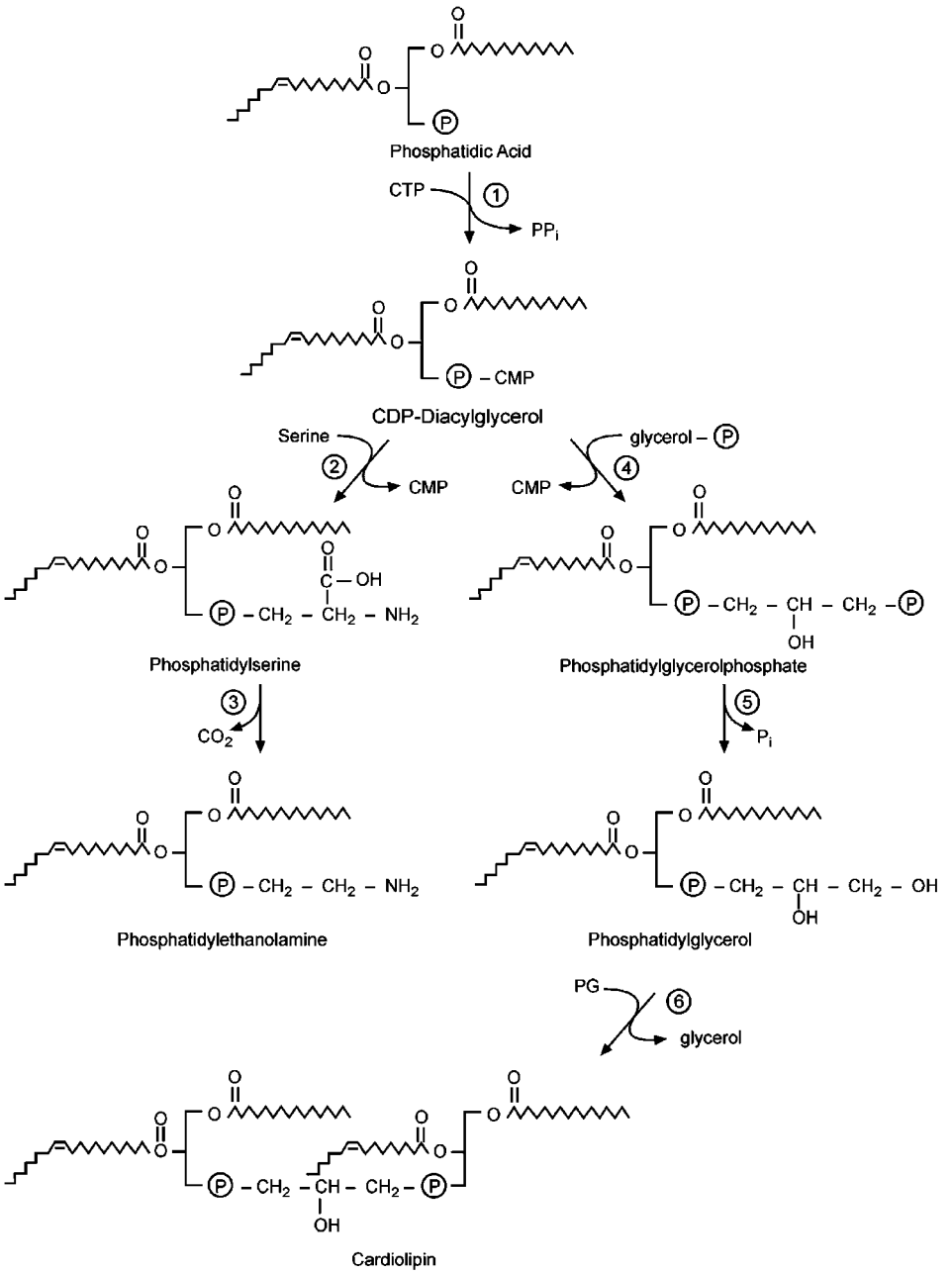

Fig. 6. Synthesis of phospholipid polar head groups. The three major phospholipid species in *E. coli* are synthesized by a total of six different enzymatic activities: (1) phosphatidate cytidylyltransferase (Cds), (2) phosphatidylserine synthase (PssA), (3) phosphatidylserine decarboxylase (Psd), (4) phosphatidylglycerol phosphate synthase (PgsA), (5) phosphatidylglycerol phosphate phosphatase (PgpA or PgpB), and (6) cardiolipin synthase (cls).

5.1. Phosphatidate cytidylyltransferase

The key activated intermediate in bacterial phospholipid synthesis, CDP-diacylglycerol, comprises only 0.05% of the total phospholipid pool. The 27.6-kDa enzyme phosphatidate cytidylyltransferase (or CDP–diacylglycerol synthase) catalyzes the conversion of phosphatidic acid to a mixture of CDP–diacylglycerol and dCDP–diacylglycerol. Strains of *E. coli* with mutations in the *cds* gene retain 5% of the normal levels of CDP–diacylglycerol synthase and grow normally under standard laboratory conditions, although are hypersensitive to erythromycin and elevated pH. Thus, CDP–diacylglycerol synthase is present in large excess of the minimum amount of enzyme required to sustain phospholipid synthesis. These mutants accumulate substantial amounts of phosphatidic acid (up to 5% of the total phospholipid). Null mutations in *cds* have not been reported, and would presumably be non-viable due to the complete lack of phospholipid synthesis.

CDP–diacylglycerol stands at the branch point between PE synthesis and PtdGro and Ptd_2Gro synthesis (Fig. 6). It has been hypothesized that the presence of both ribo and deoxyribo forms of the liponucleotide could play a role in determining the relative amount of intermediate entering these two arms of the pathway. For this to be true, the respective synthases that utilize this compound would have to be selective toward either dCDP–diacylglycerol or CDP–diacylglycerol. In vivo, the ratio of dCDP–diacylglycerol to CDP–diacylglycerol is 0.88. A change in this ratio to 3.1 has no effect on the relative rates of PE and PtdGro synthesis in vivo, arguing against selectivity of the subsequent enzymes for one form or the other. Further, both riboliponucleotide and deoxyriboliponucleotide are substrates for phosphatidylserine (PS) synthase in vitro, and thus, the significance, if any, of the two forms of liponucleotide remains to be determined.

5.2. Phosphatidylethanolamine production

5.2.1. Phosphatidylserine synthase

The first step in the synthesis of PE is the condensation of CDP–diacylglycerol with serine catalyzed by PS synthase to form PS. During cell disruption, the 58-kDa PS synthase appears associated with ribosomes, but re-attaches to the membrane vesicles once substrate is added. PS is a minor membrane constituent of *E. coli* since it is rapidly converted to PE by PS decarboxylase. Mutants in the *pss* gene encoding PS synthase are viable only when supplemented with divalent metal ions. PE is capable of forming the hexagonal (non-bilayer) H_{II} lipid phase, and Dowhan has demonstrated that the divalent cations interact with Ptd_2Gro to replace the function of PE in the formation of an H_{II} phase (Chapter 1). The cells lack PS synthase activity, and thus contain no PS or PE in their membranes. There are also perturbations in the function of permeases, electron transport, motility, and chemotaxis. The defect appears to arise from the improper folding of membrane proteins leading to the idea that specific phospholipids act as chaperones to guide the folding of membrane proteins. Whether other physiological processes are dependent on the formation of local regions of non-bilayer structure or specifically require PE remain to be elucidated, but the process of cell division, the formation of contacts between inner and outer membranes, and the translocation of molecules across the membrane are viable candidates.

5.2.2. Phosphatidylserine decarboxylase

PS is decarboxylated by PS decarboxylase to yield the zwitterionic PE. This inner membrane enzyme has a subunit molecular mass of 36 kDa. PS decarboxylase has a pyruvate prosthetic group that participates in the reaction by forming a Schiff base with PS. Overproduction of the enzyme 30–50-fold by plasmid-borne copies of the *psd* gene has no effect on membrane phospholipid composition, indicating that the level of this enzyme does not regulate the amount of PE in the membrane. The majority of the PE is found in the periplasmic leaflet of the inner membrane, and there is a rapid flipping from the inner to outer leaflet by the MsbA lipid flippase (Section 7).

Mutants with a temperature-sensitive decarboxylase accumulate PS at the non-permissive temperature. The mutants continue to grow for several hours after the shift to the non-permissive temperature despite the reduced levels of PE and the concomitant increase in PS. Complete inactivation of *psd* by insertional mutagenesis has the same divalent cation-requiring phenotype as the *pss* mutants described above. The requirement for Ptd_2Gro is consistent with the inability to introduce a null *cls* allele into *psd* strains. Thus, PE is essential for the polymorphic regulation of lipid structure.

5.3. Phosphatidylglycerol synthesis

5.3.1. Phosphatidylglycerol phosphate synthase

CDP-diacylglycerol is condensed with glycerol phosphate to form phosphatidylglycerol phosphate (PtdGroP), an intermediate in the production of the acidic phospholipids PtdGro and Ptd_2Gro (Fig. 6). The reaction is analogous to the synthesis of PS, with the product CMP being released. Mutants (*pgsA*) defective in PtdGroP synthesis contain less than 5% of normal PtdGroP synthase activity in vitro; however, there is no growth phenotype associated with these mutants. The PgsA protein is predicted to be a 20.7-kDa integral membrane protein. It has long been thought that PtdGroP synthase is essential and that cells cannot survive without acidic phospholipids. There are many important cellular functions that are affected by reduced PtdGro and/or Ptd_2Gro content of the membrane. PtdGro is required for protein translocation across the membrane, and acidic phospholipids are required for channel activity of bacterial colicins and the interaction of antibiotics with the membrane. Cell division proteins such as FtsY also apparently require acidic phospholipids for activity, as does the DnaA protein involved in chromosome segregation. However, Matsumoto has recently inactivated the *pgsA* gene with a kanamycin cassette (S. Kikuchi, 2000). This *pgsA::kan* strain has no detectable PtdGro or Ptd_2Gro, is viable, but not above 40 °C, and contains increased concentrations of PtdOH, which may at least partially compensate for the absence of PtdGro and Ptd_2Gro.

5.3.2. PtdGroP phosphatases

The second step in the synthesis of PtdGro is the dephosphorylation of PtdGroP (Fig. 6). Two independent genes have been identified, *pgpA* and *pgpB*, that encode PtdGroP phosphatases based on an in vitro assay. Both proteins are small (19.4 and 29 kDa, respectively) but share no sequence homology. In vitro, the *pgpA*-encoded phosphatase specifically hydrolyzes PtdGroP, whereas the PgpB phosphatase also hydrolyzes PtdOH. However, disruption of both of these genes in a single strain did not impair PtdGro synthesis, although

the respective phosphatase activities were reduced. Thus, neither of these phosphatases is required for PtdGro synthesis, suggesting that another phosphatase capable of operating in the PtdGro biosynthetic pathway remains to be discovered.

5.4. Cardiolipin biosynthesis

Unlike in mammalian mitochondria, where Ptd_2Gro is synthesized by the reaction of CDP–diacylglycerol with PtdGro (Chapter 8), Ptd_2Gro is produced in bacteria by the condensation of two PtdGro molecules (Fig. 6). Ptd_2Gro accumulates as the cells enter the stationary phase of growth, and is required for prolonged survival of the bacteria. Ptd_2Gro synthase is post-translationally processed from a 55-kDa precursor to a 45–46 kDa form. Mutants deficient in Ptd_2Gro synthase (*cls*) possess very low levels of Ptd_2Gro and lose viability in stationary phase (S. Kikuchi, 2000). The mutants also grow at a slower rate and to a lower density than the corresponding wild-type cells. Low, residual concentrations of Ptd_2Gro are present in the *cls* null mutants, hampering efforts to establish the role of this lipid in cell physiology. Amplification of Ptd_2Gro synthase leads to the overproduction of Ptd_2Gro, a decrease in membrane potential, and loss of viability. Therefore, *E. coli* can tolerate changes in the overall Ptd_2Gro content, but the elimination or significant overproduction of Ptd_2Gro leads to significant physiological imbalance.

5.5. Cyclopropane fatty acids

Fatty acids attached to membrane phospholipids can be post-synthetically converted to their cyclopropane derivatives during the stationary phase of bacterial growth. Their biosynthesis and function have been elucidated by the Cronan's laboratory (D.W. Grogan, 1997). *E. coli* mutants that completely lack cyclopropane fatty acid synthase activity (owing to null mutations in the *cfa* gene) grow and survive normally under virtually all conditions, except that *cfa* mutant strains are more sensitive to freeze-thaw treatment and acid shock than are isogenic cfa^+ strains. Thus, the stable cyclopropane derivative protects the reactive double bond from adverse reactions during stationary phase. Cyclopropanation involves a significant energy commitment by the cell: the reaction uses *S*-adenosylmethionine, which requires three molecules of ATP for regeneration.

The 44-kDa cylcopropane synthase protein is metabolically unstable, but protein levels peak sharply due to increased *cfa* transcription as cultures enter the stationary phase. Cfa levels drop in late-stationary-phase cultures as the enzyme is destroyed by proteolysis, probably by a protease of the heat-shock response. The *cfa* gene possesses two promoters of approximately equal strengths, with the more distal promoter functioning throughout the growth cycle. The proximal promoter requires the specialized sigma factor, σ^S (encoded by *rpoS*), for transcription, and is thus active only as cultures enter stationary phase. Indeed, the cyclopropane fatty acid content of *rpoS* strains is low and transcription from the proximal promoter is absent in these strains. As cells remain in stationary phase and phospholipid biosynthesis ceases, the low levels of Cfa that do persist no longer encounter an expanding substrate pool. Thus, increasing amounts of the fatty acyl chains are converted to their cyclopropane derivatives over time. The instability of Cfa results in little carryover of synthetic capacity when exponential growth resumes, and the existing cyclopropane fatty acids are quickly diluted by de novo phospholipid synthesis.

6. Lipid A biosynthesis

LPSs form the majority of the outermost leaflet of the membrane in most gram-negative bacteria (Fig. 4 in Chapter 1) and display a tremendous amount of structural variability. Their biosynthesis has been reviewed in detail [16]. LPS is essential to the growth of gram-negative bacteria and provides an effective hydrophobic barrier to toxic compounds. LPSs are composed of three components: the O-antigen, a core polysaccharide, and the lipid A. The O-antigen is a polysaccharide that extends from the cell surface. O-antigens are constructed from 10 to 30 repeats of specific β-6 sugar oligosaccahride units, and each is essentially unique to a given serotype of bacteria. The O-antigen is linked to the core polysaccharide region, which is common to groups of bacteria. The membrane-associated portion of LPS is lipid A. The core polysaccharide is attached to lipid A by a 2-keto-β-deoxyoctonate (KDO) disaccharide. Lipid A anchors the LPS to the outer membrane and functions as an endotoxin and a mitogen during bacterial infections. The lipid A is synthesized and ligated to the oligosaccharide core on the cytoplasmic face of the inner membrane, while the O-antigen is added in the periplasm. O-antigen is not essential for the viability of *E. coli*, and is in fact missing from *E. coli* K12, making it avirulent and safe for laboratory use. Details of the synthesis of the O-antigen and core region are outside the scope of this discussion and can be found in a review by Raetz [16].

The pathway of lipid A synthesis has been determined mainly in the laboratory of Raetz (Fig. 7) (C.R. Raetz, 2002). The first step in lipid A synthesis is the reversible transfer of the β-hydroxymyristoyl group from ACP to UDP–*N*-acetylglucosamine (UDP–GlcNAc) by the UDP–GlcNAc acyltransferase (*lpxA*). Competition between LpxA and the FabZ dehydratase of fatty acid synthesis helps to determine the rate of lipid A synthesis. The second step in the pathway is the deacetylation of UDP–*O*-acyl-GlcNAc by the zinc-dependent 34-kDa UDP–*O*-acyl-GlcNAc deacetylase (*lpxC*). The *lpxC* gene was first described as *envA*, an essential gene involved in envelope production. Mutations in *lpxC* cause a plethora of effects, including increased sensitivity to antibiotics, increased dye permeability, and defects in cell division. Null mutations in *lpxC* are lethal. The LpxA-catalyzed acyltransfer step is thermodynamically unfavorable, thus the irreversible deacetylase reaction is the first committed step in the pathway. The essential nature of LpxC has driven the development of a novel group of anti-microbial compounds active against this step (Section 12). These compounds are effective against a wide range of gram-negative bacteria in vivo.

The third step is a second β-hydroxymyristoyl-ACP acyltransferase catalyzed by UDP–β-*O*-[β-hydroxymyristoyl] GlcN acyltransferase (*lpxD*). Like the LpxA acyltransferase, the 36-kDa LpxD possesses repeating hexapeptide units and will presumably have a fold and trimeric structure similar to LpxA. The product of the LpxD reaction is UDP-2,3-diacyl-GlcN. UMP is removed from this compound to form 2,3-diacyl-GlcN-1-phosphate (lipid X), but the enzyme responsible for this reaction is not known. There is an approximately 10-fold excess of lipid X over UDP–2,3-diacyl-GlcN in wild-type cells. Mutations in *lpxB* cause a 500-fold increase in the amount of lipid X in the membrane, although LpxB is not a pyrophosphatase. LpxB, a dimer of 42-kDa monomers, catalyzes the condensation of lipid X with UDP–2,3-diacyl-GlcN to form the lipid A-disaccharide-1-phosphate. *lpxB* is present in a complex cluster with *lpxA*, *lpxD*, and *fabZ*, and other genes encoding enzymes of phospholipid synthesis, outer membrane proteins, DNA synthesis, and fatty acid synthesis.

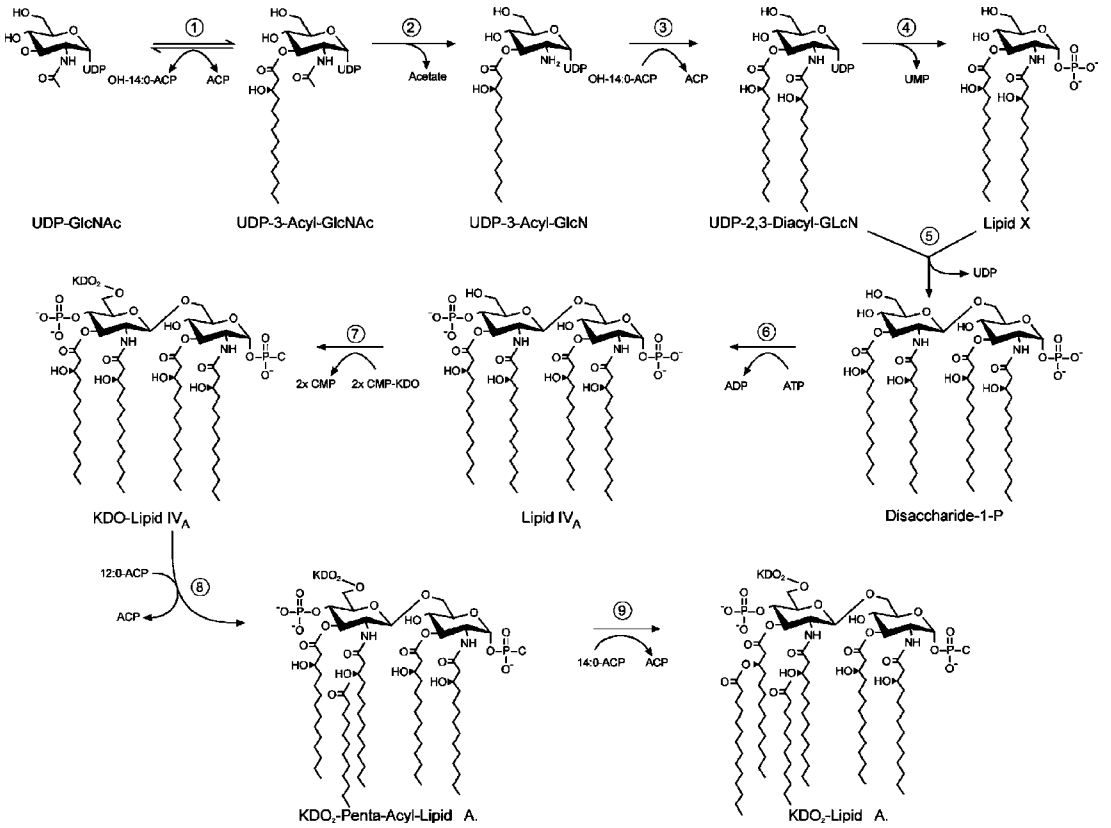

Fig. 7. Biosynthesis of endotoxin in *E. coli*. The first step (1) in the pathway is catalyzed by UDP–*N*-acetylglucosamine (UDP–GlcNAc) acyltransferase (LpxA). (2) The committed step is catalyzed by the LpxC deacetylase, followed by (3) a second acyltransferase (LpxD). (4) Lipid X is generated by the removal of UMP from UDP–2,3-diacyl-GlcN by an unknown enzyme. (5) Lipid X and UDP–2,3-diacyl-GlcN are then condensed together by LpxD to form Lipid IV$_A$. (6) A 4′-kinase phosphorylates the disaccharide to produce lipid IV$_A$. (7) Two consecutive additions of KDO by KdtA, and two *O*-acylations by (8) HtrB and (9) MsbB yield KDO$_2$–lipid A. Subsequent addition of core sugars and O-antigen chains (not shown) yield the mature LPS.

Strains mutated in *lpxK* accumulate lipid A-disaccharide-1-phosphate, which led to the cloning and characterization of lipid A-disaccharide kinase, a Mg^{2+}-dependent 4′-kinase activity stimulated by Ptd_2Gro, that makes the key intermediate, lipid IV_A. The presence of the 4′-phosphate is essential for the recognition of lipid A by mammalian cells during endotoxin stimulation. Two subsequent additions of KDO are catalyzed by the KDO transferase, the 47-kDa KdtA protein. A single protein catalyzes both additions since overexpression of *kdtA* causes a large increase in both transfers. KDO transferase activity is essential for growth, and conditional mutants accumulate massive amounts of lipid IV_A prior to cessation of growth at the non-permissive temperature.

In the final steps of lipid A synthesis, two fatty acids are transferred to the hydroxyl groups of the β-hydroxymyristoyl group on the distal unit. The first of these is usually laurate (12 carbons) added from lauroyl-ACP by the enzyme HtrB, and then MsbB adds myristate from myristoyl-ACP. During cold shock (on shifting *E. coli* from 30 to 12°C), palmitoleate appears in the lipid A at the expense of laurate. A novel gene, *lpxP*, is induced for this reaction, and null mutants in HtrB are not defective in this adaptation. Double null *htrB msbB* mutants can also be generated. Thus, the extensive acylation of the lipid A is not absolutely required for its proper insertion into the outer membrane or the formation of a hydrophobic barrier. There are numerous variations on the acylation and glycosylation scheme of LPS in gram-negative bacteria (for review see Ref. [17]).

7. Phospholipid flippase

All of the enzymes of phospholipid and lipid A biosynthesis are either cytosolic or located on the inner aspect of the inner membrane. How then do the lipids get to the outer face of the inner membrane, or to the outer membrane? The product of the *msbA* gene is a lipid translocator responsible for the movement of LPS precursors and phospholipid to the outer membrane (A. Polissi, 1996; Z. Zhou, 1998). A temperature-sensitive mutant, *msbA*(Ts), with a A270T substitution in the MsbA protein, shows a rapid and dramatic reduction in the export of all major lipid classes, including PE and core-lipid A, to the outer membrane. Lipid export is inhibited by >90% after 30 min at the non-permissive temperature, while protein transport is not affected. *E. coli* harboring this mutation possesses duplicated inner membranes at the elevated temperature. Null mutations in *msbA* are lethal, and this is the only bacterial transporter that has been shown to be essential. The MsbA protein is a member of the ATP-binding cassette (ABC) family of transporters, and has most similarity to mammalian P-glycoprotein multiple-drug resistance ABC transporters (>30% identity). MsbA exists as a homodimer of 64.6-kDa monomers, each of which has a single membrane-spanning region (composed of six transmembrane helices) and a nucleotide-binding domain. This arrangement is distinct from the mammalian multiple-drug resistance pumps, which have two membrane-spanning regions and two nucleotide-binding domains fused into a single polypeptide. Thus, MsbA is a 'half-transporter', and is actually homologous to both amino- and carboxy-terminal halves of the mammalian multiple-drug resistance protein.

A second lipid flippase that moves lyso-PE across the inner membrane was recently discovered (E.M. Harvat, 2005). This protein belongs to the major facilitator superfamily

of enzymes, illustrating that all lipid transporters do not belong to a single class of transport proteins. This transporter does not require energy and equilibrates lyso-PE across the membrane. However, the net movement is to the inside of the cell because once internalized, the lyso-PE is rapidly converted to PE by the 2-acylglycerolphosphoethanolamine acyltransferase.

8. Degradation of fatty acids and phospholipids

8.1. β-Oxidation of fatty acids

8.1.1. Transport of fatty acids across the membrane

Exogenous long-chain fatty acids are utilized by *E. coli* in two ways. First, they can be incorporated into the membrane phospholipids by the acyltransferase system (PlsB/PlsC, but not PlsX/PlsY; Section 4). Second, they can be used as the sole carbon source for growth. The CoA thioester of the fatty acid is the substrate for both of these pathways. Fatty acids greater than 10 carbons in length require the *fadL* gene product to be taken up from the growth medium in sufficient quantities to support growth. FadL is a 46-kDa outer membrane protein produced following the cleavage of a 28-residue signal peptide from the propeptide. Fatty acid uptake is closely coupled to acyl-CoA formation since very low levels of free fatty acid are found in the cells. The acyl-CoA synthetase, encoded by *fadD*, is a homodimer of 62-kDa subunits and associates with the cytoplasmic leaflet of the inner membrane. Strains mutated in *fadD* cannot produce acyl-CoA and thus cannot grow on exogenous fatty acids, nor incorporate them into their membrane phospholipids. The esterification of the free fatty acid to CoA traps the fatty acid inside the cell, driving its transport across the inner membrane, and the net accumulation of fatty acid from the medium. Medium-chain fatty acids do not require FadL to enter the cells, and may traverse the outer membrane by passive diffusion. Fatty acids are also incorporated in some gram-positive bacteria, but these organisms do not contain a recognizable acyl-CoA synthetase gene. The metabolism of exogenous fatty acids in these organisms remains shrouded in mystery.

8.1.2. Degradation of fatty acids

Degradation of fatty acids proceeds via an inducible set of enzymes that catalyze the pathway of β-oxidation [18]. β-Oxidation occurs via repeated cycles of reactions that are essentially the reverse of the reactions of fatty acid synthesis (Fig. 8). However, three major differences distinguish the two pathways. First, β-oxidation utilizes acyl-CoA thioesters and not acyl-ACPs. Second, the β-hydroxy intermediates have the opposite stereochemistry (L in β-oxidation and D in synthesis). Finally, the enzymes of β-oxidation share no homology with those of synthesis.

The first step in the pathway is the dehydrogenation of acyl-CoA by the enzyme acyl-CoA dehydrogenase. While other organisms have several dehydrogenases with different chain-length specificities (i.e., for short, medium, or long acyl chains), it has been reported that *E. coli* has one enzyme active on all chain lengths. The dehydrogenase has been linked to two mutations in the 5-min region of the *E. coli* chromosome, *fadF* and *fadG*. However, the genome sequence suggests that a single gene in this region, *yahF*, encodes for a

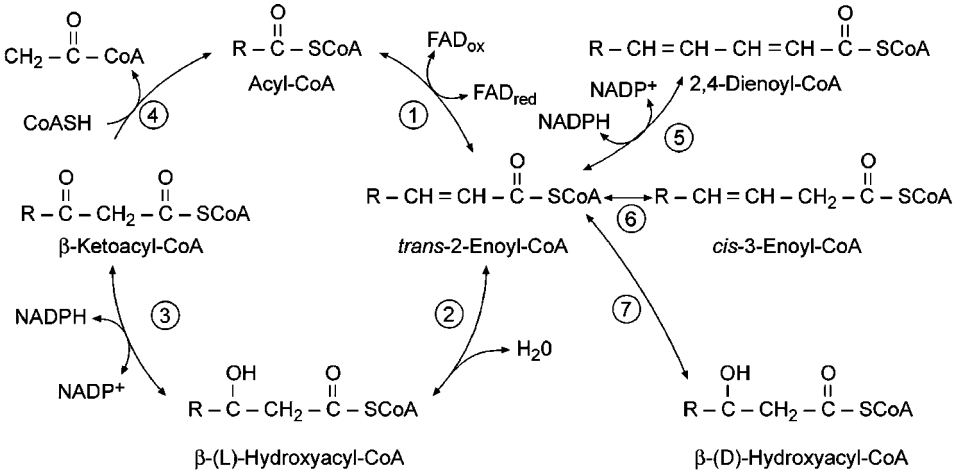

Fig. 8. β-Oxidation of fatty acids in *E. coli*. Long-chain fatty acids are transported into the cell by FadL and converted to their CoA thioesters by FadD (not shown). The acyl-CoAs are substrates for the (1) acyl-CoA dehydrogenase (YafH) to form a *trans*-2-enoyl-CoA. The double bond is reduced by (2) *trans*-2-enoyl-hydratase (crotonase) activity of FadB. The β-hydroxyacyl-CoA is then a substrate for the $NADP^+$-dependent dehydrogenase activity of FadB (3). A thiolase, FadA (4), releases acetyl-CoA from the β-ketoacyl-CoA to form an acyl-CoA for subsequent cycles. (5) Polyunsaturated fatty acyl-CoAs are reduced by the 2,4-dienoyl-CoA reductase (FadH). (6) FadB also catalyzes the isomerization of *cis*-unsaturated fatty acids to *trans*. (7) The epimerase activity of FadB converts *D*-β-hydroxy thioesters to their L-enantiomers via the *trans*-2-enoyl-CoA.

92-kDa dehydrogenases. Thus, *fadF* and *fadG* probably represent different mutations in the same YahF dehydrogenase and do not encode two distinct proteins. The acyl-CoA dehydrogenase is a flavoprotein and is linked to an electron-transferring flavoprotein (*fadE*). Further confusion exists in the literature as many acyl-CoA dehydrogenase (YafH) homologs are annotated as FadE homologs. Mutant strains of *E. coli* blocked in β-oxidation with *fadE* or *fadF* (*yahF*) mutations can accumulate acyl-CoA species, but cannot degrade them.

The second step in the cycle is enoyl-CoA hydratase, an activity commonly referred to as 'crotonase'. Traditionally, in vitro measurements of this activity utilize crotonoyl-CoA (*trans*-2-butenoyl-CoA) as the substrate. Crotonase activity in *E. coli* is one function present in a multi-functional protein encoded by *fadB*. The next step in the cycle is catalyzed by β-hydroxyacyl-CoA dehydrogenase, another function of the FadB enzyme. The β-ketoacyl-CoA produced in this reaction is a substrate for the monofunctional *fadA*-encoded β-ketoacyl thiolase, which cleaves acetyl-CoA from the β-ketoacyl-CoA to produce an acyl chain two carbons shorter than when it entered the cycle. The cycle is then repeated until the fatty acid is metabolized. The FadB protein is a homodimer of 78-kDa subunits and is purified in complex with the homodimeric 42-kDa *fadA* gene product. The total complex is thus an $\alpha_2\beta_2$ heterotetramer with an apparent mass of about 260 kDa. The *fadA* gene encodes the β-subunit, while the *fadB* gene gives the α-subunit, the confusing nomenclature a remnant of the days of classical genetics.

Unsaturated fatty acids can also be degraded by the β-oxidation pathway. The FadB protein possesses *cis*-β-enoyl-CoA isomerase activity, which converts *cis*-3 double bonds to *trans*-2 (Fig. 8). A 2,4-dienoyl-CoA reductase encoded by *fadH* is also required for the metabolism of polyunsaturated fatty acids (Fig. 8). This protein is a 73-kDa monomeric, $NADP^+$-dependent, 4Fe–4S flavoprotein. The FadH protein can utilize compounds with either *cis* or *trans* double bonds at the 4-position. An epimerase activity of FadB allows for the utilization of *D*-hydroxy fatty acids. The epimerase is actually a combination of a *D*-β-hydroxyacyl-CoA dehydratase and the crotonase (hydratase) activities, resulting in the conversion of the D to the L enantiomer (Fig. 8).

The substrate specificities of the enzyme complex in vitro suggest that all of the enzymes can utilize all chain lengths of substrates, with the possible exception of the crotonase activity. This function of FadB appears somewhat limited to short-chain substrates, and it has been suggested that a separate long-chain enoyl-CoA hydratase may exist in *E. coli*. Two open reading frames in the *E. coli* genome, discovered by bioinformatics, are predicted to encode homologs of FadA and FadB (Y.-M. Zhang, 2002). Thus, two complexes may be present with preferences for long- or short-chain acyl-CoAs.

Finally, there is also an anaerobic pathway for fatty acid degradation in *E. coli* that is shared by other gram-negative bacteria [19]. This system allows the utilization of fatty acids in the anaerobic intestinal environment.

8.2. Phospholipases

Based mainly on cell-free assays, 10 enzymatic activities that degrade phospholipids, intermediates in the phospholipid biosynthetic pathway, or triacylglycerol have been reported (Table 2). The detergent-resistant phospholipase A_1 (encoded by *pldA*) of the outer membrane, characterized by Nojima and colleagues (Y. Nakagawa, 1991), is the most studied of these enzymes. This enzyme is unusually resistant to inactivation by heat and ionic detergents

Table 2
Phospholipid degradative activities in *E. coli*

Enzyme	Gene	Location	Substrates
Phospholipase A1	*pldA*	Outer membrane	Phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and lyso derivatives
Phospholipase A		Cytoplasm	Phosphatidylglycerol
Lysophospholipase L2	*pldB*	Inner membrane	Lysophosphatidylethanolamine
Lysophospholipase		Cytoplasm	Lysophosphatidylethanolamine, lysophosphatidylglycerol
Phospholipase C		Unknown	Phosphatidylethanolamine
Phospholipase D		Cytoplasm	Cardiolipin
Phospholipase D		Cytoplasm	Phosphatidylserine
Lipase		Membrane	Triacylglycerol
CDP–diacylglycerol hydrolase		Inner membrane	CDP–diacylglycerol
Phosphatidic acid phosphatase		Membrane	Phosphatidic acid
Thioesterase I	*tesA*	Periplasm	Acyl-CoA
Thioesterase II	*tesB*	Cytoplasm	Acyl-CoA

and requires calcium for maximal activity. The mature phospholipase has a subunit molecular mass of 31 kDa. Hydrolysis of fatty acids from the 1-position of phospholipids is the most rapid reaction, but the enzyme will also hydrolyze 2-position fatty acids as well as both isomeric forms of lysophosphatides and mono- and di-acylglycerols. A detergent-sensitive phospholipase A_1 has also been described, although this activity has not been designated to a gene. This enzyme differs from the detergent-resistant protein in that it is located in the soluble fraction of the cell, is inactivated by heat and ionic detergents, and has a high degree of specificity for PtdGro. The cytoplasmic phospholipase A also requires calcium for activity. There are also inner membrane and cytoplasmic lysophospholipases. The best characterized of these is the inner membrane lysophospholipase L2 (*pldB*) which hydrolyzes 2-acyl-glycerophosphoethanolamine efficiently, but is barely active on the 1-acyl isomer. This lysophospholipase also catalyzes the transfer of fatty acids from 2-acyl-glycerophosphoethanolamine to PtdGro to form acyl-PtdGro.

The physiological role of these degradative enzymes remains largely unknown. Mutants lacking the detergent-resistant phospholipase (*pldA*), lysophospholipase L2 (*pldB*), or both enzymes do not have any obvious defects in growth, phospholipid composition, or turnover. Moreover, strains that overproduce the detergent-resistant enzyme also grow normally. It has been established that the detergent-resistant phospholipase is responsible for the release of fatty acids from phospholipids that occurs during infection with T4 and λ phages. However, phospholipid hydrolysis is not essential for the life cycle of these bacteriophages. One possible function for the hydrolytic activities with unassigned genes is that they are actually biosynthetic proteins (acyltransferases) that act as lipases in the absence of suitable acceptor molecules in the assay systems employed. An example of such an enzyme is PS synthase, which catalyzes both phospholipase D and CDP–diacylglycerol hydrolase reactions. PS synthase appears to function via a phosphatidyl–enzyme intermediate, and in the absence of a suitable acceptor such as serine or CMP, the phosphatidyl–enzyme complex is hydrolyzed by water; thus, the enzyme acts as a phospholipase D. Also, some of these enzyme activities may reflect a broad substrate specificity of a single enzyme rather than the presence of several distinct protein species. For example, the observed lipase activity that cleaves the 1-position fatty acids from triacylglycerols (a lipid usually not found in *E. coli*) may arise from the detergent-resistant phospholipase A_1 acting on triacylglycerol as an alternate substrate. Further, a lysophospholipase L1 activity has been attributed to thioesterase I. Three open reading frames (*ybaC*, *yhjY*, and *yiaL*) present in the *E. coli* genome potentially encode proteins with lipase activity, but have not been studied to date. Lipases and phospholipases are secreted by many pathogenic bacteria and are responsible for tissue damage and inflammation.

8.3. Thioesterases

Thioesterases preferentially cleave the thioester bond of acyl-CoA molecules to produce CoA and free fatty acid. *E. coli* contains two well-characterized thioesterases. Thioesterase I (encoded by *tesA*) is a periplasmic enzyme of 20.5 kDa, with a substrate specificity for acyl chains >12 carbon atoms. Thioesterase I hydrolyzes synthetic substrates used in the assay of chymotrypsin, which led to the initial conclusion that TesA was a protease ('protease I'). However, the purified protein does not cleave peptide bonds. Thioesterase

I also appears to possess lysophospholipase L1 activity. Thioesterase II is a cytosolic tetrameric protein composed of 32-kDa subunits encoded by the *tesB* gene. Thioesterase II cleaves acyl-CoAs of >6 carbons and β-hydroxyacyl-CoAs, but is unable to cleave acyl-pantetheine thioesters. The physiological function of thioesterases I and II is unknown. Null mutants have been constructed in both *tesA* and *tesB*, and the double-mutant strain generated. None of these strains has an observable growth phenotype, indicating that neither protein is essential. However, the *tesAB* double-null mutant still retains about 10% of the total wild-type thioesterase activity, indicating the existence of a third unidentified thioesterase in *E. coli*.

9. Phospholipid turnover

9.1. The diacylglycerol cycle

The polar head group of PtdGro is rapidly lost in a pulse-chase experiment, whereas that of PE is stable. The conversion of PtdGro to Ptd_2Gro, catalyzed by Ptd_2Gro synthase, does not account for all the loss of ^{32}P-labeled PtdGro observed in the pulse-chase experiments. A phosphate-containing non-lipid compound derived from the head group of PtdGro was sought, which led to the discovery of the linkage between MDOs and PtdGro metabolism by Kennedy's group (H. Schulman, 1977). These compounds are composed of *sn*-glycerol-1-phosphate (derived from PtdGro), glucose, and (usually) succinate moieties, and have molecular weights in the range of 4000–5000. They are found in the periplasm of gram-negative bacteria, an osmotically sensitive compartment. The synthesis of the MDO compounds is regulated by the osmotic pressure of the growth medium and decreased osmotic pressure gives an increased rate of MDO synthesis. Thus, MDO compounds seem to be involved in osmotic regulation.

In the synthesis of MDOs, the *sn*-glycerol-1-phosphate polar head group of PtdGro is transferred to the oligosaccharide, with 1,2-diacylglycerol as the other product (Fig. 9). The 85-kDa transmembrane protein that catalyzes this reaction is encoded by *mdoB*. Diacylglycerol kinase phosphorylates the diacylglycerol to phosphatidic acid, which re-enters the phospholipid biosynthetic pathway (Fig. 6) to complete the diacylglycerol cycle (Fig. 9). In the overall reaction, only the *sn*-glycerol-1-phosphate portion of the PtdGro molecule is consumed; the lipid portion of the molecule is recycled back into phospholipid. MDO synthesis is responsible for most of the metabolic instability of the polar group of PtdGro, since blocking MDO synthesis at the level of oligosaccharide synthesis by lack of UDP-glucose greatly reduces PtdGro turnover. Moreover, the rate of accumulation of diacylglycerol in strains lacking diacylglycerol kinase (*dgk*) correlates with the presence of both the oligosaccharide acceptor and the osmolarity of the growth medium. *dgk* is immediately upstream of *plsB* in *E. coli*. The protein is a trimer of identical 13-kDa subunits, each with three predicted transmembrane helices. Activity appears to be limited by diffusion of substrate across the membrane to the cytoplasmic active site. It should be noted that some species of MDOs contain phosphoethanolamine. Although direct proof is lacking, it is likely that the ethanolamine moiety is derived from PE, as this is the only known source of ethanolamine.

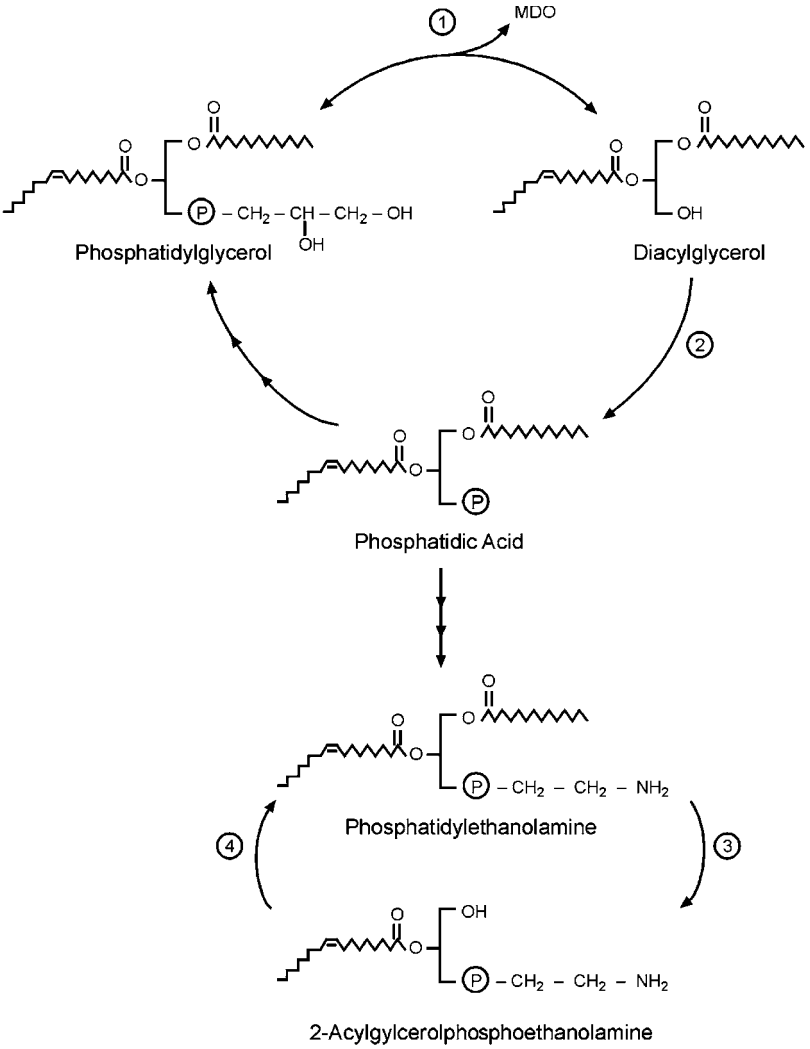

Fig. 9. Phospholipid turnover. The 1,2-diacylglycerol kinase cycle involves the (1) transfer of the *sn*-1-glycerol phosphate moiety from phosphatidylglycerol to MDO by the enzyme MdoB. (2) Diacylglycerol kinase converts the diacylglycerol to phosphatidic acid, which can regenerate the phosphatidylglycerol (see Fig. 6). Phosphatidylethanolamine cycling involves (3) the transfer of an acyl chain to membrane lipoprotein and (4) re-esterification of the 1-position by 2-acylglycerophosphoethanolamine (Aas).

There is an analogous diacylglycerol cycle operating in gram-positive bacteria. These organisms have a molecule called lipoteichoic acid that is formed by the addition of about 25 *sn*-1-glycerol phosphate moieties to the head group of glucosyldiacylglycerols. Like MDO synthesis, the glycerol phosphate is transferred from PtdGro and the resulting diacylglycerol is re-introduced into the biosynthetic pathway.

9.2. The 2-acylglycerophosphoethanolamine cycle

2-Acylglycerolphosphoethanolamine is a minor membrane lipid in *E. coli* generated from fatty acid transfer of the acyl moiety at the 1-position of PE to the outer membrane lipoprotein. The 2-Acylglycerolphosphoethanolamine acyltransferase is an inner membrane enzyme that esterifies the 1-position of 2-acylglycerolphosphoethanolamine utilizing acyl-ACP (and not acyl-CoA) as the acyl donor. The acyltransferase was first recognized as a protein called acyl-ACP synthetase that catalyzes the ligation of fatty acids to ACP, hence the gene designation *aas*. ACP acts as a bound subunit for accepting the acyl intermediate in the normal acyltransferase reaction, and high salt concentrations are required to dissociate the acyl-ACP intermediate from the enzyme in vitro. However, 2-acylglycerolphosphoethanolamine acyltransferase is the only reaction catalyzed by Aas in vivo. *aas* mutants are defective in both acyl-ACP synthetase and the 2-acylglycerolphosphoethanolamine acyltransferase activities in vitro. They do not accumulate 2-acylglycerolphosphoethanolamine in vivo unless they are also defective in the *pldB* gene which encodes a lysophospholipase that represents a second pathway for the 2-acylglycerolphosphoethanolamine metabolism. The acyl-ACP synthetase reaction has proven extremely valuable in the preparation of acyl-ACPs for use as substrates and inhibitors for the enzymes of fatty acid synthase. The 2-acylglycerolphosphoethanolamine is transported into the cell by an inner membrane lyso-PE flippase (Section 7).

10. Regulation of lipid metabolism

10.1. Regulation of fatty acid chain length

Fatty acyl chains in the membrane phospholipids of *E. coli* are normally 16 or 18 carbons in length. This specificity is a result of a combination of two factors: the poor reactivity of the β-ketoacyl-ACP synthases for longer chains and the high specificity of the acyltransferases for 16- and 18-carbon products. Overexpression of FabB leads to the overproduction of *cis*-vaccenate, which is incorporated into the membranes. Overexpression of FabH causes a decrease in the average fatty acid chain length and the appearance of significant amounts of myristic acid (14:0) in the phospholipids. This effect is attributed to an increased rate of fatty acid initiation, which leads to a deficiency in malonyl-ACP for the terminal elongation reactions. The fatty acid biosynthetic machinery has the capacity to produce longer-chain fatty acids. Under normal conditions, the 16- or 18-carbon chains are removed from the cytoplasm by the action of the acyltransferases. However, when phospholipid synthesis is blocked at the acyltransferase step, the fatty acids that accumulate have abnormally long chain lengths (e.g., 20 and 22 carbons). Conversely, overproduction of the acyltransferase results in a somewhat decreased average chain length, represented mainly by an increase in myristic acid. Thus, competition among the elongation synthases, the supply of malonyl-ACP, and the utilization of acyl-ACPs by the acyltransferase are the most significant determinants of fatty acid chain length. Any process that alters the competitive balance between these enzymes alters the chain-length composition, and this is most efficiently accomplished by altering the levels of one or more of the enzymes by transcriptional regulation (Section 10.3).

10.2. Temperature modulation of fatty acid composition

All organisms regulate the fluidity of their membranes to maintain a membrane bilayer in a largely fluid state. As temperatures are lowered, membranes undergo a reversible change from a fluid (disordered) to a non-fluid (ordered) state. In *E. coli*, the temperature of the transition point depends on the fatty acid composition of the membrane phospholipids [12]. At lower temperatures, the amount of *cis*-vaccenic acid is rapidly (within 30 s) increased due to the increased activity of FabF. Synthesis of mRNA and protein are not required. Mutants that lack FabF are unable to modulate their fatty acid composition in a temperature-dependent manner. Thus FabF, and not FabB, is involved in the thermal regulation of the fatty acid composition of the membranes.

Other bacteria use different systems to regulate fluidity. For example, gram-positive bacteria alter the ratio of iso- to anteiso-branched-chain fatty acids in response to temperature [20]. However, the biochemical mechanisms that govern this universal response to environmental temperature are largely unknown in most bacterial species.

10.3. Transcriptional regulation of the genes of fatty acid synthesis and degradation

The known genes of fatty acid synthesis are organized very differently in different bacteria and range from being either scattered along the genome or present in a single cluster. In *E. coli*, the organization is between these two extremes. There is an *accBC* operon and a *fab* cluster that contains some, but not all, of the genes. The *fab* cluster contains the *fabH*, *fabD*, *fabG*, *acpP*, and *fabF* genes and may have functional significance because Cronan has demonstrated that several genes are co-transcribed. *S. pneumoniae* is an example of the other extreme. All of the *fab* genes are located in a contiguous region of DNA and are co-regulated.

For balanced production of each member of the ACC complex, one might expect each gene to be regulated in the same manner. However, while transcription of all four *acc* genes is under growth rate control, with the rate of transcription decreasing with decreased growth rate, the *accBC* operon seems to be regulated by a mechanism that differs from the regulation of the *accA* and *accD* genes (S.-J. Li, 1993). The *accBC* operon is transcribed from a promoter located unusually far upstream of the *accB* gene. The major *accA* promoter lies within the coding sequence of the upstream *polC* (*dnaE)* gene, although transcription through *polC* and perhaps other upstream genes also reads through the *accA* sequence. The *accD* gene is transcribed from a promoter located within the upstream *dedA* gene.

Transcriptional regulation of the other genes of fatty acid synthesis is no less complicated. The FadR protein, which was first identified as a repressor of transcription of genes in the *fad* regulon of β-oxidation and fatty acid transport, also positively regulates *fabA* transcription [21]. FadR binds to DNA in the absence of acyl-CoA to repress the β-oxidation regulon and activate *fabA*. Acyl-CoA (formed from exogenous fatty acids transported into the cell) binds FadR and the protein is released from the DNA. The molecular details of these interactions have been examined by the crystallization of FadR and the FadR·acyl-CoA and FadR·DNA complexes (D.M. van Aalten, 2001; Y. Xu, 2001). Whether FadR activates or represses transcription depends on the location of its binding site within the promoter region. For repression, FadR binds in the –30 to +10 region of the promoter and prevents binding of DNA polymerase. For activation, the FadR operator site is located in

a 17-bp region at –40 of the *fabA* promoter, and FadR binding promotes DNA polymerase binding. In *fadR* null mutants, the *fabA* gene is transcribed from two weak promoters of about equal strength, whereas in wild-type strains a 20-fold increase in transcription from the proximal promoter is seen in the absence of acyl-CoA. Thus, FadR monitors the intracellular concentration of acyl-CoA and coordinately regulates fatty acid synthesis and β-oxidation in response to these compounds.

The *fabB* gene, encoding β-ketoacyl-ACP synthase I, possesses a nucleotide sequence in its promoter region that functions as a combinatorial lock that controls the expression of this key component. There is a FadR site which, like in *fabA*, functions as a transcriptional activator. A transcriptional repressor, termed FabR, has a binding site located downstream of the FadR site and is the most important controller of *fabB* expression. A major gap in our knowledge is that we do not know the ligand that regulates FabR binding and thus do not understand the physiological signals that govern *fabB* expression.

Transcriptional regulation of the *fab* genes in gram-positive bacteria is quite diverse. In *B. subtilis*, the transcriptional repressor is called FapR and it controls all of the *fab* genes which are dispersed through the genome [22]. DNA binding of FapR is regulated by malonyl-CoA to increase the expression of the *fab* genes when the concentration of malonyl-CoA increases. The *fab* gene cluster in *S. pneumoniae* is controlled by a transcription factor called FabT. Whereas all of the transcriptional regulators discussed above belong to the TetR protein superfamily, FabT belongs to a completely different group of helix-turn-helix regulators (the MarR family). FabT acts as a repressor of all the genes in the *fab* cluster by binding to two locations. One site is upstream of the *acpP* gene and the other downstream of *acpP*. The ligand that controls the binding of FabT to DNA is unknown.

10.4. Regulation of phospholipid head-group composition

Within a particular strain of *E. coli*, the phospholipid ratio (PE:PtdGro:Ptd_2Gro) is maintained under a variety of growth rates and conditions. The exception to this is the increased conversion of PtdGro to Ptd_2Gro during the stationary phase (Section 5.4). Thus, a mechanism must exist to maintain phospholipid homeostasis. Regulatory mutants resulting in the overexpression of PS synthase (*pssR*) and diacylglycerol kinase (*dgkR*) have been identified, suggesting the existence of *trans* acting factors that control the expression of these key enzymes, but their significance is unclear. The hypothetical 32-kDa PssR protein is similar to the LysR family of transcriptional regulators. Overexpression of PS synthase, PtdGroP synthase, or Ptd_2Gro synthase in plasmid-based systems does not lead to dramatic changes in the membrane phospholipid composition. Thus, modulation of protein level is unlikely to have a role in the regulatory scheme.

So, how is phospholipid homeostasis maintained? Control of the individual enzymes at the level of activity by feedback regulation appears a more probable mechanism. Perturbations in the ratio of phospholipids were attempted experimentally by the activation of phosphoglycerol transferase I (*mdoB*). This enzyme, involved in MDO synthesis, catalyzes the transfer of glycerol phosphate from PtdGro to the extracellular arbutin (4-hydroxyphenyl-*O*-β-D-glucoside). Treatment with arbutin (an MDO substrate analog) causes a 7-fold increase in the rate of PtdGro synthesis without a concomitant increase in PtdGroP synthase proteins or significant changes in membrane phospholipid composition. Thus, PS synthase

and PtdGroP synthase are independently regulated by phospholipid composition. Similarly, purified Ptd_2Gro synthase is strongly feedback inhibited by Ptd_2Gro, and this inhibition is partially relieved by PE. Thus, the regulation of phospholipid content in *E. coli* appears to be an intrinsic property of the enzymes.

10.5. Coordinate regulation of fatty acid and phospholipid synthesis with macromolecular biosynthesis

Fatty acid biosynthesis is coordinately regulated with phospholipid synthesis. Labeling the ACP moiety of the fatty acid intermediates by growth of a *panD* strain on medium containing tritiated β-alanine, a precursor of the 4′-phosphopantetheine ACP prosthetic group, shows that long-chain acyl-ACPs accumulate for a short period following the cessation of phospholipid synthesis. This accumulation does not continue indefinitely, however, and reaches a plateau after about 20 min following inhibition of phospholipid synthesis. Thus, de novo fatty acid synthesis ceases, probably by a feedback inhibition mechanism involving long-chain acyl-ACPs inhibiting early steps in the fatty acid biosynthesis pathway (Fig. 10). A significant finding in support of this idea is that overexpression of a thioesterase (which prevents the accumulation of acyl-ACP by cleavage of the thioester linkage and releases the acyl chain) allows continued fatty acid synthesis following cessation of phospholipid synthesis. This further suggests that acyl-ACP and not free fatty acids mediate the regulation. A reduction in total ACP is not responsible for the inhibition of fatty acid synthesis, since the free ACP pools of the glycerol-starved *plsB* mutants are not significantly depleted, and overproduction of ACP fails to relieve inhibition of fatty acid synthesis. A *fadD* mutant strain, which cannot produce acyl-CoA, overexpressing a thioesterase gave the same results

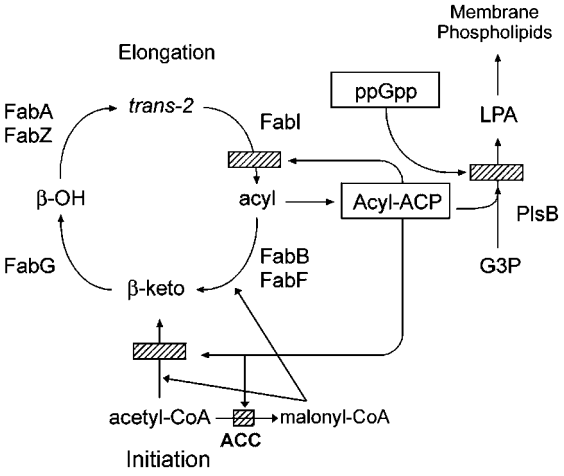

Fig. 10. Coordinate regulation of fatty acid and phospholipid metabolism. The pleiotropic regulator ppGpp regulates transfer of fatty acids to the membrane via inhibition of the PlsB acyltransferase step, coordinating phospholipid synthesis with macromolecular synthesis. PlsB inhibition leads to the accumulation of long-chain acyl-ACPs that feedback inhibit their own synthesis at the point of initiation (inhibition of acetyl-CoA carboxylase and FabH) and elongation, by inhibition of FabI. LPA, lysophosphatidic acid; G3P, glycerol-3-phosphate.

as strains blocked elsewhere in β-oxidation or wild-type strains, thus ruling out a role for acyl-CoA.

There are several target enzymes for acyl-ACP feedback inhibition (Fig. 10). ACC is an obvious target and indeed long-chain acyl-ACP inhibits the activity of this enzyme. Because malonyl-CoA is required for both initiation and elongation of fatty acids, a blockade at this step is very effective. Acyl-ACP also inhibits FabH, which catalyzes the first step in the pathway. Inhibition of this enzyme would halt initiation of new acyl chains, but would allow the elongation of existing fatty acid intermediates. Inhibition of FabH by physiologically relevant concentrations of long-chain acyl-ACPs has been demonstrated in vitro. Finally, enoyl-ACP reductase (FabI) is an acyl-ACP target and is important because the activity of this enzyme is a determining factor in completing rounds of fatty acid elongation. Acyl-ACP acts as a product inhibitor of FabI. The relative contributions of each of these regulatory points to the control of fatty acid synthesis in vivo is not clear, but it is likely that they all contribute to the cessation of acetate incorporation following the accumulation of acyl-ACP.

Fatty acid synthesis in *E. coli* is also regulated by an unusual nucleotide, guanosine 5′-diphosphate-3′-diphosphate (ppGpp) [23]. Wild-type strains of *E. coli* undergo the so-called 'stringent response' following starvation for a required amino acid, an effect also mediated by increased intracellular ppGpp. Increased levels of ppGpp cause a strong inhibition of stable RNA synthesis and inhibition of protein and phospholipid synthesis. Mutant strains (*relA*) do not undergo the stringent response following amino acid starvation, due to the lack of ppGpp synthase I, a ribosomal protein that produces pppGpp in response to uncharged tRNA. The interaction of ppGpp with RNA polymerase mediates the inhibitory effects on stable RNA synthesis. ppGpp directly inhibits phospholipid biosynthesis by inhibition of the PlsB and causes an accumulation of long-chain acyl-ACPs, which in turn lead to the inhibition of fatty acid biosynthesis. Overexpression of the acyltransferase relieves the inhibition on both fatty acid and phospholipid synthesis. These events are regulated by ppGpp formation by ppGpp synthase II, or SpoT. Intriguingly, a direct interaction occurs between ACP and SpoT that is likely to play a key role in regulating ppGpp production in response to the status of fatty acid biosynthesis. However, there are many biochemical details that need to be worked out to establish the mechanism for this regulation.

11. Lipid metabolism in other bacteria

11.1. Analysis of lipid metabolism by genomic inference

The availability of genomic sequences for a variety of bacteria allows for the rapid assessment of the lipid metabolic pathways present. At the time of writing, there are over 400 genomic sequences available on the web, and by the time this chapter is being read, another 100 or so will have appeared. This rich bioinformatic database is a powerful tool driving research into the diversity of bacteria. Bacterial diversity is truly mind-boggling, and this short review barely scratches the surface of what is known. Open reading frames encoding species-specific isoforms of known genes can be amplified by the polymerase chain reaction, the proteins expressed in *E. coli*, and the properties of the enzymes compared to the

E. coli or other known proteins. This approach has been used extensively in our laboratory for the genes of fatty acid synthesis, with isoforms from pathogenic bacteria being isolated to assess their unique biochemical characteristics and for use in drug screening programs. Subtle differences in cofactor specificity can be detected that are not obvious from the primary sequences.

Most importantly, novel proteins and new biochemical reactions have been identified using genomic information. The *fabI*-encoded enzyme is the sole enoyl-ACP reductase present in *E. coli*, and it was assumed that this was probably true for all bacteria. However, analysis of the genome of *S. pneumoniae* did not reveal a *fabI* homolog, while genes for all of the other enzymes required for saturated fatty acid biosynthesis were identified in a gene cluster. Thus, a novel enoyl-ACP reductase isoform was sought and the *fabK*-encoded enoyl-ACP reductase II identified [24]. The *fabK*-encoded protein possesses no homology to the FabI protein, utilizes flavin mononucleotide as a cofactor, and is resistant to inhibition by the antibiotic triclosan. The most interesting finding based on genomic analysis was the discovery of fatty acid acylphosphates as intermediates in lipid synthesis (Fig. 5) (Y.-J. Lu, 2006). New mechanisms for unsaturated fatty acid synthesis have also been uncovered using this approach. There will certainly be many new discoveries on the horizon as investigators continue to mine this rich resource. Other examples are the discovery of *fabM* and *fabN* (see Section 11.3).

11.2. Branched-chain fatty acid biosynthesis

Not all bacteria regulate membrane fluidity through the production of straight-chain unsaturated fatty acids. In fact gram-positive bacteria often use branched-chain fatty acids to modulate membrane fluidity [20]. The branch is a methyl group in the iso- or anteiso-position in the chain (i.e., the second or third carbon from the distal end of the chain). Based on the concept of acetyl-CoA as a primer for straight-chain fatty acid synthesis in *E. coli*, it can be seen that the methyl group could be introduced using a 'branched-chain' primer. Indeed, isotope labeling and biochemical analysis identify precursors of the branched-chain amino acids, valine, and isoleucine (isobutyryl-CoA or 2-methylvaleryl-CoA, respectively) as the primers for branched-chain fatty acid synthesis. As in straight-chain synthesis, the primer is condensed with malonyl-ACP by the action of FabH. The substrate specificities of the FabH enzyme(s) present in the bacteria determine the relative amounts of the respective fatty acids produced. *B. subtilis* contains a high proportion of branched-chain fatty acids in its membranes, and has two FabH enzymes, each of which prefers the branched-chain substrates over acetyl-CoA. *E. coli* FabH cannot use branched-chain primers. Why *B. subtilis* possesses two FabH enzymes, with only minor differences in substrate specificity, is not understood.

11.3. Other ways to make unsaturated fatty acids

The *fabA* gene encodes the dehydratase or isomerase specifically required for the production of unsaturated fatty acids in gram-negative bacteria. Gram-positive bacteria do not contain an identifiable *fabA* homolog in their genome, but do possess unsaturated fatty acids. In *Bacillus*, it has been shown that a cold shock-induced gene (*desA*) encodes a desaturase

that is active on the existing fatty acids present in membrane phospholipids. Desaturase activities are dependent on oxygen, and thus an aerobic lifestyle. This mechanism is analogous to that observed in plants (Chapter 4). *S. pneumoniae*, on the other hand, has a fatty acid profile similar to *E. coli*, but possesses neither an identifiable isomerase nor a desaturase. This bacteria uses *fabM* isomerase that is more related to the isomerase of fatty acid oxidation than *fabA* [25]. Members of the *Enterococcus* genus use *fabN*, a *fabZ*-like dehydratase that functions like a *fabA* dehydratase or isomerase [26]. Finally, *P. aeruginosa* has three ways to make unsaturated fatty acids [27]: the anaerobic FabA pathway, and two aerobic pathways — a phospholipid desaturase (DesA) and an acyl-CoA desaturase (DesB).

11.4. Bacteria with other phospholipid head groups

E. coli has a very simple phospholipid composition with just three major forms: PE, PtdGro, and Ptd_2Gro. However, the prokaryotic kingdom possesses a wide array of head groups that defy adequate description in this short space; hence, the reader is referred to Goldfine's review [28] for a more comprehensive treatment of bacterial phospholipid structures. The phosphocholine head group stands worthy of mention for its uniqueness and distinct mechanisms of synthesis. Phosphatidylcholine (PC) had long been considered a eukaryotic phospholipid, where it is synthesized by transfer of choline from CDP–choline to diacylglycerol, or by methylation of PE (Chapter 8).

Rhodopseudomonas spheroides, *Bradyrhizobium japonicum*, and a few other specialized photosynthetic or nitrogen-fixing bacteria synthesize PC by three subsequent methylations of PE. The first methyltransferase, encoded by *pmtA*, has been disrupted in *B. japonicum*, and the mutants, which contain significantly reduced PC, are less able to fix nitrogen in colonization assays (A.C. Minder, 2001). Thus, PC seems to be involved in host–bacteria interactions to establish symbiosis. The prokaryotic PE methyltransferases share weak homology with other bacterial methyltransferases, but no homology with their eukaryotic counterparts. *R. spheroides* are also somewhat unique among bacteria in that they contain intracellular membranes that hold the photosynthetic machinery. The amount of the intracellular membrane correlates to the amount of incident light, indicating a light-specific regulation of phospholipid synthesis in these organisms.

Sinorhizobium meliloti synthesizes PC by direct condensation of choline with CDP–diacylglycerol as well as by the methyltransferase pathway. The *pcs* gene was identified and expression in *E. coli* demonstrates that it does code for a PC synthase [29]. The genomes of *P. aeruginosa* and *Borrelia burgdorferi* contain similar genes and have been reported to possess PC in their membranes. The PC synthase protein shares weak homology with PS synthase (a CDP–diacylglycerol:serine *O*-phosphatidyltransferase) from other bacteria, but not with any eukaryotic proteins.

A genus of bacteria, termed the *Sphingobacterium*, produces sphingolipids by a pathway similar to that in mammals. Clostridia produce plasmalogens (1-alk-1′-enyl lipids) by an anaerobic pathway clearly different from the O_2-dependent pathway in mammals (Chapter 9). Branched-chain fatty acids are also found in which the methyl group is inserted post-synthetically into the middle of the chain, in a manner analogous to cyclopropane fatty acid synthesis (Section 5.5). *S*-adenosylmethionine is also the methyl donor for these reactions. The biochemistry surrounding the formation of these and many other bacterial phospholipids remains to be elucidated.

11.5. Bacteria with a type I fatty acid synthase

A general distinction between prokaryotic and eukaryotic fatty acid synthases is that bacteria possess the dissociated enzymes described above (type II), while higher organisms have a single, multi-functional, protein (type I) that catalyzes all of the reactions. There are exceptions to this rule, however. Mycobacteria, for example, possess a type I fatty acid synthase for the production of their membrane fatty acids. This enzyme is a homohexamer of 290-kDa subunits. Each subunit possesses the six different active sites required to generate a fatty acid. Unlike the type II system, the products of a type I enzyme are acyl-CoAs. For the mycobacterial enzyme, the saturated acyl chains produced are between 16 and 24 carbons in length. Even more unusual is that the *Mycobacterium* possesses a type II synthase system for the further elongation of the fatty acyl products of the type I system into the 70–80 carbon mycolic acids. *Brevibacterium ammoniagenes*, a highly developed bacterium thought to be a progenitor of the fungi, possesses a type I fatty acid synthase that is capable of producing both saturated and unsaturated fatty acids anaerobically.

11.6. Lipid synthesis in Archaea

Archaea are a group of organisms, previously classified as bacteria, from which eubacteria and other life may have evolved. A melavonic acid (six carbon)-building block is used for synthesis instead of acetic acid. The generated phytanyl chains are attached to glycerol moieties of complex lipids by ether linkages. Thus, these lipids are unlike anything found in eubacteria or eukaryotes today.

11.7. Other organisms with a bacteria-like fatty acid synthase system

The dissociated enzymes that form the fatty acid synthesis system of most bacteria are not limited to the prokaryotic kingdom [2]. Plants utilize a homologous series of enzymes for synthesis of their fatty acids (Chapter 4). Although the genes are present on the nuclear chromosomes, fatty acid biosynthesis occurs in the chloroplasts of plants. It is thus hypothesized that the pathway evolved from the endosymbiont bacteria that became the chloroplast in plants. Recently, it has also been shown that the apicomplexans, a group of intracellular parasites including *Plasmodium falciparum*, the causative organism of malaria, possess a bacterial-like fatty acid synthase system [30]. These eukaryotic organisms possess an organelle described as a vestigial chloroplast (the apiplast), and thus are presumably evolved from chloroplast-containing algae. Plasmodium is sensitive to the antibiotics thiolactomycin and triclosan, indicating that this pathway could be exploited for the development of novel anti-malaria drugs.

12. Inhibitors of lipid metabolism

Although the reactions catalyzed by bacterial type II system and the multi-functional type I synthase found in mammals are the same, there exist important structural differences in the enzymes that allow the identification of inhibitors that selectively target the bacterial system (for review see Ref. [7]). The individual genes of bacterial fatty acid synthesis have

all been cloned from the model organism *E. coli* as well as from various other human pathogens. Also, high-resolution structures of all members of the pathway are now known [8], and this facilitates the structure-based design of new inhibitory compounds. Most of the *fab* enzymes are essential for bacterial viability, and are thus in principle suitable targets for anti-bacterial drug discovery. Furthermore, there are a number of different natural products identified that target the elongation condensing enzymes, illustrating that evolution has selected fatty acid biosynthesis as a viable target for antibiotics. The emergence of multi-drug-resistant pathogens has spurred the investigation of new targets for anti-bacterial drug discovery and led to an explosion of research in this area that is too large in scope to adequately cover in this review. Readers are referred to a recent review that highlights progress in the field, and evaluates the suitability and future prospects for each pathway step as a drug discovery target [7].

13. Future directions

Considerable progress has been made over the last several years in elucidating the details of the enzymes of fatty acid biosynthesis. This pathway has become a major target for therapeutic intervention in bacteria-mediated disease. Significant inroads have also been made into the molecular mechanisms that regulate fatty acid synthesis. The availability of genomic data has allowed the facile translation of many of the findings made in *E. coli* to other bacteria facilitating the discovery of novel genes and unique biochemistry involved in lipid biosynthesis.

Many of the details of the regulation of fatty acid biosynthesis are still to be elucidated, including the discovery of new transcription factors and the identification of the effector molecules that control the activity of the known factors. The fine details of the enzymatic mechanisms, and the comparison of the biochemical properties and functions of different isoforms, will continue in an effort, in part, to elucidate probable activity spectra of next-generation antibiotics that will surely be generated against this pathway. The rush to study fatty acid synthesis has somewhat overshadowed bacterial phospholipid synthesis in recent years, and discoveries in this area are more difficult since the enzymes and substrates involved are membrane-associated. However, techniques to study the structure and function of membrane proteins are rapidly evolving and will certainly be applied to resolving outstanding issues in bacterial lipid biogenesis.

Abbreviations

ABC	ATP-binding cassette
ACP	acyl carrier protein
BCCP	biotin carboxyl carrier protein
CoA	coenzyme A
FabA, FabA	lowercase italics indicates gene, while uppercase Roman type indicates the protein product of the gene
KDO	2-keto-β-deoxyoctonate

LPS	lipopolysaccharide
MDOs	membrane-derived oligosaccharides
PE	phosphatidylethanolamine
PS	phosphatidylserine
Ptd_2Gro	cardiolipin
PtdGro	phosphatidylglycerol
PtdGroP	phosphatidylglycerol phosphate

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CHAPTER 4

Lipid metabolism in plants

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Contents

1. Introduction

Plants produce the majority of the world's lipids, and most animals, including humans, depend on these lipids as a major source of calories and essential fatty acids. Like other eukaryotes, plants require lipids for membrane biogenesis, as signal molecules, and as a form of stored carbon and energy. In addition, leaves and other aerial surfaces, bark, herbaceous shoots, and roots each have distinctive protective lipids that help prevent desiccation and infection. To what extent does the biochemistry of plant lipid metabolism resemble that in other organisms? This chapter mentions a number of similarities, but emphasizes aspects unique to plants. Major differences between lipid metabolism in plants and other organisms are summarized in Table 1.

The presence of chloroplasts and related organelles in plants has a profound effect on both gross lipid composition and the flow of lipid within the cell. Fatty acid synthesis occurs not in the cytosol as in animals and fungi, but in the chloroplast and other plastids. Acyl groups must then be distributed to multiple compartments, and the complex interactions between alternative pathways are a major focus of plant lipid biochemists. It is also significant that the lipid bilayers of chloroplasts are largely composed of galactolipids rather than phospholipids. As a result, galactolipids are the most abundant acyl lipids in green tissues and probably on earth.

Plant lipids also have a substantial impact on the world economy and human nutrition. More than three-quarters of the edible and industrial oils marketed annually are derived from seed and fruit triacylglycerols (TGs). These figures are particularly impressive given that, on a whole organism basis, plants store more carbon as carbohydrate than as lipid.

Table 1
Comparison of plant, mammalian, and bacterial lipid metabolism

	Higher Plants	Mammals	*E. coli*
Fatty acid synthase			
Structure	Type II (multicomponent)	Type I (multifunctional)	Type II (multicomponent)
Location	Plastids	Cytosol	Cytosol
Acetyl-CoA carboxylase(s)	Multisubunit and multifunctional	Multifunctional	Multisubunit
Primary desaturase substrates			
Δ^9	18:0-ACP	18:0-CoA	None
ω^6	18:1 on glycerolipids	None	None
ω^3	18:2 on glycerolipids	None	None
Primary substrate(s) for phosphatidic acid synthesis	Acyl-ACP and Acyl-CoA	Acyl-CoA	Acyl-ACP
Prominent bilayer lipids	Galactolipid > phospholipid	Phospholipid	Phospholipid
Main β-oxidation function	Provides acetyl-CoA for glyoxylate cycle	Provides acetyl-CoA for TCA cycle	Provides acetyl-CoA for TCA cycle
Major sterols	Sitosterol and others via cycloartenol	Cholesterol via lanosterol	None

Since plants are not mobile, and since photosynthesis provides fixed carbon on a regular basis, plant requirements for storage lipid as an efficient, light weight energy reserve are less acute than those of animals.

Finally, hundreds of genes required for plant lipid biosynthesis, utilization, and turnover have now been cloned. In addition to providing valuable information on enzyme structure and function, these genes are being exploited to design new, more valuable plant oils. The coordination of lipid metabolic genes with each other and with their potential regulators is now becoming better understood, as DNA microarray and other genomic technologies mature [1].

2. Plant lipid geography

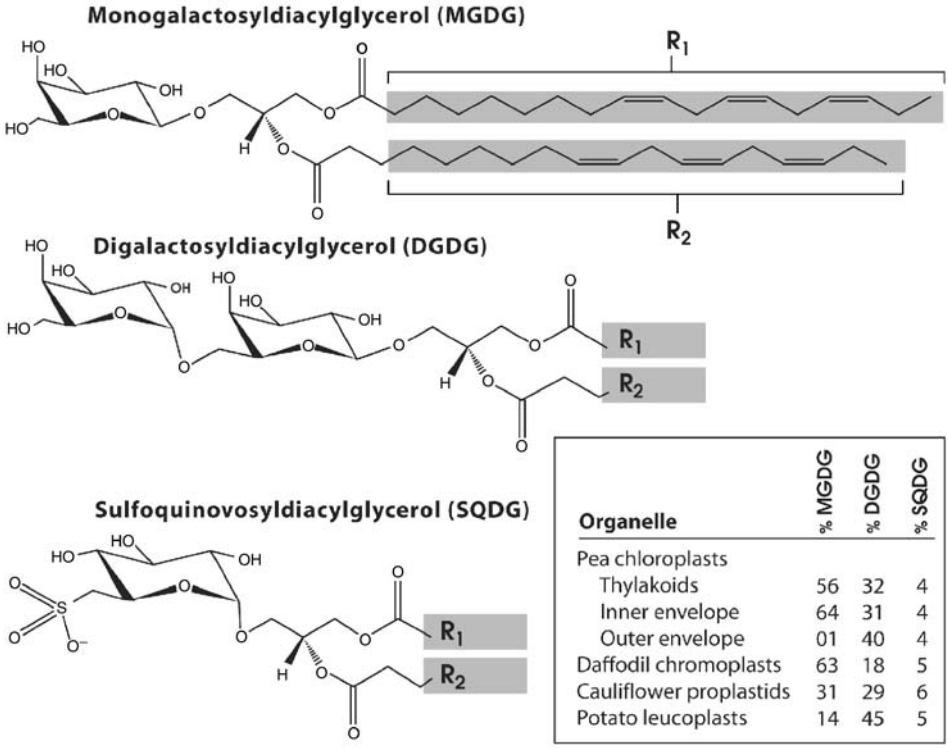
Although all eukaryotic cells have much in common, the ultrastructure of a plant cell differs from that of the typical mammalian cell in three major ways. First, all living plant cells contain plastids. Second, the plasma membrane of plant cells is shielded by the cellulosic cell wall, preventing lysis in the naturally hypotonic environment but making preparation of cell fractions more difficult. Finally, the nucleus, cytosol, and organelles are pressed against the cell wall by the tonoplast, the membrane of the large, central vacuole that can occupy 80% or more of the cell's volume.

2.1. Plastids

The plastids are a family of organelles containing a circular chromosome present in multiple copies. Young or undifferentiated cells contain tiny proplastids that, depending on the tissue, may differentiate into photosynthetic chloroplasts, carotenoid-rich chromoplasts, or any of several varieties of colorless leucoplasts, including plastids specialized for starch storage [2]. These different types of plastids, which may be interconverted in vivo, have varying amounts of internal membrane but invariably are bounded by two membranes. The internal structure of chloroplasts is dominated by the flattened green membrane sacks known as thylakoids. The thylakoid membranes contain chlorophyll and are the site of the major energy capturing light reactions of photosynthesis.

As noted above, chloroplasts and other plastids are enriched in galactolipids (Fig. 1). They also contain a unique sulfolipid, sulfoquinovosyldiacylglycerol, whose head group is a modified galactose. The phospholipid components of plastids are less abundant. Phosphatidylglycerol, the most prominent phospholipid contributor to the thylakoid membrane system, comprises less than 10% of chloroplast glycerolipids, while plastidial phosphatidylcholine is limited primarily to the organelle's outer membrane.

Representatives of each type of plastid have been isolated and found to incorporate acetate into long-chain fatty acids, to desaturate 18:0 to 18:1, and to assemble phosphatidic acid and galactolipids. Chloroplasts have also been shown to synthesize phosphatidylglycerol, including molecular species containing the unusual *trans*-3-hexadecenoic acid at the 2-position. In addition to the components normally retained within the plastids, large quantities of fatty acids, particularly 18:1 and 16:0, are produced for export to the rest of the cell. An acyl-CoA synthetase on the outer membrane of plastids is thought to facilitate release of acyl groups into the cytosol. It should also be noted that, although net lipid



Organelle	% MGDG	% DGDG	% SQDG
Pea chloroplasts			
Thylakoids	56	32	4
Inner envelope	64	31	4
Outer envelope	01	40	4
Daffodil chromoplasts	63	18	5
Cauliflower proplastids	31	29	6
Potato leucoplasts	14	45	5

Fig. 1. Composition of plastid membranes. Figures given are percentages (percentage of total lipid) of the pictured lipid in the membranes specified. Data from Refs. [34,35].

traffic is from the plastids, this organelle can likewise be on the receiving end. In addition to small quantities of plastidial phospholipids whose head groups are not known to arise in that compartment, there may be considerable flow of extraplastidially constructed diacylglycerol backbones into the galactolipid synthesis pathway. The quantitative significance of this backflow depends on the plant species, as will be discussed in Section 5.3.

2.2. Endoplasmic reticulum and lipid bodies

The endoplasmic reticulum (ER) has traditionally been viewed as the primary source of phospholipids in plant cells. With the exception of cardiolipin, all of the common phospholipids can be produced by microsomal fractions. The ER also serves as the major site of fatty acid diversification. Although plastids do have the ability to synthesize polyunsaturated fatty acids, they are formed on acyl lipid substrates and are not typically exported. Thus, the ER desaturation pathways are of particular importance for developing seeds that store large quantities of 18:2 and 18:3. Pathways for the production of unusual fatty acids found primarily in seed oils have likewise been described in microsomes. Not surprisingly, the ER also appears to be instrumental in the formation of TGs and the lipid bodies in which they are stored (Section 7).

2.3. Mitochondria

Next to plastids and the ER, the plant mitochondrion is probably the organelle investigated most thoroughly with respect to lipid metabolism. Its ability to synthesize phosphatidylglycerol and cardiolipin is well established, although cardiolipin can accumulate to normal levels in the absence of the mitochondrial phosphatidylglycerol phosphate synthase (E. Babiychuk, 2003). While most fatty acids for mitochondrial membranes are imported from the plastids or the ER, recently mitochondria have been shown to synthesize low levels of fatty acids from malonate. Octanoate is a major product of this pathway and serves as a precursor for the lipoic acid cofactor needed by glycine decarboxylase and pyruvate dehydrogenase [3] (Chapter 6).

2.4. Glyoxysomes and peroxisomes

A discussion of the compartmentation of lipids and their metabolism would be incomplete without reference to the organelles responsible for fatty acid oxidation. As in mammals, there is evidence for both mitochondrial and peroxisomal β-oxidation systems. In plants, the peroxisomal system appears to be the more significant [4]. Unlike mammals (Chapter 5), plants can use the peroxisomal enzymes to catabolize long-chain fatty acids all the way to acetyl-CoA. Under certain conditions, such as oilseed germination, plants also differentiate specialized peroxisomes called glyoxysomes. In addition to the β-oxidation pathway, glyoxysomes contain the enzymes of the glyoxylate cycle, a pathway absent from animals. Plants are able to use the glyoxylate cycle to feed the acetyl-CoA produced by β-oxidation into carbohydrate synthesis. Since plants cannot transport fatty acids over long distances, only this conversion of acetate to sucrose, which can be transported by the plant vascular system, makes lipid a practical carbon reserve for the growing shoots and roots of seedlings.

3. Acyl-ACP synthesis in plants

Fatty acid synthases may be classified into two groups. 'Type I' fatty acid synthases are characterized by the large, multifunctional proteins typical of yeast and mammals (Chapter 6), while 'Type II' synthases of most prokaryotes are dissociable into components that catalyze individual reactions (Chapter 3). Plants, while certainly eukaryotic, appear to have inherited a Type II fatty acid synthase from the photosynthetic prokaryotes from which plastids originated.

Early studies by Overath and Stumpf (P. Overath, 1964) established not only that the constituents of the avocado fatty acid synthesis system could be dissociated and reconstituted, but also that the heat stable fraction from *E. coli* known as acyl carrier protein (ACP) could replace the corresponding fraction from avocado. Plant ACPs share both extensive sequence homology and significant elements of three-dimensional structure with their bacterial counterparts. In plants, this small, acidic protein not only holds the growing acyl chain during fatty acid synthesis, but also is required for synthesis of monounsaturated fatty acids and plastidial glycerolipids.

3.1. Components of plant fatty acid synthase

Fatty acid synthase is generally defined as including all polypeptides required for the conversion of acetyl-CoA and malonyl-CoA to the corresponding ACP derivatives, the acyl-ACP elongation cycle diagrammed in Chapter 3, and the cleavage of ACP from completed fatty acids by enzymes termed thioesterases or acyl-ACP hydrolases [5]. All components of fatty acid synthase occur in plastids, although they are encoded in the nuclear genome and synthesized on cytosolic ribosomes. Most of the 8–10 enzymes of the pathway are soluble when isolated from homogenates. Nevertheless, ACP and some subunits of acetyl-CoA carboxylase (ACC) may be associated with the plastid membranes.

Despite the presence of acetyl-CoA:ACP acyltransferase activity in plant fatty acid synthase preparations, acetyl-ACP does not appear to play a major role in plant fatty acid synthesis (J. Jaworski, 1993). Instead, the first condensation takes place between acetyl-CoA and malonyl-ACP. This reaction is catalyzed by β-ketoacyl-ACP synthase III, one of three ketoacyl synthases in plant systems (Fig. 2). The acetoacetyl-ACP product then undergoes the standard reduction–dehydration–reduction sequence to produce 4:0-ACP, the initial substrate of ketoacyl-ACP synthase I. KAS I is responsible for the condensations in each elongation cycle up through that producing 16:0-ACP. The third ketoacyl synthase, KAS II, is dedicated to the final plastidial elongation, that of 16:0-ACP to 18:0-ACP.

3.2. The first double bond is introduced by soluble acyl-ACP desaturases

The major components of the long-chain acyl-ACP pool in most plant tissues are 16:0-ACP, 18:0-ACP, and 18:1-ACP. This finding highlights the importance of stearoyl-ACP desaturase, the plastidial enzyme responsible for Δ^9-desaturation in plants. In contrast to the desaturation system of *E. coli* (Chapter 3), the plant enzyme introduces the double bond directly into the Δ^9 position. Unlike yeast and mammalian Δ^9-desaturases (Chapter 7), the plant desaturase is a soluble enzyme and is specific for acyl-ACPs rather than acyl-CoAs. In recent years, work on stearoyl-ACP desaturase has progressed rapidly and is now providing a more detailed understanding of the fundamental mechanisms of oxygenic fatty acid desaturation and related reactions (J. Guy, 2006). The gene encoding the enzyme has been cloned from a number of species, and the structure of the castor

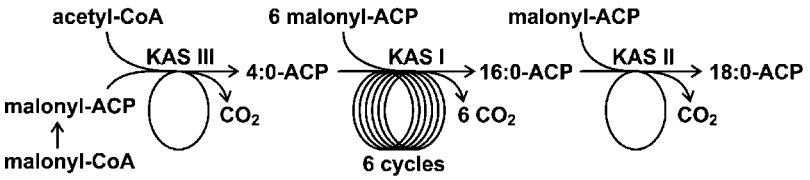


Fig. 2. Contribution of the three ketoacyl synthases (KASI, II, and III) to fatty acid elongation. Each circle represents one round of the elongation cycle catalyzed by ketoacyl-ACP synthase, enoyl-ACP reductase, hydroxyacyl-ACP dehydrase, and acyl-ACP reductase.

bean Δ^9-desaturase has been determined to 2.4 Å resolution. A combination of the crystal structure and spectroscopic methods has revealed two identical monomers, each with an active site containing a diiron-oxo cluster. Reduction of the iron by ferredoxin leads to its binding of molecular oxygen. The resulting complex ultimately removes electrons at the Δ^9 position, resulting in double bond formation [6].

Although the most common unsaturated fatty acids in plants are derived from oleic acid, a wide range of unusual fatty acids are found in the seed oils of different species. Divergent plastid acyl-ACP desaturases have been shown to account for some of this diversity. For example, *Coriandrum sativum* achieves seed oils rich in Δ^6-18:1 (petroselinic acid) by desaturation of 16:0 at the Δ^4 position followed by elongation, while *Thunbergia alata* attains a similar oil by direct Δ^6-desaturation of 16:0. A single acyl-ACP desaturase from *Hedera helix* can even produce $\Delta^{4,9}$-dienes (E. Whittle, 2005).

Several structure–function relationships suggested by unusual desaturases have been tested in the acyl-ACP desaturase system. Shortening the acyl binding pocket of the Δ^9-18:0 desaturase by altering a single amino acid, as in *Doxantha unguis-cati*, shifts substrate specificity in favor of 16:0-ACP (E. Cahoon, 1998). In addition, a set of five specific amino acids suggested by the *Thunbergia* gene transforms the castor bean Δ^9-desaturase to a Δ^6-desaturase, while enzymes with certain subsets of the five amino acids can desaturate at either position [6].

3.3. Acyl-ACP thioesterases terminate fatty acid synthesis

Among most prokaryotes, all acyl groups exiting the fatty acid synthase are transferred directly from ACP to glycerol-3-phosphate to form polar lipids. However, plants must also release sufficient fatty acid from ACP to supply membrane synthesis by compartments outside the plastid site of fatty acid synthesis. Since the typical chloroplast exports primarily 18:1 and 16:0, the same fatty acids that comprise the greatest fraction of long-chain acyl-ACPs, it might be assumed that a relatively non-specific thioesterase releases 16- and 18-carbon fatty acids from ACP. However, molecular and biochemical analyses of cloned plant thioesterases indicate that plants possess individual thioesterases with specificity either for 18:1 or for one or more saturated fatty acids [7]. The most prominent thioesterase in most plants has a strong preference for 18:1-ACP, making 18:1 the fatty acid most available for extraplastidial glycerolipid synthesis. In contrast, mangosteen, a plant with seed oil particularly high in 18:0, contains an 18:0-ACP thioesterase gene that has been used to engineer rapeseed with high 18:0 content (D. Hawkins, 1998).

Plants that synthesize certain unusual fatty acids have additional or modified thioesterases. For example, several plant species that produce storage oils containing large amounts of 8- to 14-carbon acyl chains contain thioesterases specific for those chain lengths. By removing acyl groups from ACP prematurely, the medium-chain thioesterases simultaneously prevent further elongation of acyl chains and release them for TG synthesis outside the plastids. In addition, both the standard Δ^9-18:1 thioesterase and a Δ^6-18:1 thioesterase have been purified from the Δ^6-18:1 accumulating coriander plant (P. Dörmann, 1994). Thus plants, by regulating expression of different thioesterases, can both fine tune and radically modify the exported fatty acid pool.

4. Acetyl-CoA carboxylase and control of fatty acid synthesis

4.1. Two forms of acetyl-CoA carboxylase

The malonyl-CoA that supplies two carbon units for fatty acid synthesis is produced from acetyl-CoA and bicarbonate by ACC. In plants, malonyl-CoA for fatty acid synthesis is provided by a plastid-localized ACC, while a cytosolic ACC contributes malonyl units for fatty acid elongation beyond C18 as well as for synthesis of flavonoids, polyketides, and other metabolites. Like fatty acid synthase, ACCase forms may be categorized either as 'eukaryotic' enzymes, which are dimers of a multifunctional polypeptide (Chapter 6), or 'prokaryotic' enzymes, which are heteromers of four subunits: biotin carboxyl carrier protein, biotin carboxylase, and two subunits of carboxyltransferase (Chapter 3). In the grass family, both plastids and cytosol house 'eukaryotic' enzymes. However, dicots and monocots other than grasses appear to have both forms, with the 'eukaryotic' form limited primarily to the cytosol, and 'prokaryotic' enzymes predominating in the plastids [8]. Assembly of the 'prokaryotic' form requires participation of both the nuclear genome, which encodes biotin carboxyl carrier protein, biotin carboxylase, and the alpha subunit of carboxyltransferase, and the plastid genome, which has retained the gene for the carboxyltransferase beta subunit, perhaps due to a requirement for RNA editing.

4.2. Acetyl-CoA carboxylase is a control point for fatty acid synthesis

In other kingdoms, ACC is a major control point for fatty acid biosynthesis. Although the mechanisms acting in plants are incompletely characterized, there is evidence that plant ACCs are also tightly regulated [8,9]. For example, both redox regulation via thioredoxin and phosphorylation of the carboxyltransferase have been implicated in up-regulation of the chloroplast ACC by light. Conversely, feedback inhibition is observed at the level of ACC when tobacco cell cultures are given exogenous fatty acids (D. Shintani, 1995). Due to its impact on the rate of fatty acid synthesis, ACC is considered a promising target in oilseed improvement programs, and some increases in oil content have been obtained by engineering a cytosolic ACC gene to be expressed in plastids (D. Klaus, 2004).

5. Phosphatidic acid synthesis: 'prokaryotic' and 'eukaryotic' acyltransferases

Since phosphatidic acid serves as a precursor of phospholipids, galactolipids, and TGs, it is not surprising that its own synthesis has been reported in four plant compartments: plastids, ER, mitochondria, and Golgi bodies. In each case, esterification of the first acyl group to the *sn*-1 position of glycerol-3-phosphate is catalyzed by glycerol-3-phosphate acyltransferase. Lysophosphatidic acid acyltransferase then completes the synthesis by acylating the *sn*-2 position. However, plastidial and extraplastidial acyltransferases show distinct differences in structure and specificity. Analysis of these differences and the different compositions of plastid and non-plastid membranes led to the 'prokaryotic/ eukaryotic two-pathway' scheme for plant lipid synthesis shown in Fig. 3.

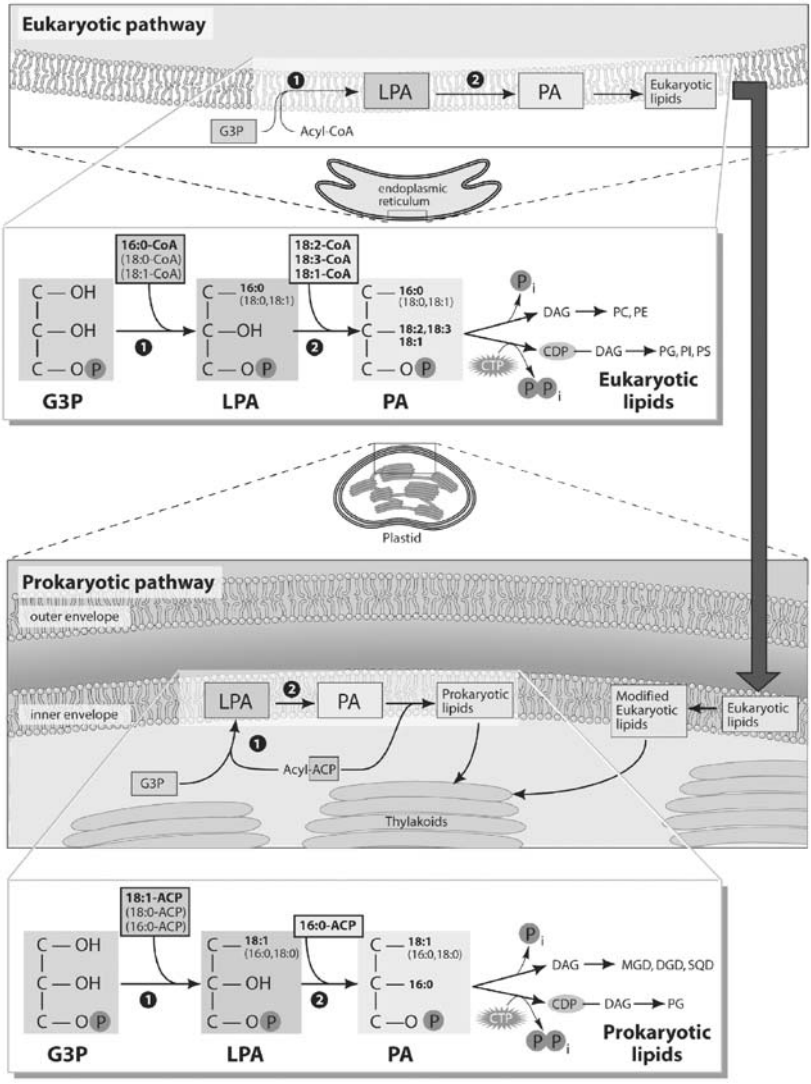


Fig. 3. The prokaryotic and eukaryotic pathways of plant glycerolipid synthesis. The prokaryotic pathway takes place in plastids and esterifies mainly palmitate to the *sn*-2 position of lysophosphatidate (LPA). The eukaryotic pathway occurs outside the plastid, primarily in the ER and results in 18-carbon fatty acids esterified to the *sn*-2 position of glycerolipids. In the prokaryotic pathway, acyl-ACP is condensed with glycerol-3-phosphate (G3P) by a soluble enzyme, G3P acyltransferase (reaction 1). The product, LPA, partitions into the membranes where LPA is converted to phosphatidic acid (PA) by a membrane localized LPA-acyltransferase (reaction 2). PA is then converted to the other lipids found in chloroplasts. It is thought that most of the reactions of lipid synthesis by the prokaryotic pathway take place in the inner envelope of the plastids. The initial reactions of the eukaryotic pathway are similar except that acyl-CoA substrates are used and the G3P acyltransferase is thought to be associated with the ER. After desaturation of 18:1 to 18:2, lipids move from the ER to the other organelles, including the outer envelope of plastids. Eukaryotic lipids in the outer envelope are transferred into the inner membranes and modified by the replacement of headgroups and by the action of additional desaturases. CDP-DG, cytidine diphosphate-diacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidyserine; DGD, digalactosyl diacylglycerol; MDG, monogalactosyl diacylglycerol; SQD, sulfoquinovosyl diacylglycerol. (See color plate section, plate no. 2.)

5.1. Plastidial acyltransferases

In the plastids, acyltransferases provide a direct route for entrance of acyl groups from ACP to membrane lipids. Since this is the standard pathway for phosphatidic acid synthesis in *E. coli* and cyanobacteria, both the enzymes of phosphatidic acid synthesis in plastids and the glycerolipid backbones they produce are termed 'prokaryotic'. In both chloroplasts and non-green plastids, the glycerol-3-phosphate acyltransferase is a soluble enzyme that, unlike the *E. coli* enzyme, shows preference for 18:1-ACP over 16:0-ACP. The lysophosphatidic acid acyltransferase, which is a component of the inner envelope of plastids, is extremely selective for 16:0-ACP. The presence of a 16-carbon fatty acid at the 2-position is therefore considered diagnostic for lipids synthesized in the plastids.

5.2. Extraplastidial acyltransferases

At least superficially, the mitochondrial and Golgi acyltransferase activities resemble those of the better-studied ER system. All three compartments have membrane-bound glycerol-3-phosphate and lysophosphatidic acid acyltransferases that utilize acyl-CoA substrates. In the ER, which is quantitatively the most significant of the extraplastidial sites for phosphatidic acid synthesis, saturated fatty acids are almost entirely excluded from the *sn*-2 position. The glycerol-3-phosphate acyltransferase is less selective, but, due to substrate availability, more often fills the *sn*-1 position with 18:1 than with 16:0. It is therefore possible to judge the relative contributions of the prokaryotic and eukaryotic pathways by comparing the proportions of eukaryotic 18/18 or 16/18 glycerolipids with prokaryotic 18/16 or 16/16 glycerolipids [5].

5.3. Lipid trafficking across the chloroplast envelope is a major flux

The most abundant membranes in nature are the thylakoids inside chloroplasts of green plants. A surprising amount of lipid traffic is involved in the assembly of these membranes. Almost all the acyl chains that form the core of the photosynthetic membranes are first produced by fatty acid synthase in the chloroplast. In most plants these acyl chains are then exported to the ER where they become esterified to glycerol, desaturated while they are part of phosphatidylcholine and then are returned to the plastid. The exact mechanisms for the export and return of acyl chains are still uncertain although much has been learned (Chapter 17) [10]. The export from plastids across the chloroplast envelope membranes is known to involve a fatty acid intermediate, and probably is a channeled or facilitated process rather than free diffusion because only a tiny pool of free fatty acid is ever detected (A. Koo, 2004). An acyl-CoA synthetase on the envelope membrane is believed to quickly convert the exported fatty acid to a thioester form that is then a substrate for acyltransferases. Transfer of acyl groups to the ER may occur via diffusion of the acyl-CoAs; however, recent evidence suggests this initial acyl transfer reaction involves acylation of lyso-phosphatidylcholine and may occur at the chloroplast envelope.

After the desaturation of 18:1 groups of phosphatidylcholine to 18:2 and 18:3, a major proportion of the leaf cell acyl chains returns to the chloroplast and becomes part of the glycolipid core of the photosynthetic membranes. Most research suggests that two acyl

chains return together esterified to glycerol. The exact molecule(s) that moves from the ER and crosses the envelope is not known, but mutants of an inner envelope ATP-binding cassette transporter involved in this process have been characterized and a second component of this transport system binds to phosphatidic acid (K. Awai, 2006). Phosphatidylcholine from the ER could therefore be first converted to phosphatidic acid by phospholipase D. Phosphatidic acid is then transported to the chloroplast inner envelope, where phosphatidic acid phosphatase produces the diacylglycerol substrate for galactolipid and sulfolipid synthesis. Thus, not only does the plastid envelope play a central role in many reactions of plant lipid metabolism, but almost all acyl chains in photosynthetic cells must cross the outer and inner envelope membranes not just once but twice. In terms of lipid trafficking it is likely that the plastid envelope is the most dynamic organellar membrane in nature.

5.4. 16:3- and 18:3-plants have different proportions of prokaryotic flux

Relative fluxes through the prokaryotic and eukaryotic pathways vary among organisms and tissues. Plastids have the potential to use phosphatidic acid from the prokaryotic pathway for all of their glycerolipid syntheses. However, not all plants do so; in some cases, the prokaryotic acyl chain arrangement is found only in plastidial phosphatidylglycerol, whereas galactolipids are derived from diacylglycerol imported into the plastids from the ER. As indicated above, the eukaryotic acyltransferases of the ER produce substantially more 18/18 than 16/18 lipids, and it is chiefly the 18/18 units that are assembled into galactolipids by plants having a minor prokaryotic pathway. Because galactolipids become highly unsaturated, plants that import diacylglycerol for galactolipids are rich in 18:3 and are called ‘18:3 plants’. Species in which most galactolipid is derived from the prokaryotic 18/16 or 16/16 diacylglycerol contain substantial 16:3 and are known as ‘16:3 plants’.

Kunst et al. [11] have demonstrated that a 16:3 plant, *Arabidopsis thaliana*, may be converted to a de facto 18:3 plant by a single mutation in plastidial glycerol-3-phosphate acyltransferase. Under these conditions, 16:3 content is reduced dramatically, and when isolated chloroplasts are labeled with glycerol-3-phosphate, only phosphatidylglycerol is labeled. Nevertheless, the percentage of galactolipids in mutant plants is nearly identical to that in wild-type plants, emphasizing the ability of plants to compensate for reduction of the prokaryotic pathway. Other studies in mutants have confirmed that plants have an amazing capacity to adapt to many, but not all, perturbations of lipid metabolism (Section 11).

6. Glycerolipid biosynthetic pathways

In plants, glycerolipid biosynthesis involves a complex web of reactions distributed among multiple compartments [11,12]. As in mammals (Chapters 8 and 10), the synthesis of individual glycerolipids is initiated either by the formation of CDP-diacylglycerol from phosphatidic acid and CTP, or by cleavage of phosphate from phosphatidic acid to produce diacylglycerol.

CTP:phosphatidate cytidylyltransferase has been observed in plastids, mitochondria, and ER. In all three compartments, the CDP-diacylglycerol derived from phosphatidic acid is used in the synthesis of phosphatidylglycerol; in mitochondria, the reaction of phosphatidylglycerol with a second CDP-diacylglycerol then produces cardiolipin. The ER can also incorporate CDP-diacylglycerol into phosphatidylinositol and phosphatidylserine.

Phosphatidic acid phosphatase is present in the same three compartments. In the ER and mitochondria, diacylglycerol combines with CDP-ethanolamine or CDP-choline to produce phosphatidylethanolamine or phosphatidylcholine respectively. Although separate enzymes catalyze ethanolamine and choline transfer in animals and yeast, there is evidence that non-specific aminoalcoholphosphotransferases produce both phospholipids in plants (Q. Qi, 2003). Flux into phosphatidylcholine is at least partially determined by regulation of the phosphocholine cytidyltransferase that generates CDP-choline, as is also observed in mammals (Chapter 8). The production of CDP-ethanolamine from phosphoethanolamine is less well studied, but is also considered a probable regulatory step. In addition, there is clear evidence that phosphethanolamine can be methylated to monomethylphosphoethanolamine, dimethylphosphoethanolamine, and phosphocholine, and that this pathway is inhibited by exogenous choline at the initial methylation step (S. McNeil, 2001). The methylation of phosphoethanolamine in plants is frequently contrasted with the methylation of phosphatidylethanolamine to phosphatidylcholine in animals and yeast. In general, no significant methylation of phosphatidylethanolamine itself occurs in plants. However, phosphatidylmonomethylethanolamine is synthesized from monomethylphosphoethanolamine and converted to phosphatidylcholine [12].

The diacylglycerol released in plastids reacts either with UDP-galactose or with UDP-sulfoquinovose to generate sulfolipid or monogalactosyldiacylglycerol (Fig. 1). Production of digalactosyldiacylglycerol has recently been confirmed to involve reaction of monogalactosyl diacylglycerol with a second UDP-galactose, despite the ability of chloroplast envelopes to transfer galactose from one monogalactosyldiacylglycerol to another (A. Kelly, 2003). Phosphate is often a limiting nutrient for plant growth and there is now evidence that plants replace some of their extraplastidial phospholipids with galactolipids when suffering from phospholipid deficiency [13].

6.1. Glycerolipids are substrates for plant fatty acid desaturases

In addition to the soluble acyl-ACP desaturases, plants contain a number of membrane-bound enzymes that desaturate fatty acids while they are esterified within glycerolipids [5,6]. The recent cloning and characterization of these desaturases is of great interest to the scientific community because the products of the membrane-bound systems include $\Delta^{9,12}$-18:2 and $\Delta^{9,12,15}$-18:3, both of which are essential to the human diet, and are thought to play a major role in human health and disease.

Once again, separate pathways occur in plastids and ER, although, as should be evident from the discussion above, fatty acids from the ER may make their way back to the plastids. Clarification of the number of desaturases involved in plant lipid metabolism and isolation of their genes has been greatly assisted by the isolation of a large number of mutants in *A. thaliana*, a small weed of the mustard family used as a model organism by

plant geneticists and molecular biologists. Briefly, three membrane-bound desaturation sequences are evident in *Arabidopsis* [5,6].

(1) In chloroplasts, 16:0 at the 2-position of phosphatidylglycerol is desaturated to *trans*-Δ^3-16:1. This desaturase is most likely encoded by the *FAD4* gene.
(2) Plastids are able to convert 18:1 to 18:3 and 16:0 to 16:3 using a combination of three membrane-bound desaturases. One of them, encoded by *FAD5*, is relatively specific for the conversion of 16:0 on monogalactosyldiacylglycerol to Δ^7-16:1. This 16:1 and Δ^9-18:1 may then be given a second and third double bond by the *FAD6* and the *FAD7* or *FAD8* gene products respectively. The latter two desaturases are less selective in their choice of glycerolipid substrate, and will accept appropriate fatty acids on phosphatidylglycerol, sulfolipid, or either of the major galactolipids.
(3) In the ER, 18:1 esterified to phosphatidylcholine or occasionally phosphatidy lethanolamine may be desaturated to 18:2 by *FAD2* and to 18:3 by *FAD3*.

It should be noted that fatty acids entering one of the multistep desaturation pathways listed above are not necessarily committed to completing that set of reactions. It is particularly common for 18:2 to be an end product of ER desaturation. This 18:2 may remain in phospholipid, be incorporated into TG, or enter the galactolipid pathway and receive a third double bond in the chloroplast.

7. Lipid storage in plants

A plant stores reserve material in its seeds in order to allow seedling growth of the next generation until photosynthetic capacity can be established. The three major storage materials are oil, protein, and carbohydrate, and almost all seeds contain some of each. However, their proportions vary greatly. For example, the amount of oil in different species may range from as little as 1–2% of the total dry weight in grasses such as wheat, to as much as 60% in the castor seed. With the exception of the jojoba plant, which accumulates wax esters in seeds, plants store oil as TG.

7.1. Lipid body structure and biogenesis

In the mature seed, TG is stored in densely packed lipid bodies, which are roughly spherical in shape with an average diameter of 1 μm (Fig. 4) [14]. This size does not change during seed development, and accumulation of oil is accompanied by an increase in the number of lipid bodies. The very large number of lipid bodies in an oilseed cell (often >1000) contrasts strikingly with animal adipose tissue where oil droplets produced in the cytosol can coalesce into a few or only one droplet. The plant lipid bodies appear to be surrounded by a phospholipid monolayer in which the polar headgroups face the cytosol, while the non-polar acyl groups are associated with the non-polar TG within. The membranes of isolated lipid bodies, which comprise less than 5% of a lipid body's weight, contain both phospholipids and characteristic proteins known as caleosins and oleosins. The recently discovered caleosins are calcium-binding proteins that may be involved in mobilization of stored TG during germination (M. Poxleitner, 2006). Oleosins are small (15–26 kDa) proteins that are believed to preserve individual lipid bodies as discrete entities (K. Hsieh, 2004).

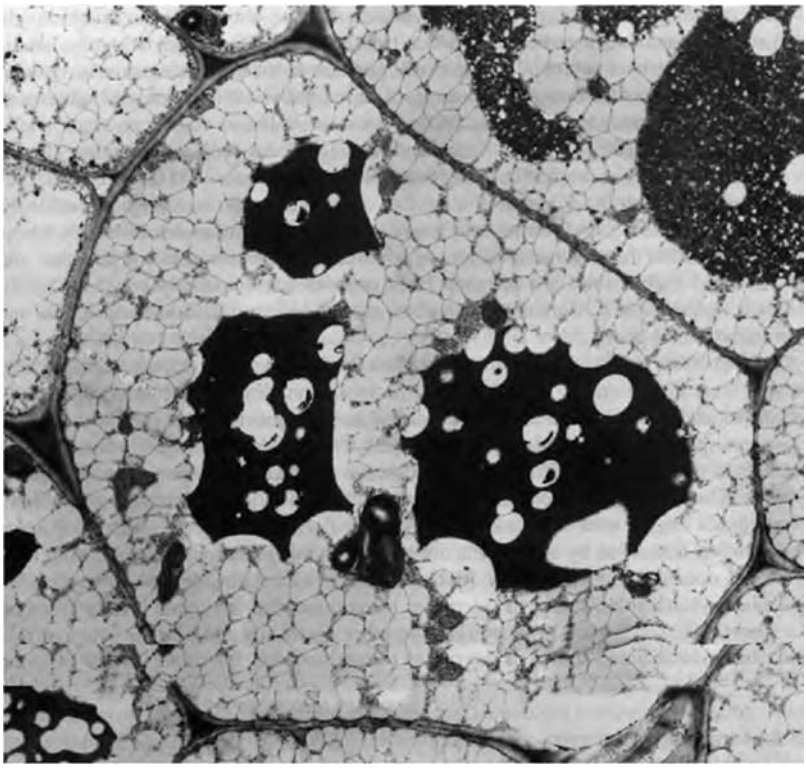


Fig. 4. Thin-sectional view of cells in a cotyledon of a developing cotton embryo harvested 42 days after anthesis. The cells are densely packed with lipid bodies and several large storage protein bodies (dark). Magnification ×9000. Photo courtesy of Richard Trelease, Arizona State University.

Desiccated seeds lacking oleosins undergo lipid body fusion and cell disruption when rehydrated (O. Leprince, 1998). The cDNAs encoding many oleosins have been cloned and each has a sequence encoding a totally hydrophobic domain of 68–74 amino acids which is likely to be the longest hydrophobic sequence found in any organism. Structurally, oleosins are roughly analogous to the animal apolipoproteins which coat the surface of lipid droplets during their transport between tissues (Chapter 17).

When a seed germinates, the TG stored in the lipid bodies becomes the substrate for lipases. In at least some cases, peroxidation of polyunsaturated fatty acids by a lipid body lipoxygenase precedes the release of fatty acids from TG. Typically lipases and lipid body lipoxygenase are active only after germination is triggered by imbibition and other environmental signals. Fatty acids released by the lipid bodies are further metabolized through the β-oxidation pathway and glyoxylate cycle in the glyoxysomes (Section 2.4).

7.2. Seed triacylglycerols often contain unusual fatty acids

The structural glycerolipids of all plant membranes contain predominantly five fatty acids (18:1, 18:2, 18:3, 16:0, and in some species, 16:3). However, the fatty acid composition of storage oils varies far more than in membrane glycerolipids. Altogether more than 300

Table 2
Some unusual fatty acids produced in plant seeds

Fatty acid type	Example	Major sources	Major or potential uses	Approximate U.S. market size, $10^6
Medium chain	Lauric acid (12:0)	Coconut, palm kernel	Soaps, detergents, surfactants	350
Long chain	Erucic acid (22:1)	Rapeseed	Lubricants, anti-slip agents	100
Epoxy	Vernolic acid 18:1 Δ^9epoxy12,13	Epoxidized soybean oil, *Vernonia*	Plasticizers	70
Hydroxy	Ricinoleic acid 18:1 Δ^9,12OH	Castor bean	Coatings, lubricants	80
Acetylenic	Crepenynic acid 18:2Δ^9,12yne	*Crepis foetida*	Polymers	–
Cyclopropene	Sterculic acid 19:1	*Sterculia foetida*	Lubricants, polymers	–
Conjugated	Parinaric 18:4 Δ^9c11t13t15c	*Impatiens balsamina*	Coatings	–
Trienoic	Linolenic acid (α18:3)	Flax	Paints, varnishes, coatings	45
Wax esters	Jojoba oil	Jojoba	Lubricants, cosmetics	10

different fatty acids are known to occur in seed TG (Table 2) [15]. Chain length may range from less than 8 to over 22 carbons. The position and number of double bonds may also be unusual, and hydroxy, epoxy, or other functional groups can modify the acyl chain. Many of the different fatty acid structures, including hydroxy, epoxy, acetylenic, and conjugated varieties, are now known to originate from minor modifications in the amino acid sequence of the oleate desaturase. For example, only four amino acid changes have been shown to convert a desaturase into a hydroxylase (P. Broun, 1998).

The reason for the great diversity in plant storage oils is unknown. However, the special physical or chemical properties of the 'unusual' plant fatty acids have been exploited for centuries. In fact, approximately one-third of all vegetable oil is used for non-food purposes (Table 2). Reading the ingredients of a soap or shampoo container reveals one of the major end uses of high lauric acid specialty plant oils. Other major applications include the use of erucic acid (22:1) derivatives to provide lubricants and as a coating for plastic films. Hydroxy fatty acids from the castor bean have over 100 industrial applications including plastic and lubricant manufacture. As discussed in Section 12, the ability of genetic engineering to transfer genes for some unusual fatty acid production from exotic wild species to high-yielding oil crops is now providing the ability to produce new renewable agricultural products and to replace feedstocks derived from petroleum.

7.3. The pathway of triacylglycerol biosynthesis

As in animal tissues, it has been suggested that TGs are produced by a relatively simple four-reaction pathway. According to this model, phosphatidic acid is synthesized by the extraplastidial pathway (Section 5) and dephosphorylated to diacylglycerol. A third fatty acid is then transferred from CoA to the vacant third hydroxyl of the diacylglycerol, producing TG. This last and single committed step is catalyzed by diacylglycerol

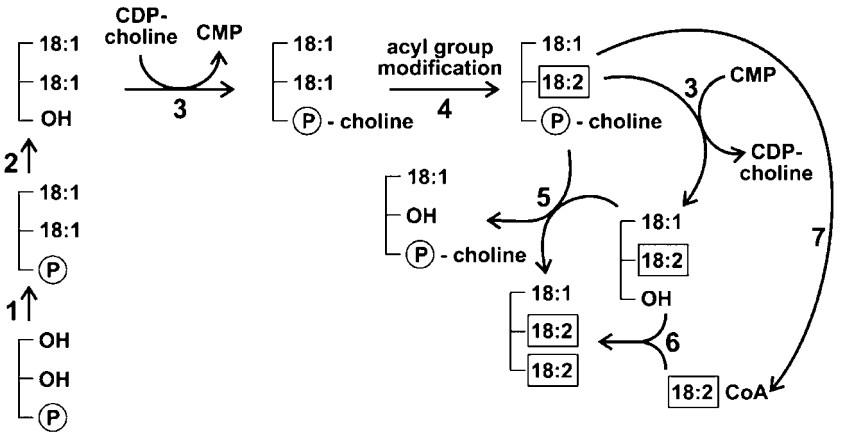


Fig. 5. Pathway depicting how flux through phosphatidylcholine (product of reaction 3) can promote acyl group diversity in plant triacylglycerols. Production of 18:2 (boxed) at the *sn*-2 position and its transfer to TG is used as a sample modification. Other fatty acid alterations may be substituted. Enzymes: 1, glycerol-3-phosphate:acyl-CoA acyltransferase and lysophosphatidic acid:acyl-CoA acyltransferase; 2, phosphatidic acid phosphatase 3, diacylglycerol:CDP-aminoalcohol aminoalcoholphosphotransferase; 4, ω^6 18:1-desaturase or other fatty acid modifying enzyme; 5, phosphlipid:diacylglycerol acyltransferase; 6, diacylglycerol acyltransferase; 7, acyl-CoA:phosphatidylcholine acyltransferase or phospholipase plus acyl-CoA synthetase.

acyltransferase (Fig. 5, reaction 6). Although plants possess all of the enzymes required for the reactions above, the assembly of three fatty acids onto a glycerol backbone is not always as straightforward as suggested by the above pathway. In many oilseeds, pulse-chase labeling has revealed that fatty acids reach TG only after passing through phosphatidylcholine, (or phosphatidylethanolamine to a lesser extent) (E. Cahoon, 1994). Given the range of desaturation and other modification reactions that can take place on phosphatidylcholine, transit through this phospholipid helps to explain some of the fatty acid diversity in TG.

Fatty acids from phosphatidylcholine may become available for TG synthesis in several ways [16,17]. In some plants, a phospholipid:diacylglycerol acyltransferase produces TG by direct transfer of a fatty acid from the 2-position of phospholipid to diacylglycerol (Fig. 5, reaction 5) [18]. The second mechanism by which phosphatidylcholine can participate in TG synthesis is by donation of its entire diacylglycerol unit. In many plants, the synthesis of phosphatidylcholine from diacylglycerol and CDP-choline appears to be rapidly reversible. As shown in Fig. 5 (reaction 3), the activity of cholinephosphotransferase allows diacylglycerol moieties modified on phosphatidylcholine to be incorporated into TAG via the diacylglycerol acyltransferase or the more recently observed diacyglycerol:diacylglycerol acyltransferase [18]. Finally, a fatty acid removed from phosphatidylcholine may subsequently be used for TG synthesis. Such an 'acyl exchange' may provide acyl-CoA either by the combined reverse and forward reactions of an acyl-CoA:phosphatidylcholine acyltransferase, or by a combination of phospholipase and acyl-CoA synthetase.

7.4. Challenges in understanding triacylglycerol synthesis

Although the basic reactions of TG biosynthesis have been determined, several fundamental and potentially related questions persist. As highlighted above, TG and membrane lipids frequently have radically different fatty acid compositions. How do plants control which fatty acids are stored in TG as opposed to which fatty acids are restricted to membranes? Are unusual fatty acids excluded from membranes because their physical and chemical idiosyncracies would perturb membrane fluidity or other physical characteristics? Is TG synthesis spatially distinct from the synthesis of membrane lipids (J. Shockey 2006), or do enzyme specificities dictate the partitioning of fatty acid species among glycerolipids? Although all of these factors may be significant, selectivity for unusual fatty acids by enzymes such as diacylglycerol and phospholipid:diacylglycerol acyltransferases, and editing of unusual fatty acids from phospholipids, are currently the best documented [17,18].

8. Protective lipids: cutin, suberin, and waxes

All above-ground tissues of plants are protected against desiccation and pathogens by a thin layer of lipids called the cuticle (Fig. 6). The cuticle is produced by epidermal cells and consists of two major types of lipids, cutin and waxes. Cutin is a complex, insoluble polyester of glycerol and fatty acid derivatives with a range of oxygen-containing functional groups and may be anchored to the cell wall. 16 and 18-carbon fatty acids with one or more hydroxyl groups are particularly common [19]. Many details of the three-dimensional structure and the nature of cross-linking in cutin remain a mystery. These details are clearly significant because mutants in enzymes involved in cutin biosynthesis, and also *Arabidopsis* transformed with cutinase from a fungal plant pathogen, not only develop a leaky cuticle, but also suffer from fusions between leaves and flower parts (P. Sieber, 2000).

Waxes form the other major component of the cuticle and are both embedded into the cutin and occur on the surface forming crystals of epicuticular wax that influence the appearance of the plant surface. These waxes are complex mixtures including a range of very long-chain alkanes, aldehydes, and ketones as well as wax esters and their building blocks. Visual screening of plant surfaces has allowed isolation of mutants blocked in many of the biosynthetic steps of wax synthesis and a number of genes encoding these enzymes have now been isolated [20]. For example, the cDNA encoding an elongase required for extension of wax acyl units beyond 24 carbons has been cloned, and resembles the condensing enzymes involved in synthesis of erucic acid in seed oil and wax ester precursors in jojoba seeds (A.A. Millar, 1999). Transport of cuticular lipids to the surface of epidermal cells involves an ATP-binding cassette transporter (Chapters 16 and 19) [21].

Bark, wound callus, and specialized tissues such as the endodermis that controls entry into the root vascular system, have walls lined with suberin. Suberin, like cutin, is a

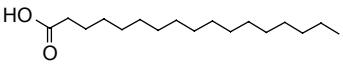
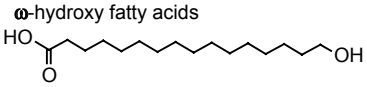
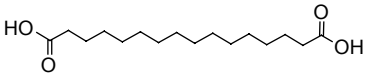
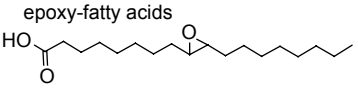
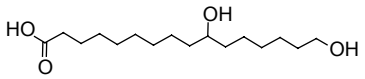
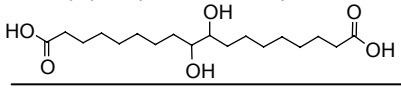
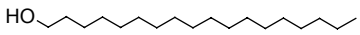
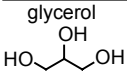
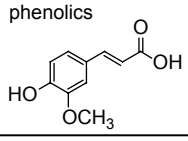
Monomer type	Abundance% and common monomers	
Example	Cutin	Suberin
unsubstituted fatty acids 	1 to 25% 16:0, 18:0, 18:1, 18:2	1 to 10% 18:0 to 24:0
ω-hydroxy fatty acids 	1 to 32% 16:0, 18:1, 18:2	11 to 43% 18:1,16:0 to 26:0
α,ω-dicarboxylic acids 	usually <5% but >50% in Arabidopsis 16:0, 18:0, 18:1, 18:2	24 to 45% 18:1, 18:2, 16:0 to 26:0
mid-chain functionalized monomers		
epoxy-fatty acids 	0 to 34% 18:0, 18:1 (9,10-epoxy)	0 to 30% 18:1 (9-epoxy-18-hydroxy) 18:0 (9-epoxy-1, 18-diacid)
polyhydroxy-fatty acids 	16 to 92% 16:0 (10, 16 diol) 18:0 (9, 10, 18 triol)	0 to 2% 18:0 (9, 10, 18 triol)
polyhydroxy- **α,ω**-dicarboxylic acids 	traces	0 to 8% 18:0 (9, 10 diol)
fatty alcohols and **α,ω**-diols 	<2% 18.0-1-1-diol	1 to 20% 18:0 to 26:0 (1-ol)
glycerol 	1 to 14%	14 to 26%
phenolics 	<1% ferulate	≤10% ferulate, smaller amounts of coumarate, sinapate, caffeate

Fig. 6. Structures of common cutin and suberin monomers, and ranges of typical composition values. Non-substituted fatty acids are not represented. There are overlaps in some classes of monomers (e.g. some monomers are epoxy hydroxy–fatty acids, of epoxy dicarboxylic acids).

polyester of glycerol and fatty acids enriched in carboxyl and hydroxyl groups. In addition to placement on the inner surface of cell walls rather than outside, the tough, waterproof suberin differs from cutin in its preference for longer fatty acids and in its incorporation of large amounts of phenylpropanoids [22]. A mutant in suberin biosynthesis has allowed identification of a class of glycerol acyltransferases involved in extracellular lipid polyester biosynthesis (F. Beisson, 2007).

9. Sterol, isoprenoid, and sphingolipid biosynthesis

In the plant kingdom, isoprenoids represent the most diverse range of natural products with over 25,000 lipophilic structures known, ranging from small, volatile compounds to rubber. Quantitatively, the photosynthetic apparatus is probably the primary consumer of isoprenoids, since carotenoids, plastoquinone, and the phytol tail of chlorophyll all belong to this group. Given that vital plant hormones such as gibberellin and abscisic acid, plus many defensive compounds, are isoprenoids, the early steps of this pathway have been studied intensely. However, surprisingly, it was not until the late 1990s that researchers realized that plants have two very different pathways for production of isopentenyl pyrophosphate, the five-carbon central precursor of all isoprenoids [23]. For several decades it was known that, as in other organisms, plants join three molecules of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA followed by the highly regulated reduction of that compound to mevalonic acid (Chapter 14). Furthermore, plants contain multiple well-studied 3-hydroxy-3-methylglutaryl-CoA reductase genes that are differentially expressed during development and in response to such stimuli as light, wounding, and infection. It was incorrectly suspected that this 'mevalonate' pathway was localized in both the cytosol and the plastids and produced all classes of isoprenoids. The story has now been clarified with the discovery that plastids produce isopentenyl pyrophosphate by a 'non-mevalonate' pathway that begins with the condensation of pyruvate with glyceraldehyde-3 phosphate to produce 1-deoxy-D-xylulose-5-P. At least three additional enzymes are required to produce isopentenyl pyrophosphate in the plastids. In parallel to work in plants, this pathway has also been demonstrated in bacteria and algae. The non-mevalonate pathway in plastids is responsible for production of the classic plant photosynthetic isoprenoids such as phytols and carotenoids, as well as mono- and diterpenes [23].

The mevalonate pathway in the cytosol is responsible for biosynthesis of sterols, sesquiterpenes, and triterpenoids. After conversion of mevalonic acid to isopentenyl pyrophosphate, three C5 units can be joined head to tail to produce a C15 compound, farnesyl pyrophosphate. Two farnesyl pyrophosphates are then united head to head to form squalene, the progenitor of the C30 isoprenoids from which sterols are derived. The plant squalene synthetase, like its mammalian homologue, is found in the ER and the reaction proceeds via a presqualene pyrophosphate intermediate (Chapter 14). In the last step prior to cyclization, squalene is converted to squalene 2,3-epoxide.

It is also in the cyclization step that photosynthetic and non-photosynthetic organisms diverge. While animals and fungi produce lanosterol, sterol synthesis in organisms with a photosynthetic heritage proceeds via cycloartenol despite the presence of lanosterol synthase genes in higher plants (M. Kolesnikova, 2006). Cycolartenol and lanosterol synthases share less than 40% identity, but may be interconverted functionally by alteration of as few as two amino acids (S. Lodeiro, 2005).

A complex series of reactions including opening of the cyloartenol cyclopropane ring, double bond formation and isomerization, demethylation of ring carbons, and methylation of the side chain result in formation of a number of different plant sterols [24]. Sitosterol is the most common plant sterol (Fig. 7); however, plants normally contain mixtures of sterols whose proportions differ from tissue to tissue. In addition, sterol esters, sterol

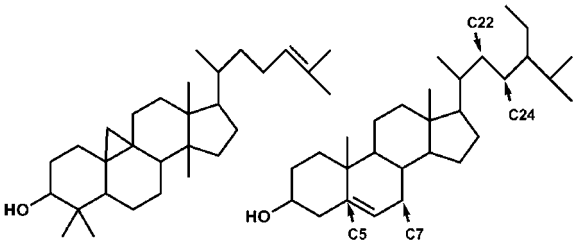


Fig. 7. Cycloartenol, rather than lanosterol, is the first cyclized precursor of sterols and plants. Sitosterol (24α-ethylcholesterol), shown here, is the most common plant sterol, but plants generally contain complex mixtures of sterols. Other prominent phytosterols differ from sitosterol as follows. Campesterol, 24α-methyl; stigmasterol, C22 double bond; dihydrospinasterol, move double bond from C5 to C7; spinasterol, move C5 double bond to C7, add C22 double bond; dihydrobrassicasterol, 24β-methyl; brassicasterol, 24β-methyl, add C22 double bond.

glycosides, and acylated sterol glycosides are common plant constituents whose physiological significance is under scrutiny. Both cold adaptation and pathogenesis drastically alter free and derivatized sterol pools. Plants also produce a steroid hormone, brassinolide, required for both light-induced development and fertility. Interestingly, the gene encoding a 5α-reductase in the brassinolide pathway can complement the corresponding reductase in the testosterone pathway (J. Li, 1997).

Sphingolipids are usually considered minor constituents of plant lipids, accounting for 5% or less of most lipid extracts. This fact, and the more complex methods needed for their identification and characterization, have resulted in a comparative lack of information on plant sphingolipid biosynthesis and function. Nevertheless, sphingolipids make up a substantial proportion (25% or more) of the composition of plasma and tonoplast membranes, with the glucosylceramides and inositolphosphorylceramides constituting major fractions (J. Markham, 2006). Moreover, sphingolipids have been implicated in signal transduction pathways and programmed cell death in plants [25].

As in animals (Chapter 13), sphingolipid biosynthesis begins with condensation of palmitoyl-CoA with serine to form 3-keto-sphinganine. The plant enzyme is a heteromer homologous to the fungal and animal proteins, and deletions of single subunits are lethal in *Arabidopsis* [26]. 3-keto-sphinganine is typically reduced to sphinganine, and further modifications including desaturation or hydroxylation at Δ^4 and desaturation at Δ^8 are catalyzed by a family of related enzymes [25]. Less information is available on the synthesis of ceramides and glucosylceramides, although there is evidence that both sterol glucoside and UDP-glucose could serve as glucose donors for the latter [25]. In addition, inositolphosphorylceramide synthase activity has now been detected in at least one plant (P. Bromley, 2003).

10. Oxylipins as plant hormones

Jasmonate is one of several lipid-derived plant growth regulators referred to as oxylipins [27]. The structure and biosynthesis of jasmonate have intrigued plant biologists because

of the parallels to some eicosanoids (Chapter 13), which are central to inflammatory responses and other physiological processes in mammals. In plants, jasmonate derives from α-linolenic acid, presumably released from membrane lipids by a phospholipase A_2. The linolenic acid is oxidized by lipoxygenase and the resulting products, 9-hydroperoxylinolenic acid or 13-hydroperoxylinolenic acid may be further metabolized by one of three routes to produce a wide variety of oxylipins (Fig. 8). The pathways by which 13-hydroperoxylinoleic acid may be metabolized include hydroperoxide lyase-catalyzed

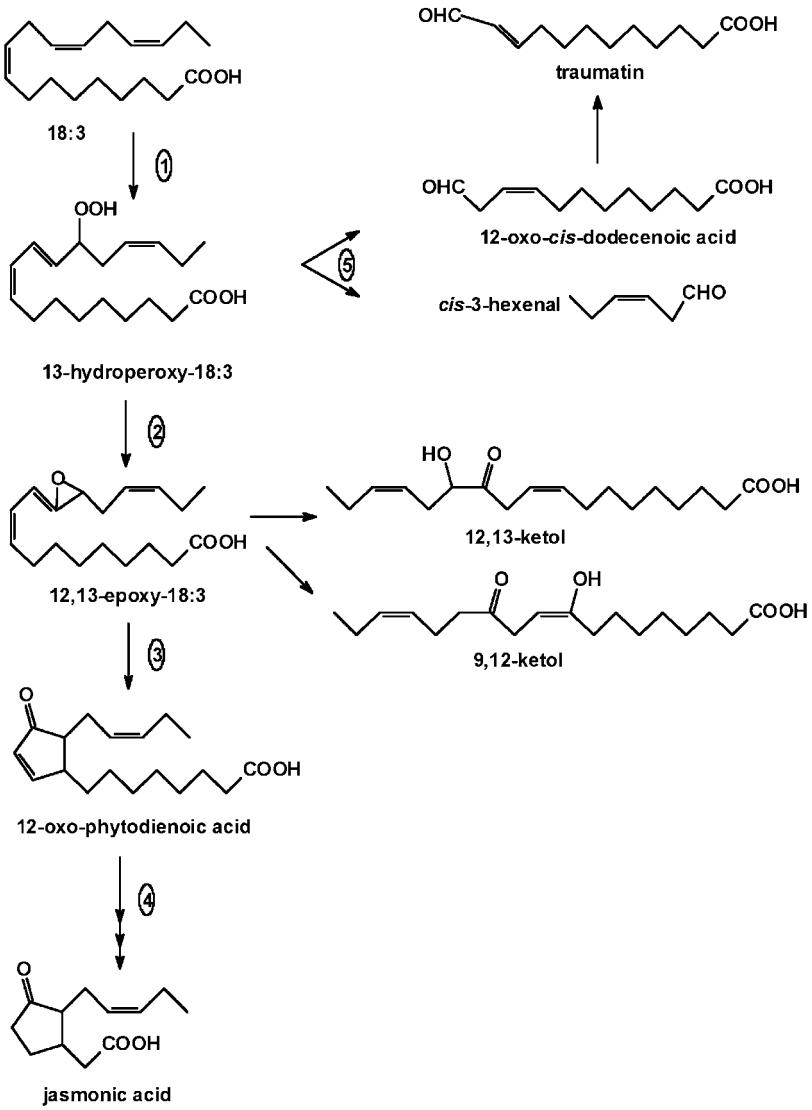


Fig. 8. Metabolism of 18:3 fatty acid to oxylipins. 1. Lipoxygenase; 2, alleneoxide synthase; 3, allene oxide cyclase; 4, 12-oxo-phytodienoic acid reductase, β-oxidation; 5, hydroperoxide lyase.

scission of the *trans*-11,12-double bond to produce a C6 aldehyde, *cis*-3-hexenal, and the C12 compound, 12-oxo-*cis*-9-dodecenoic acid (Fig. 8, reaction 5). The acid is subsequently metabolized to 12-oxo-*trans*-10-dodecenoic acid, the wound hormone traumatin. The enzyme hydroperoxide dehydratase (allene oxide synthase) (Fig. 8, reaction 2) catalyzes the dehydration of hydroperoxides to unstable allene oxides that readily decompose to form a 9,12-ketol or a 12,13-ketol. The allene oxide of 13-hydroperoxylinolenic acid may also be converted by allene oxide cyclase (Fig. 8, reaction 3) to 12-oxo-phytodienoic acid which can be further metabolized to 7-iso-jasmonic acid. In the last few years, it has become clear that jasmonate is a key component of a wound-signaling pathway that allows plants to protect themselves against insect attack (T. Schilmiller, 2005). When experimentally applied to plants at low concentrations, jasmonate leads to the induction of protease inhibitors and other defense compounds. Furthermore, mutants of tomato and *Arabidopsis* that are deficient in jasmonate synthesis are much more susceptible to insect damage. In addition to jasmonate, a number of the other oxylipins have been reported to function as signal molecules. In particular, the oxylipin traumatin has been suggested to trigger cell division at the site of wounds, leading to the development of a protective callus. The lipoxygenase product 13-hydroxylinolenic acid triggers phytoalexin production. Similarly, C6-C10 alkenals act as volatile elicitors of a defense response in cotton.

11. Progress in plant lipid research: the value of mutants

Biochemical approaches toward understanding plant lipid biosynthesis and function provided much of the information summarized above. However, in recent years, the isolation of mutants in plant lipid metabolism has been extremely fruitful in providing new insights and new methods for gene isolation. Much of the progress in the genetic dissection of plant lipid metabolism has evolved from the extensive studies of Somerville, Browse and coworkers with *A. thaliana*, which has one of the smallest genomes of higher plants. By using gas chromatography to screen several thousand randomly selected plants from a mutagenized population, Somerville and Browse were able to obtain an extensive collection of mutants showing altered leaf or seed fatty acid compositions. As described above, these mutants were instrumental in confirming the relationships between prokaryotic and eukaryotic phosphatidic acid synthesis and in the analysis and cloning of membrane-bound desaturases.

11.1. Mutants in lipid metabolism have helped link lipid structure and function

Two other major benefits have been derived from the *Arabidopsis* lipid mutants. First, the physiological effects of the mutations have provided the opportunity to evaluate the relationships between lipid structure and function. There has been a long-term assumption, based on the strong association of high levels of polyunsaturated fatty acids with photosynthetic membranes and the conservation of this property among higher and lower plant species, that these fatty acids must be essential for photosynthesis. However, many attempts to understand the relationships between membrane fatty acid composition and cell physiology or photosynthesis have led to equivocal results. The isolation of mutants

totally lacking certain unsaturated fatty acids has now provided much more convincing evaluations of their function and indeed, the results have forced re-evaluation of several previous hypotheses. For example, Δ^3-*trans*-hexadecenoate is an unusual plant fatty acid which is associated with phosphatidylglycerol of chloroplast membranes, is evolutionarily conserved, and is synthesized in coordination with the assembly of the photosynthetic apparatus. These observations led to the suggestion that Δ^3-*trans*-hexadecenoate is an essential component of photosynthesis. However, mutants which contain no detectable Δ^3-*trans*-hexadecenoate grow as well as wild-type plants, and all photosynthetic parameters examined appear normal (J. Browse, 1985). A minor difference in stability of some components of the photosystem can be detected by polyacrylamide gel electrophoresis. It has been concluded from such analyses that, although Δ^3-*trans*-hexadecenoate may facilitate assembly of the light harvesting complex into thylakoids, a more obvious phenotype could be restricted to certain unusual environmental conditions.

As mentioned above, a number of mutants blocked in the production of polyunsaturated fatty acid biosynthesis have also been isolated (Table 3). Because leaves have desaturases both in chloroplasts and in the ER, single mutations lead to only partial reduction of polyunsaturated fatty acid levels. Again, these mutants grow normally under most conditions and have normal photosynthetic parameters. However, several alterations in physiology are observed including changes in chloroplast ultrastructure, a reduction in the cross-sectional area of chloroplasts, and increased stability to thermal disruption of photosynthesis. Moreover, whereas wild-type *Arabidopsis* plants are chilling resistant and can reproduce normally at temperatures as low as 6°C, the mutants blocked in plastidial Δ^7 (*fad5*) and ω^6 (*fad6*) desaturation lose chlorophyll at 6°C and show a 20–30% reduction in growth rate relative to the wild-type plant. The *fad2* mutants, in which the ER ω^6-desaturase is blocked, are even more sensitive to 6°C and die if left at this temperature for several days. These results demonstrate that polyunsaturated fatty acids are essential for maintaining cellular function and plant viability at low temperatures (J. Okuley, 1994).

While most mutants which are reduced only in polyunsaturated fatty acid synthesis grow and develop normally at 22°C, a high-stearate mutant with 14% 18:0 is strikingly abnormal (Fig. 9) [28]. Many cell types fail to expand, resulting in mutant plants growing to less than one tenth the size of wild-type. At higher growth temperatures (36°C), the effects are less dramatic, suggesting that the physical properties or fluidity of highly saturated membranes are less impaired under these conditions. Mutants with a reduced content of saturated fatty acids are also impaired in growth, seed germination, and surface wax synthesis reflecting the essential role of these structures in a number of functions [29].

Other large-scale alterations in membrane fatty acid composition and phenotypes have been obtained by creation of multiple-mutant lines. When mutants defective in ER ω^6-desaturase were crossed with plants defective in the plastid ω^6-desaturase, double mutants could be recovered only on sucrose-supplemented media. The sucrose grown plants, which contained less than 6% polyunsaturated fatty acids, were chlorotic and unable to perform photosynthesis but otherwise were remarkably normal. These results, while confirming the significance of polyunsaturated fatty acids to photosynthesis, indicate that the vast majority of membrane functions can proceed despite drastically reduced levels of polyunsaturated fatty acids [30].

Table 3
Biochemical and physiological responses of selected *Arabidopsis* lipid mutants[a]

Mutant	Enzyme blocked[a]	Fatty acid or lipid phenotype[b]	Physiological response
fab1	3-ketoacyl-ACP synthase II	16:0↑	Death of plants after prolonged exposure to 2°C
fab2	18:0-ACP Δ^9-desaturase	18:0↑	Dwarf at 22°C
fad4	tΔ^3,16:0 desaturase?	tΔ^3,16:1↓	Altered stability of photosystem?
fad5	16:0 Δ^7-desaturase	16:0↑; 16:3↓	Enhanced growth rate at high temperatures; leaf chlorosis, reduced growth rate and impaired chloroplast development at low temperature
fad6	Plastid ω^6-desaturase	16:1↑;16:3↓	Leaf chlorosis, reduced growth rate and impaired chloroplast development at low temperature; enhanced thermotolerance of photosynthetic electron transport at high temperatures
fad7	Plastid ω^3-desaturase	16:2↑; 18:2↑ 16:3↓; 18:3↓	Reduced chloroplast size and altered chloroplast ultrastructure
fad2	ER ω^6-desaturase	18:1↑; 18:2↓	Greatly reduced stem elongation at 12°C; death at 6°C
fad2/*fad6*	Plastid and ER ω^6-desaturases	<6% polyunsaturated	Loss of photosynthesis
fad3/*fad7*/ *fad8*	Plastid and ER ω^3-desaturases	<1% trienoic	Male sterile, insect resistance decreased
dgd1	Digalactosyldiacyl-glycerol synthase	Digalactosyldiacyl-glycerol↓	Dwarfism, abnormal chloroplast size
act1	Plastid acyl-ACP:G3P acyltransferase	Phosphatidylglycerol↓, 16:3↓	Altered chloroplast structure
pgp1	Plastid & mitochondrial phosphatidylglycerol phosphate synthase	Phosphatidylglycerol↓	Loss of photosynthesis
mgd1	Diacylglycerol glycosyltransferase	Monogalactosyldiacyl-glycerol↓	Abnormal chloroplast development
AS11	Diacylglycerol acyltransferase	50% reduction in seed TAG	Slow germination
wri1	Transcription factor; glycolysis impaired	80% reduction in seed TAG	Slow germination

[a] In some cases the enzyme defect in the mutation is not known and this table lists the most likely possibility.
[b] Arrows indicate increase or decrease in component in mutant.

Triunsaturated fatty acids normally dominate chloroplast membranes and thus are the most abundant fatty acids on the planet. By constructing a triple mutant of *fad3*, *fad7*, and *fad8*, it has been possible to eliminate triunsaturated fatty acids from *Arabidopsis* without affecting 16:2 and 18:2 production (M. McConn, 1996). Surprisingly, these plants are able to grow, photosynthesize, and even flower. However, they are male sterile and therefore cannot produce seeds. This observation led to the discovery of a very different

Fig. 9. Increased stearic acid causes severe dwarfing of *Arabidopsis* [28]. A wild-type *Arabidopsis* plant (left) is compared to the *fab2* mutant (right) in which leaf stearic acid content has increased from 1 to 14%. Photo courtesy of John Browse, Washington State University.

role for jasmonate. This mutant cannot synthesize jasmonate because it lacks the 18:3 precursor (Section 10), and the plants are male sterile because pollen does not mature properly and is not released from the anthers. Application of jasmonate or linolenic acid to the anthers restores fertility, demonstrating that jasmonate is a key signal for pollen development (M. McConn, 1996). This result is a dramatic example of a change in fatty acid composition having a very specific effect on an essential developmental and tissue-specific reproductive process.

11.2. Arabidopsis *mutants have allowed cloning of desaturases and elongases*

A. thaliana mutants have also provided a means of cloning genes difficult to isolate by other methods. As in other kingdoms, the membrane-bound enzymes of plants have been

notoriously difficult to purify and characterize. However, cDNAs or genes encoding a number of these enzymes have now been isolated using molecular genetic strategies based on mutations. Several approaches have been successful. A cDNA encoding the ω^3-desaturase which converts linoleic to linolenic acid was isolated in 1992 by Arondel et al. [31] after a mutation leading to the loss of function was genetically mapped. The genomic region was then used to screen a cDNA library, and some of the clones had sequence similarity to cyanobacterial desaturases. These clones subsequently were shown to complement the loss of 18:3.

Gene 'tagging' strategies have also proved enormously valuable in identifying clones of membrane-bound enzymes. *Arabidopsis* and other plant genes can be 'tagged' by insertional inactivation when 'T-DNA' (a fragment from a plasmid carried by the plant pathogen, *Agrobacterium tumefaciens*) inserts randomly in the genome. When a promising phenotype is observed, the inactivated gene can be identified by probing with the T-DNA sequence. This method was used to identify the ω^6-desaturase required for the 18:1 to 18:2 conversion in the ER (J. Okuley, 1994). In addition, transposon tagging led to the cloning of a gene which controls elongation of oleic acid to 20:1 and 22:1 in developing *Arabidopsis* seeds (D.W. James, 1995). Since no membrane-bound fatty acid elongase had ever been completely purified or cloned from a eukaryotic organism, the finding that this gene encodes a 60-kDa condensing enzyme provided the first direct evidence that membrane fatty acid elongation is not catalyzed by a type I multifunctional fatty acid synthase.

12. Design of new plant oils

In recent years, progress has occurred not only in the isolation of many plant lipid biosynthetic genes, but also in the use of these genes to manipulate plant oil composition. As shown in Table 4, both substantial changes in seed oil composition and introduction of unusual fatty acids to heterologous species have been achieved. Progress in this area has been accelerated by several industrial laboratories whose goal has been the production of higher value oilseeds. Interest in renewable fuels has also stimulated efforts to increase oil content of seeds to provide greater supplies of biodiesel.

12.1. Design of new edible oils

12.1.1. Reduction in saturated fatty acids and improved stability of vegetable oils

Vegetable oils have gradually replaced animal fats as the major source of lipids in human diets and now constitute 15–25% of total caloric intake by industrialized nations. As shown in Fig. 10, vegetable oils display a wide range in the relative proportions of saturated and unsaturated fatty acid acids although in the United States, up to 80% of vegetable oil consumed is soybean oil making it the single largest source of calories in American diets. Most nutritionists recommend a reduction in saturated fat content in diets, and genetic engineering of plant oils can substantially help achieve this goal. Most of the saturated fatty acid in common plant oils is palmitic acid, and its occurrence is largely related to the action of a palmitoyl-ACP thioesterase. Reduction of the expression of this activity

Table 4
Some examples of genetic engineering of plant lipid metabolism

Modification achieved	Enzyme(s) engineered	Source of gene	Plants transformed
Omega-3 polyunsaturated fatty acids	phosphatidylcholine and acyl-CoA desaturases and elongases	Plant, fungal, and algal	*Arabidopsis*, soybean, canola, flax
Lauric acid production	Acyl-ACP thioesterase	California Bay	Rapeseed
Increased stearic acid	Antisense of stearoyl-ACP desaturase	Rapeseed	Rapeseed
Reduced saturated fatty acids	Stearoyl-CoA desaturase	Rat, yeast	Tobacco
	Acyl-ACP thioesterase	Soybean	Soybean
Increased and reduced 18:3	Omega-3 desaturase	Soybean, Canola, *Arabidopsis*	Soybean, rapeseed, *Arabidopsis*
Altered lauric acid distribution in TG	1-acyl-glycerol-3-phosphate acyltransferase	Coconut	Rapeseed
Altered cold tolerance	Acyl-ACP:glycerol-3-phosphate acyltransferase	*E. coli*, squash, *Arabidopsis*	Tobacco, *Arabidopsis*
Petroselinic acid production	Acyl-ACP desaturase	Coriander	Tobacco
Cyclopropane fatty acid production	Cyclopropane synthase	*E. coli*	Tobacco
γ-linolenic acid production	Linolenic Δ^6-desaturase	*Synechocystis*	Tobacco
Increased long-chain fatty acids	Fatty acid elongase	Jojoba	Rapeseed
Wax ester synthesis	Wax ester synthase, fatty acid reductase, fatty acid elongase	Jojoba	*Arabidopsis*

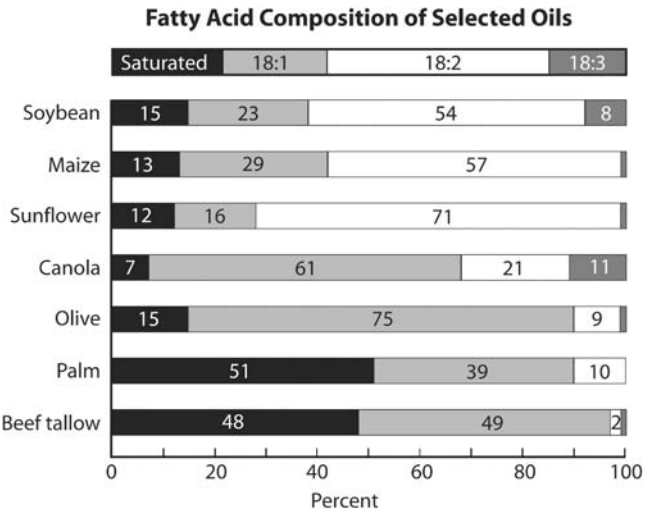


Fig. 10. Fatty acid composition of dietary vegetable oils and beef tallow. The values shown represent typical compositions of varieties grown commercially. Lines modified substantially through breeding or genetic engineering are available for soybean, canola, corn, and sunflower.

in transgenic soybean using co-suppression has led to decreases of the palmitic acid content from 15 to 6%.

A liquid soybean oil with very high oxidative stability and with low saturated fatty acids was also produced in soybean by suppression of the oleoyl desaturase (A. Kinney, 1996). Oleic acid content was increased up to 89%, 18:2 content reduced from 57 to less than 3%, and saturated fatty acid content was reduced to less than 7%. This oil has been produced commercially and is extremely stable for high-temperature frying applications. In addition, its stability matches that of mineral oil-derived lubricants, and therefore non-food uses as biodegradable lubricants are underway. One added consumer benefit to wide future use of the engineered high oleic acid oils may be reduction in the pathologies associated with high ω^6 consumption.

12.1.2. Engineering plants to replace fish oils

Diets high in the omega-3 polyunsaturated fatty acids typical of fish oils are believed to be highly beneficial in preventing heart disease (W.E. Lands, 2003). Is it possible for plants to produce these as an alternative to limited supplies of fish? A number of recent impressive advances in engineering plants to produce health-beneficial fatty acids in seed oils have been achieved. Eicosapentaenoic, docosohexaenoic, and arachidonic acid have now been produced in transgenic oilseeds by introducing desaturases and elongases from fungi and algae (J. Napier, 2006; I.A. Graham, 2007). Assuming that levels of these fatty acids can be further optimized, genetically engineered plants that produce omega-3 polyunsaturated fatty acids will provide a low-cost dietary replacement for fish oils and may contribute significantly to improved health.

Two other potential health-promoting fatty acids are stearidonic, ($18{:}4\Delta^{6,9,12,15}$), an ω^3 fatty acid precursor of the ω^3 family found in fish oils, and γ-linolenic acid ($18{:}3\Delta^{6,9,12}$), which is implicated in relieving arthritis and other conditions [32]. γ-linolenic acid is produced in some fungi and the seeds of a few plant species by the desaturation of linoleic acid to γ-linolenic acid by an ER-localized Δ^6-desaturase. Identification of cDNAs encoding this desaturase from borage and the fungus *Mortierella alpina* has led to their heterologous expression in plants. Expression of the *Mortierella* gene in *Brassica napus* seed resulted in 47% γ-linolenic acid production (V.M. Ursin, 2003). Expression of the borage Δ^6-desaturase together with an *Arabidopsis* Δ^{15}-desaturase in soybean resulted in 29% stearidonic and over 60% total omega-3 content in the oil (H. Eckert, 2006). These examples demonstrate that a very substantial level of high-value fatty acid end products can be achieved in crops.

12.2. Design of new industrial oils

As described above, plants have evolved the ability to produce a diverse range of fatty acid structures. Several specialty fatty acids have already been extensively exploited for industrial uses such as lubricants, plasticizers, and surfactants (Table 2). In fact, approximately one-third of all vegetable oil is now used for non-food products, and this figure is expected to increase as petroleum reserves are depleted. Thus, in addition to providing food, oilseed crops can be considered efficient, low polluting chemical factories which are

able to harness energy from sunlight and transform it into a variety of valuable chemical structures with a multitude of non-food uses.

12.2.1. High laurate and caprate oils

One of the major non-food uses of vegetable oils (approximately 500 million pounds of oil per annum in the US) is the production of soaps, detergents, and other surfactants. The solubility and other physical properties of medium-chain fatty acids and their derivatives make them especially suited for surfactant manufacture. Coconut and palm kernel oils, which contain 40–60% lauric acid (12:0), are current major feedstocks for the surfactant industry. The mechanism of synthesis of lauric and other medium-chain fatty acids in plants involves the action of a medium-chain acyl-ACP thioesterase which terminates fatty acid synthesis after a 10 or 12 carbon chain has been assembled (M. Pollard, 1991). A cDNA encoding such a thioesterase was isolated from seeds of the California Bay tree and transformed into rapeseed. As shown in Fig. 11, the introduction of this specialized thioesterase resulted in transgenic seeds that produced up to 60 mol% lauric acid. The plants grow normally and oil yields are very similar to those of the untransformed cultivars. Commercial production of high lauric rapeseed oil began in 1995. Although this crop has the potential to provide a new, non-tropical source of lauric oils for

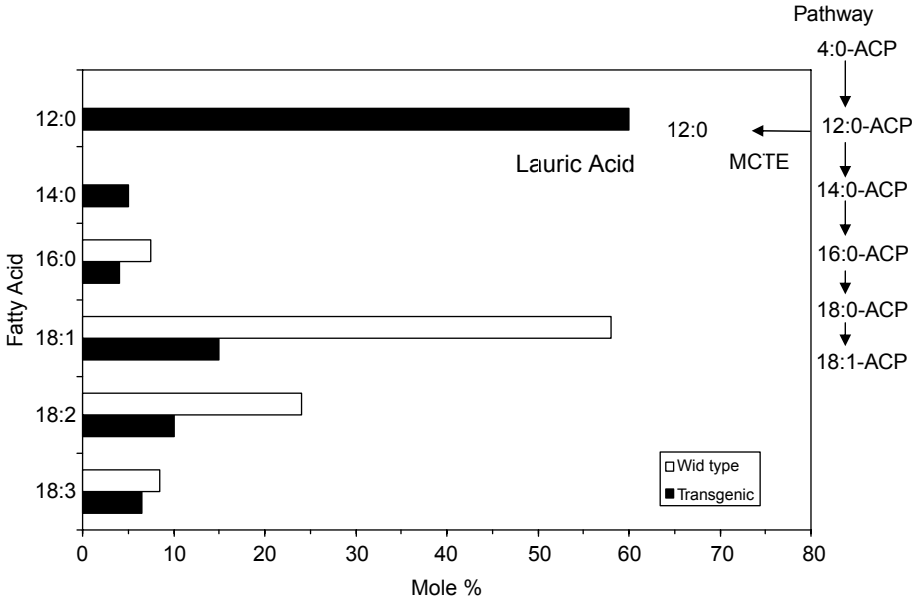


Fig. 11. Genetic engineering of rapeseed oil. A high level of lauric acid was achieved by expressing a medium-chain acyl-ACP thioesterase (MCTE) from California Bay in the transgenic seeds. This enzyme intercepts the fatty acid synthesis pathway at 12 carbons and hydrolyzes the fatty acid from its ACP carrier. Mol% of major fatty acids in a typical canola cultivar are compared to the composition achieved through genetic engineering.

the surfactant industry issues of cost-competitiveness and production of non-food oils in a food crop have limited its development.

Since current oil crops do not produce significant amounts of caprate (10:0) and caprylate (8:0), the cloning of thioesterases from plants accumulating these species raised hopes for development of commercial sources of 8:0 and 10:0. Surprisingly, medium-chain thioesterase from elm, a 10:0 accumulator, recognized either 10:0 or 16:0. However, a thioesterase from a C_{8-10}-rich *Cuphea* species, although showing strong selectivity for medium-chain fatty acids, gave disappointing results in transgenic plants. Part of this discrepancy was resolved with the discovery that medium-chain fatty acid production in *Cuphea* also involves a specific ketoacyl-ACP synthase. Co-transformation with the medium-chain thioesterase and the specialized ketoacyl-ACP synthase gave plants with substantial content of caprate and caprylate (K. Dehesh, 1998). Further improvements may be possible with addition of acyltransferases better able to introduce 8:0 and 10:0 fatty acids to underrepresented positions in TG (D.S. Knutzon, 1999).

12.2.2. Production of jojoba waxes in transgenic plants

In the past, long-chain wax esters were harvested from sperm whales and were a major ingredient of industrial lubricants and transmission fluids. Banning of whale harvests led to searches for alternative biological sources of such compounds. Jojoba, a desert shrub found in the American southwest, is the only plant species known to accumulate waxes (up to 60% of seed weight) rather than TG as a seed storage reserve. These waxes are mostly derived from C20 to C24 monounsaturated fatty acids and alcohols and are synthesized by the elongation of oleate followed by reduction to the alcohol by a fatty acid reductase. The wax storage lipid is formed by a fatty acyl-CoA: fatty alcohol acyltransferase, also referred to as wax synthase. The reductase and acyltransferase were purified from jojoba and the cognate cDNAs cloned. Coordinated expression of three genes, a *Lunaria annua* long-chain acyl-CoA elongase, and the jojoba reductase and acyltransferase in *Arabidopsis*, resulted in wax esters becoming the major component of the oil present in mature seeds (K. Laradizabal, 2000). The accumulation of waxes indicated that all the genes necessary for this trait were identified. If this trait could be successfully transferred to commercial crops, it would represent a large potential source of waxes used for a variety of applications including cosmetics and industrial lubricants.

12.2.3. Other industrial oils

Many of the unusual fatty acids produced by plants would have substantial value as industrial feedstocks if they were available in sufficient quantities at low prices. Examples of fatty acids in this category include those with hydroxy, epoxy, cyclopropane, or branched chains. These specialty fatty acids are often produced in wild species which have not been optimized for high agronomic and oil yields, and therefore production of such specialty oils is expensive. An alternative to the long-term effort required for domestication of such plants is the introduction of genes relevant to unusual fatty acid production into existing high-yielding oil crops. A step in this direction was made by the isolation of a cDNA encoding a fatty acid hydroxylase from the castor oil plant. When this gene was introduced into plants, approximately 20% of the fatty acids were hydroxy fatty acids (P. Broun, 1997). Genes required for specialty fatty acid production in plants need not be

isolated only from plants. As mentioned above, the stearoyl-CoA desaturases from animals and yeast are active in plants. Furthermore, the cyclopropane synthase of *E. coli* (K. Schmid, 1995) and desaturases from cyanobacteria and fungi have been successfully expressed in transgenic plants. Thus, in principle there are no fundamental barriers to producing a wide range of oil compositions using genes borrowed from diverse organisms. Furthermore, protein engineering offers even more possibilities to tailor the substrates and products of plant enzymes to produce 'designer oil crops' for specific end uses [6].

13. Future prospects

It is tempting to say that the biosynthetic pathways have been determined for all major plant lipids and most of the genes identified for enzymes in these pathways have been cloned. Clearly there has been great progress, although the biosynthetic pathways of cutin, suberin, sphingolipids, *trans*-Δ^3-16:1, and details of several other pathways, remain elusive. In addition, the enzymes involved in the production of many unusual fatty acids found in seed oils are largely unexplored. It is also intriguing to note that plants contain more genes for putative lipases, presumably involved in lipid breakdown and turnover, than they do biosynthetic enzymes [33]. Almost nothing is known about the specific role of most of these.

One area of expanding interest is the production of lipid hormones and signaling molecules (W. van Leeuwen, 2004). Several lipids, including phosphatidylinositol phosphates, diacylglycerol and *N*-acylphosphatidylethanolamine, have been implicated in signal transduction in plants. Another intriguing similarity between plants and animals is their use of oxygenated fatty acids in response to wounding. As mentioned in Section 10, jasmonate, a plant growth regulator derived from 18:3, is able at femtomolar concentrations to induce proteinase inhibitors and other plant defense genes. Like leukotriene synthesis in animals, jasmonate biosynthesis begins with the generation of a hydroperoxide by lipoxygenase. Jasmonate itself contains a cyclopentane ring comparable to those of prostaglandins. The common roles and origins of oxygenated fatty acids in plants and animals suggest a very early common ancestor for these pathways.

Application of molecular genetics and genomics to problems in lipid biochemistry will continue to expand. A particularly stimulating advance has been the complete sequencing of the *Arabidopsis* and other plant genomes and the availability of over several million expressed sequence tags sequences from a variety of plants. A recent survey of *Arabidopsis* genes involved in plant acyl lipid metabolism identified over 600 genes (http://www.plantbiology.msu.edu/lipids/genesurvey/). Although most of these genes could be assigned a tentative function based on sequence similarity to previously characterized genes, only a handful have been examined individually at an experimental level. Therefore, the precise functions of hundreds of genes awaits further work. For example, several genes are similar to acyl-CoA desaturases whose function has not yet been identified, and there are 19 genes encoding lipid transfer proteins and 51 additional lipid transfer protein-like genes. The physiological reasons underlying the existence of these large gene families for lipid transfer proteins, plastid ACPs, and stearoyl desaturases, but only one gene for ketoacyl-ACP synthase III and for most fatty acid synthase and ACC subunits remain to be elucidated.

Table 5
Internet resources related to plant lipid metabolism

Organization	Web site content	URL
National Plant Lipid Consortium (NPLC)	Program and abstracts of NPLC meetings	http://www.plantlipids.org/
	Directory of scientists involved in plant lipid research	http://www.msu.edu/user/ohlrogge/
	E-mail newsgroup for information on plant lipids	
Michigan State University	Survey and catalog of genes for plant lipid metabolism.	http://www.plantbiology.msu.edu/lipids/genesurvey/
	Gene expression profiles based on plant lipid ESTs and microarrays	
Kathy Schmid, Butler University	Links to many plant lipid research labs and web sites	http://blue.butler.edu/~kschmid/lipids.html
German Research Centre for Nutrition and Food — Institute for Lipid Research	Large database of seed oil fatty acids, sterols, literature, and other information	http://sofa.bfel.de/

The availability of T-DNA insertion mutants for almost all *Arabidopsis* genes means that the impact of gene disruptions can be tested. However, because 60% of *Arabidopsis* genes are present as duplicates, such gene disruptions must be supplemented by other strategies of functional genomics. Some websites related to these efforts in the plant lipid field are presented in Table 5.

Much of past lipid research has focused on a reductionist approach in which cells are taken apart and their pieces analyzed. The overall success of this approach and the wealth of new clones and sequence information have given us unprecedented knowledge of the pieces of the puzzle which represent lipid metabolism. However, as in any puzzle, it is not just complete knowledge of the pieces, but an understanding of how (and when) they fit together that defines the challenge. Microarrays that permit simultaneous monitoring of expression of many genes have begun to provide a more global overview of how genes work together to control seed metabolism [1]. Together with the ability to over- and under-express genes rapidly in transgenic plants and the strengths of classical biochemistry, such recent advances in analytical techniques should allow us to enter a new stage of lipid research emphasizing the interplay between metabolic compartments and the control of lipid synthesis during the plant life cycle.

Abbreviations

ACC acetyl-CoA carboxylase
ACP acyl carrier protein
ER endoplasmic reticulum
TG triacylglycerol

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CHAPTER 5

Oxidation of fatty acids in eukaryotes

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Contents

1. The pathway of β-oxidation: a historical account

Fatty acids are a major source of energy in animals. The study of their biological degradation began in 1904 when Knoop [1] performed the classical experiments that led him to formulate the theory of β-oxidation. In his experiments, Knoop used fatty acids with phenyl residues in place of the terminal methyl groups. The phenyl residue served as a *reporter group* because it was not metabolized, but instead was excreted in the urine. When Knoop fed phenyl-substituted fatty acids with an odd number of carbon atoms, like phenylpropionic acid (C_6H_5–CH_2–CH_2–COOH) or phenylvaleric acid (C_6H_5–CH_2–CH_2–CH_2–CH_2–COOH), to dogs, he isolated from their urine hippuric acid (C_6H_5–CO–NH–CH_2–COOH), the conjugate of benzoic acid and glycine. In contrast, phenyl-substituted fatty acids with an even number of carbon atoms, such as phenylbutyric acid (C_6H_5–CH_2–CH_2–CH_2–COOH), were degraded to phenylacetic acid (C_6H_5–CH_2–COOH) and excreted as phenylaceturic acid (C_6H_5–CH_2–CO–NH–CH_2–COOH). These observations led Knoop to propose that the oxidation of fatty acids begins at carbon atom 3, the β-carbon, and the resulting β-keto acids are cleaved between the α- and β-carbon to yield fatty acids shortened by two carbon atoms. Knoop's experiments prompted the idea that fatty acids are degraded in a stepwise manner by successive β-oxidation. In the years following Knoop's initial study, Dakin [2] performed similar experiments with phenylpropionic acid. Besides hippuric acid, he isolated the glycine conjugates of the following β-oxidation

intermediates: phenylacrylic acid (C_6H_5–CH=CH–COOH), β-phenyl-β-hydroxypropionic acid (C_6H_5–CHOH–CH_2–COOH), and benzoylacetic acid (C_6H_5–CO–CH_2–COOH). At the same time Embden and coworkers demonstrated that in perfused livers, unsubstituted fatty acids are degraded by β-oxidation and converted to ketone bodies. Thus, by 1910 the basic information necessary for formulating the pathway of β-oxidation was available.

After a 30-year period of little progress, Munoz and Leloir in 1943, and Lehninger in 1944, demonstrated the oxidation of fatty acids in cell-free preparations from liver. Their works set the stage for the complete elucidation of β-oxidation. Detailed investigations with cell-free systems demonstrated the need for energy to 'spark' the oxidation of fatty acids. ATP turned out to meet this requirement and to be essential for the activation of fatty acids (A.L. Lehninger, 1944). Activated fatty acids were shown to be thioesters formed from fatty acids and coenzyme A (CoA) (A. Kornberg, 1953; S. Wakil, 1953). This advance was made possible by earlier studies of Lipmann and coworkers, who isolated and characterized CoA (F. Lipmann, 1950), and Lynen [3], who proved the structure of 'active acetate' to be acetyl-CoA. Acetyl-CoA was found to be identical with the two-carbon fragment removed from fatty acids during their degradation. The subcellular location of the β-oxidation system was finally established by Kennedy and Lehninger (E.P. Kennedy, 1948), who demonstrated that mitochondria are the cellular components most active in fatty acid oxidation. The mitochondrial location of this pathway agreed with the observed coupling of fatty acid oxidation to the citric acid cycle and to oxidative phosphorylation. The most direct evidence for the proposed β-oxidation cycle emerged from enzyme studies in the 1950s primarily in the laboratories of Green in Wisconsin, Lynen in Munich, and Ochoa in New York. Their studies were greatly facilitated by newly developed methods of protein purification and by the use of spectrophotometric enzyme assays with chemically synthesized intermediates of β-oxidation as substrates.

2. *Uptake and activation of fatty acids in animal cells*

Fatty acids are transported between organs either as unesterified fatty acids complexed to serum albumin or in the form of triacylglycerols associated with lipoproteins. Triacylglycerols are hydrolyzed outside cells by lipoprotein lipase to yield free fatty acids (Chapter 19). The mechanism by which fatty acids enter cells remains poorly understood despite a number of studies performed with isolated cells from various tissues [4]. Kinetic evidence has been obtained for both a saturable and a non-saturable uptake of fatty acids. The saturable uptake predominates at nanomolar concentrations of fatty acids and is thought to be mediated, or assisted, by proteins. In contrast, the non-saturable uptake that is effective at higher concentrations of fatty acids has been attributed to passive diffusion of fatty acids across the membrane. Several suspected fatty acid transport proteins have been identified [5]. Although their specific functions in fatty acid uptake remain to be elucidated, these proteins may assist in the desorption of fatty acids from albumin and/or function in uptake coupled to the esterification of fatty acids with CoA, in a process referred to as vectorial acylation.

Once long-chain fatty acids have crossed the plasma membrane, they either diffuse or are transported to mitochondria, peroxisomes, and the endoplasmic reticulum where they

are activated by conversion to their CoA thioesters. Whether this transfer of fatty acids between membranes is a facilitated process or occurs by simple diffusion is an unresolved issue. The identification of low-molecular-weight (14–15 kDa) fatty acid binding proteins (FABPs) in the cytosol of various animal tissues prompted the suggestion that these proteins might function as carriers of fatty acids in the cytosolic compartment [6]. FABPs may also be involved in the cellular uptake of fatty acids, their intracellular storage, or the delivery of fatty acids to sites of their utilization. The importance of FABPs in fatty acid metabolism is supported by the observation that the uptake and utilization of long-chain fatty acids are reduced in knockout mice lacking heart FABP (B. Binas, 1999). These animals exhibit exercise intolerance and, at old age, develop cardiac hypertrophy. Also, ablation of the gene for liver FABP reduces fatty acid oxidation and ketogenesis in mice (B. Binas, 2004). Nonetheless, the molecular mechanism of FABP function remains to be elucidated.

The metabolism of fatty acids requires their prior activation by conversion to fatty acyl-CoA thioesters. The activating enzymes are ATP-dependent acyl-CoA synthetases, which catalyze the formation of acyl-CoA by the following two-step mechanism in which E represents the enzyme:

$$\mathrm{E + R{-}COOH + ATP \xrightarrow{Mg^{2+}} (E{:}R{-}CO{-}AMP) + PPi}$$
$$\mathrm{(E{:}R{-}CO{-}AMP) + CoASH \rightarrow R{-}CO{-}SCoA + AMP + E}$$

The evidence for this mechanism was primarily derived from a study of acetyl-CoA synthetase. Although the postulated intermediate, acetyl-AMP, does not accumulate in solution, and therefore exists only bound to the enzyme, the indirect evidence for this intermediate is very compelling. Other fatty acids are assumed to be activated by a similar mechanism, even though less evidence in support of this hypothesis has been obtained. The activation of fatty acids is catalyzed by a group of acyl-CoA synthetases that differ with respect to their subcellular locations and their specificities for fatty acids of different chain lengths [7]. Their chain-length specificities are the basis for classifying these enzymes as short-chain, medium-chain, long-chain, and very-long-chain acyl-CoA synthetases.

A short-chain specific acetyl-CoA synthetase that is present in mammalian mitochondria has been purified and its cDNA has been cloned. This 71-kDa enzyme, which is most active with acetate as a substrate but exhibits some activity towards propionate, has been detected in mitochondria of heart, skeletal muscle, kidney, adipose tissue, and intestine, but not in mitochondria of liver. A cytosolic 78-kDa acetyl-CoA synthetase has been identified in liver, intestine, adipose tissue, and mammary gland, all of which have high lipogenic activities. Expression studies support the hypothesis that the cytosolic enzyme synthesizes acetyl-CoA for lipogenesis, whereas the mitochondrial acetyl-CoA synthetase activates acetate destined for oxidation (T.T. Yamamoto, 2001).

Medium-chain acyl-CoA synthetases exist in mitochondria of various mammalian tissues. The enzyme present in heart mitochondria acts on fatty acids with three to seven carbon atoms, but is most active with butyrate. In contrast, a 62-kDa enzyme of liver mitochondria activates fatty acids with 4–14 carbon atoms with octanoate being the best substrate. This enzyme also activates aromatic carboxylic acids such as benzoic acid and

its substituted derivatives. Overall, the medium-chain acyl-CoA synthetase of liver mitochondria has a broader and more diverse substrate specificity than the enzyme in heart mitochondria. Additionally, liver mitochondria contain a medium-chain acyl-CoA synthetase that is most active with isobutyrate, a metabolite of valine.

Long-chain acyl-CoA synthetase (ACSL) is a membrane-bound enzyme that is associated with the endoplasmic reticulum, peroxisomes, and the outer mitochondrial membrane. The enzyme acts efficiently on saturated fatty acids with 10–20 carbon atoms and on common unsaturated fatty acids having 16–20 carbon atoms. Molecular cloning and expression studies revealed the existence of five different long-chain acyl-CoA synthetases (ACSL 1, 3–6) in mammals (R.A. Coleman, 2004). The five ACSL isozymes differ with regard to their tissue distributions, subcellular locations, kinetic properties, substrate specificities, and regulatory responses. The existence of ACSL isoforms with different properties has been interpreted in terms of distinct metabolic functions that assure the channeling of fatty acids toward degradation or lipid biosynthesis. Such functional commitments of isozymes have previously been recognized. For example, long-chain acyl-CoA synthetases ACS I and ACS II of *Candida lipolytica* are thought to activate long-chain fatty acids for complex lipid synthesis and peroxisomal β-oxidation, respectively (S. Numa, 1981).

Very-long-chain acyl-CoA synthetase activates fatty acids with 22 or more carbon atoms and also acts on long-chain and branched-chain fatty acids and on trihydroxycholestanoic acid. This membrane-bound enzyme is strongly expressed in liver where it is associated with the endoplasmic reticulum and peroxisomes but not with mitochondria. Purification of the 70-kDa very-long-chain acyl-CoA synthetase enabled the cloning of rat and human cDNAs encoding this enzyme (T. Hashimoto, 1996). Sequence homologies with other cDNAs aided in the identification of choloyl-CoA synthetase and in the demonstration that fatty acid transport protein 1, a suggested transporter of fatty acids across the plasma membrane, exhibits very-long-chain acyl-CoA synthetase activity.

3. Fatty acid oxidation in mitochondria

In animal cells, fatty acids are degraded both in mitochondria and peroxisomes, whereas in lower eukaryotes, β-oxidation is confined to peroxisomes. Mitochondrial β-oxidation provides energy for oxidative phosphorylation and generates acetyl-CoA for ketogenesis in liver. The oxidation of fatty acids with odd numbers of carbon atoms also yields propionyl-CoA that is metabolized to succinate.

3.1. Mitochondrial uptake of fatty acids

Fatty acyl-CoA thioesters that are formed at the outer mitochondrial membrane cannot directly enter the mitochondrial matrix where the enzymes of β-oxidation are located, because CoA and its derivatives are unable to pass rapidly through the inner mitochondrial membrane. Instead, carnitine carries the acyl residues of acyl-CoA thioesters across the inner mitochondrial membrane. The carnitine-dependent translocation of fatty acids across the inner mitochondrial membrane is schematically shown in Fig. 1 [8]. The reversible

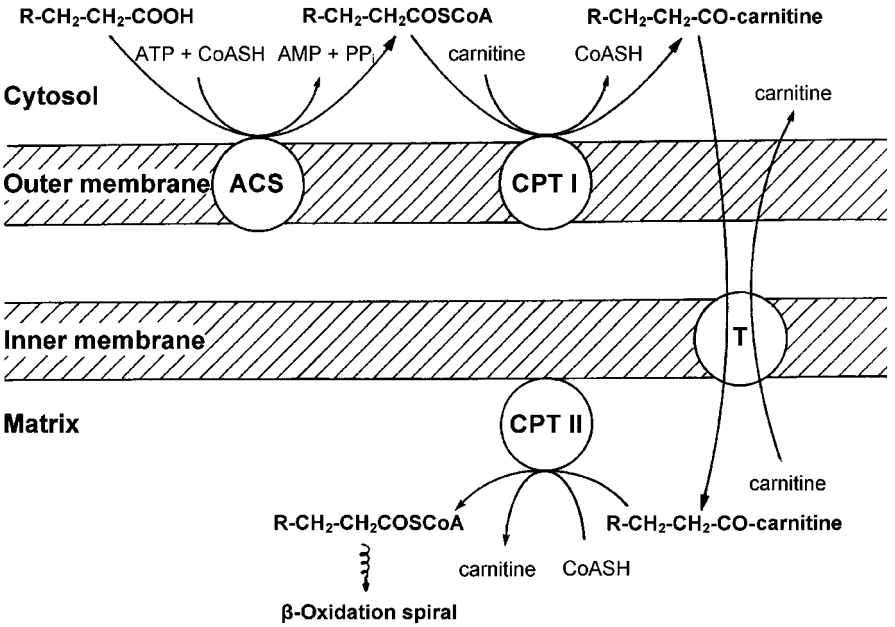

Fig. 1. Carnitine-dependent transfer of acyl groups across the inner mitochondrial membrane. Abbreviations: ACS, acyl-CoA synthetase; CPT I and CPT II, carnitine palmitoyltransferase I and II, respectively; T, carnitine: acylcarnitine translocase.

transfer of fatty acyl residues from CoA to carnitine is catalyzed by carnitine palmitoyltransferase I (CPT I), which is an enzyme of the outer mitochondrial membrane. The resultant acylcarnitines cross the inner mitochondrial membrane via the carnitine:acylcarnitine translocase. This carrier protein catalyzes a rapid mole-for-mole exchange of acylcarnitine for carnitine, carnitine for carnitine, and acylcarnitine for acylcarnitine. This exchange, especially of acylcarnitine for carnitine, is essential for the translocation of long-chain fatty acids from the cytosol into mitochondria. In addition, the translocase facilitates a slow unidirectional flux of carnitine across the inner mitochondrial membrane. This unidirectional flux of carnitine may be important for mitochondria of organs other than liver to acquire carnitine, which is synthesized in the liver. The rat liver translocase, which has a subunit molecular mass of 32.5 kDa, has been purified, its cDNA has been cloned, and the recombinant protein has been functionally reconstituted into proteoliposomes (F. Palmieri, 1997).

In the mitochondrial matrix, carnitine palmitoyltransferase II (CPT II) catalyzes the reversible transfer of acyl residues with 10–18 carbon atoms between carnitine and CoA to form acyl-CoA thioesters that are the substrates of β-oxidation [4]. CPT II purified from mitochondria of bovine heart and rat liver has a subunit molecular mass of approximately 70 kDa. The crystal structure of CPT II revealed the presence of two antiparallel helices that are absent from soluble carnitine acyltransferases and are believed to facilitate the association with the inner mitochondrial membrane (M. Henning, 2006).

CPT I, in contrast to CPT II, is reversibly inhibited by malonyl-CoA, its natural regulator, and is covalently modified and inactivated by CoA derivatives of certain alkyl glycidic acids [8]. The latter property was utilized to label this protein for generating sequence information that permitted the molecular cloning of CPT I. Human and rat cDNAs encode 88-kDa proteins that are highly homologous (88%) to each other and also are very similar (50%) to CPT II. An isoform of liver CPT I (L-CPT I) is present in skeletal muscle (M-CPT I), while both isoforms are expressed in heart mitochondria. L-CPT I and M-CPT I are products of different genes and have different kinetic properties. M-CPT I is much more sensitive toward malonyl-CoA ($K_i \approx 20$ nM vs. 2 μM) than is L-CPT I but has a lower affinity for carnitine ($K_m \approx 500$ μM vs. 30 μM). CPT I is anchored in the outer mitochondrial membrane via two transmembrane segments so that the 46 N-terminal residues and the large C-terminal catalytic domain remain on the cytosolic side of the membrane. CPT I together with CPT II and ACS seem to be concentrated at contact sites between the inner and outer mitochondrial membrane. The structure–function relationship of CPT I was studied by expressing it in the yeast *Pichia pastoris* that normally does not contain this enzyme. That approach revealed positive as well as negative regulatory elements in the cytosolic N-terminal region of CPT I, which affect the sensitivity of L-CPT I toward malonyl-CoA and the affinity of M-CPT I for carnitine [9]. Additionally, the intermembrane loop region and a single glutamate residue in the C-terminal region were observed to affect the sensitivity of CPT I toward malonyl-CoA.

Mitochondria contain carnitine acetyltransferase in addition to CPT I and CPT II. The purified enzyme from bovine heart consists of a single polypeptide chain with an estimated molecular mass of 60 kDa. This enzyme catalyzes the transfer of acyl groups with 2–10 carbon atoms between CoA and carnitine at an active site that is located in a deep channel at the interface of two domains with similar backbone fold (L. Tong, 2003). The function of this enzyme has not been established conclusively. Perhaps, the enzyme regenerates free CoA in the mitochondrial matrix by transferring acetyl groups and other short-chain or medium-chain acyl residues from CoA to carnitine. The resultant acylcarnitines can leave mitochondria via the carnitine:acylcarnitine translocase and can be metabolized by the same or other tissues, or can be excreted in urine. In addition, carnitine acetyltransferase together with CPT II may convert acylcarnitines that were formed by the partial β-oxidation of fatty acids in peroxisomes to acyl-CoAs for further oxidation in mitochondria.

Short-chain and medium-chain fatty acids with less than 10 carbon atoms can enter mitochondria as free acids independent of carnitine. They are activated by short-chain and medium-chain acyl-CoA synthetases that are present in the mitochondrial matrix.

3.2. Enzymes of β-oxidation in mitochondria

The enzymes of β-oxidation either are associated with the inner mitochondrial membrane or are located in the mitochondrial matrix. The reactions catalyzed by these enzymes are shown schematically in Fig. 2, which also provides a hypothetical view of the physical and functional organization of these enzymes.

In the first of four reactions that constitute one cycle of the β-oxidation spiral, acyl-CoA is dehydrogenated to 2-*trans*-enoyl-CoA according to the following equation.

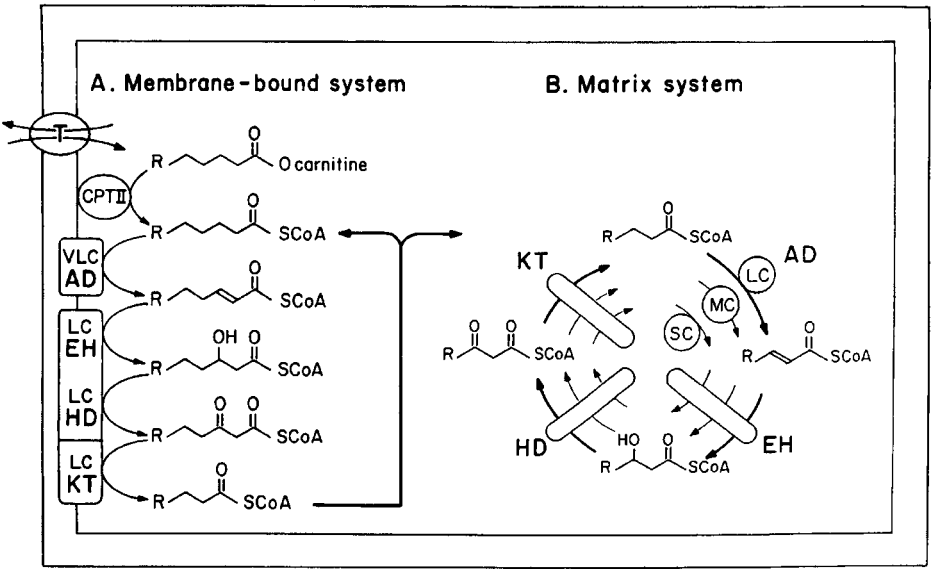

Fig. 2. Model of the functional and physical organization of β-oxidation enzymes in mitochondria. (A) β-Oxidation system active with long-chain (LC) acyl-CoAs; (B) β-oxidation system active with medium-chain (MC) and short-chain (SC) acyl-CoAs. Abbreviations: T, carnitine:acylcarnitine translocase; CPT II, carnitine palmitoyltransferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; VLC, very-long-chain.

$$R-CH_2-CH_2-CO-SCoA + FAD \rightarrow R-CH=CH-CO-SCoA + FADH_2$$

Four acyl-CoA dehydrogenases with different but overlapping chain length specificities cooperate to assure the complete degradation of all fatty acids that can be metabolized by mitochondrial β-oxidation. The names of the four dehydrogenases — short-chain, medium-chain, long-chain, and very-long-chain acyl-CoA dehydrogenases — reflect their chain length specificities. Purification of these enzymes permitted detailed studies of their molecular and mechanistic properties [10,11]. The first three dehydrogenases are soluble matrix enzymes with similar molecular masses between 170 and 190 kDa. They are composed of four identical subunits, each of which carries a tightly, but non-covalently-bound flavin adenine dinucleotide (FAD). Cloning and sequencing of their cDNAs revealed high degrees of homology (close to 90%) for the same type of enzyme from man and rat and significant homologies (30–35%) for different enzymes from the same organism. The crystal structures of medium-chain acyl-CoA dehydrogenase and other acyl-CoA dehydrogenases confirmed the homotetrameric structure of these enzymes with one FAD bound per subunit in an extended conformation [12]. Very-long-chain acyl-CoA dehydrogenase, in contrast to the three other dehydrogenases, is a protein of the inner mitochondrial membrane. Purification of this enzyme and its molecular cloning established that it is a 133-kDa homodimer with one FAD bound per subunit. The four dehydrogenases differ with respect to their specificities for substrates of various chain lengths.

Short-chain acyl-CoA dehydrogenase acts only on short-chain substrates like butyryl-CoA and hexanoyl-CoA. Medium-chain acyl-CoA dehydrogenase is most active with substrates from hexanoyl-CoA to dodecanoyl-CoA, whereas long-chain acyl-CoA dehydrogenase preferentially acts on octanoyl-CoA and longer chain substrates. Very-long-chain acyl-CoA dehydrogenase extends the activity spectrum to longer chain substrates, including those having acyl chains with 22 and 24 carbon atoms. However, long-chain acyl-CoA dehydrogenase may have a specific function in the β-oxidation of unsaturated fatty acids because this enzyme, in contrast to very-long-chain acyl-CoA dehydrogenase, effectively dehydrogenates 4-enoyl-CoAs and 5-enoyl-CoAs, which are β-oxidation intermediates of unsaturated fatty acids. This hypothesis is supported by the observed accumulation of intermediates of unsaturated fatty acids, but not of saturated fatty acids, in knockout mice lacking long-chain acyl-CoA dehydrogenase (P.A. Wood, 1998). Also, acyl-CoA dehydrogenase 9, a recently identified homolog of very-long-chain acyl-CoA dehydrogenase, is suspected of playing a role in the β-oxidation of unsaturated fatty acids, especially in brain (J. Vockley, 2005).

The dehydrogenation of acyl-CoA thioesters involves the removal of a proton from the α-carbon of the substrate and hydride transfer from the β-carbon to the FAD cofactor of the enzyme to yield 2-*trans*-enoyl-CoA and enzyme-bound $FADH_2$ [13]. Studies based on X-ray crystallography, chemical modifications, and site-specific mutagenesis established that a glutamate residue is responsible for the α-proton abstraction in medium-chain acyl-CoA dehydrogenase and other acyl-CoA dehydrogenases. Reoxidation of $FADH_2$ occurs by two successive single-electron transfers from the dehydrogenase to the FAD prosthetic group of a second flavoprotein named electron-transferring flavoprotein (ETF), which donates electrons to an iron–sulfur flavoprotein named ETF:ubiquinone oxidoreductase. The latter enzyme, a component of the inner mitochondrial membrane, feeds electrons into the mitochondrial electron transport chain via ubiquinone. The flow of electrons from acyl-CoA to oxygen is schematically shown below:

$$\mathrm{R{-}CH_2{-}CH_2{-}CO{-}SCoA} \rightarrow \mathrm{FAD(acyl\text{-}CoA\ dehydrogenase)} \rightarrow \mathrm{FAD(ETF)}$$
$$\rightarrow \mathrm{FAD/[4Fe4S](ETF{:}ubiquinone\ oxidoreductase)} \rightarrow \mathrm{ubiquinone} \rightarrow \mathrm{oxygen}$$

ETF is a soluble matrix protein with a molecular mass of close to 60 kDa. It is composed of two non-identical subunits of similar molecular masses with one FAD per protein dimer. The crystal structure of ETF revealed the location of FAD in a cleft between the two subunits (J.-J. Kim, 1996).

In addition to the four acyl-CoA dehydrogenases involved in fatty acid oxidation, three acyl-CoA dehydrogenases specific for metabolites of branched-chain amino acids have been characterized. They are isovaleryl-CoA dehydrogenase, 2-methyl-branched-chain acyl-CoA dehydrogenase, and isobutyryl-CoA dehydrogenase, which may also function in the β-oxidation of branched-chain carboxylic acids.

In the second step of β-oxidation, 2-*trans*-enoyl-CoA is reversibly hydrated by enoyl-CoA hydratase to L-3-hydroxyacyl-CoA as shown below.

$$\mathrm{R{-}CH{=}CH{-}CO{-}SCoA + H_2O} \rightarrow \mathrm{R{-}CH(OH){-}CH_2{-}CO{-}SCoA}$$

Two enoyl-CoA hydratases have been identified in mitochondria [4]. The better characterized of the two enzymes is enoyl-CoA hydratase or crotonase, which is a 161-kDa homohexamer. The best substrate of crotonase is crotonyl-CoA (CH_3–CH= CH–CO–SCoA), which is hydrated to form L(*S*)-3-hydroxybutyryl-CoA. The activity of the enzyme decreases with increasing chain length of the substrate so that the activity with 2-*trans*-hexadecenoyl-CoA is only 1–2% of the activity achieved with crotonyl-CoA. Crotonase also hydrates 2-*cis*-enoyl-CoA to D-3-hydroxyacyl-CoA and exhibits very low Δ^3,Δ^2-enoyl-CoA isomerase activity. The crystal structure of crotonase revealed that it belongs to the hydratase/isomerase superfamily with two active site glutamate residues that function as general acid and general base in the *syn* addition of water to crotonyl-CoA (R.K. Wierenga, 1998). The second enoyl-CoA hydratase, referred to as long-chain enoyl-CoA hydratase, is virtually inactive with crotonyl-CoA, but effectively hydrates medium-chain and long-chain substrates. The activities of crotonase and long-chain enoyl-CoA hydratase complement each other thereby assuring high rates of hydration of all enoyl-CoA intermediates. Long-chain enoyl-CoA hydratase is a component enzyme of the trifunctional β-oxidation complex, which additionally exhibits long-chain activities of L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase [14]. This β-oxidation complex is a protein of the inner mitochondrial membrane. It consists of equimolar amounts of a large α-subunit with a molecular mass of close to 80 kDa and of a small β-subunit with a molecular mass of approximately 48 kDa. Cloning and cDNA sequencing revealed significant homologies of the amino-terminal and central regions of the large subunit with enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase, respectively, and of the small subunit with 3-ketoacyl-CoA thiolase. These homologies are indicative of the locations of the component enzymes on the complex.

The third reaction in the β-oxidation cycle is the reversible dehydrogenation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenase as shown in the following equation.

$$R-CH(OH)-CH_2-CO-SCoA + NAD^+ \rightarrow R-CO-CH_2-CO-SCoA + NADH + H^+$$

Three L-3-hydroxyacyl-CoA dehydrogenases have been identified in mitochondria. L-3-Hydroxyacyl-CoA dehydrogenase is a soluble matrix enzyme that has a molecular mass of approximately 65 kDa and is composed of two identical subunits [4,15]. The crystal structure of the pig heart enzyme revealed a bi-lobal structure with the NAD^+ binding site in the N-terminal region and the substrate binding site in the cleft between the C-terminal and N-terminal domains. The enzyme is specific for NAD^+ as a coenzyme and acts on L-3-hydroxyacyl-CoAs of various chain lengths but is most active with medium-chain and short-chain substrates. The second L-3-hydroxyacyl-CoA dehydrogenase is long-chain L-3-hydroxyacyl-CoA dehydrogenase, a component enzyme of the trifunctional β-oxidation complex or trifunctional protein that is associated with the inner mitochondrial membrane [14]. This dehydrogenase is active with medium- and long-chain substrates, but not with 3-hydroxybutyryl-CoA, and hence complements the soluble dehydrogenase to assure high rates of dehydrogenation over the whole spectrum of β-oxidation intermediates. The third dehydrogenase is short-chain L-3-hydroxyacyl-CoA dehydrogenase [15], a homotetramer with a subunit molecular mass of 27 kDa. The primary

and crystal structures of this enzyme confirmed it as a member of the short-chain dehydrogenase/reductase family. This enzyme catalyzes the NAD^+-dependent dehydrogenation of short-chain L-3-hydroxyacyl-CoAs, but is most active with branched-chain substrates like 3-hydroxy-2-methylbutyryl-CoA, a metabolite of isoleucine degradation. The essential function of this dehydrogenase in isoleucine degradation was demonstrated by the excretion of isoleucine metabolites in a patient with a mutation in the short-chain L-3-hydroxyacyl-CoA dehydrogenase gene. This enzyme may have another major function in androgen metabolism because it exhibits significant 17β-hydroxysteroid dehydrogenase activity.

In the last reaction of the β-oxidation cycle, 3-ketoacyl-CoA is cleaved by thiolase as shown below.

$$R-CO-CH_2-CO-SCoA + CoASH \rightarrow R-CO-SCoA + CH_3-CO-SCoA$$

The products of the reaction are acetyl-CoA and an acyl-CoA shortened by two carbon atoms. The equilibrium of the reaction is more to the side of the thiolytic cleavage products thereby driving β-oxidation to completion. All thiolases that have been studied in detail contain an essential sulfhydryl group, which participates directly in the carbon–carbon bond cleavage as outlined in the following equations where E–SH represents thiolase [4].

$$E-SH + R-CO-CH_2-CO-SCoA \rightarrow R-CO-S-E + CH_3-CO-SCoA$$
$$R-CO-S-E + CoASH \rightarrow R-CO-SCoA + E\text{-}SH$$

According to this mechanism, 3-ketoacyl-CoA binds to the enzyme and is cleaved between its α and β carbon atoms. An acyl residue, which is two carbons shorter than the substrate, is transiently bound to the enzyme via a thioester bond, while acetyl-CoA is released from the enzyme. Finally, the acyl residue is transferred from the sulfhydryl group of the enzyme to CoA to yield acyl-CoA.

Several types of thiolases have been identified, some of which exist in multiple forms [4]. Mitochondria contain three classes of thiolases: (i) acetoacetyl-CoA thiolase or acetyl-CoA acetyltransferase, which is specific for acetoacetyl-CoA (C_4) as a substrate; (ii) 3-ketoacyl-CoA thiolase or acetyl-CoA acyltransferase, which acts on 3-ketoacyl-CoA thioesters of various chain lengths (C_4–C_{16}); and (iii) long-chain 3-ketoacyl-CoA thiolase, which acts on medium- and long-chain 3-ketoacyl-CoA thioesters but not on acetoacetyl-CoA. The latter two enzymes are essential for fatty acid β-oxidation, whereas acetoacetyl-CoA thiolase most likely functions only in ketone body and isoleucine metabolism. Long-chain 3-ketoacyl-CoA thiolase is a component enzyme of the membrane-bound trifunctional β-oxidation complex [14], whereas the other two thiolases are soluble matrix enzymes. All mitochondrial thiolases have been purified and their cDNAs have been cloned and sequenced. A comparison of amino acid sequences proved all mitochondrial thiolases to be different, but homologous, enzymes. 3-Ketoacyl-CoA thiolase is composed of four identical subunits with a molecular mass ≈42 kDa. This enzyme acts equally well on all substrates tested except for acetoacetyl-CoA, which is cleaved at half the maximal rate observed with longer chain substrates.

The absence or near absence of intermediates of β-oxidation from mitochondria prompted the idea of intermediate channeling due to the existence of a multienzyme complex of β-oxidation enzymes in intact mitochondria. The identification and characterization of at least two isozymes for each of the four reactions of the β-oxidation spiral led to the presentation of a model for their physical and functional organization as shown in Fig. 2 [16]. By this model, the membrane-bound, long-chain specific β-oxidation system, consisting of very-long-chain acyl-CoA dehydrogenase and the trifunctional β-oxidation complex, converts long-chain fatty acyl-CoAs to medium-chain acyl-CoAs, which are completely degraded by the matrix system of soluble enzymes that have a preference for medium-chain and short-chain substrates. An assumption underlying this model is that all enzymes thought to function in fatty acid β-oxidation are essential for this process. So far this assumption has proven to be correct. The characterization of inherited disorders of fat metabolism in humans has revealed that each of the many β-oxidation enzymes found to be deficient in a patient is essential for the normal degradation of fatty acids (Section 5).

3.3. β-Oxidation of unsaturated fatty acids

Unsaturated fatty acids, which usually contain *cis* double bonds, also are degraded by β-oxidation. However, additional (auxiliary) enzymes are required to act on the preexisting double bonds once they are in close proximity to the thioester group as a result of chain-shortening [4]. All double bonds present in unsaturated and polyunsaturated fatty acids can be classified either as odd-numbered double bonds, like the 9-*cis* double bond of oleic acid and linoleic acid or as even-numbered double bonds like the 12-*cis* double bond of linoleic acid. Since both classes of double bonds are present in linoleic acid, its degradation illustrates the breakdown of all unsaturated fatty acids. The β-oxidation of linoleic acid is shown in Fig. 3. Linoleic acid, after conversion to its CoA thioester (I), undergoes three cycles of β-oxidation to yield 3-*cis*,6-*cis*-dodecadienoyl-CoA (II), which is isomerized to 2-*trans*,6-*cis*-dodecadienoyl-CoA (III) by Δ^3,Δ^2-*trans*-enoyl-CoA isomerase, an auxiliary enzyme of β-oxidation. 2-*trans*,6-*cis*-Dodecadienoyl-CoA (III) is a substrate of β-oxidation and can complete one cycle to yield 4-*cis*-decenoyl-CoA (IV), which is dehydrogenated to 2-*trans*,4-*cis*-decadienoyl-CoA (V) by medium-chain acyl-CoA dehydrogenase. 2-*trans*,4-*cis*-Decadienoyl-CoA (V) cannot continue on its course through the β-oxidation spiral, but instead is reduced by NADPH in a reaction catalyzed by 2,4-dienoyl-CoA reductase. The product of this reduction, 3-*trans*-decenoyl-CoA (VI), is isomerized by Δ^3,Δ^2-enoyl-CoA isomerase to 2-*trans*-decenoyl-CoA (VII), which can be completely degraded by completing four cycles of β-oxidation. Altogether, the degradation of unsaturated fatty acids in mitochondria involves at least Δ^3,Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase as auxiliary enzymes in addition to the enzymes of the β-oxidation spiral.

More recent is the observation that odd-numbered double bonds can be reduced at the stage of 5-enoyl-CoA intermediates that are formed during the β-oxidation of unsaturated fatty acids. Shown in Fig. 4 is the sequence of reactions that explains the NADPH-dependent reduction of 5-*cis*-enoyl-CoA (I) [17]. The introduction of a 2-*trans* double bond by acyl-CoA dehydrogenase yields 2,5-dienoyl-CoA (II), part of which is converted

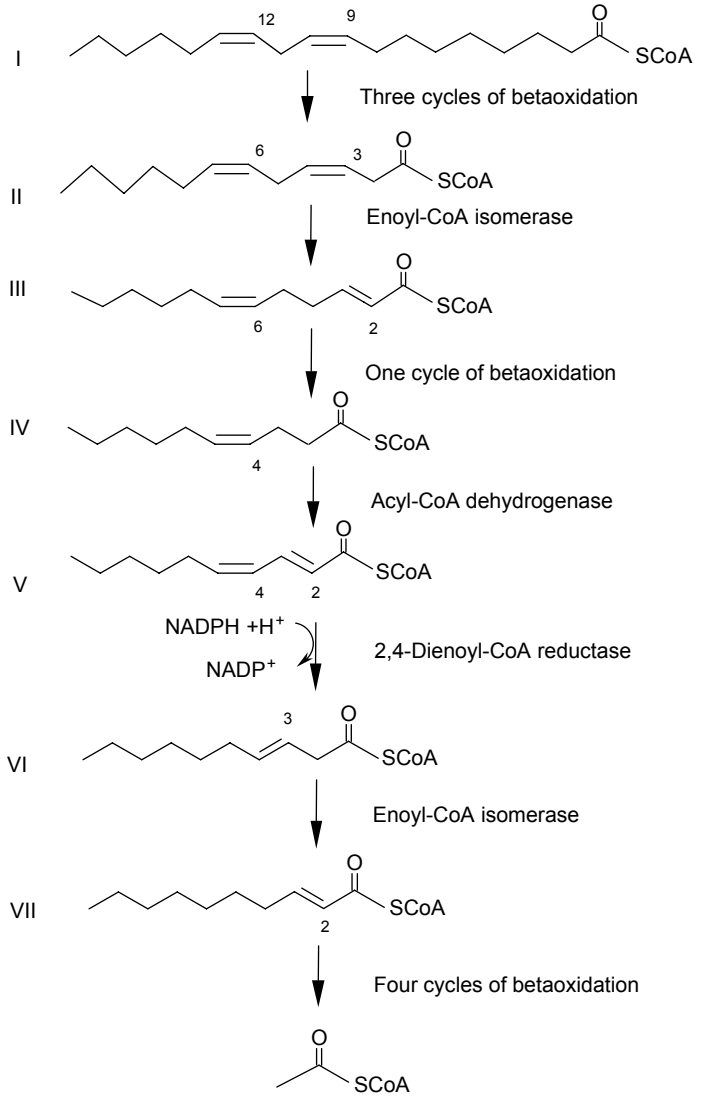

Fig. 3. β-Oxidation of linoleoyl-CoA.

to 3,5-dienoyl-CoA (III) by Δ^3,Δ^2-enoyl-CoA isomerase. A novel enzyme, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, converts 3,5-dienoyl-CoA (III) to 2-*trans,*4-*trans*-dienoyl-CoA (IV) by a concerted shift of both double bonds. Finally, 2,4-dienoyl-CoA reductase catalyzes the NADPH-dependent reduction of one double bond to produce 3-*trans*-enoyl-CoA (V), which, after isomerization to 2-*trans*-enoyl-CoA (VI) by Δ^3,Δ^2-enoyl-CoA isomerase, can reenter the β-oxidation spiral. It has been estimated that in rat liver less than 20% of oleic acid is degraded by this pathway, while most of this fatty acid is broken

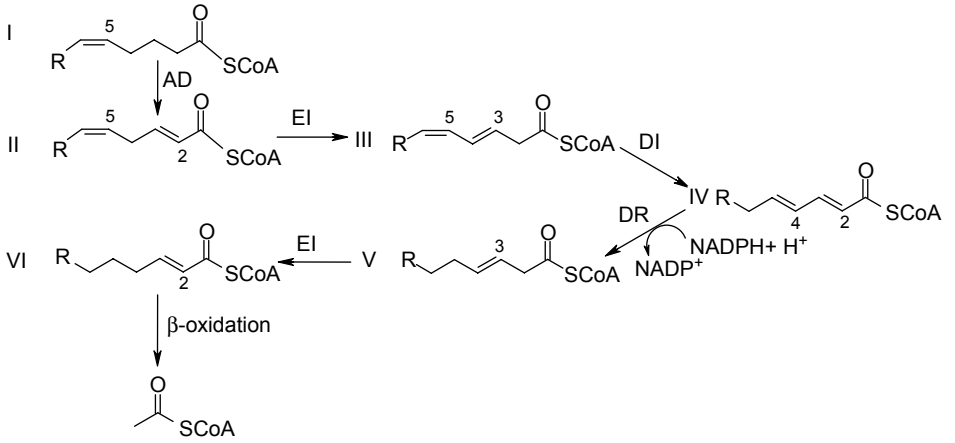

Fig. 4. β-Oxidation of 5-*cis*-enoyl-CoA. Abbreviations: AD, acyl-CoA dehydrogenase; EI, Δ^3,Δ^2-enoyl-CoA isomerase; DI, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase; DR, 2,4-dienoyl-CoA reductase.

down via the pathway outlined in Fig. 3 (H. Schulz, 2003). The most important metabolic function of the minor pathway might be its facility to degrade and thereby dispose of 3,5-dienoyl-CoAs that otherwise might accumulate and inhibit fatty acid β-oxidation. 3,5-Dienoyl-CoAs might be generated fortuitously by Δ^3,Δ^2-enoyl-CoA isomerase acting on 2,5-dienoyl-CoAs that are normal β-oxidation intermediates of unsaturated fatty acids with odd-numbered double bonds. However, 3,5-dodecadienoyl-CoA is an obligatory metabolite of 9-*cis*,11-*trans*-octadecadienoic acid, also referred to as conjugated linoleic acid, which is a minor fatty acid of the human diet.

Two Δ^3,Δ^2-enoyl-CoA isomerases exist in rat mitochondria. One is mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase that has been purified and its cDNA has been cloned [18]. The crystal structure revealed that this enzyme is a homotrimer with a subunit molecular mass of 30 kDa. In addition to converting the CoA derivatives of 3-*cis*-enoic acids and 3-*trans*-enoic acids with 6–16 carbon atoms to the corresponding 2-*trans*-enoyl-CoAs, the enzyme catalyzes the conversion of 2,5-dienoyl-CoA to 3,5-dienoyl-CoA and of 3-ynoyl-CoA to 2,3-dienoyl-CoA. A second Δ^3,Δ^2-enoyl-CoA isomerase has been identified in mitochondria (H. Schulz, 2002). This isomerase is identical with peroxisomal Δ^3,Δ^2-enoyl-CoA isomerase and therefore has a dual subcellular localization. It is more active with long-chain substrates than with medium-chain substrates and has a preference for 3-*trans*-enoyl-CoA compared to 3-*cis*-enoyl-CoAs.

The mitochondrial NADPH-dependent 2,4-dienoyl-CoA reductase has been purified and its cDNA has been cloned [4]. The crystal structure of the enzyme confirmed its proposed homotetrameric arrangement with a native molecular mass of 124 kDa (W.N. Hunter, 2005).

$\Delta^{3,5}$,$\Delta^{2,4}$-Dienoyl-CoA isomerase is a member of the hydratase/isomerase superfamily with a 32-kDa subunit in a homohexameric arrangement (R.K. Wierenga, 1998). It is present in both mitochondria and peroxisomes because of mitochondrial and peroxisomal targeting signals at its N-terminus and C-terminus, respectively. The crystal structure of

this isomerase revealed the presence of one active site glutamate and aspartate residue, which catalyze simultaneous proton transfers that facilitate the 3,5 → 2,4 double bond isomerization with substrates having acyl chains with 8–20 carbon atoms. This enzyme also catalyzes the isomerization of 3,5,7-trienoyl-CoA to 2,4,6-trienoyl-CoA, which may be formed during the β-oxidation of 9-*cis*,11-*trans*-octadecadienoic acid (conjugated linoleic acid) (H. Schulz, 1999).

3.4. Regulation of fatty acid oxidation in mitochondria

The rate of fatty acid oxidation is a function of the plasma concentration of unesterified fatty acids. Unesterified fatty acids are generated by lipoprotein lipase or are released from adipose tissue into the circulatory system, which carries them to other tissues or organs. Hormones like glucagon and insulin regulate the lipolysis of triacylglycerols in adipose tissue (Chapter 10). The utilization of fatty acids for either oxidation or lipid synthesis depends on the nutritional state of the animal, more specifically on the availability of carbohydrates. Because of the close relationship among lipid metabolism, carbohydrate metabolism, and ketogenesis, the regulation of fatty acid oxidation in liver differs from that in tissues like heart and skeletal muscle, which have an overwhelming catabolic function. For this reason, the regulation of fatty acid oxidation in liver and heart will be discussed separately.

The direction of fatty acid metabolism in liver depends on the nutritional state of the animal. In the fed animal, the liver converts carbohydrates to fatty acids, while in fasted animals fatty acid oxidation, ketogenesis, and gluconeogenesis are the more active processes. Clearly, there exists a reciprocal relationship between fatty acid synthesis and fatty acid oxidation. Although it is well established that lipid and carbohydrate metabolisms are under hormonal control, it has been more difficult to identify the mechanism that regulates fatty acid synthesis and oxidation. McGarry and Foster [19] demonstrated that the concentration of malonyl-CoA, the first committed intermediate in fatty acid biosynthesis, determines the rate of fatty acid oxidation. The essential features of their hypothesis are presented in Fig. 5. In the fed animal, where glucose is actively converted to fatty acids, the concentration of cytosolic malonyl-CoA is elevated. Malonyl-CoA at micromolar concentrations inhibits hepatic CPT I thereby decreasing the transfer of fatty acyl residues from CoA to carnitine and their translocation into mitochondria. Consequently, β-oxidation is depressed. When the animal changes from the fed to the fasted state, hepatic metabolism shifts from glucose breakdown to gluconeogenesis with a resultant decrease in fatty acid synthesis. The concentration of malonyl-CoA decreases and the inhibition of CPT I is relieved. Furthermore, starvation causes an increase in the total CPT I activity and a decrease in the sensitivity of CPT I toward malonyl-CoA. Altogether, during starvation acylcarnitines are more rapidly formed and translocated into mitochondria thereby stimulating β-oxidation and ketogenesis.

It appears that the cellular concentration of malonyl-CoA is directly related to the activity of acetyl-CoA carboxylase, which is hormonally regulated (Chapter 6). The short-term regulation of acetyl-CoA carboxylase involves the phosphorylation and dephosphorylation of the enzyme. In the fasting animal, a high [glucagon]/[insulin] ratio causes the phosphorylation and inactivation of acetyl-CoA carboxylase. As a consequence, the concentration of

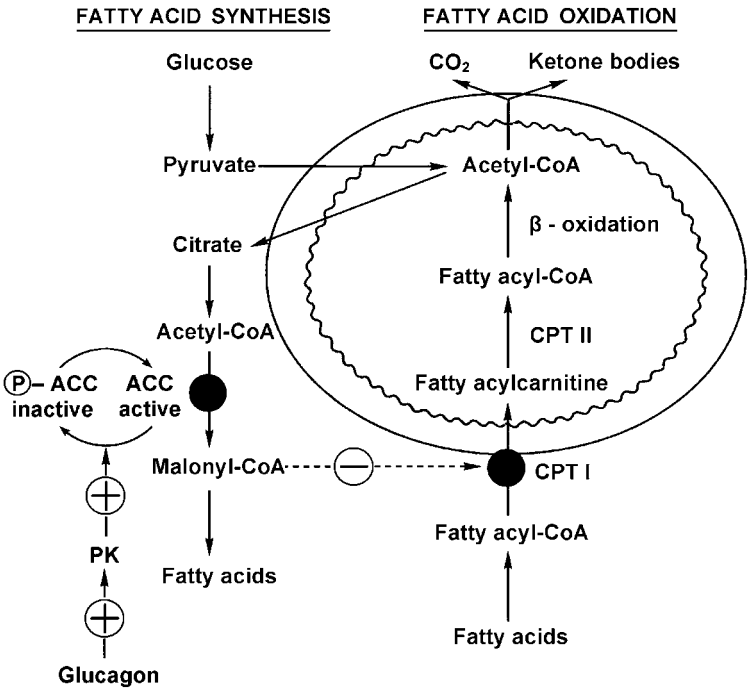

Fig. 5. Proposed regulation of fatty acid oxidation in liver. ⊕ Stimulation; ⊖ inhibition; ● enzymes subject to regulation. Abbreviations: ACC, acetyl-CoA carboxylase; CPT, carnitine palmitoyltransferase; PK, protein kinase.

malonyl-CoA and the rate of fatty acid synthesis decrease, while the rate of β-oxidation increases. A decrease of the [glucagon]/[insulin] ratio reverses these effects. Thus, both fatty acid synthesis and fatty acid oxidation are regulated by the ratio of [glucagon]/[insulin].

It has been suggested that malonyl-CoA also regulates fatty acid oxidation in non-hepatic tissues like heart and skeletal muscle [20–22]. The formation of malonyl-CoA in these tissues is catalyzed by a 280-kDa isoform (ACC2) of the 265-kDa acetyl-CoA carboxylase (ACC1) that is the predominant form of ACC in lipogenic tissues. The proposed role of ACC2 in the control of fatty acid oxidation in heart and muscle is supported by the observations that the concentration of malonyl-CoA in heart and muscle of *Acc2* knockout mice is lower than in wild-type mice and that the rate of fatty acid oxidation in muscle is higher [23]. The disposal of malonyl-CoA is thought to be catalyzed by cytosolic malonyl-CoA decarboxylase. Thus, the tissue concentration of malonyl-CoA is determined by the activities of both the carboxylase and decarboxylase (G.D. Lopaschuk, 2000). ACC2 is phosphorylated and inactivated by AMP-dependent kinase in response to stress caused by ischemia/hypoxia and exercise and is activated allosterically by citrate [22]. A concern about this model for the regulation of fatty acid oxidation in heart and skeletal muscle is the discrepancy between the micromolar tissue concentration of malonyl-CoA and the nanomolar K_i of muscle CPT I (M-CPT I) for malonyl-CoA. Consequently, CPT I is expected to be fully inhibited at all times unless the effective malonyl-CoA concentration is lower due to its binding to other proteins or due to intracellular compartmentation.

In heart, and possibly in other tissues, the rate of fatty acid oxidation is tuned to the cellular energy demand in addition to being dependent on the concentration of plasma fatty acids [22]. At sufficiently high concentrations (>0.6 mM) of fatty acids, the rate of fatty acid oxidation is a function only of the cellular energy demand. Studies with perfused hearts and isolated heart mitochondria have shown that a decrease in the energy demand results in elevated concentrations of acetyl-CoA and NADH and in lower concentrations of CoA and NAD^+. The resultant increases in the ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] in the mitochondrial matrix may be the cause for the reduced rate of β-oxidation. Experiments with isolated heart mitochondria support this view. Moreover, these experiments suggested that the ratio of [acetyl-CoA]/[CoA] may be more important than the ratio of [NADH]/[NAD^+] in controlling the rate of β-oxidation. The site of this regulation has not been identified unequivocally.

4. Fatty acid oxidation in peroxisomes

Peroxisomes and glyoxysomes, collectively referred to as microbodies, are subcellular organelles capable of respiration. They do not have an energy-coupled electron transport system like mitochondria, but instead contain flavin oxidases, which catalyze the substrate-dependent reduction of oxygen to H_2O_2. Because catalase is present in these organelles, H_2O_2 is rapidly reduced to water. Thus, peroxisomes and glyoxysomes are organelles with a primitive respiratory chain where energy released during the reduction of oxygen is lost as heat. Glyoxysomes are peroxisomes that contain the enzymes of the glyoxylate pathway in addition to flavin oxidases and catalase. Peroxisomes or glyoxysomes are found in all major groups of eukaryotic organisms including yeasts, fungi, protozoa, plants, and animals.

An extramitochondrial system capable of oxidizing fatty acids was first detected in glyoxysomes of germinating seeds. When rat liver cells were shown to contain a β-oxidation system in peroxisomes (P.B. Lazarow, 1978), the interest in the peroxisomal pathway was greatly stimulated and one set of β-oxidation enzymes was soon identified and characterized [24]. It should be noted that peroxisomal β-oxidation occurs in all eukaryotic organisms, whereas mitochondrial β-oxidation seems to be restricted to animals. Studies of peroxisomal β-oxidation were aided by the use of certain drugs, e.g., clofibrate and di(2-ethylhexyl)phthalate, which induce the expression of the enzymes of peroxisomal β-oxidation and in addition cause the proliferation of peroxisomes in rodents. The induction of peroxisomal β-oxidation by xenobiotic proliferators or fatty acids involves the peroxisomal proliferator-activated receptors (PPARs), which are members of the nuclear hormone receptor family and which recognize peroxisomal proliferator response elements upstream of the affected structural genes [4]. Although rat liver peroxisomes are capable of chain-shortening regular long-chain fatty acids, their main function seems to be the partial β-oxidation of very-long-chain fatty acids, methyl-branched carboxylic acids like pristanic acid, prostaglandins, dicarboxylic acids, xenobiotic compounds like phenyl fatty acids, and hydroxylated 5-β-cholestanoic acids, formed during the conversion of cholesterol to cholic acid.

4.1. Uptake of fatty acids by peroxisomes

The mechanism of fatty acid uptake by peroxisomes is poorly understood. Although small molecules like substrates and cofactors can freely cross the membrane of peroxisomes isolated from animals, it seems that in vivo the peroxisomal membrane constitutes a permeability barrier that would require transporters to facilitate the uptake of substrates and cofactors [25,26]. In animal and yeast cells, long-chain fatty acids are activated outside of the peroxisomal membrane. Long-chain acyl-CoAs are thought to enter peroxisomes in a facilitated process involving half ATP-binding cassette transporters like ALDP, ALDRP, PMP70, and PMP69 in animal cells and Pxa1p and Pxa2p in yeast [25]. In contrast, very-long-chain fatty acids in animal cells and medium-chain fatty acids in yeast can be activated in the peroxisomal matrix. The β-oxidation of medium-chain fatty acids in yeast depends on the facilitated uptake of ATP that provides the energy for the intraperoxisomal activation of medium-chain fatty acids by fatty ACS Faa2p.

4.2. Pathways and enzymology of peroxisomal α- and β-oxidation

The first step in peroxisomal β-oxidation (Fig. 6) is the dehydrogenation of acyl-CoA to 2-*trans*-enoyl-CoA catalyzed by acyl-CoA oxidase. This enzyme, in contrast to the mitochondrial dehydrogenases, transfers two hydrogens from the substrate to its FAD cofactor and then to O_2, which is reduced to H_2O_2. Rat liver contains three acyl-CoA oxidases with different substrate specificities. Their names, palmitoyl-CoA oxidase, pristanoyl-CoA oxidase, and trihydroxycoprostanoyl-CoA (an intermediate in bile acid synthesis) oxidase, are indicative of their preferred substrates [27]. Interestingly, human liver contains only one branched-chain acyl-CoA oxidase besides palmitoyl-CoA oxidase. Cloning and sequencing of the gene and cDNAs encoding rat palmitoyl-CoA oxidase revealed two isoforms of this enzyme that are produced by the alternative use of two exons. The rat liver palmitoyl-CoA oxidase is a homodimer with a molecular mass of close to 150 kDa. Ligands of PPARs induce the expression of this enzyme but not of the other two acyl-CoA oxidases. Palmitoyl-CoA oxidase is inactive with butyryl-CoA and hexanoyl-CoA as substrates, but dehydrogenates all longer chain substrates with similar maximal velocities. Acyl-CoA oxidases from organisms other than mammals are either active with substrates of all chain lengths or the organisms express more than one acyl-CoA oxidase with complementing chain length specificities. Consequently, fatty acids can be completely degraded in peroxisomes of yeasts, plants, and other lower eukaryotic organisms, but not in mammals.

The next two reactions of β-oxidation, the hydration of 2-enoyl-CoA to 3-hydroxyacyl-CoA and the NAD^+-dependent dehydrogenation of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA, are catalyzed in mammalian peroxisomes by multifunctional enzyme 1 (MFE1) and multifunctional enzyme 2 (MFE2) [27]. Both multifunctional enzymes harbor enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. MFE1 additionally exhibits Δ^3,Δ^2-enoyl-CoA isomerase activity. 3-Hydroxyacyl-CoA intermediates formed and acted on by MFE1 have the L-configuration, whereas the hydroxy intermediates produced by MFE2 have the D-configuration. Both MFE1 and MFE2 convert medium-chain and long-chain 2-*trans*-enoyl-CoAs to 3-ketoacyl-CoAs but show little or no activity with short-chain substrates. MFE2 is slightly more active than MFE1 with longer chain substrates and it alone

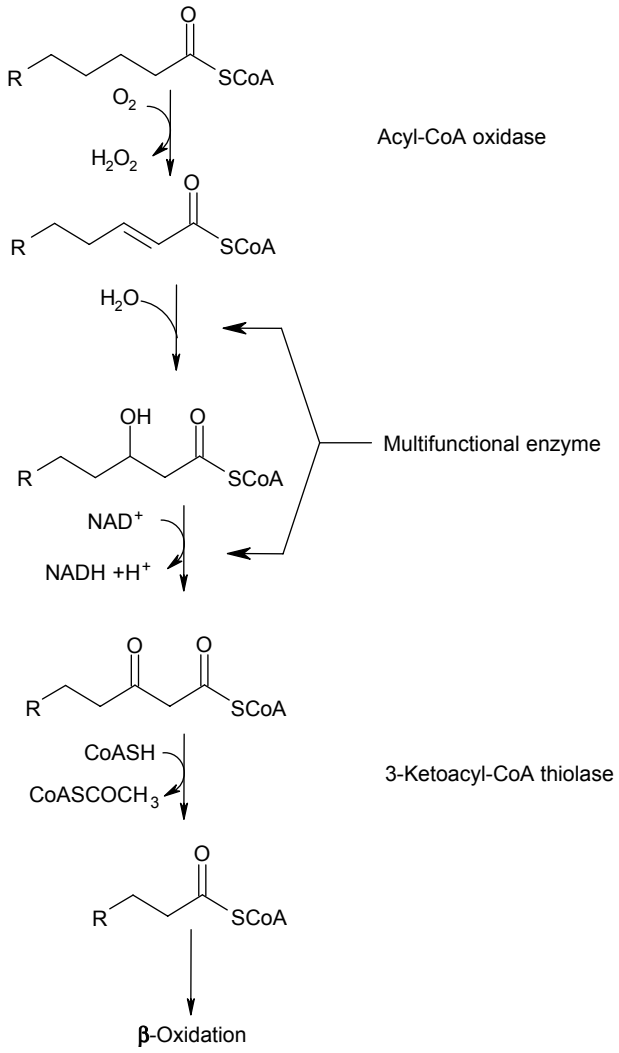

Fig. 6. Pathway of β-oxidation in peroxisomes.

acts on substrates with 2-methyl branches such as those formed during the β-oxidation of pristanic acid and hydroxylated 5-β-cholestanoic acid. MFE1 is a monomeric 80-kDa protein with an N-terminal hydratase/isomerase domain and a dehydrogenase domain located in its central region. In contrast, mammalian MFE2 is a 150-kDa homodimer with a dehydrogenase domain at the N-terminus followed by hydratase and sterol carrier protein-2-like domains. Yeast and fungi contain only one multifunctional enzyme each with D-specific enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities.

The last reaction of β-oxidation, the CoA-dependent cleavage of 3-ketoacyl-CoA, is catalyzed by 3-ketoacyl-CoA thiolase. Three 3-ketoacyl-CoA thiolases encoded by different genes have been detected in rat [27]. Thiolase A is constitutively expressed, whereas the expression of thiolase B is highly induced in response to peroxisomal

proliferators. Both enzymes are 80-kDa homodimers that exhibit little activity toward acetoacetyl-CoA, but are active with all longer chain substrates except branched-chain intermediates of pristanic acid and hydroxylated 5-β-cholestanoic acid β-oxidation. However, these intermediates are acted upon by another 3-ketoacyl-CoA thiolase (SCP_x-thiolase) that was identified in peroxisomes during a study of the 58-kDa precursor of sterol carrier protein-2 (U. Seedorf, 1994). The C-terminal segment of this 58-kDa protein is identical with sterol carrier protein-2, whereas the N-terminal domain harbors the thiolase, which is most active with medium-chain substrates. Human peroxisomes contain only an inducible 3-ketoacyl-CoA thiolase and SCP_x-thiolase. The crystal structure of the peroxisomal 3-ketoacyl-CoA thiolase from *S. cerevisiae* at 2.8-Å resolution shows two cysteine residues in close proximity at the active site [4].

Because mammalian peroxisomes contain at least two enzymes for each step of β-oxidation, specific functions for these enzymes were inferred from their substrate specificities. Most of the predictions were verified by analyzing fatty acids that accumulate in patients and/or knockout mice deficient in the individual enzymes [26,27]. Together these data support the proposal that branched-chain acyl-CoA oxidase, MFE2, and SCP_x-thiolase are essential for the degradation of pristanic acid and hydroxylated 5-β-cholestanoic acid. The β-oxidation of very-long-chain fatty acids involves palmitoyl-CoA oxidase, MFE2, and both thiolases. Surprisingly, the knockout mouse for MFE1 does not exhibit an obvious phenotype, leaving the function of this enzyme unresolved. However, evidence has recently been obtained for the involvement of MFE1 in the β-oxidation of dicarboxylic acids [27].

Unsaturated fatty acids are degraded in peroxisomes by the pathways outlined in Figs. 3 and 4 [4,18,27]. Two Δ^3,Δ^2-enoyl-CoA isomerases are present in rat liver peroxisomes. One is the monofunctional Δ^3,Δ^2-enoyl-CoA isomerase that is also present in mitochondria (Section 3.3), the other is the Δ^3,Δ^2-enoyl-CoA isomerase activity of multifunctional enzyme 1. The monofunctional Δ^3,Δ^2-enoyl-CoA isomerase has a preference for long-chain substrates and may play the major role in the partial β-oxidation of long-chain unsaturated fatty acids. So far this isomerase has been identified only by cloning and heterologous expression based on its homology with the only Δ^3,Δ^2-enoyl-CoA isomerase of yeast. The crystal structure of yeast Δ^3,Δ^2-enoyl-CoA isomerase revealed the presence of a single glutamate residue at the active site, which catalyzes a 1,3-proton transfer that results in the shift of the double bond (R.K. Wierenga, 2001). A 2,4-dienoyl-CoA reductase distinct from the mitochondrial reductase but homologous with the yeast 2,4-dienoyl-CoA reductase has been identified in mammalian peroxisomes by a cloning and expression approach. This enzyme acts on a wide spectrum of 2,4-dienoyl-CoAs but is most active with medium-chain substrates (S.J. Gould, 1999). $\Delta^{3,5}\Delta^{2,4}$-Dienoyl-CoA isomerase of mammalian peroxisomes is the same enzyme that is present in mitochondria (Section 3.3).

The products of peroxisomal β-oxidation in animals are acetyl-CoA, NADH, and chain-shortened acyl-CoAs that are completely degraded in mitochondria. Chain-shortened acyl residues and acetyl groups are thought to leave peroxisomes as acylcarnitines that can be formed from acyl-CoAs by peroxisomal carnitine octanoyltransferase and/or carnitine acetyltransferase [25,26]. The conversion of acyl-CoAs to acylcarnitines regenerates CoA in peroxisomes as does the hydrolysis of acyl-CoAs by thioesterases. The recycling of cofactors in peroxisomes and the transport of substrates, cofactors, and metabolites across the peroxisomal membrane are aspects of β-oxidation that remain to be investigated.

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a component of the human diet that is derived from phytol, a constituent of chlorophyll, is not degraded by β-oxidation because its 3-methyl group interferes with this process. Instead, phytanic acid is chain-shortened by α-oxidation in peroxisomes as outlined in Fig. 7 [28]. Activation

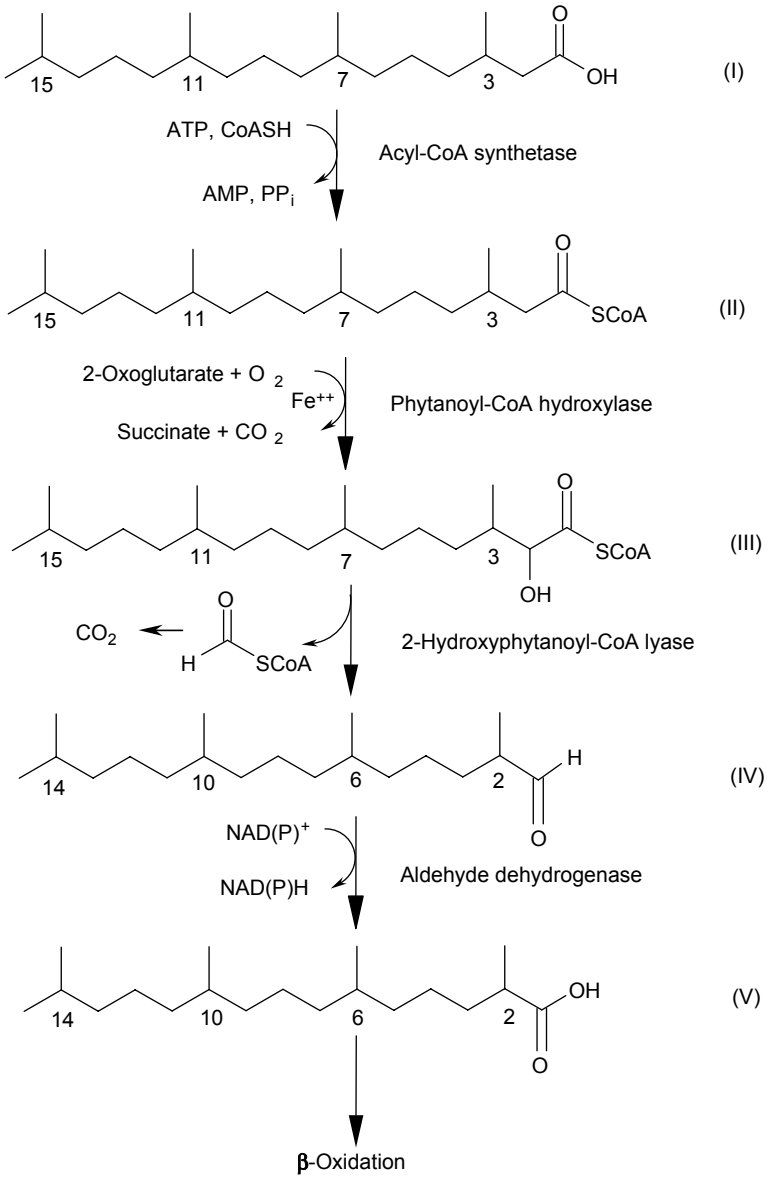

Fig. 7. α-Oxidation of phytanic acid. Compound I, phytanic acid; compound II, phytanoyl-CoA; compound III, 2-hydroxyphytanoyl-CoA; compound IV, pristanal; compound V, pristanic acid.

of phytanic acid (I) to phytanoyl-CoA (II) by long-chain or very-long-chain acyl-CoA synthetase converts it to a substrate of phytanoyl-CoA hydroxylase. Cleavage of the resultant 2-hydroxyphytanoyl-CoA (III) by 2-hydroxyphytanoyl-CoA lyase yields pristanal (IV) and formyl-CoA that are oxidized to pristanic acid (V) and CO_2, respectively. Pristanic acid after activation to pristanoyl-CoA is a substrate of β-oxidation because the 2-methyl group does not interfere with the process as long as the 2-methyl group has the *S* configuration. A 2-methylacyl-CoA racemase that is present in both peroxisomes and mitochondria epimerizes (2*R*)-pristanoyl-CoA and (25*R*)-trihydroxycholestanoyl-CoA to their *S* isomers, which are substrates of peroxisomal β-oxidation [27].

5. Inherited diseases of fatty acid oxidation

Disorders of fatty acid oxidation in mitochondria [29] were first described in 1973 when deficiencies of carnitine and carnitine palmitoyltransferase (CPT II) were identified as causes of muscle weakness. Patients with low levels (5–45% of normal) of CPT II have recurrent episodes of muscle weakness and myoglobinuria, often precipitated by prolonged exercise and/or fasting. Almost a decade later, a deficiency of medium-chain dehydrogenase was identified in patients with a disorder of fasting adaptation [29]. This relatively common disorder is characterized by episodes of non-ketotic hypoglycemia provoked by fasting or infection during the first 2 years of life. Between episodes, patients with medium-chain acyl-CoA dehydrogenase deficiency appear normal. Therapy is aimed at preventing fasting, if necessary by the intravenous administration of glucose, and includes carnitine supplementation. The molecular basis of medium-chain acyl-CoA dehydrogenase deficiency is an A → G base transition in 90% of the disease causing alleles. This mutation results in the replacement of lysine 329 by a glutamate residue, which impairs the assembly of subunits into the functional tetrameric enzyme. In the years following the identification of medium-chain acyl-CoA dehydrogenase deficiency, fatty acid oxidation disorders due to the following enzymes deficiencies have been described: short-chain acyl-CoA dehydrogenase, very-long-chain acyl-CoA dehydrogenase, ETF, ETF:ubiquinone oxidoreductase, 3-hydroxyacyl-CoA dehydrogenase, long-chain 3-hydroxyacyl-CoA dehydrogenase, trifunctional β-oxidation complex, 2,4-dienoyl-CoA reductase, CPT I, and carnitine:acylcarnitine translocase. A deficiency of mitochondrial acetoacetyl-CoA thiolase (β-ketothiolase) impairs isoleucine and ketone body metabolism, but not fatty acid oxidation.

Many of these disorders are associated with the urinary excretion of acylcarnitines, acyl conjugates of glycine, and dicarboxylic acids that are characteristic of the metabolic block. A general conclusion derived from studies of these disorders is that an impairment of β-oxidation makes fatty acids available for microsomal ω-oxidation by which fatty acids are oxidized at their terminal (ω) methyl group or at their penultimate (ω – 1) carbon atom. Molecular oxygen is required for this oxidation and the hydroxylated fatty acids are further oxidized to dicarboxylic acids. Long-chain dicarboxylic acids can be

chain-shortened by peroxisomal β-oxidation to medium-chain dicarboxylic acids, which are excreted in urine.

Several disorders associated with an impairment of peroxisomal β-oxidation have been described [30,31]. Of these, Zellweger syndrome and neonatal adrenoleukodystrophy are characterized by the absence, or low levels, of peroxisomes due to the defective biogenesis of this organelle [30]. As a result of this deficiency, compounds that are normally metabolized in peroxisomes, for example, very-long-chain fatty acids, dicarboxylic acids, hydroxylated 5-β-cholestanoic acids, and also phytanic acid, accumulate in plasma. Infants with Zellweger syndrome rarely survive longer than a few months due to hypotonia, seizures and frequently cardiac defects. In addition to disorders of peroxisome biogenesis, defects of individual enzymes required for peroxisomal β-oxidation have been described [31]. This group of diseases includes X-linked adrenoleukodystrophy that is caused by mutations in the *ABCD1* gene, which leads to the accumulation of very-long-chain fatty acids. Many of the patients with a defect in a single enzyme of β-oxidation or related to β-oxidation are hypotonic, develop seizures, fail to make psychomotor gains, and die in early childhood. The importance of α-oxidation in humans has been established as a result of studying Refsum's disease, a rare and inherited neurological disorder. Patients afflicted with this disease accumulate large amounts of phytanic acid due to a deficiency of phytanoyl-CoA hydroxylase [31].

6. Future directions

Fatty acid oxidation has been studied for a century with the result that a fairly detailed view of this process has emerged. The biochemical and molecular characterization of most β-oxidation enzymes has yielded a wealth of functional and structural information. However, the dynamics of the process remain poorly understood due to limited information about the impact of changes in gene expression on the flux through the pathway. Also, the organization of β-oxidation enzymes has not been sufficiently characterized to evaluate the impact that such organization has on the control of the process. A number of questions about the regulation of this process have not been completely resolved, especially questions about the regulation in extrahepatic tissues. Even the extensively studied regulation of hepatic fatty acid oxidation by malonyl-CoA continues to be further investigated to provide an understanding of the regulatory mechanism at the molecular level. In spite of impressive progress in the area of peroxisomal β-oxidation, aspects of this process remain unresolved. For example, it is unclear how fatty acids and some cofactors enter peroxisomes and how products exit from this organelle. Also, the transcriptional regulation of this process has not been fully explored. Moreover, the cooperation between peroxisomes and mitochondria in fatty acid oxidation remains to be further studied. Not all of the reactions of the β-oxidation spiral have been verified experimentally and hence some may not take place as envisioned. Finally, the complete characterization of known disorders of β-oxidation in humans and the identification of new disorders will raise questions about some accepted features of this process and will prompt re-investigations of issues thought to be resolved.

Abbreviations

ACC	acetyl-CoA carboxylase
ACS	acyl-CoA synthetase
ACSL	long-chain acyl-CoA synthetase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
CPT	carnitine palmitoyltransferase
ETF	electron-transferring flavoprotein
FABP	fatty acid binding protein
FAD	flavin adenine dinucleotide, oxidized form
$FADH_2$	flavin adenine dinucleotide, reduced form
GTP	guanosine triphosphate
MFE	multifunctional enzyme
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
PPAR	peroxisomal proliferator-activated receptor
SCP	sterol carrier protein

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CHAPTER 6

Fatty acid synthesis in eukaryotes

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Contents

1. Introduction

Fatty acids fulfill a number of crucial roles in animals. They represent a major storage form of energy (Chapter 10), they constitute essential structural components of membranes (Chapter 1), they are used to modify and regulate the properties of many proteins through direct covalent linkage (Chapter 2), and they perform important roles as signaling molecules in metabolic regulation. This chapter focuses primarily on the structure, mechanism of action, and regulation of the enzymes responsible for the biosynthesis of saturated fatty acids de novo in eukaryotes. The field has its origins in the 1950s when it was first demonstrated that the thermodynamic barrier posed by condensation of two acetyl-CoA molecules is circumvented by the introduction of an energy-dependent carboxylation step that generates malonyl-CoA as a co-substrate (S.J. Wakil, P.R. Vagelos, and F. Lynen). Much of the early progress in identifying the individual enzymes involved in the pathway was made using the *Escherichia coli* system, and it was not until the mid-1970s that it became clear that in the cytosol of eukaryotes the enzymes required for the conversion of malonyl-CoA to fatty acids are covalently linked in 'multifunctional' polypeptides. This cytosolic fatty acid synthase (FAS) is responsible for the bulk synthesis of fatty acids and in animals is most active in the 'lipogenic tissues' such as liver, adipose, and lactating mammary glands. Recent studies have revealed that eukaryotes contain a second system for the biosynthesis of saturated fatty acids de novo, this one in the mitochondria. The exact role of the mitochondrial FAS is unclear, but it may provide the octanoyl precursor required for the de novo biosynthesis of lipoyl moieties that are utilized for the covalent modification of several mitochondrial enzymes, and/or fatty acids required for the remodeling of mitochondrial lipids. In both systems the carbon source for chain extension is derived from malonyl-CoA but the enzymes involved are organized into quite different architectural forms. In the mitochondrial system, the enzymes exist as separate, freestanding proteins (type II) whereas in the cytosolic system, the enzymes are covalently linked in large multifunctional polypeptides (type I). The mitochondrial proteins closely resemble their prokaryotic type II counterparts (Chapter 3), consistent with the hypothesis that mitochondria originated from free-living bacteria, but are nuclear encoded and possess N-terminal targeting sequences that direct them into the mitochondrial compartment.

In the animal and fungal cytosolic fatty acid synthesizing systems, the catalytic components required for the entire pathway are integrated into two multifunctional polypeptide proteins, acetyl-CoA carboxylase (ACC) and FAS. Both proteins are post-translationally modified by the covalent attachment of vitamin derivatives that function as 'swinging arms' in the translocation of intermediates between different catalytic sites. In ACC, a biotin moiety, attached to the ε-amino group of a lysine residue, serves as a carboxyl carrier between the carboxylase and transcarboxylase catalytic domains and in the FAS, a phosphopantetheine moiety, attached to a serine hydroxyl, serves to transport reaction intermediates through the various catalytic centers of the complex. Details of the architectural organization of the eukaryotic ACC and FAS have been difficult to establish and, particularly in the case of the animal FAS system, resulted in the proposal of radically different models. However, in recent years, the picture for the FAS has been clarified considerably by biochemical studies, electron microscopy, and X-ray crystallographic analysis [1].

In animals, expression of both ACC and FAS is regulated at the transcriptional level, in a tissue-specific manner, in response to various developmental, nutritional, and hormonal signals. In addition, ACC is subject to short-term regulation by allosteric and phosphorylation mechanisms and is commonly regarded as the pace-setting enzyme for fatty acid synthesis. The only free intermediate in the entire biosynthetic pathway is malonyl-CoA; all other intermediates exist only as covalently bound acyl–enzyme complexes. Malonyl-CoA plays a critical role in a fuel sensing and signaling mechanism that regulates food intake and energy metabolism.

2. Acetyl-CoA carboxylase

2.1. The reaction sequence

The formation of malonyl-CoA from acetyl-CoA is a two-step reaction involving first, the ATP-dependent carboxylation of the N1 atom in the ureido ring of biotin, using bicarbonate as the carboxyl donor (Fig. 1). This reaction requires ATP and a divalent cation and proceeds via an activated carboxyphosphate intermediate that breaks down to produce phosphate and CO_2. One of the phosphate oxygens then acts as a general base to deprotonate the N1 atom of biotin, which attacks the CO_2 to produce carboxybiotin. In the second step, the carboxybiotin engages the carboxyltransferase active site and breaks down to give CO_2 and the N1 atom of biotin, again acting as a general base, extracts a proton from the methyl group of acetyl-CoA, which then reacts with the CO_2 to form malonyl-CoA [2].

2.2. Structural organization

Animal and fungal ACCs are comprised of large multifunctional polypeptides containing the biotin carboxylase, biotinyl carboxyl carrier protein, and carboxyltransferase

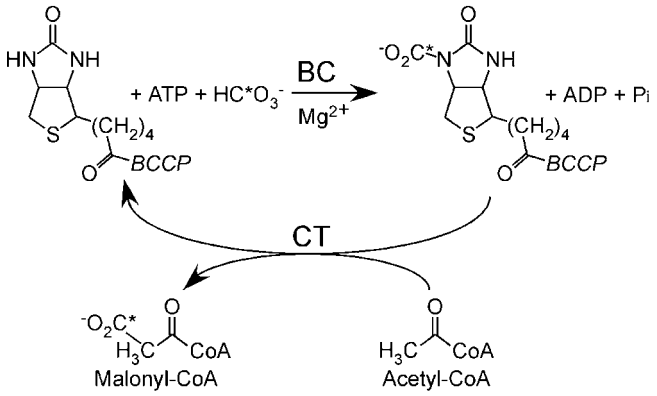

Fig. 1. Reactions catalyzed by acetyl-CoA carboxylase. BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; CT, carboxyl transferase.

domains (Fig. 2A). In prokaryotes, these functional components reside on three discrete polypeptides (Chapter 3). The biotinyl moiety is covalently linked through an amide bond between the valeryl carboxyl of biotin and the ammonium group in the side chain of a lysine residue in the biotin carboxyl carrier protein domain. With multiple rotatable bonds between the ring structure and the protein backbone, biotin functions as a swinging arm to effect the transfer of the carboxyl moiety between the active sites of the two catalytic domains, a distance of ~7 Å.

The amino acid sequences of the catalytic domains of ACC are highly conserved in eukaryotes, exhibiting more than 50% identity between yeast and human such that antibodies raised against animal and fungal forms cross-react with each other. The minimal functional unit (protomer) of ACC is a homodimer. In the presence of citrate, the $\alpha_2\beta_2$ protomeric unit of ACC rapidly assumes a catalytically active conformation and undergoes a slower, reversible polymerization to a long filamentous polymeric structure, ~100 Å wide and up to 5000 Å long, containing as many as 20 protomers (Fig. 2B). The polymeric structure may stabilize the enzyme protomers in an active conformation. Depolymerization and inactivation of the enzyme is promoted by long-chain acyl-CoA thioesters, which are direct competitors with citrate, and by malonyl-CoA.

The crystal structures of the yeast biotin carboxylase and carboxyltransferase domains have been solved [2] and likely are representative of their mammalian counterparts (Fig. 2C and D). The active form of prokaryotic biotin carboxylases is the dimer and isolated biotin carboxylase domains of eukaryotic ACCs are inactive monomers, suggesting that they too are probably active as dimers in the context of the full-length ACC. The structure of the biotin carboxylase complexed with soraphen A, a potent polyketide inhibitor of eukaryotic ACCs, was solved by X-ray crystallography. The biotin carboxylase domain exhibits an 'ATP-grasp' fold and consists of three subdomains, A, B, and C and an AB linker (Fig. 2C). The A and C domains and AB-linker form a cylindrical structure with the soraphen and ATP-binding sites located at opposite ends; the B-domain forms a lid on the cylinder. Based on the structure of the *E. coli* biotin carboxylase–ATP complex, residues likely to be involved in ATP binding have been identified. Soraphen A, a non-competitive inhibitor, engages in multiple interactions at the interface between the A- and C-domains, consistent with its very low K_D value, ~1 nM, and is thought to disrupt the essential dimerization of the biotin carboxylase domain.

The carboxyltransferase domain crystallizes as a dimer, each polypeptide composed of intimately associated subunits orientated 'head-to-tail' (Fig. 2D). The dimer contains two active sites located at the interface between the N-terminal and C-terminal domains of opposed subunits. Acetyl-CoA is bound at the active site through hydrogen bonds between the adenine base and the main chain of residues in the N-terminal domain and through interaction of the phosphate groups with basic residues associated with both the N- and C-domains. The carboxybiotin substrate most likely docks with a short β-sheet in the C-domain adjacent to acetyl-CoA bound to the N-domain of the other subunit. The proposed reaction mechanism is supported by the results of experiments involving mutagenesis of many of the residues in the active-site region. Recently the structure of the biotin carboxyl carrier apoprotein domain of the human ACC2 has been solved by NMR (Fig. 2E). The core structure of this domain is characterized as a 'capped β-sandwich' flanked by long linkers. The biotinylation site is located on a β-hairpin turn, as in the prokaryotic counterparts.

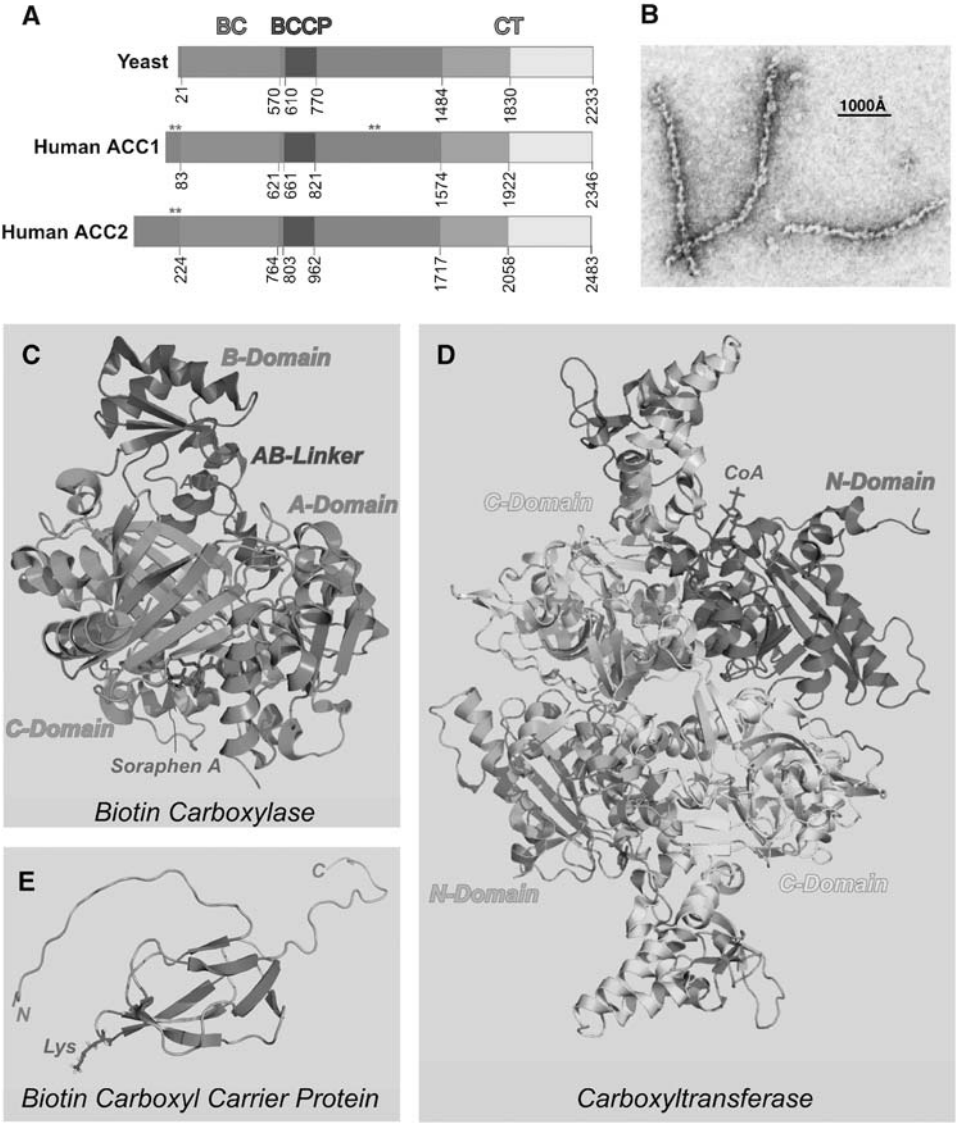

Fig. 2. Acetyl-CoA carboxylase. (A) Eukaryotic ACCs contain ~2300 residues organized into three functional domains — biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT). The role of the region between the biotin carboxyl carrier and carboxyltransferase domains is unknown. The biotin carboxyl carrier protein contains a typical conserved biotin attachment-site motif, VM**K**MV. The sites of phosphorylation are indicated by asterisks. (B) Electron micrograph of polymerized rat acetyl-CoA carboxylase (F. Ahmad, 1978). (C) Crystal structure of the biotin carboxylase domain of the yeast enzyme. In the presence of soraphen A, the biotin carboxyl carrier protein domain forms an inactive monomer; the likely position of the modeled ATP-binding site is shown (adapted from Ref. [2]). (D) Crystal structure of the dimeric carboxyltransferase domain of the yeast enzyme. Although acetyl-CoA was included in the crystallization, density was observed only for CoA at one site and adenine at the other (adapted from Ref. [2]). (E) NMR structure of the biotin carboxyl carrier apoprotein domain of the human ACC2 The lysine attachment site for biotin is shown (RIKEN Structural Genomics/Proteomics Initiative, 2006). (See color plate section, plate no. 3.)

ACCs represent potential targets for the development of new therapeutic agents against obesity and the structural information now available may assist in optimization of inhibitor design.

2.3. Isoforms

The two major isoforms of ACC1 and ACC2, also referred to as α and β, have been described in animals that, paradoxically, have distinctly different roles in metabolism [3–5]. The 265 kDa ACC1-isoform is the predominant form expressed in the soluble cytoplasm of lipogenic tissues such as adipose, liver, and mammary gland, where it plays an essential role in providing malonyl-CoA as a carbon source for fatty acid synthesis. The 280 kDa ACC2 isoform, on the other hand, is associated with the outer mitochondrial membrane and expressed mainly in heart and skeletal muscle, tissues with very low lipogenic capacity, but also to some extent in liver. In these tissues, malonyl-CoA produced by the ACC2 isoform functions primarily as a negative regulator of carnitine palmitoyltransferase-I (CPT-1) and, in so doing, controls the flux of fatty acids into the mitochondria for β oxidation (Chapter 5). The two isoforms exhibit extensive sequence similarity, the major difference being the presence of an aminoterminal extension in ACC2 that anchors the enzyme in the outer mitochondrial membrane (Fig. 2A).

2.4. Posttranslational modification

In animals, carboxylase enzymes for acetyl-CoA, propionyl-CoA, methylcrotonyl-CoA, and pyruvate require biotin as a cofactor. All four enzymes are biotinylated by the same biotin protein ligase (also called holocarboxylase synthetase) that recognizes a 60–90 residue region of the biotin carboxyl carrier apoprotein. The catalytic domain of the biotin protein ligases has been highly conserved through evolution and indeed the enzymes are functionally interchangeable, even between prokaryotes and eukaryotes. The crystal structures have been determined only for the enzyme from prokaryotes. In humans, inherited deficiency of the enzyme leads to depressed activity of all four carboxylases, a condition known as ‘early onset multiple carboxylase deficiency’ that can usually be successfully treated by high doses of biotin (J. Thoene, 1981).

The regulation of ACC activity is discussed in Section 5.

3. The cytosolic fatty acid synthase

3.1. The reaction sequence

The overall reaction catalyzed by the animal FAS can be summarized thus:

$$\text{Acetyl-CoA} + 7\text{Malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{Palmitic Acid} + 7\text{HCO}_3^- + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O}$$

The pathway can be visualized as a cyclic process in which the acetyl primer undergoes a series of decarboxylative condensation reactions with seven malonyl extender molecules

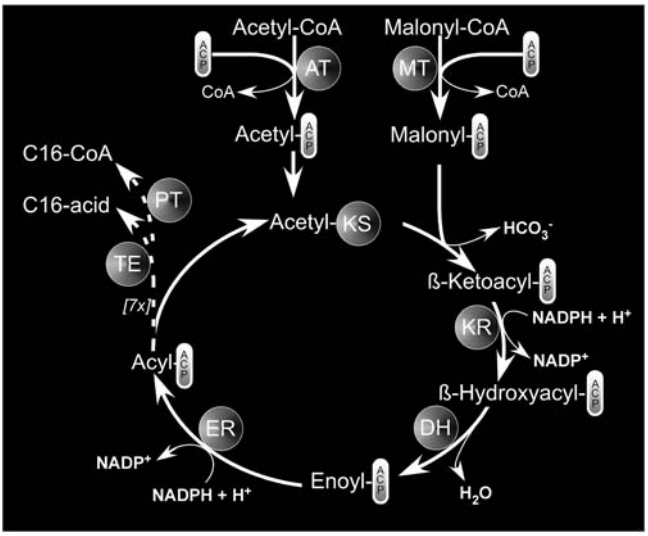

Fig. 3. Generic reaction sequence for the FASs. ACP, acyl carrier protein; AT, acetyltransferase; MT, malonyl transferase; KS, β-ketoacyl synthase; KR, β-ketoacyl reductase; DH, dehydrase; ER, enoyl reductase; TE, thioesterase; PT, palmitoyl transferase. In the animal FAS the acetyl and malonyl loading reactions are catalyzed by the same acyl transferase and the chain-termination reaction is catalyzed by a thioesterase. In the fungal FAS, the malonyl loading and palmitoyl unloading reactions are catalyzed by the same acyl transferase. Stereochemical analyses in the laboratories of Cornforth and Hammes established that in both animal and fungal FASs the KS-catalyzed condensation reaction proceeds with inversion of configuration at the malonyl C2 position, followed by KR-catalyzed reduction of the 3-keto moiety to the 3*R* alcohol by transfer of the pro-4S hydride from NADPH, and DH-catalyzed dehydration to a *trans*-enoyl moiety by the *syn* elimination of the 2*S* hydrogen and the 3*R* hydroxyl as water. However, the stereochemistry of the final reduction reaction catalyzed by ER domain proceeds with different stereochemistry. The animal FAS transfers the pro-4R hydride of NADPH to the pro-3*R* position with simultaneous addition of a solvent proton to the pro-2*S* position, whereas the fungal FAS takes the pro-4*S* hydride of NADPH into the pro-3*S* position and the solvent proton is incorporated at the pro-2*S* position.

and, following each condensation, the β-carbon of the β-ketoacyl moiety formed is completely reduced by a three-step ketoreduction-dehydration-enoylreduction process [6]. The saturated acyl chain product of one cycle becomes the primer substrate for the following cycle, so that two saturated carbon atoms are added to the primer with each turn of the cycle (Fig. 3). The final product is released as a free fatty acid by the animal FAS and as a CoA ester by the yeast complex.

3.2. The catalytic components

Several catalytic elements are required for the biosynthetic process: acyltransferases that load the primer and extender substrates onto the FAS complex; a posttranslationally phosphopantetheinylated acyl carrier protein (ACP) that translocates the various thioester intermediates between catalytic sites; a β-ketoacyl synthase (KS) that performs the condensation reaction; the β-ketoacylreductase, dehydrase, and enoylreductase enzymes that are responsible for the beta-carbon-processing reactions; and a chain-terminating enzyme

that is responsible for product release. Although the animal and yeast FASs employ essentially the same overall reaction scheme, they exhibit minor differences in the enzymatic details and major differences in their macromolecular architecture [7,8]. (i) Whereas the animal FAS utilizes the same acyltransferase for loading of both the acetyl and malonyl substrates, the yeast FAS uses two different enzymes, one specific for acetyl moieties, the other able to accept malonyl. (ii) The animal FAS releases the product as a free fatty acid, through the action of a thioesterase, which severs the bond between fatty acid and phosphopantetheine thiol whereas the yeast FAS uses the same acyltransferase responsible for malonyl loading to transfer the product to a CoA acceptor so that the product is an acyl-CoA thioester. (iii) The enoylreductase of the animal FAS utilizes only NADPH, but the yeast enzyme requires both NADPH and FMN as cofactors. (iv) Posttranslational modification of the ACP domain of the fungal FAS is catalyzed by a resident phosphopantetheinyl transferase (PPT) domain whereas this role is performed by a separate freestanding PPT in animals.

3.3. Structural organization

In eukaryotes, the enzymes required for fatty acid synthesis de novo are integrated into large multifunctional polypeptides that are located in the cytosol. Remarkably, unlike the ACCs, the animal and fungal FASs have evolved along quite different themes (Fig. 4). Thus, the order of the fused catalytic domains, the number of subunits, and the overall architectural design are radically different; the fungal FAS is a 2.6-mDa dodecamer comprised of two different subunits ($\alpha_6\beta_6$) whereas the animal FAS is a 0.54-mDa homodimer (α_2). In 2006, Nenad Ban and colleagues derived ~5 Å resolution electron density maps of the two types of FAS by X-ray crystallography [9,10]. The structures agree well with lower resolution electron micrographic structures obtained earlier [11,12]. As the resolution was insufficient to identify amino acid side chains, or trace the complete backbone of individual subunits, the authors fitted high-resolution three-dimensional structures of homologous

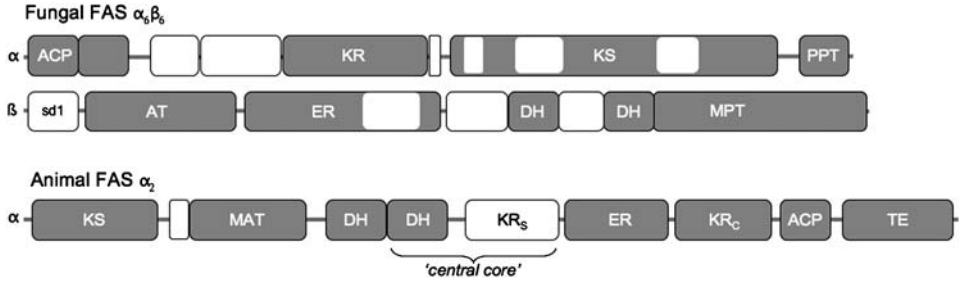

Fig. 4. Domain maps of the fungal and animal FAS. Catalytic domains are shown as gray boxes, structural elements in white, the location of the β-chain structural domain 1 (sd1) is identified in Fig. 5B and D. The region tentatively assigned to the second DH pseudosubunit and the structural domain of the KR has been referred to as the central core. MPT, malonyl/palmitoyltransferase; PPT, phosphopantetheinyl transferase; MAT, malonyl/acetyltransferase; KRs and KRc are structural and catalytic subdomains of the β-ketoacyl reductase; other abreviations as in Fig. 2. The yeast alpha-subunit contains 1887 residues and the β subunit, 1845. The animal FAS polypeptide contains ~2500 residues.

individual bacterial proteins into the electron density map to reveal the locations of most of the catalytic domains. More recently, the resolution of the fungal FAS structure was improved sufficiently to allow tracing of the entire backbone and position 50% of the protein side chains [13,14].

The fungal FAS is a large barrel-shaped protein with an extraordinarily complex architecture supported by many structural elements that constitute almost half of the entire molecule (Fig. 5A and B). The sides of the barrel are comprised of two trimeric β-chains, each chain containing four catalytic domains, acetyltransferase (AT), enoylreductase (ER),

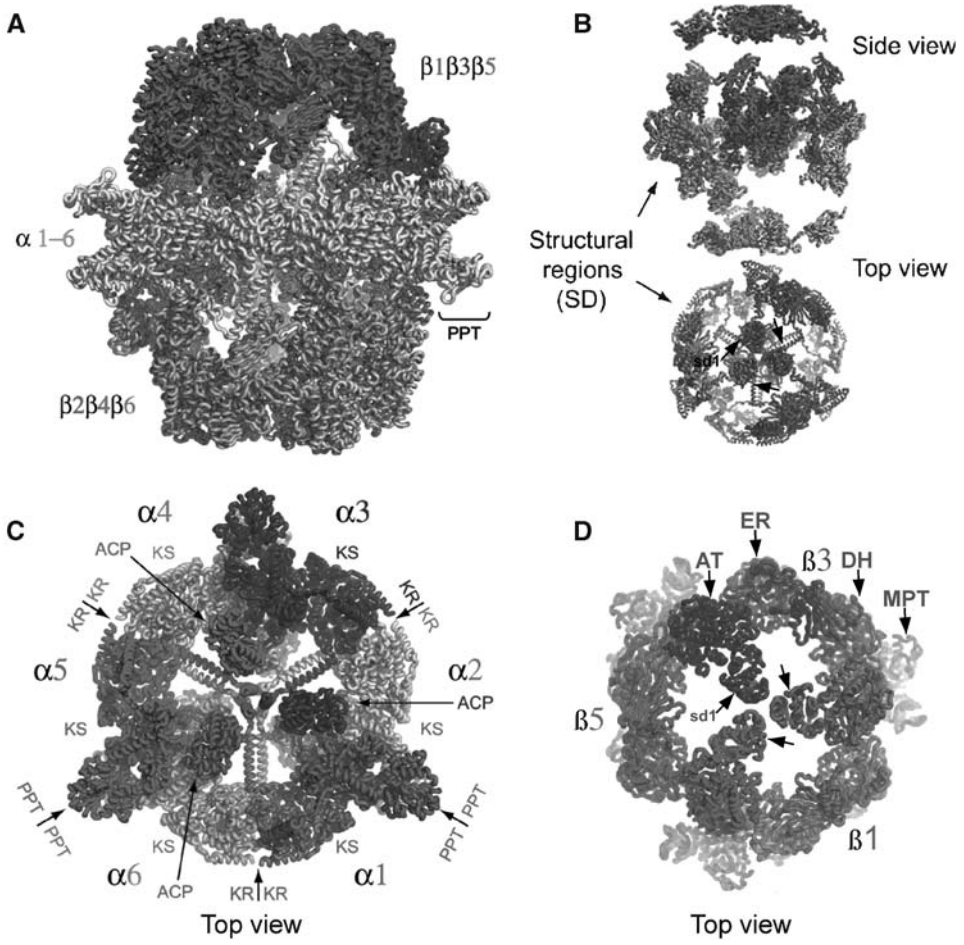

Fig. 5. Architecture of the fungal FAS. (A) Structural overview of the barrel-shaped molecule showing the location of the equatorial wheel composed of the six alpha subunits flanked by two domes, each composed of a β subunit trimer. The barrel is 270 Å long and 230 Å wide at the equator. (B) Location of the structural underpinnings of the molecule with the catalytic domains removed. (C) Organization of the alpha-subunit hexamer. (D) Organization of one β-subunit trimer (adapted from Lomakin et al. [13] with permission). (See color plate section, plate no. 4.)

dehydrase (DH), and malonyl/palmitoyltransferase (MPT), together with four discreet structural domains (Fig. 5D). The DH domain adopts a double hot-dog fold with each of the two subdomains, or pseudosubunits, contributed by the same polypeptide. Six alpha-chains form a central wheel-like structure that divides the barrel into two reaction chambers and are comprised of an N-terminal ACP domain, a ketoreductase (KR) domain, a KS domain, two discreet structural domains, and a C-terminal PPT domain (Fig. 5C). The KR, KS, and PPT domains are all dimeric. The structural domains form the axle, hub, and spokes of the central wheel with the catalytic domains arranged around the rim. Three ACP domains are located in each chamber and are tethered via a structural subdomain and a flexible hinge to the center of the molecule with the phosphopantetheinylated subdomain facing the active centers of the catalytic domains in the chamber wall. Remarkably, the KS catalytic domain is fragmented by several structural elements, a phenomenon unique to the fungal FAS. The PPT domains, which are located at the C-terminus of each alpha chain, form three protuberances from the central wheel and from this position on the outside of the barrel are unable to contact the ACP domains once the structure has been assembled. The architecture of the molecule effectively forms two reaction chambers, each containing three complete sets of catalytic domains that are sealed off from the exterior, allowing only the entry of substrates and exit of the palmitoyl-CoA product through small pores in the barrel sides.

The animal FAS can be conveniently characterized as a body consisting of dimeric KS, ER, and DH domains, with two arms (KR monomers) and legs (malonyl/acetyltransferase (MAT) monomers) (Fig. 6A). The pairs of ACP and thioesterase domains, which because of inherent mobility could not be assigned unambiguously to the electron density map, likely are located at the ends of the two arms. Paradoxically, these were the only two domains for which high-resolution structures had previously been determined (M.A.C. Reed, 2003; F.A. Quiocho, 2004). Prior to 2006, a substantial region of the animal FAS, the ~600 residue 'central core' between the DH and ER domains had no assigned function [7]. The FAS structure derived in Ban's laboratory [9] suggested that the aminoterminal section of the central core constitutes a subdomain of a pseudodimeric DH domain, with each pseudosubunit contributed by adjacent regions of the same FAS subunit. This hypothesis was recently confirmed by expression and characterization of several constructs containing different lengths of the putative pseudodimeric DH domain (S. Smith, 2007). Mutagenesis experiments have revealed that the active site is formed at the DH pseudosubunit interface by the cooperation of a histidine residue in the first pseudosubunit with an aspartate in the second (S. Smith, 1993, 2007). A crystal structure derived for the KR region of a modular polyketide synthase indicates that this domain also may be pseudodimeric, with one pseudosubunit contributed by the carboxyterminal region of the central core. In this case, however, the two pseudosubunits are contributed by polypeptide regions interrupted by the ER domain and only one contains a nucleotide-binding domain suggesting that one pseudosubunit plays a structural role in support of the catalytic pseudosubunit (A. Keatinge-Clay, 2006). While this claim requires confirmation by biochemical studies, if validated, the entire region of the previously unassigned 'central core' could be assigned to subdomains of the DH and KR. It appears likely then that, compared to the fungal FAS, in which almost half of the residues are required to provide the infrastructure to support the activities of the catalytic domains, the animal FAS is relatively parsimonious

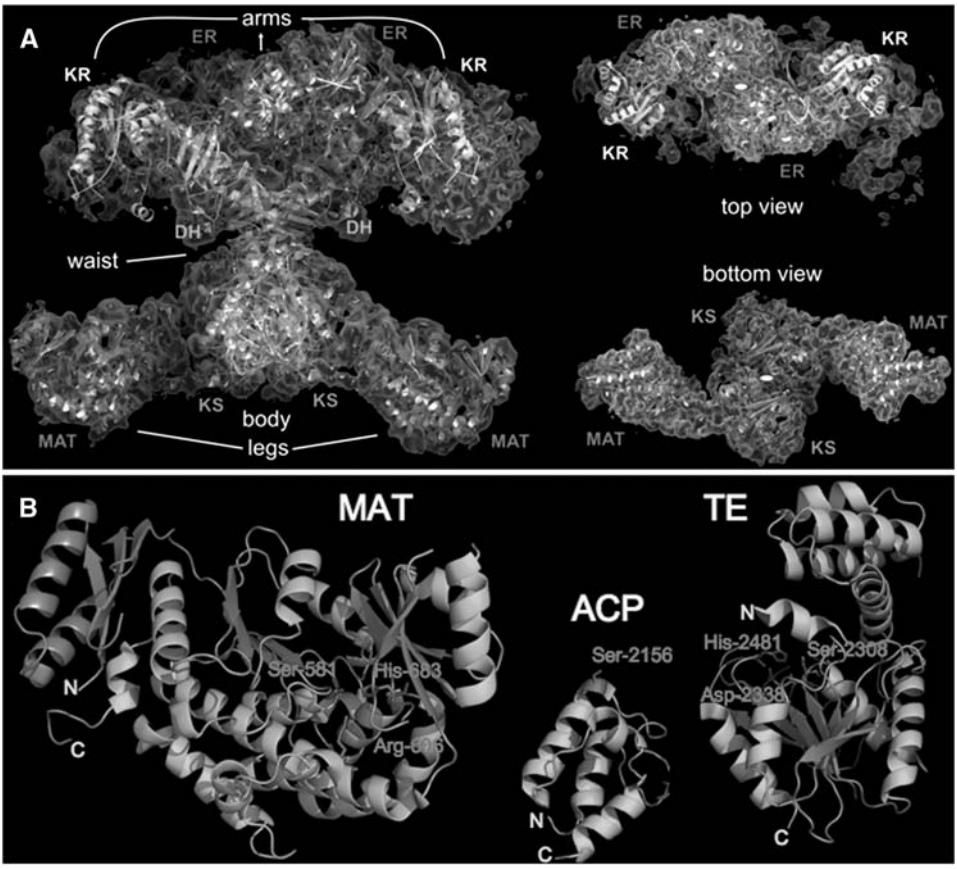

Fig. 6. Structure of the animal FAS. (A) An overview of the entire complex (reproduced with permission of Maier et al. [9]). Fitted homologous domains are shown with a semi-transparent surface representation of the experimental 4.5-Å-resolution electron density. Two white stars indicate the pseudosymmetry-related suggested attachment regions for the ACP and thioesterase ACP and TE, where more density is visible on the right side. MAT, malonyl/acetyltransferase. (B) High-resolution structures of the ACP and MAT domains (Structural Genomics Consortium) and the TE domain (F.A. Quiocho, 2004) of the human FAS showing locations of active-site residues. The gray region of the MAT (residues 422–484) represents a structured linker region that probably interacts with the adjacent KS domain and is not part of the MAT catalytic domain. (See color plate section, plate no. 5.)

with most of the structure devoted to the individual catalytic elements. Analysis of the orientation of the structures fitted into the electron density map indicates that the active sites of the two sets of catalytic domains face inward to each of the lateral clefts in the structure, which presumably constitute two reaction chambers. Interestingly, as in the electron microscope reconstruction, the structure is not symmetrical and the distance between the arms and legs is different on each side. It has been suggested that the two centers for fatty acid synthesis may function asynchronously, with one center engaged in chain-elongation while the other in β-carbon processing [12]. The different conformations in the two

chambers revealed by electron microscopy and X-ray crystallography are consistent with this hypothesis and indicate that substantial conformational changes may be necessary to allow access of the ACP domains to each of the catalytic centers. Indeed, cross-linking and mutant complementation studies have revealed that the ACP domains can access the KS and MAT domains of either subunit (S. Smith, 2001) and, in the absence of any electron density that can clearly be assigned to the ACP domain, it remains unclear how this apparent redundancy is achieved. The new structures for the animal FAS derived from electron microscopic and crystallographic analyses reveal unambiguously that the subunits are coiled in a head-to-head orientation so that the long-held view that this megasynthase consists of fully extended head-to-tail-oriented subunits is incorrect [15].

3.4. Chain initiation

Since the animal FASs utilize a common loading site for both the primer and chain-extender substrates, acetyl- and malonyl-CoA are mutually competitive inhibitors of each other. These FASs cannot order the sequential loading of one acetyl moiety and seven malonyl moieties but instead rely on a self-editing process, in which both substrates are rapidly exchanged between CoA-ester- and enzyme-bound forms [16,17]. Only when the appropriate pair of substrates is bound, that is an acetyl, or longer saturated acyl moiety, at the cysteine-active site and a malonyl moiety at the phosphopantetheine site, does condensation take place. Nonproductive binding, for example when the malonyl moiety binds before the acetyl moiety, or when two acetyl moieties are bound sequentially, results in rapid translocation of the substrates back to their CoA ester form. Paradoxically, for the system to function efficiently, free CoA, which appears on the product side of the equation, must be available at all times (S. Smith, 1982). The structure of the substrate-loading acyltransferase has recently been solved at the Structural Genomics Consortium in Oxford (Fig. 6B) and confirms the identity of three critical residues at the active site previously implicated by mutagenesis (S. Smith, 1996, 1997): the serine nucleophile, a histidine that supports the nucleophilicity of the serine and an arginine residue that is essential for the binding of malonyl, but not acetyl, moieties, and makes direct contact with the 3-carboxylate of the malonyl substrate. Replacement of the active-site arginine residue with alanine eliminates binding of malonyl moieties but actually enhances binding of acetyl moieties (S. Smith, 1997) but the structural basis for this unusual dual substrate specificity of the MAT domain remains unclear.

The yeast FAS employs two different acyltransferases for loading of acetyl and malonyl substrates and does not rely on a self-editing mechanism to facilitate translocation of the appropriate substrates to the condensation sites [18]. Nevertheless, since these complexes terminate acyl-chain growth by transfer of the acyl moiety to a CoA acceptor, free CoA is also required for efficient operation of the pathway. The AT domain of the fungal FAS exhibits a similar overall fold to the MPT domain at the opposite end of the β-subunit and the MAT domain of FAS. These domains are characterized by an α/β core structure capped by a small ferridoxin-like subdomain and an active site located in a gorge between the two subdomains. Both AT and MPT employ a nucleophilic serine residue supported by a histidine at the active site. However, in the fungal AT domain, the gorge is relatively narrow and the arginine residue that typically interacts with the 3-carboxylate of malonyl moieties

in the other enzymes is replaced by an isoleucine, perhaps accounting for the unique specificity for acetyl substrates [9,13].

The KS domains of the fungal and animal FASs are homodimeric, adopting a thiolase fold analogous to their prokaryotic counterparts in type II FASs. These enzymes are all characterized by an active-site triad consisting of the cysteine nucleophile and two histidine residues. Details of the reaction catalyzed by the type I KS have been best studied in the animal FAS (S. Smith, 2002). Nucleophilicity of the active-site serine appears to result primarily from a helix dipole effect resulting from its location at the N-terminus of an alpha-helix. One of the active-site histidine residues activates a water molecule that attacks the malonyl C3 carboxylate releasing bicarbonate. The second active-site histidine residue assists in the formation of a carbanion at C2 by stabilizing the enol intermediate. In the final step of the reaction, the carbanion attacks the acyl-thioester, forming a tetrahedral intermediate that breaks down to form the β-ketoacyl product.

3.5. β-Carbon-processing reactions

The KR domains of the fungal FAS and the catalytic subdomain of the animal FAS closely resemble their type II counterparts, exhibiting typical characteristics of an NADPH-binding Rossmann fold [13]. However, the fungal KR domains form true dimers whereas the KR domains associated with different subunits of the animal FAS are widely separated at the ends of the two 'arms' of the molecule and do not interact (Fig. 6A). Nevertheless, it appears likely that these KR domains may be pseudodimeric in that a second subdomain, which does not exhibit the properties of a complete Rossmann fold, provides structural support by interacting with the catalytic KR subdomain (Keatinge-Clay, 2006). This trend is repeated in the DH domains of both the fungal and animal FASs, which are pseudo-dimeric, double hot-dog structures. The reaction catalyzed by the DH domains are readily reversible and indeed the equilibrium has been shown to favor the reverse hydration reaction, at least in the case of the animal FAS (S. Smith, 2004). The ER domain of the fungal FAS is unique among enoyl reductases in that it requires both FMN and NADPH, it is monomeric and adopts a TIM-barrel fold consisting of an eight-stranded β-barrel surrounded by eight helices [13]. In contrast, the ER domains of the animal FAS use only NADPH, they are dimeric and are members of the medium-chain dehydrogenase family [9].

3.6. Chain termination and product specificity

The eukaryotic FASs synthesize predominantly the 16-carbon saturated product with smaller amounts of 14- and 18-carbon products. The enzymatic basis of product specificity of the animal FAS has been studied in some detail in G. Hammes' and S. Smith's laboratories. The KS has a relatively broad chain-length specificity and is able to transfer efficiently saturated acyl moieties with 2–14 carbon atoms from the phosphopantetheine thiol to the active-site cysteine thiol. However, longer chain-length acyl moieties are transferred between thiols with increasing difficulty [17]. In contrast, the chain-terminating thioesterase has very limited ability to remove acyl moieties with less than 16 carbon atoms from the phosphopantetheine thiol (S. Smith, 1978, 1991). Thus the specificities of

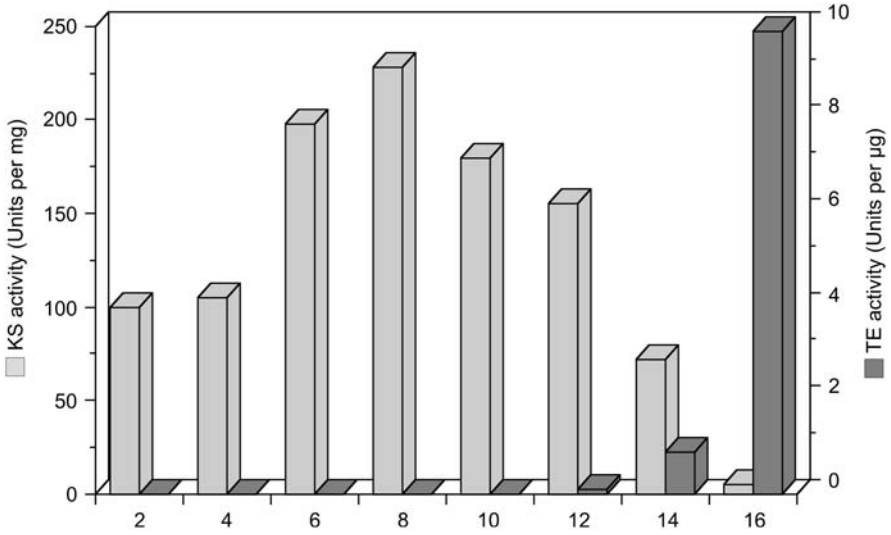

Fig. 7. Chain-length specificities of the KS and thioesterase components of the mammalian FAS. The activities were measured using model substrates and reflect the ability of the KS to translocate saturated acyl chains from the phosphopantetheine thiol of the ACP to the active-site cysteine residue of the KS and the ability of the TE domain to hydrolyze saturated acyl chains from thioester linkage to the phosphopantetheine moiety.

the chain-elongating and chain-terminating enzymes complement each other perfectly, ensuring that the 16-carbon fatty acid is the major product (Fig. 7). The KS cannot transfer incompletely reduced intermediates (β-keto-, β-hydroxy, or enoyl) from the phosphopantetheine thiol to the active-site cysteine, so only the saturated intermediates are elongated (S. Smith, 1997). Recently, a crystal structure has been solved for the thioesterase domain of the human FAS (F.A. Quiocho, 2004). The protein consists of two subdomains, one exhibiting an α/β hydrolase fold and the other a four-helix bundle that caps the active-site region (Fig. 6B). The active site consists of a typical serine–histidine–aspartate triad. Mutagenesis experiments and modeling of acyl chains into the putative substrate-binding pocket indicates that the first 12 C-atoms are likely exposed to solvent with only about the last 4–6 C-atoms making contact with the closed-end hydrophobic pocket, consistent with the high specificity of the FAS for producing predominantly the 16 C-atom fatty acid.

The fungal FAS terminates acyl chain elongation at the 16–18 C-atom stage by direct transfer of the acyl chain to a CoA acceptor. Adjacent to the active site of the MPT domain is a gorge that is long enough to accommodate a 16–18 carbon long chain. As in the thioesterase domain of the animal FAS, only the distal end of the gorge is hydrophobic, so that only long-chain acyl moieties are able to dock into the catalytic center. The partly open side of the binding pocket is immediately adjacent to a pore in the barrel wall that presumably allows release and diffusion of the long-chain acyl-CoA to the exterior.

The same acyltransferase is responsible for loading the acetyl primer substrate and unloading the palmitoyl product and this enzyme is unique in that the active site possesses

both a hydrophobic pocket capable of binding long-chain acyl moieties and an arginine residue able to interact with the 3-carboxylate of the malonyl chain extender substrate.

3.7. Interdomain communication

The 20-Å-long phosphopantetheine 'swinging arm' of the animal and fungal FASs has long been accepted as a key factor in allowing each of the six catalytic center's access to the ACP domains. However, the distances between some of the catalytic sites of the resting animal FAS are clearly considerably greater than 20 Å, as revealed by fluorescence energy transfer (P.E. Kolattukudy, 1985; G.G. Hammes, 1986) and more recently by the crystallographic studies. Moreover, as pointed out by Ban and colleagues, a 20-Å-long phosphopantetheine arm is sufficient only to reach from the perimeter of the catalytic domains into the active site. These observations suggest that cooperation between the acyl carrier domain and the catalytic domains during fatty acid biosynthesis must involve significant conformational changes within the complex. Such conformational changes may be mediated in part through the presence of flexible linker regions between the catalytic domains. Based on the observed asymmetry of the animal FAS structure, Ban and colleagues [9] have identified several putative hinge regions that may contribute to the conformational flexibility of the structure. In contrast, the fungal FAS appears to be a relatively rigid structure. In this FAS, the distances between Ser180, the point of attachment of the 20-Å-long phosphopantetheine to the ACP, and the catalytic centers range from 18 to 80 Å. Thus, in this FAS too, spanning of the longer distances cannot be achieved by movement of the 20-Å-long phosphopantetheine arm alone. However, in the fungal FAS, the ACP consists of two subdomains, a typical four-helix bundle that includes the phosphopantetheine moiety and a second subdomain that is tethered via a flexible hinge to the hub of the central wheel. The distance from the phosphopantetheinylated site to the hinge is such that swinging of the entire ACP structure is likely sufficient to allow access to each of the distantly located catalytic centers [13].

3.8. Posttranslational modification

Most prokaryotes employ dedicated PPTs active toward specific apoprotein substrates. Fungi also utilize separate PPTs for modification of the ACPs associated with the cytosolic and mitochondrial FAS systems, as well as the α-aminoadipate reductase involved in lysine biosynthesis [8]. The former is unusual in that it is a constituent domain of the multifunctional α-subunit (Fig. 4). Surprisingly, animals appear to employ a single PPT for servicing three different apoproteins: the ACP domain of the cytosolic FAS, the ACP component of the mitochondrial FAS, and the α-aminoadipate semialdehyde dehydrogenase involved in lysine catabolism. The human PPT has recently been identified and characterized (S. Smith, 2003) and its crystal structure determined in complex with Mg^{2+}, CoA, and the ACP domain of the cytosolic FAS (Structural Genomics Consortium). The ACP domain is comprised of a four-helix bundle, as are the ACPs associated with type II systems. The conserved serine residue that is the site of posttranslational modification lies at the N-terminal end of helix-2 (Fig. 6B), closely juxtaposed with the pyrophosphate of CoA that is cleaved during the phosphopantetheine transfer. Helix-2 makes multiple

contacts with the PPT and indeed this structural element is regarded as a universal 'recognition helix' that plays a pivotal role in the interaction of ACPs with their cognate enzymes in fatty acid synthesis. Surprisingly, hydrophobic interactions appear to play an important role in the association of human PPT and ACP, whereas in prokaryotes the interaction of ACPs with their cognate catalytic proteins are typically mediated by ionic interactions. Extensive mutagenesis of residues implicated in binding of substrate and Mg^{2+} has led to proposal of a detailed mechanism for this reaction (U. Oppermann, 2007).

In the fungal FAS, the PPT domains appear to be distorted by interaction with a structural element in such a way as to disrupt the CoA-binding site. This observation, together with the location of the PPT domains on the outside of the barrel, implies that posttranslational modification of the ACP domains occurs prior to assembly of the FAS molecule.

4. The mitochondrial FAS

The existence of a mitochondrial pathway for de novo fatty acid synthesis was first reported 50 years ago, when it was generally assumed that fatty acid synthesis proceeded by reversal of the mitochondrial pathway for fatty acid β-oxidation (F. Lynen, 1957). However, the discovery of the cytosolic malonyl-CoA pathway (R.O. Brady, 1958; S.J Wakil, 1958) casted doubt on these claims and interest in this system waned until the discovery that mitochondria of both *Neurospora crassa* and *Saccharomyces cerevisiae* contain nuclear-encoded mitochondrial proteins that function as a type II FAS system. Disruption of the genes encoding these enzymes in both *N. crassa* and *S. cerevisiae* produces respiratory-deficient phenotypes and in *S. cerevisiae* cellular lipoic acid is reduced to less than 10% of that of the wild-type strain (R. Schneider, 1995; E. Schweizer, 1997). These observations suggested that in fungi one of the roles of this pathway might be to generate the lipoyl moieties required for mitochondrial function.

Recently, several of the components of a putative type II FAS system have also been identified in human mitochondria, including ACP, malonyltransferase, KS (S. Smith, 2003, 2005), and ER (J.K. Hiltunen, 2003). All are nuclear encoded and possess aminoterminal extension sequence elements that target the proteins into mitochondria. The mitochondrial ACP is phosphopantetheinylated by the same enzyme that services the ACP domain of the cytosolic FAS, presumably prior to import into the mitochondria. The crystal structures of all of these enzymes have been solved (Structural Genomics Consortium, J.K. Hiltunen, 2003) and reveal that these human mitochondrial proteins bear a striking resemblance to their prokaryotic counterparts.

Recent studies with bovine heart mitochondrial matrix preparations indicate that one of the major products of this pathway is octanoyl-ACP and these newly synthesized octanoyl moieties can be translocated directly to the lipoylation site of the glycine cleavage apo-H protein (S. Smith, 2007). Octanoylated mitochondrial proteins are the substrates for the enzyme lipoic acid synthase, which inserts two sulfur atoms at the C6 and C8 positions of the octanoyl moiety. These results are consistent with the hypothesis that one of the major roles of the mitochondrial FAS pathway in all eukaryotes is to ensure that an adequate supply of lipoyl moieties is always available to service the glycine cleavage enzyme and the alpha-ketoacid dehydrogenases that are essential to mitochondrial function.

5. Regulation of fatty acid synthesis

5.1. Significance of de novo lipogenesis

Although synthesis of medium-chain fatty acids by mammary gland is important for milk production, liver and adipose tissues are the major anatomic sites for de novo synthesis of fatty acids from carbohydrate or protein. A major proportion of fatty acids made in liver is used for synthesis of triacylglycerols for very low density lipoproteins that are then secreted into the blood stream (Chapter 18). In contrast, fatty acids synthesized in adipose tissue are used for synthesis of triacylglycerol to be stored in adipose tissue (Chapter 10). Very low density lipoprotein synthesis/secretion in liver and triacylglycerol storage in adipose tissue are increased upon feeding or during positive energy balance. De novo lipogenesis is suppressed by high-fat diets and elevated by high-carbohydrate diets. Thus, de novo lipogenesis is tightly regulated by different dietary components as well as by hormones whose secretion in turn is influenced by nutritional states and by dietary components [19].

In humans, unlike rodents, the extent of de novo lipogenesis in liver and adipose tissue is less clear. Fatty acids produced by de novo lipogenesis in either tissue may not make a major contribution quantitatively in humans, due to a high fat content (~40% of total energy) in modern diets. Furthermore, the degree of de novo lipogenesis in adipose tissue has been considered to be even lower than that in liver. Recent in vivo measurements, however, suggest that human liver and adipose tissue may have similar degrees of fatty acid synthesis. Genetic, dietary, or other factors may influence de novo lipogenesis. De novo lipogenesis has been shown to be higher in obese and hyperinsulinemic humans in whom fatty acids from de novo lipogenesis can contribute up to 25% of triacylglycerol in circulating very low density lipoproteins. The increased contribution of de novo lipogenesis can be demonstrated by the increased palmitate level in triacylglycerol of very low density lipoproteins.

In addition to the provision of fatty acids for triacylglycerol synthesis, the low but regulated rates of lipogenesis may be critical for overall control of fatty acid metabolism in humans. As discussed above, malonyl-CoA, which is the product of the ACC reaction and the only free intermediate of fatty acid synthesis, inhibits CPT-1 activity, and thereby prevents transport of fatty acids into mitochondria. Thus, the dual role of malonyl-CoA as an intermediate of fatty acid synthesis and as a regulator of fatty acid oxidation, prevents the operation of the futile cycle, in tissues where both fatty acid synthesis and oxidation can be active.

De novo lipogenesis may also play a significant role in certain physiologic and pathophysiologic conditions. For example, developmental needs for lipid in the fetus may be met by de novo lipogenesis, as demonstrated by the extremely high rate of lipogenesis in premature infants. De novo lipogenesis also contributes significantly to the hypertriglyceridemia and hepatic steatosis in alcoholic liver disease, obesity-associated insulin resistance, and type II diabetes. Furthermore, upregulated lipogenic enzymes and elevated lipogenesis have been shown to occur in many cancers [20,21]. Thus, increased fatty acid synthesis may even be required for carcinogenesis and has been linked to other cancer-associated metabolic changes.

5.2. Generation of substrates for fatty acid synthesis

The major site for fatty acid synthesis is the cytosol. However, acetyl-CoA, the substrate for fatty acid synthesis, is formed from pyruvate in the mitochondria and, since the inner membrane is impermeable to acetyl-CoA, is translocated to the cytoplasm indirectly as citrate (Fig. 8). When the rate of production of acetyl-CoA from pyruvate is high, the rate of formation of citrate, catalyzed by citrate synthase in the citric acid cycle, is also elevated, and citrate accumulates in mitochondria. Citrate is then translocated into the

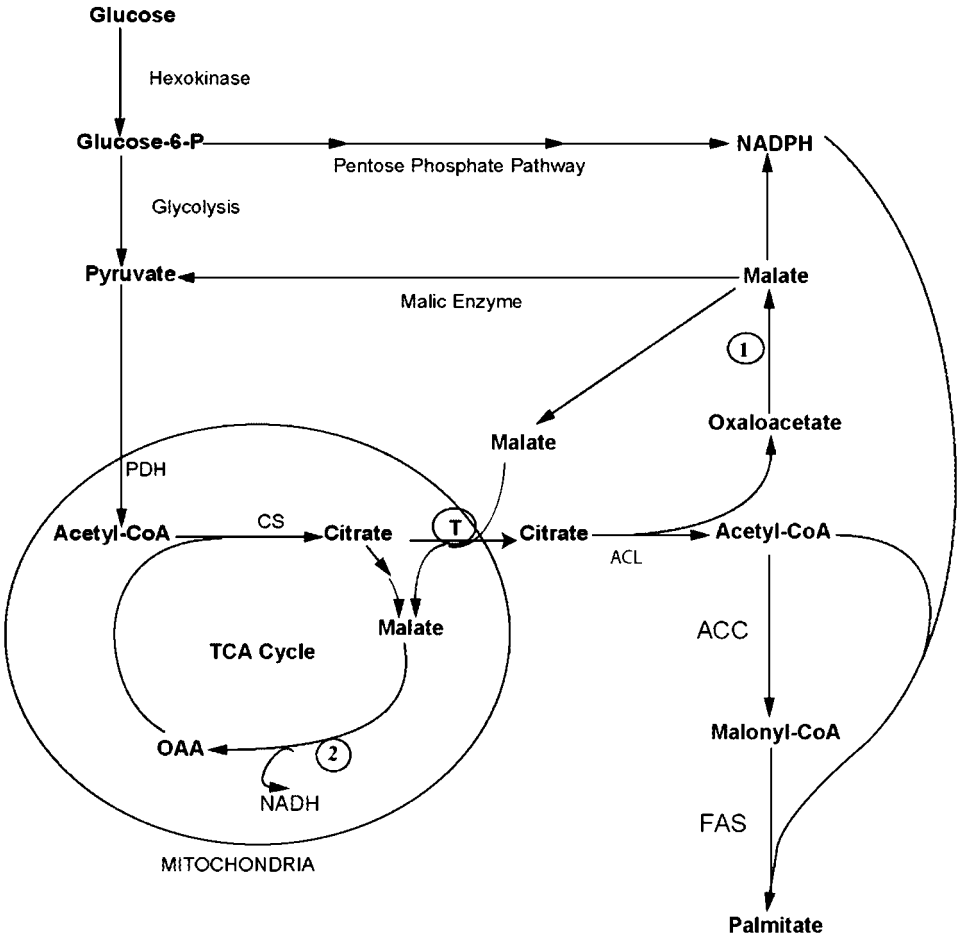

Fig. 8. Pathways involved in the conversion of glucose to fatty acid. Reaction (1) is catalyzed by cytosolic malate dehydrogenase. Reaction (2) is catalyzed by mitochondrial malate dehydrogenase. (T) designates tricarboxylate anion transporter. Reactions catalyzed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the pentose phosphate pathway produce NADPH. CS, citrate synthase; ACL, ATP citrate lyase; PDH, pyruvate dehydrogenase complex; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase.

cytosol in exchange for a dicarboxylate anion, malate, by the tricarboxylate anion carrier at the inner mitochondrial membrane. Citrate, as a feed-forward activator of ACC, also plays a key role in the regulation of fatty acid synthesis. In the cytosol, fatty acid synthesis proceeds by cleavage of citrate to acetyl-CoA and oxaloacetate catalyzed by ATP-citrate lyase. Oxaloacetate, in turn, is reduced to malate by cytosolic malate dehydrogenase, then malate is returned to the mitochondria via the tricarboxylate anion carrier in exchange for another molecule of citrate and is converted back to oxaloacetate via the tricarboxylate cycle and mitochondrial malate dehydrogenase. This cycle results in the net transport of acetyl-CoA into cytosol and NADH into mitochondria. Alternatively, malate is oxidized to pyruvate in a reaction catalyzed by malic enzyme in the cytosol. This reaction converts NADP to NADPH, which is required for the reductive step in fatty acid synthesis. Thus, acetyl-CoA is translocated into the cytosol and reducing equivalents, NADPH, along with NADPH produced by the pentose phosphate pathway, are used for fatty acid synthesis.

5.3. Allosteric regulation of ACC

The reaction catalyzed by ACC is the first committed step in fatty acid synthesis and is highly regulated by an array of control mechanisms, thereby facilitating adjustments in the rate of fatty acid synthesis in response to various physiological and developmental conditions. At the same time, the rate of fatty acid oxidation in mitochondria is reciprocally regulated since malonyl-CoA, the product of ACC, is a potent inhibitor of CPT-I. As stated in Section 2.3, cytosolic ACC1 provides malonyl moieties for use as the chain extender substrate in fatty acid synthesis in lipogenic tissues, such as liver and adipose tissue. ACC2 provides malonyl-CoA that functions as a regulator of fatty acid oxidation, primarily in muscle, but also in liver, where fatty acid oxidation and synthesis are both important. ACC2, which is anchored to the outer mitochondrial membrane via a unique N-terminal hydrophobic domain, generates malonyl-CoA on the mitochondrial surface where it allosterically inhibits CPT-1 thereby controlling transfer of cytoplasmic long-chain fatty acyl-CoA to the mitochondria. ACC2-null mice, lacking the pool of malonyl-CoA that normally is generated by ACC2 (to inhibit CPT-1 activity), continuously oxidize fatty acids and accumulate less fat than wild-type mice (S.J. Wakil, 2005). ACC1-null mice are not viable because of the essential role of de novo lipogenesis during embryonic development. Liver-specific deletion of ACC1 causes reduced hepatic triacylglycerol content, but does not alter fatty acid oxidation or glucose homeostasis, evidence for the function of the ACC1 isoform in providing malonyl-CoA for fatty acid synthesis only (S.J. Wakil, 2005).

The activity of ACC1 and ACC2 is stimulated by citrate, an allosteric activator, and is inhibited by long-chain acyl-CoA, an allosteric inhibitor, thus representing feed-forward and feedback regulation, respectively [22,23] (Fig. 9). Under conditions favoring fatty acid synthesis, production of cytosolic citrate is increased. Although details of the mechanism of binding are poorly understood, citrate and possibly other carboxylic acids activate ACC by increasing the V_{max} without affecting the K_m for acetyl-CoA. Citrate also prevents the binding of the ACC inhibitor, fatty acyl-CoA. Citrate causes polymerization of ACC (Section 2.2 and Fig. 2B), but it is not clear whether the polymerization precedes the

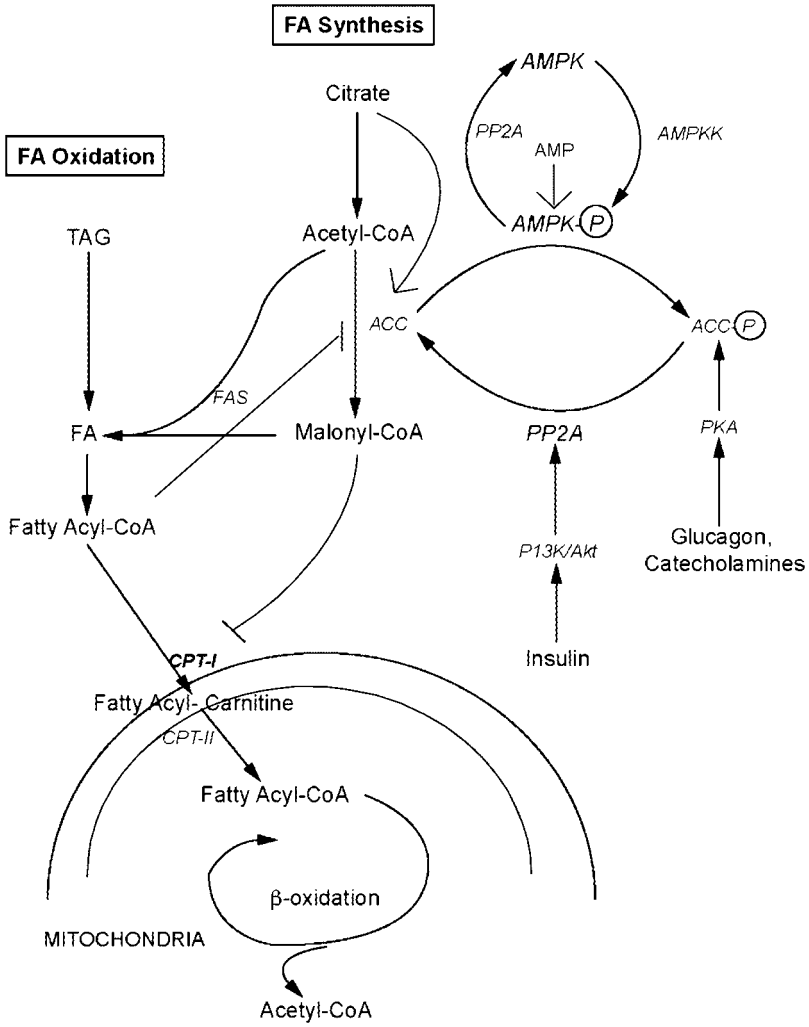

Fig. 9. Reciprocal regulation of fatty acid synthesis and oxidation. Malonyl-CoA, the product of the ACC reaction, inhibits CPT-1, which is localized at the outer mitochondrial membrane and catalyzes the conversion of fatty acyl-CoA to fatty acyl-carnitine for mitochondrial fatty acid import and oxidation. At the inner mitochondrial membrane, fatty acyl moieties are converted to CoA thioesters by CPT-II before undergoing β-oxidation. ACC is activated by citrate and inhibited by fatty acyl-CoA. AMPK is activated by AMP and the high AMP level reflects the low energy state of the cell. Activation of AMPK in response to increases in AMP involves phosphorylation by an upstream AMPK kinase (AMPKK), the tumor suppressor LKB1, and AMPK is inactivated/dephosphorylated by protein phosphatase 2A (PP2A), which is first activated by insulin via PI3K/Akt pathway. ACC is dephosphorylated/activated by PP2A and is inactivated upon phosphorylation by AMPK. ACC can also be phosphorylated/inactivated by PKA. TAG, triacylglycerol; FA, fatty acid.

activation of ACC. Polymerization of ACC in the presence of citrate may protect ACC from inactivation via the phosphorylation mechanism described in Section 5.4. ACC2 does not polymerize as readily as ACC1, probably because ACC2 is localized to the outer mitochondrial membrane. During starvation, when fatty acid synthesis is inhibited, the level of long-chain fatty acyl-CoA increases. Conversely, in the fed state when fatty acid synthesis is stimulated, the long-chain acyl-CoA level decreases. The K_i of long-chain fatty acyl-CoA for ACC, with saturated 16–20 carbon fatty acyl-CoA being most effective, is approximately 5 nM. Although fatty acyl-CoA levels can reach intracellular concentrations of 200 nM, they are mostly sequestered by acyl-CoA binding protein. Thus, the free fatty acyl-CoA concentration is probably limited to about 2–10 nM, which falls within the range of the K_i for ACC, an evidence that fatty acyl-CoA is a physiological regulator.

5.4. Regulation of ACC by multisite phosphorylation

In addition to allosteric regulation, ACC is also regulated by covalent modification. In hepatocytes and adipocytes, ACC1 is phosphorylated at four different sites in response to hormonal stimuli (M.R. Munday, 2002). Glucagon and epinephrine induce rapid phosphorylation of ACC1 (Fig. 9). The phosphorylated enzyme is less active and less sensitive to the stimulatory effect of citrate, while more sensitive to the inhibitory effect of long-chain fatty acyl-CoA. A number of different protein kinases catalyze the phosphorylation of ACC1 in vitro. For example, cAMP-dependent protein kinase (PKA) phosphorylates ACC1 at Ser77 and Ser1200. However, phosphorylation of ACC1 at Ser79, Ser1200, and Ser1215 is catalyzed largely by AMP-activated protein kinase (AMPK). Furthermore, mutual exclusivity has been described for the two adjacent phosphorylation sites of Ser77 and Ser79: phosphorylation of Ser77 by PKA blocks, phosphorylation of Ser79 by AMPK, and vice versa. Removal of the N-terminal 200 amino acid residues that include Ser77 and Ser79 reverses the effects of phosphorylation by PKA and AMPK on ACC activity, respectively, providing evidence for the importance of these phosphorylation sites. PKA mediates the effects of glucagon and β-adrenergic stimulation through G_s and the adenylyl cyclase system to increase cAMP levels, while AMPK mediates the effects of the intracellular energy state on metabolism. AMPK is composed of catalytic α and regulatory β and γ. subunits. Each subunit is encoded by multiple genes. AMPK serves as a sensor of the cellular energy state, i.e., the AMP/ATP ratio, and is activated by AMP and inhibited by ATP. AMPK is also controlled by upstream kinases, which have recently been identified as LKB1 and Ca^{2+}/calmodulin-dependent protein kinase. AMPK has received much attention because many of the metabolic abnormalities in type II diabetes are expected to be reversed by activation of AMPK. Along these lines, metformin, a mainstay in the treatment of type II diabetes, activates AMPK by enhancing phosphorylation of AMPK by LKB1 (L.C. Cantley, 2005). In peripheral tissues, AMPK is activated in a variety of conditions, such as starvation, exercise, and hypoxia. Once activated, AMPK affects overall metabolism by inhibiting anabolic pathways, while stimulating catabolic pathways and restoring ATP levels. Thus, when the cellular energy state is low, activated AMPK phosphorylates ACC1, resulting in decreased ACC activity and a low rate of fatty acid synthesis.

ACC2 also was shown to be phosphorylated in the ischemic perfused heart or in exercising skeletal muscle when AMPK is activated. Similar to ACC1, ACC2 also is phosphorylated at multiple sites by PKA and phosphorylation desensitizes ACC2 to allosteric activation by citrate and sensitizes the enzyme to inhibition by long-chain fatty acyl-CoAs. It is possible that, even if the kinetics of ACC2 were not altered by phosphorylation, the charge effect of phosphorylation might decrease ACC2 association with mitochondria. Regardless, the end result is a decrease in production of malonyl-CoA and, by prevention of malonyl-CoA inhibition of CPT-1, an increase in fatty acid oxidation in mitochondria.

Although the molecular mechanisms are not clear, insulin causes phosphorylation as well as dephosphorylation at different sites of ACC, but the net effect is activation of ACC. By the use of inhibitors, protein phosphatase 2A class of Ser/Thr phosphatase was shown to be involved in the dephosphorylation of ACC. Thus, activation of ACC by insulin might be independent of cAMP or AMP concentrations. However, blocking the phosphatidylinositol 3-kinase (PI3K) signaling pathway prevents the insulin-mediated activation of ACC. Insulin has recently been reported to inhibit AMPK activity by PKB/Akt-mediated phosphorylation of AMPK at Ser485/491 of the catalytic $\alpha 1/\alpha 2$ subunit in cardiac muscle. Phosphorylation at Ser485/491 antagonizes and reduces phosphorylation of AMPK at Thr172, a site that accounts for most of the AMPK activation by upstream AMPK kinases.

5.5. Role of hypothalamic malonyl-CoA in controlling food intake and energy expenditure

As discussed above, malonyl-CoA is a critical metabolite in fatty acid metabolism in peripheral tissues because it represents the main carbon source for fatty acid synthesis and is a key regulator of fatty acid oxidation. Recently, malonyl-CoA in the hypothalamus has been implicated in the regulation of food intake and energy expenditure [24,25]. Lipogenic enzymes such as ACC and FAS are present at a high level in neurons of many brain regions, including the hypothalamic neurons that regulate feeding behavior. Both ACC1 and ACC2 are present in hypothalamic neurons. The potential role of malonyl-CoA in the central nervous system was first suggested by the observation that small molecule inhibitors of FAS — the mycotoxin cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienamide) and the chemically synthesized compound C75 (α-methylene-2-octyl-5-oxo-tetrahydrofuran-3-carboxylic acid) — cause accumulation of malonyl-CoA, suppression of food intake, and loss of adipose tissue and body weight in mice (F.P. Kuhajda, 2000). The rapid and dose-dependent reduction in food intake occurred either with peripheral (intraperitoneal) or central (intracerebroventricular) administration of the inhibitors. Central administration of these inhibitors increased the hypothalamic malonyl-CoA concentrations that were associated with rapid suppression of food intake. However, these pharmacological inhibitors have additional effects. For example, C75 not only increases the malonyl-CoA level by inhibiting FAS activity but, by acting as a malonyl-CoA analog, C75 prevents the inhibitory effect of malonyl-CoA on CPT-1 activity. Conversely, a recent report showed that C75-CoA can be formed from C75 and, by binding to the substrate (palmitoyl-CoA) pocket, inhibits CPT-1 activity (F. G. Hegardt, 2006). Regardless, a conditional knockdown

of FAS in the hypothalamus of mice decreased food intake and energy expenditure and markedly increased the malonyl-CoA level in hypothalamus. Administration of an ACC inhibitor, TOFA [5-(tetradecycloxy)-2-furoic acid], blunted the rise in malonyl-CoA and suppression of food intake. Furthermore, viral overexpression of malonyl-CoA decarboxylase in hypothalamic regions increased food intake and reversed the C75-mediated suppression of food intake. Malonyl-CoA decarboxylase decreases the malonyl-CoA level by converting malonyl-CoA to acetyl-CoA (N.B. Ruderman, 2001). Malonyl-CoA decarboxylase is usually found in tissues, such as muscle, with low lipogenic capacity and in which ACC2 predominates over ACC1. Overall, inhibition of FAS in the hypothalamus causes accumulation of its substrate, malonyl-CoA, and suppresses food intake (Fig. 10).

Nutritional conditions that control food intake affect hypothalamic malonyl-CoA levels: in the fasted state, the malonyl-CoA level is low but in the fed state it rises. How is food intake regulated by changes in malonyl-CoA concentration? The malonyl-CoA concentration detected in hypothalamus is within the range of the K_d for CPT-1c, a brain-specific CPT-1 isoform which has been suggested to catalyze acyl transfer from fatty acyl-CoA to carnitine. CPT-1c null mice have lower food intake and body weight than wild-type mice. When fed a high-fat diet, CPT-1c null mice are resistant to diet-induced obesity but, paradoxically, have decreased fatty acid oxidation. The function of CPT-1c in mediating control of food intake is yet to be understood. A rise in malonyl-CoA level after intracerebroventricular administration of C75 activates neurons at the major feeding behavior center, the arcuate nucleus. Although not consistently shown in all studies, C75 and cerulenin can decrease the levels of the major orexigenic (increased appetite) neuropeptides, neuropeptide Y, and Agouti-related peptide, and increase the levels of anorexigenic (decreased appetite) neuropeptides, α-melanocyte-stimulating hormone, and cocaine-amphetamine-regulated transcript in the hypothalamus.

In the physiological context, the malonyl-CoA level is controlled by the activity of ACC, which is phosphorylated/inactivated by AMPK. Changes in hypothalamic AMPK activity are consistent with the predicted changes in the malonyl-CoA level and food intake. Furthermore, central administration of the AMP analog, AICAR (5′-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside), which activates AMPK, or expression of an activated mutant form of AMPK in the hypothalamus, increases food intake in mice. Conversely, expression of a dominant-negative inhibitory mutant of AMPK decreases food intake. In this respect, the adipose tissue hormone leptin (Chapter 10) has been suggested to stimulate the dephosphorylation and inactivation of AMPK in the hypothalamus. In turn, the decrease in AMPK activity can change levels of neuropeptides that regulate food intake. These effects of AMPK on food intake are consistent with the idea that AMPK phosphorylates and inhibits ACC, causing a decrease in the level of malonyl-CoA. It has been speculated that during fasting, the AMP/ATP ratio increases and AMPK is activated, which in turn phosphorylates and inactivates ACC, and decreases the malonyl-CoA level. Therefore, it is also possible that activation of AMPK may directly contribute, at least in part, to changes in neuropeptide levels in the hypothalamus thereby altering food intake.

Administration of FAS inhibitors not only alters food intake but also changes peripheral energy expenditure. Thus, administration of C75 by either peripheral or central routes increases fatty acid oxidation. As mentioned above, C75 appears to activate CPT-1,

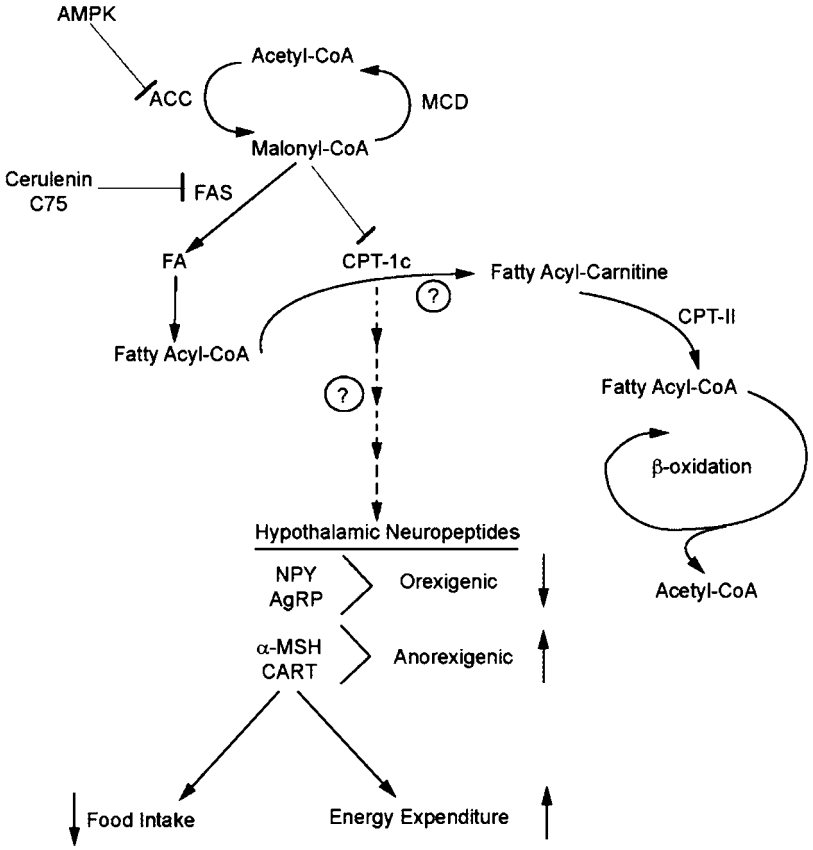

Fig. 10. Regulation of food intake and energy expenditure by hypothalamic malonyl-CoA. In the fed state, especially when a high-carbohydrate diet is consumed, excess glucose is converted to acetyl-CoA and de novo lipogenesis is increased. Acetyl-CoA is first converted to malonyl-CoA catalyzed by ACC, which is activated by citrate and inhibited by fatty acyl-CoA. ACC also is phosphorylated and inactivated by AMPK. Malonyl-CoA is decarboxylated in a reaction catalyzed by malonyl-CoA decarboxylase (MCD) or is utilized by FAS for the synthesis of long-chain fatty acids. Fatty acids are activated to form fatty acyl-CoA that can be transported into mitochondria by first being converted into fatty acyl carnitine in a reaction catalyzed by CPT-1 at the outer mitochondrial membrane to be transported into and used for β-oxidation in mitochondria. Cerulenin and C75 are inhibitors of FAS activity and cause accumulation of malonyl-CoA, resulting in inhibition of CPT-1, decreased transport of fatty acyl moieties into the mitochondria and decreased fatty acid oxidation. However, it is not yet known if the CPT-1c isoform has catalytic activity. C75 decreases the level of orexigenic peptides and increases the level of anorexigenic peptides, which in turn decreases food intake and increases peripheral energy expenditure. NPY, neuropeptide Y; AgRP, Agouti related peptide; α-MSH, α-melanocyte-stimulating hormone; CART, cocaine-amphetamine-regulated transcript.

increase fatty acid import into mitochondria, and stimulate fatty acid oxidation in peripheral tissues. However, this idea is controversial since C75-CoA, which inhibits CPT-1 activity, can be produced from C75. Nevertheless, since central and peripheral administration of C75 have a similar effect on peripheral energy expenditure, it is likely that hypothalamic malonyl-CoA is involved. As hypothesized for control of food intake,

malonyl-CoA and/or AMPK/neuropeptides might affect energy expenditure through the sympathetic outflow to the periphery. Recently, both peripheral and central administration of C75 have been shown to trigger mitochondrial biogenesis in skeletal muscle, accompanying the induction of peroxisome proliferator-activated receptor gamma coactivator-1α, as well as estrogen receptor related receptor-α that facilitates peroxisome proliferator-activated receptor gamma coactivator-1α action (M.D. Lane, 2006). These transcription factors in turn induce expression of key mitochondrial oxidative enzymes and uncoupling protein-3, increasing fatty acid oxidation, thermogenesis, thus increasing the energy expenditure.

6. Long-term regulation of fatty acid synthesis

6.1. Coordinate regulation of lipogenic enzymes by hormones and nutrients

As discussed in Section 5, ACC is under exquisite short-term regulation involving allosteric as well as phosphorylation–dephosphorylation mechanisms. In contrast, neither allosteric regulation nor phosphorylation–dephosphorylation mechanisms appear to contribute to the regulation of FAS activity. A recent report has, however, suggested that a mechanism by which insulin acutely reduces hepatic FAS activity is by causing phosphorylation of carcinoembryonic antigen-related cell adhesion molecule 1 and its interaction with FAS (S.M. Najjar, 2006). ACC catalyzes the pace-setting step of fatty acid synthesis, especially in the short term, while FAS and ACC both may govern long-term fatty acid synthesis through changes in gene expression. ACC, FAS, and enzymes indirectly involved in fatty acid synthesis — ATP-citrate lyase, malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase — are coordinately regulated in response to nutritional/hormonal stimuli via changes in the number of these protein molecules in the cell. The concentration of these enzymes is low in livers of starved animals and high in livers of fed animals, especially if the diet is high in carbohydrate. Induction of these enzymes during high carbohydrate feeding has been attributed to the high glucose level and insulin secretion that accompanies increased blood glucose level. It is well documented that in untreated diabetes, fatty acid synthesis is impaired and that administration of insulin restores normal rates of FA synthesis. Furthermore, lipogenesis is higher in rodent obesity models with elevated circulating glucose and insulin levels. In addition, at the onset of feeding, insulin release might stimulate the conversion of thyroxine to the more potent T3 and also increase thyroid hormone receptor expression. On the other hand, during fasting the circulating glucagon level increases as lipogenesis is suppressed. In cultured cells, insulin, glucose, and T3 increase, whereas cAMP decreases the amounts of the enzymes involved in fatty acid synthesis. Fat, particularly polyunsaturated fatty acids, in the diet decreases the amounts of lipogenic enzymes although its mode of action has been elusive. Overall, the interplay of hormones and dietary components including insulin, glucose, cAMP as well as polyunsaturated fatty acids achieves an optimal degree of de novo lipogenesis in response to changing metabolic needs. Regulation is at the level of protein concentration, and both transcriptional and posttranscriptional mechanisms are involved [26]. For example, a recent report suggests

that in the fasted state the degradation of ACC protein is accelerated by the interaction with the homolog of *Drosophila* tribbles3 which acts as an adaptor protein for ACC and triggers ubiquitination (M. Montminy, 2006). Recent research has focused on elucidating the transcriptional regulation of lipogenic genes. Transcriptional regulation of ACC, FAS, and other lipogenic enzymes is coordinated and the genes share common transcription factors and regulatory mechanisms.

6.2. ACC and FAS

ACC1 and ACC2 are encoded by two separate genes that are transcribed from multiple promoters. For the ACC1 gene, at least three promoters —PI, PII, and PIII — have been identified. PI and PII of the ACC1 gene generate the same protein products, whereas PIII is used in humans to produce a N-terminal variant of ACC1 in some tissues such as lung, liver, and mammary gland, but not in muscle or adipose tissue. The N-terminal 75 amino acid residues are replaced by 17 amino acid residues in this variant, resulting in modification of an AMPK phosphorylation site, potentially altering the kinetics of phosphorylation of ACC1. At least 2 promoters for ACC2 are used in various tissues including muscle. Because multiple ACC genes/promoters from various mammalian and avian species have been studied, it is difficult to provide simple insights into the transcriptional regulation of ACC.

FAS is coded by a single copy gene transcribed from a single promoter in most species. Human FAS, however, is transcribed from two promoters. The promoter PI contains TATA and CAAT boxes, whereas the promoter PII, which contains neither TATA nor CAAT boxes, initiates transcription from two sites, one in intron 1 and the other 49 bp upstream of the start codon in exon 2. Most studies have focused on the PI promoter, which has stronger activity and appears to be utilized preferentially when demand for elevated lipogenesis is high. PII appears to play a role in the constitutive expression of FAS at a low level. The rat FAS promoter, which exhibits high identity with the human PI, contains a TATA box, Sp1 sites, and an inverted CCAAT box where a heterotrimeric transcription factor NF-Y binds. NF-Y binding to the inverted CCAAT box appears to be involved in the cAMP-mediated downregulation of FAS transcription during fasting (S. Smith, 1999). Studies from several laboratories employing cultured cells revealed binding sites for Sp1, NF-Y, upstream stimulatory factor (USF), and sterol regulatory element binding protein (SREBP) at the proximal promoter region of the FAS gene and potential cooperative binding and function of these transcription factors in regulating the FAS promoter activity [27,28] (Fig. 11).

6.3. Upstream stimulatory factor

USF belongs to the family of class C bHLH (basic helix-loop-helix) transcription factors that recognize the 5′-CANNTG-3′ E-box core sequence. USF, which contains an additional leucine zipper of protein–protein interaction domain, was originally identified by its ability to bind to the adenovirus major late promoter to stimulate transcription in vitro. Two polypeptides of 43 and 44 kDa, termed USF1 and USF2, are encoded by separate genes, while alternative splicing gives rise to two USF2 isoforms. USF interacts to form

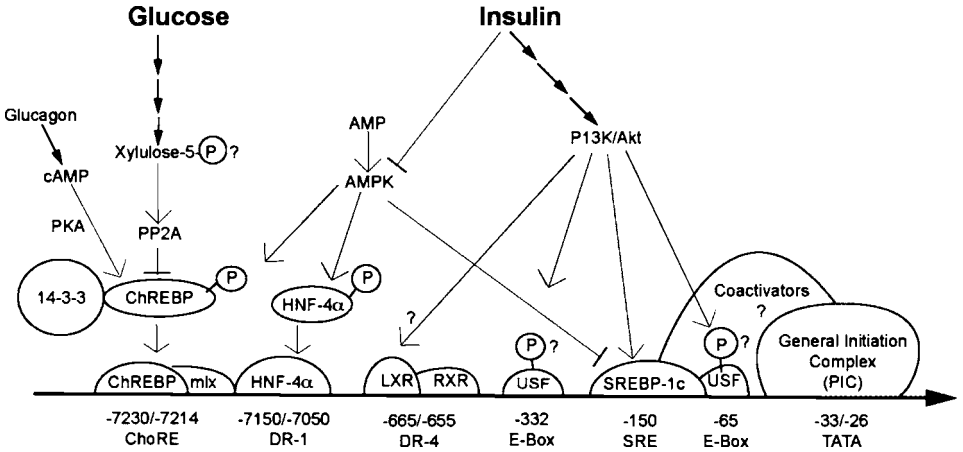

Fig. 11. Transcriptional regulation of a model lipogenic enzyme, FAS. Both glucose and insulin activate the FAS promoter upon feeding of a high-carbohydrate diet. Insulin, through PI3K/Akt pathway, increases the level of SREBP-1c and SREBP-1c binds to the –150 sterol regulatory element (SRE). USF binds to the –65 E-box and –332 E-box but USF binding does not change upon insulin treatment. USF may be phosphorylated upon insulin treatment. SREBP-1c and USF interact directly and may recruit coactivators to activate FAS promoter. LXR is largely responsible for enhanced expression of SREBP-1c in the fed state. LXR forms heterodimers with RXR and increases FAS promoter activity by binding to the –665 direct repeat-4 (DR-4). AMPK may phosphorylate transcription factors that bind to the FAS promoter, including USF, SREBP-1c, hepatocyte nuclear factor-4α (HNF-4α) and ChREBP. AMPK phosphorylates HNF-4α to prevent binding to the FAS promoter at –7150 direct repeat-1 (DR-1). Both PKA and AMPK phosphorylate ChREBP, which then interacts with 14-3-3 to be retained in the cytosol. Upon feeding, glucose metabolism and xylulose 5-phosphate (or possibly other metabolite) levels increase to activate protein PP2A, which catalyzes the dephosphorylation of ChREBP. ChREBP then dissociates from 14-3-3, translocates to the nucleus, heterodimerzes with Mlx, and binds to the –7230 carbohydrate response element (ChoRE) to activate FAS promoter. The ChRBP/Mlx complex appears to interact with HNF-4α that binds to the nearby DR-1 at –7150. Sites present but not indicated in this diagram include multiple Sp1 sites at the proximal region as well as an inverted CCAAT box (–98/–92) where NF-Y binds and mediates cAMP suppression of the FAS promoter activity. Other abbreviations are as cited in the text.

homo- or hetero-dimers, and also interacts with several bHLH leucine zipper and other transcription factors. Although USF is expressed ubiquitously, it is implicated in the expression of some tissue-specific and developmentally regulated genes. USF is a phosphoprotein but functional significance of the phosphorylation has not been extensively studied. In 3T3-L1 adipocytes, the activation of the FAS promoter by insulin requires the –65 E-box where USF binds (H.S. Sul, 1994). By overexpression of wild-type and dominant negative forms of USF, the function of USF in insulin-mediated activation of the FAS promoter has been elucidated. However, studies of transgenic mice expressing the chloramphenicol AT reporter, driven by the FAS promoter containing various 5′-deletions, showed that the region of –131/–278 containing a SREBP binding site at –150 was required for mediation of the feeding and insulin responsiveness, although the degree of induction did not approach the physiological level. Maximal activation of FAS transcription requires a region between –278 and –444 that includes an E-box at –332 where USF binds (H.S. Sul, 2000). Chromatin immunoprecipitation in mice transgenic for various

5′-deletions, as well as for mutations, of the FAS promoter clearly showed in vivo binding of USF at these two E-boxes of the FAS promoter [29]. The requirement for USF was confirmed by observations that USF1 or USF2 knockout mice exhibit severely impaired induction of FAS and other lipogenic enzymes when fed a high carbohydrate diet. However, the nuclear levels of USF and USF binding to the E-boxes do not change upon fasting/refeeding. Overall, USF binding to the –65 E-box is required for activation of FAS transcription by feeding/insulin. Nevertheless, full transcriptional activation also requires the binding of SREBP-1c to the –150 sterol regulatory element (Section 6.4), as well as additional binding of USF to an upstream –332 E-box (H.S. Sul, 2000). Similar to the lipogenic enzymes, SREBP-1c itself is induced by feeding/insulin but as the USF level and binding do not change, USF might undergo changes in phosphorylation–dephosphorylation during feeding/insulin to mediate transcription activation of lipogenic enzymes. Given the available functional data and the presence of closely spaced E-boxes and sterol regulatory elements at the promoter regions of several lipogenic genes including FAS and ACC, a general cooperative role for USF and SREBP in coordinate induction by feeding/insulin is likely. Indeed, USF and SREBP directly interact to activate the FAS promoter in a synergistic manner (Section 6.4). Similarly, studies on thyroid hormone regulation of the PII promoter of the ACC1 gene in avian hepatocytes indicate that SREBP-1 directly interacts with thyroid hormone receptor to enhance ACC transcription. In this case, a thyroid response element composed of direct repeat of 5′-AGGTCA-3′ spaced by four nucleotides at –101 to –86 appears to confer transcriptional activation by T3. The human FAS promoter I also contains a thyroid response element that confers T3 responsiveness. T3 increases binding of the thyroid hormone receptor/retinoid X receptor heterodimer to the thyroid response element of the PII promoter of ACC1 gene in avian hepatocytes. T3 also increases the binding of SREBP-1 to the –80 to –71 sterol regulatory element located immediately downstream of the thyroid response element, resulting in thyroid hormone receptor/SREBP-1 interaction (F.B. Hillgartner, 2002).

6.4. Sterol regulatory element binding protein

SREBP, which belongs to the family of class C bHLH transcription factors, binds to the sterol regulatory element and activates the genes involved in the synthesis and uptake of lipids (Chapter 14). There are three isoforms of SREBP, 1a, 1c, and 2. SREBP-1a and 1c are produced from different promoters by alternative splicing of the first exon into a common second exon, whereas SREBP-2 is encoded by a separate gene. SREBPs are synthesized as transmembrane protein precursors that are embedded in the endoplasmic reticulum membrane [30]. Nuclear entry of SREBP requires proteolytic cleavage of the cytosolic N-terminal domain, which constitutes a bHLH-zip transcription factor. SREBP binds the sterol regulatory element (and related sequences) or the E-box element in vitro although the binding affinity may differ for different isoforms. This dual binding specificity of SREBP is attributed to the unique tyrosine residue at the basic DNA-binding domain of SREBP that replaces the arginine residue of other E-box-binding bHLH transcription factors. The critical role of SREBP in inducing the transcription of FAS and other lipogenic enzymes in response to fasting/refeeding was demonstrated by the absence of this induction in SREBP-1 knockout mice (H. Shimano, 1999). Furthermore,

studies on transgenic mice overexpressing SREBP isoforms suggest that SREBP-1a and 2 play important roles in cholesterol metabolism, whereas SREBP-1c serves primarily as a transcriptional activator of genes involved in fatty acid biosynthesis (J.L. Goldstein, 1997). Adenovirus-mediated overexpression of SREBP-1c mimics the effect of insulin on the expression of FAS in primary hepatocytes and diabetic mouse liver. Mice deficient in SREBP-1c exhibit diminished expression of ACC, FAS, and other lipogenic enzymes resulting in lower hepatic fatty acid synthesis. SREBP-2 is expressed ubiquitously and SREBP-1a is predominant in most cultured cells. In contrast, SREBP-1c is the major form in lipogenic tissues, such as liver and adipose tissue. Expression of SREBP-1c, similar to the lipogenic enzymes, is increased by feeding/insulin and decreased by polyunsaturated fatty acids. SREBP-1c transcription is under the control of SREBP itself, forming an autoloop. SREBP-1c transcription, at least in part, is controlled by LXR also (Section 6.5).

Regulation of the FAS promoter activity in cultured cells has been proposed to occur by binding of SREBP to the overlapping SRE or E-box that are present at the –65 region of the promoter, although USF binding to this E-box is responsible for insulin regulation in 3T3-L1 adipocytes. However, in vivo studies with transgenic mice harboring various 5′-deletions and mutations of the FAS promoter region, as well as chromatin immunoprecipitation assays, indicated that SREBP binds to the –150 SRE of the FAS promoter. SREBP binding to the –150 SRE is induced by feeding and by insulin. In order for SREBP to activate the FAS promoter during cellular sterol depletion, Sp1 and NF-Y appear to be critical [31]. Thus, SREBPs may generally require auxiliary factors for maximal transactivation. Futhermore, SREBP binding alone is not sufficient because binding of USF to the –65 E-box and to the –332 E-box is required for maximal activation of the FAS promoter during feeding/insulin. The discrepancies between studies in vitro in cultured cells and in vivo in transgenic mice underscore the importance of an in vivo approach in addressing transcriptional regulation of FAS as well as other genes (R.D. Palmiter, 1987; S.R. Ross, 1990). The strong activation of the FAS promoter by USF and SREBP-1c in cells lacking Sp1 and NF-Y suggests that USF, rather than Sp1 or NF-Y, may be the principal co-regulator of SREBP-1c in the transcriptional activation responsible for increased lipogenesis in response to feeding/insulin. In this respect, when USF binding to the –65 E-box was prevented by mutation, SREBP-1 did not bind the –150 SRE, suggesting a functional interaction between these two transcription factors. It has recently been shown that USF and SREBP-1 directly interact in a process that requires bHLH domains of both proteins (H.S. Sul, 2007). Furthermore, USF and SREBP synergistically activate the FAS promoter as a result of their interaction. Thus, upon their direct interaction, USF and SREBP might recruit a mutual coactivator complex to FAS and other lipogenic genes, which may play a critical role in the coordinate induction during feeding/insulin.

6.5. Liver X receptor

The LXR belongs to a subclass of nuclear hormone receptors that form obligate heterodimers with RXR and is implicated in the regulation of lipogenic enzymes. LXR, as a sensor of cellular cholesterol, is activated by oxysterols, and antagonized by small lipophilic agents including some unsaturated fatty acids. There are two isoforms of LXR — LXRα and LXRβ, LXRα is expressed abundantly in lipogenic tissues such as

liver and adipose tissue whereas LXR is abundant in the brain. In LXR knockout mice, lipogenic enzyme induction is insensitive to insulin, a finding that demonstrates a requirement for LXR in this process. In SREBP-1c knockout mice, induction of lipogenic enzymes during fasting/feeding is markedly blunted by administration of LXR agonists, indicating an essential role for SREBP-1c in the LXR response. Two LXR binding sites are present in the SREBP-1c promoter and disruption of the LXR binding sites abolishes the response to LXR agonists. Results of experiments with cultured cells indicate that LXR also regulates FAS expression directly by interacting with an upstream LXR site of direct repeat-4 at –669 to –655, as well as indirectly by inducing SREBP-1c expression (P. Tontonoz, 2002). Thus, FAS and ACC may be direct, as well as indirect, targets (via induction of SREBP-1c) of LXR. Studies of knockout mice indicate that of the LXRα and LXRβ isoforms, LXRα plays a more prominent role in the induction of lipogenic enzymes. However, the LXR expression pattern showed conflicting results: in rodents, the expression of neither LXRα nor LXRβ changes during fasting/feeding when SREBP-1c as well as ACC and FAS are markedly regulated. In contrast, in primary hepatocytes, LXRα is strongly upregulated by insulin. Furthermore, LXRα is induced robustly by fatty acids, with unsaturated fatty acids being more effective than saturated fatty acids. Thus, although LXR is an important regulator of SREBP-1c expression, it is unclear whether or not changes in LXR expression cause SREBP-1c induction in the fed state. In this respect, LXRα is a target gene for the peroxisome proliferator activated receptor (PPAR)α in liver and PPARγ in adipocytes, an indication of cross regulation between nuclear receptors that have lipid ligands. The interplay between nuclear receptors during insulin/glucose/fatty acid regulation of lipogenic enzymes needs further study.

6.6. Carbohydrate response element binding protein

In addition to insulin, glucose itself provides a signal for glycolytic and lipogenic gene induction during feeding. In primary hepatocytes, addition of glucose at a constant level of insulin increases FAS gene expression in a process that requires glucose metabolism (J. Girard, 1995). Although the distinct mechanisms underlying insulin and glucose regulation are not clear, both insulin and glucose are required for full activation of lipogenic genes. As described in Section 6.5, SREBP-1c, which is induced by LXR and by SREBP itself, is induced by insulin. The carbohydrate responsive element binding protein (ChREBP) is likely a pivotal transcription factor in carbohydrate/glucose regulation [32]. SREBP-1c and ChREBP might function synergistically to induce lipogenic genes in the fed state. Although ChREBP functions primarily in hepatocytes, it is also found, albeit at a lower level, in other tissues such as adipose tissue, where its importance is not understood. ChREBP belongs to the bHLH-zip family of transcription factors. It interacts with Max-like protein X (Mlx) to form a heterotetramer that binds to the L-pyruvate kinase promoter at an element consisting of two E-boxes separated by 5 bp. Mlx is an essential component of the ChREBP/Mlx complex because of its ability to enhance DNA binding. This complex is relatively stable and abundant and thus may not confer regulatory properties in nutrient or hormone signaling. ChREBP, on the other hand, is a labile protein that undergoes phosphorylation and is the metabolic sensor of the ChREBP/Mlx complex. A carbohydrate response element at –144 to –128 of the PI promoter of the rat ACC1 gene confers strong glucose-mediated activation in primary hepatocytes. A carbohydrate

response element far upstream of the FAS promoter at –7230 to –7214 has also been identified (S.D. Clarke, 2001). A recent report suggests that hepatocyte nuclear factor-4α binds downstream to the carbohydrate response element at –7150 to –7050 and directly interacts with ChREBP to form a complex. The proposed mechanism for the action of the ChREBP is that at low glucose concentrations, when cAMP and AMP levels are elevated in liver, ChREBP is phosphorylated by PKA and AMPK and is localized in the cytosol by its association with the protein 14-3-3; this protein serves as an adaptor for various signaling molecules and phosphoproteins including transcription factors. ChREBP undergoes multisite phosphorylation by PKA as well as a distinct site phosphorylation by AMPK. Exposure of cells to glucose elevates glucose metabolism and it is proposed that a pentose phosphate pathway intermediate, xylulose-5-phosphate, activates protein phosphatase 2A that dephosphorylates ChREBP. Dephosphorylated ChREBP is released from 14-3-3 and undergoes nuclear translocation, thereby allowing the binding of ChREBP/Mlx to the promoter regions of various glycolytic and lipogenic genes including ACC and FAS. The importance of ChREBP in glucose-mediated transcription has been shown by deletion and over-expression studies in cultured cells. ChREBP-deficient mice have larger, glycogen-laden livers and smaller adipose depots with decreased expression of lipogenic enzymes and reduced fatty acid synthesis. The exact nature of the signaling molecule(s) generated from glucose metabolism, and the regulation of ChREBP by phosphorylation–dephosphorylation, need further investigation in a physiological context.

6.7. Suppression of lipogenic gene expression by polyunsaturated fatty acids

While de novo lipogenesis is almost completely repressed in fasting, feeding a high-carbohydrate diet to rodents increases lipogenesis dramatically. A high-fat diet, on the other hand, suppresses de novo lipogenesis. Although the suppressive effect of fat, especially by polyunsaturated fatty acids, has been known for long time, understanding the mechanisms underlying polyunsaturated fatty acid suppression of lipogenic gene transcription has been difficult [33,34]. During the last decade, many transcription factors have been implicated as potential mediators of polyunsaturated fatty acid action. Those factors include PPARα, β, and γ; SREBP-1c; hepatocyte nuclear factor-4α; LXRα; and ChREBP. PPARα SREBP-1c and ChREBP have been established in vivo as regulators of hepatic genes involved in fatty acid metabolism. Polyunsaturated fatty acids activate PPARα by direct binding, leading to induction of hepatic fatty acid oxidation, but PPARα may not control fatty acid synthesis. Polyunsaturated fatty acids inhibit hepatic fatty acid synthesis by suppressing nuclear levels of mature SREBP-1c through several mechanisms. Polyunsaturated fatty acids not only suppress proteolytic processing and generation of mature SREBP-1c but also suppress SREBP transcription, enhance mRNA decay, and increase SREBP-1c degradation by the proteasome. Furthermore, certain unsaturated fatty acids are competitive antagonists for LXR, thereby suppressing SREBP expression. Polyunsaturated fatty acids might also suppress LXR expression by acting as, or by producing, ligands for PPAR. Consistent with this idea, liver-specific FAS knockout mice showed a defect in PPARα-mediated signaling, suggesting that newly synthesized fatty acids provide PPARα ligands in the liver (C.F. Semenkovich, 2005). Thus, polyunsaturated fatty acids appear to require multiple mechanisms for full suppression of the FAS promoter activity (H.S. Sul, 2002).

6.8. Signaling pathways and molecular mechanisms for transcriptional regulation of lipogenic enzymes

Lipogenesis, which occurs predominantly in the liver and adipose tissue, is not detectable in fasted rodents, but feeding a high-carbohydrate fat-free diet dramatically induces lipogenic genes (Fig. 11). During feeding, insulin secretion rises accompanying the increased blood glucose level. The role of insulin in lipogenesis is demonstrated by the ability of insulin to restore lipogenesis in streptozotocin-treated diabetic animals. It is well established that insulin binding to its receptor causes tyrosine phosphorylation of the IRSs which serve as docking sites for SH2-containing molecules. Most of the metabolic effects of insulin, including glucose transport and lipogenesis, occur through recruitment of the p85 regulatory subunit of PI3K. Increased SREBP-1c levels mediate, at least in part, insulin induction of transcription of FAS and ACC. Increased FAS promoter activity by insulin in 3T3-L1 adipocytes occurs via the PI3K and PKB/Akt pathway, but not via the MAP kinase/ERK pathway (H.S. Sul, 1998). Recently, AMPK has emerged as a key player in the glucose signal for transcription. As discussed in Section 5.4, AMPK is activated by cellular ATP depletion and consequently elevation of AMP. Accordingly, AMPK activation inhibits ATP-consuming processes including fatty acid synthesis. However, whether AMPK action is completely independent of insulin action on lipogenic enzymes is not clear.

Multiple mechanisms could account for the action of SREBP, USF, and other transcription factors including LXRα in regulation of FAS promoter activity. The change in the level of these transcription factors may be responsible for lipogenic induction by activated signaling pathways. In this regard, USF level is not altered, whereas expression of SREBP-1c is induced by feeding/insulin. Induction of SREBP-1c by insulin occurs via the PI3K pathway. Acute activation of PI3K/Akt is sufficient for induction of SREBP-1 mRNA accumulation in primary hepatocytes. This observation, combined with the observed activation of the FAS promoter via the PI3K/Akt pathway, support the notion that activation of PI3K/Akt leads to induction of SREBP-1c and subsequent activation of the FAS promoter. In addition, LXRα itself can be upregulated by insulin (Section 6.5). AMPK activation by metformin which is LKB1 dependent, on the other hand, decreases expression of SREBP-1c.

Insulin increases SREBP-1c expression and the amount of the mature form of SREBP-1c in nucleus. While overexpression of SREBP-1c increases FAS promoter activity, an increase in SREBP-1c in the nucleus is not a prerequisite for the rapid insulin effect on transcription of genes, suggesting additional modes of action of insulin, including activation of nuclear SREBP-1c or other partner(s). Cooperative interactions between transcription factors and/or with coactivators might be critical for transcriptional regulation. For example, SREBP-1c directly interacts with USF and the thyroid hormone receptor. Covalent modification, such as phosphorylation and acetylation, of these transcription factors might affect their activities and/or association with other factors. In support of this idea, treatment of cells with protein phosphatase inhibitors prevents the glucose effect on lipogenic gene expression. ChREBP is phosphorylated by PKA and AMPK, causing loss of DNA binding and activation. AMPK also phosphorylates HNF-4α thereby reducing homodimerization and DNA binding. USF can be phosphorylated by various kinases.

USF phosphorylation by PI3K increases, whereas phosphorylation by p70 S6K decreases DNA-binding capacity. On the other hand, phosphorylation of USF by PKA increases binding of USF to E-box while dephosphorylation by PP1 decreases binding. However, USF binding to the FAS promoter does not change in fasted and fed states, suggesting that USF phosphorylation activates the FAS promoter via a mechanism other than DNA binding. SREBP-1a is also a phosphoprotein. Phosphorylation of SREBP-1a by ERK1/2 in vitro does not change DNA binding but increases transcriptional activation. SREBP-1a and SREBP-1c are phosphorylated at a distinct site by PKA, resulting in lowered capacity to bind to sterol-regulatory elements. However, since SREBP-1c expression is suppressed in fasting, the physiological significance of PKA phosphorylation of SREBP-1c in the fasted state is questionable.

SREBP-1a interacts with CBP/p300 and is acetylated. CBP/p300 has histone AT activity and recruits P/CAF with distinct histone AT activity. Acetylation might regulate the stability of SREBP-1a. Furthermore, interaction of SREBP with CBP/p300 links SREBP binding to histone acetylation and altered chromatin conformation. CBP/p300 is a substrate for AMPK, at least in vitro, and phosphorylation of CBP/p300 by AMPK dramatically reduces its interaction with nuclear receptors. Furthermore, SREBP-1a, through its activation domain, was reported to interact with the activator-recruited cofactor 105 (A.M. Naar, 2006). Activator-recruited cofactor/mediator complex, which serves as a key integrator of signaling, could recruit RNA polymerase II for activation of the pre-initiation complex. In the case of SREBP-1c, however, coactivator or mediator complex formation has remained elusive. For example, in contrast to SREBP-1a, interaction of SREBP-1c with CBP/p300 or activator-recruited cofactor 105 is, at best, a weak interaction.

7. Future directions

Studies completed in the last few years have brought many advances in our understanding of de novo fatty acid synthesis. In particular, the successful crystallization of the fungal and animal FASs has provided new insights into the operation of these extraordinary macromolecular machines. The baroque architecture of the rigid fungal FAS contrasts sharply with the flexible, parsimonius design of the animal FAS. Nevertheless, important questions regarding how communication between the catalytic elements of the animal FAS is achieved remain to be answered. Progress in elucidating the organization of the animal ACC also has been slow and, although we now have a sense of the structure of the catalytic domains, little is known about the architecture of the entire complex. Both ACC2 and FAS have been identified as potential targets for the development of novel anti-obesity agents and higher resolution structures for the proteins could facilitate this process. After decades of uncertainty, the existence of an independent fatty acid biosynthetic pathway in mitochondria is now established beyond doubt. However, the importance of the pathway to mitochondrial function in animals needs to be clarified. Is the pathway the major supplier of lipoyl precursors in mitochondria, or can exogenous octanoyl or lipoyl moieties be utilized for lipoylation of mitochondrial proteins under some circumstances?

Many questions about lipogenic gene transcription remain unanswered. The most important is: How do multiple transcription factors acting on FAS and ACC promoters work together to control transcription? More specifically: (i) What is the mode of action of transcription factors including USF and SREBP? What is the mechanism of covalent modification such as phosphorylation and acetylation? (ii) What is the nature of the transcription complexes formed? What coactivators or chromatin remodeling proteins are involved in FAS and ACC transcription? (iii) What are the signaling pathway(s) involved in insulin binding to its receptor? How does glucose metabolism lead to the concerted stimulation of lipogenic gene transcription? What are the effectors in polyunsaturated fatty acids suppression of lipogenic gene transcription? The molecular events leading to fine control of lipogenic gene transcription downstream of signaling remain to be further elucidated.

Abbreviations

ACC	acetyl-CoA carboxylase
ACP	acyl carrier protein
Akt/PKB	protein kinase B
AMPK	AMP-activated protein kinase
BHLH	basic helix-loop-helix leucine zipper
ChREBP	carbohydrate response element binding protein
CPT-1	cartinine palmitoyl transferase-1
DH	dehydrase
ER	enoyl reductase
FAS	fatty acid synthase
KR	β-ketoacyl reductase
KS	β-ketoacyl synthase
LXR	liver X receptor
Mlx	max-like protein X
P/CAF	P300/CBP-associated factor
PI3K	phosphatidylinositol-3-kinase
PKA	cAMP-dependent protein kinase
PPAR	peroxisome proliferator activated receptor
RXR	retinoid X receptor
SREBP	sterol response element binding protein
TE	thioesterase
USF	upstream stimulatory factor

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CHAPTER 7

Fatty acid desaturation and chain elongation in mammals

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Contents

1. Introduction

Fatty acids synthesized de novo as well as those derived from the diet are modified in the membranes of the endoplasmic reticulum (ER), mitochondria, and peroxisomes through metabolic pathways that include desaturation and elongation, producing a variety of long-chain saturated, monounsaturated, and polyunsaturated fatty acids (SFA, MUFA, and PUFA, respectively) consisting of 16 carbons or more. Esterified long-chain fatty acyls in triacylglycerols (TG) constitute the body's major and most efficient energy stores.

The phospholipid (PL) fatty acyl chain length and the number and position of double bonds can markedly influence fluidity, permeability, and stability of biological membranes. By influencing membrane physical properties, fatty acids can alter the function of integral membrane proteins including receptors and those proteins involved in ion channeling, endocytosis, and exocytosis. The release of specific fatty acids from membrane PL stores can also lead to the generation of precursors for certain signaling molecules such as eicosanoids, pheromones, growth regulators, and hormones. In addition to their effects on specific nuclear receptors and other proteins involved in the regulation of gene expression, long-chain fatty acids are also involved in the covalent modification of numerous proteins (Chapter 2) reported to be crucial for the formation and infectivity of certain viruses as well as other cellular functions.

The proliferation of analytical techniques, the greater availability of appropriate substrates and precursors labeled with radioisotopes and the advent of molecular and genetic approaches coupled to the use of knockout and transgenic mouse models are greatly contributing to our understanding of the regulation of fatty acid elongation and desaturation pathways. Our knowledge of the role of particular fatty acids in human health and disease continues to evolve. This chapter will focus on regulation of fatty acid chain elongation and desaturation in mammals.

1.1. Nomenclature and sources of long-chain fatty acids

Fatty acids are carboxylic acids containing variable lengths of hydrocarbon chains. The length can vary from 4 to 36 carbons or more depending on the mammalian species. Fatty acids can be further defined by the degree of unsaturation (double and triple bonds), and the number and position, as well as the stereochemistry (*cis* or *trans*) of the double bonds with respect to the carboxyl terminus. The systematic name of oleic acid is 9-octadecenoic acid and the condensed formula is 18:1Δ9c, which denotes an 18-carbon fatty acid with one double bond at position 9 in the *cis* configuration. This compact nomenclature is particularly useful for describing PUFA, which contain more than one double bond. However, since the physiological properties of PUFA largely depend on the position of the first unsaturation relative to the end position and not the carboxylate group, the position is signified by (ω minus a number). The terms ω – 3 and ω – 6 signify, therefore, that the first double bond exists as the third and sixth carbon–carbon bond, respectively, from the terminal CH_3 end (ω) of the carbon chain. The number of carbons and the number of double bonds are also listed. Thus, ω – 3 18:3 (α-linolenic acid) or 18:3 ω – 3 or more commonly 18:3n – 3 indicates an 18-carbon chain with three double bonds, with the first double bond in the third position from the CH_3 end. Double bonds are *cis* and separated by a single methylene (CH_2) group unless otherwise noted (Table 1).

Most mammalian cells have the capacity to synthesize fatty acids from glucose de novo in a pathway that uses products from glycolysis and two key cytosolic enzymes, acetyl-CoA carboxylase and fatty acid synthase (Chapter 6). This pathway generates long-chain SFA, mainly palmitate (16:0). The de novo synthesized palmitate and the palmitate derived from dietary sources are transported to the ER membranes. In the membranes, two major fatty acid enzymatic modifications of chain elongation and desaturation occur to yield longer chain SFA and unsaturated fatty acids of the n – 9 series. The n – 3 and n – 6 series of PUFA can be synthesized only from dietary fats, as animal cells do not have the

Table 1
Nomenclature and bond positions of major long-chain fatty acids

Common name	Abbreviation	Double bond positions
Palmitic acid	16:0	
Palmitoleic acid	16:1n – 7	Δ9
Spienoic acid	16:1n – 10	Δ6
Stearic acid	18:0	
Oleic acid	18:1n – 9	Δ9
Vaccenic acid	18:1n – 7	Δ11
Linoleic acid	18:2n – 6	Δ9,12
α-Linolenic acid	18:3n – 3	Δ9,12,15
γ-Linolenic acid	18:3n – 6	Δ6,9,12
Stearidonic acid	18:4n – 3	Δ6,9,12,15
Arachidic acid	20:0	
Paullinic acid	20:1n – 7	Δ13
Gondoic acid	20:1n – 9	Δ11
Dihomo-γ-linolenic acid	20:3n – 6	Δ8,11,14
Mead acid	20:3n – 9	Δ5,8,11
Arachidonic acid	20:4n – 6	Δ5,8,11,14
Eicosapentaenoic acid	20:5n – 3	Δ5,8,11,14,17
Behenic acid	22:0	
Erucic acid	22:1n – 9	Δ13
Adrenic acid	22:4n – 6	Δ7,10,13,16
n – 6 Docosapentaenoic acid	22:5n – 6	Δ4,7,10,13,16
n – 3 Docosapentaenoic acid	22:5n – 3	Δ7,10,13,16,19
Docosahexaenoic acid	22:6n – 3	Δ4,7,10,13,16,19
Lignoceric acid	24:0	
Nervonic acid	24:1n – 9	Δ15

All bonds are of a *cis* geometric configuration.

ability to introduce double bonds between the C-10 and the methyl terminal end. Because they are necessary precursors for the synthesis of other products, linoleate, and α-linolenate are essential fatty acids for mammals and must be obtained from dietary sources.

The types of fatty acids obtained through dietary intake and de novo synthesis are insufficient to meet the varied demands of cells, so there is substantial metabolism and rearrangement in the structures of the fatty acids as development, growth, and aging proceed. Knowledge of how the array of fatty acyl chains is derived and modified, and what regulates the metabolism of fatty acyl chains by elongation and desaturation are described in the sections that follow.

2. Elongation reactions of long-chain fatty acids

2.1. Microsomal fatty acid elongation

The predominant pathway for fatty acid elongation occurs in the ER and uses malonyl-CoA and fatty acyl-CoAs as substrates for addition of two carbon atoms to the existing

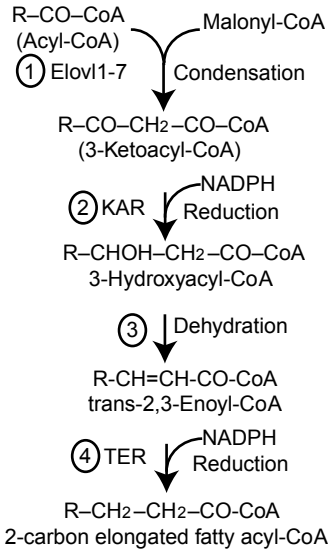

Fig. 1. Reactions in the two-carbon chain elongation of long-chain fatty acids in the ER. Elovl, elongation of long-chain fatty acids; KAR, 3-ketoacyl-CoA reductase; TER, *trans*-2,3-enoyl-CoA reductase.

fatty acid substrates. First, the fatty acids are activated to the co-enzyme A (CoA) form by fatty acyl-CoA synthetases, six of which (Acs1–6) have been identified with specificities for chain length and degree of unsaturation. The precise subcellular locations of the fatty acyl-CoA synthetases and the links to the synthesis of particular lipids have been recently described. Unlike cytosolic fatty acid synthesis that uses the fatty acid synthase complex of enzymes that reside on the same polypeptide chain (Chapter 6), separate enzymes catalyze the four well-known sequential reactions of fatty acid elongation. Microsomal chain elongation is active with both SFA and PUFA with γ-linolenate (18:3n – 6) being the most active substrate. The four consecutive and independent reactions are (i) condensation of an acyl-CoA molecule and the three-carbon malonyl-CoA substrate to form ketoacyl-CoA, (ii) a reduction by the 3-keto acyl-CoA reductase using NADPH to form 3-hydroxyacyl-CoA, (iii) a dehydration of 3-hydroxyacyl-CoA to generate *trans*-2, 3-enoyl-CoA, and (iv) a reduction of enoyl-CoA to generate a two-carbon-extended acyl-CoA product (Fig. 1).

2.1.1. Ketoacyl-CoA synthase

Ketoacyl-CoA synthases perform the condensing step, which is the initial and rate-limiting step that determines fatty acyl specificity and results in the addition of the two-carbon moiety. These enzymes have been identified in both mice and humans. They share common evolutionarily conserved motifs in a central stretch of the amino acid sequence, although the overall sequence identity is not very high. None of the identified proteins has been crystallized or structurally determined, but structure predictions based on hydropathy plots indicate that they are polytropic proteins with five to seven transmembrane

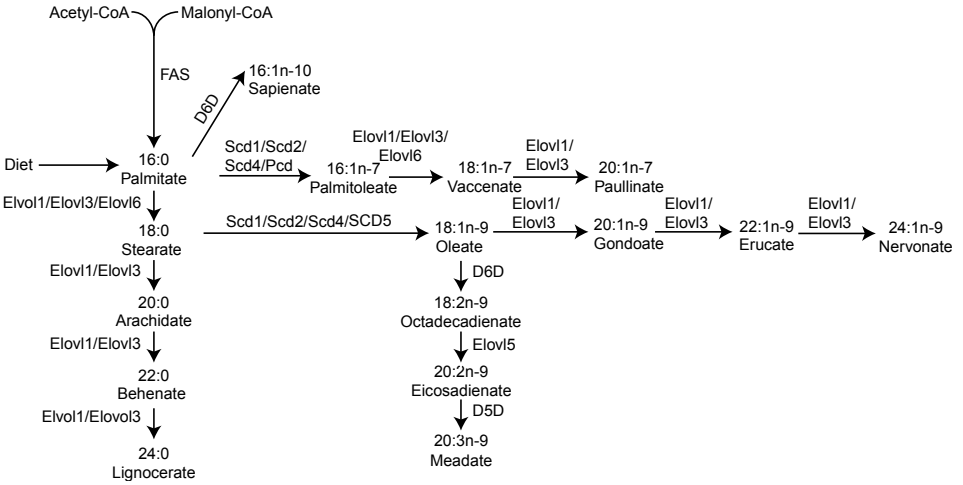

Fig. 2. Synthesis of saturated and n – 9 series of MUFA and PUFA by desaturation and elongation from dietary and de novo synthesized palmitate. FAS, fatty acid synthase.

spanning regions [1–5]. The membrane-bound elongation activities are oriented towards the cytoplasmic surface of the ER membrane. Seven distinct fatty acid elongase subtypes (Elovl-1–7) are present in the mouse, rat, and human genomes. The Elovls are divided into three major classes: (i) isoforms that elongate SFA and MUFA (Elvol-1, -3, and -6); (ii) isoforms that are involved in PUFA synthesis (Elvol-2 and -4), and (iii) Elovl-5 isoform that uses a broader array of substrates (16–22 carbons) (Figs. 1 and 2).

Elovl-3 (originally cloned as *Cig30*) is a glycoprotein and was the first elongase identified in mammals. Its expression is highly induced in brown adipose tissue upon cold exposure, by administration of norepinephrine and by high calorie intake. The physiological role of Elovl-3 is the most extensively studied since mice with targeted disruption of *Elovl-3* were generated (R. Westerberg, 2004, 2006). This enzyme is functionally equivalent to yeast Elo2p and is able to elongate SFA and MUFA containing up to 24 carbons. Consistent with complementation studies using yeast mutants, the *Elovl-3*-deficient mice show decreased activity in the elongation of C16–C22 saturated acyl-CoAs after short-term (3 days) cold exposure. Despite the tremendous induction of Elovl-3 by cold exposure, its deficiency in mice does not show any obvious phenotype in the cold. While under thermo-neutral conditions (30°C), there is depletion of fat in the brown adipose tissue of *Elovl-3*-deficient mice suggesting that Elovl-3 plays an important role in TG metabolism [6]. The most prominent dysfunction in *Elovl-3*-deficient mice is evident in the skin. Elovl-3 is expressed in the sebocytes of the sebaceous glands, which produce the lubricant sebum; consequently, *Elovl-3*-deficient mice have a sparse hair coat with hyperplastic sebaceous glands. In addition, defective water repulsion and structural abnormalities in the skin barrier are present in the *Elovl-3*-deficient mice, indicating the important role of long-chain fatty acids in skin and hair function as well as skin barrier formation. In the neutral lipids of hair, which are generally reflected by the lipid content

of sebum, *Elovl-3*-deficient mice abnormally accumulate gondoic acid (20:1n – 9), suggesting that gondonyl-CoA is a preferred substrate for Elovl-3 [7]. The detailed substrate specificity of Elovl-3 and whether or not the accumulation of gondoate (20:1n – 9) is the cause of dysfunction of skin and hair await further study.

Elovl-1 (previously named *Ssc1*) is the mammalian ortholog of yeast Elo3p that is involved in the biosynthesis of very-long-chain SFA. The overlapping functions of the well-characterized yeast Elo3p and mammalian Elovl-1 suggests that an alteration in acyl chain length of membrane PL and especially sphingolipids in mammals is crucial in membrane-associated function including membrane fusion/budding and several membrane-bound enzyme activities. *Elovl-1* is ubiquitously expressed in mammalian tissues with very high levels of expression found in the myelin of the central nervous system. Significant reductions of Elovl-1 activity and mRNA occur in the brain of Quaking and Jumpy mice, two models of myelination deficiency, suggesting that very-long-chain SFA and sphingolipid formation are essential in myelination.

Elovl-2 (formerly denoted as *Ssc2*) is involved in the biosynthesis of the n – 3 and n – 6 series of highly unsaturated fatty acids (HUFA). Expression studies in yeast demonstrated that Elovl-2 is able to efficiently elongate 20- and 22-carbon PUFA but is unable to elongate 18-carbon SFA, MUFA, and PUFA. *Elovl-2* is predominantly expressed in the testis, a tissue in which HUFA are synthesized in high levels (A. Leonard, 2002).

Mutations in *Elovl-4* were identified as being responsible for Stargardt's and autosomal dominant macular dystrophy (K. Zhang, 2001). Elovl-4 is a glycosylated protein that forms multi-subunit complexes and is highly expressed in rod and cone photoreceptor cells in the retina, a tissue with high content of docosahexaenoic acid (DHA) (22:6n – 3). Therefore, Elovl-4 is believed to be involved in the biosynthesis of PUFA and that is perhaps why plasma PUFA levels are often lower in patients with photoreceptor degeneration. Mice that over-express mutant human Elovl-4 synthesize a truncated peptide of the enzyme, which also causes photoreceptor degradation raising the possibility that either the lack of Elovl-4 function (PUFA synthesis) or the accumulation of the truncated form of the protein causes macular dystrophy (G. Karan, 2005).

Elovl-5 (formerly designated HELO1) was identified based on sequence homology with yeast Elo2p and indeed its function overlaps with that of Elovl-2. Elovl-5 is capable of elongating 18–22 carbons of PUFA but not those with more than 22 carbons. Although Elovl-5 is found in most human tissues, the highest expression of *Elovl-5* is in the testis and adrenal gland where significant amounts of adrenic acid (22:4n – 6) and docosapentaenoic acid (22:5n – 3) are found. *Elovl-5* is regulated during neonatal development by dietary fat and by the peroxisome proliferator activated receptor alpha (PPAR-α) agonist WY14643, suggesting that this enzyme may be important for the maintenance of levels of cellular long-chain fatty acids.

Elovl-6 (formerly designated *LCE*, Chapter 15) was identified as a sterol regulatory element binding protein (SREBP) target gene by DNA microarray analysis. This enzyme is highly expressed in lipogenic tissues including liver and adipose tissue. It catalyzes the elongation of saturated and monounsaturated acyl-CoAs with 12–16 carbons. The regulation of its expression is similar to that of lipogenic genes such as fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase-1 (*SCD1*), which are known SREBP-1-target genes. Thus, *Elovl-6* is suppressed in liver of fasted mice and is

extensively induced in the livers of fasted mice when they are given a high-carbohydrate, low-fat diet (T. Matsuzaka, 2002).

2.1.2. Ketoacyl-CoA reductase

Ketoacyl-CoA reductase (KAR) utilizes NADPH (in preference to NADH) to form β-hydroxy acyl-CoA. The flow of reducing equivalents from NADPH or NADH to the β-keto reductase appears to involve two other ER membrane proteins, cytochrome b_5 and cytochrome p450 reductase. Unlike Elovl family of enzymes, only a single KAR has been identified in mammals by sequence homology with yeast KAR, YBR159p [8]. The cDNAs encode proteins that are predicted to contain 312 amino acids with a conserved dilysine ER retention motif at the C-terminus and four putative transmembrane domains. The enzyme is present in all human and mouse tissues with the highest expression in tissues that are directly involved in fatty acid metabolism. Unlike other genes involved in fatty acid metabolism, *KAR* is not regulated by SREBPs.

2.1.3. β-Hydroxyacyl-CoA dehydrase

β-Hydroxyacyl-CoA dehydrase catalyzes the third reaction in chain elongation and involves dehydration of 3-hydroxyacyl-CoA to enoyl-CoA (Fig. 1). The gene that encodes this enzyme has not been cloned although the enzymatic activity has been described in microsomal preparations. In contrast to the membrane-bound elongation activities that have a cytosolic orientation, the β-hydroxyacyl-CoA dehydrase activity is embedded within the ER membrane (J. Bernert, 1979).

2.1.4. trans-2,3-Enoyl-CoA reductase

At the same time as KAR was cloned, Moon and Horton identified a *trans*-2,3-enoyl-CoA reductase using a homology search with the yeast enzyme TSC13 [8]. The *trans*-2, 3-enoyl-CoA reductase catalyzes the final step of the chain elongation pathway and requires NADPH as electron donor. The reductase protein is presumed to be 308 amino acids in length with five transmembrane domains. The tissue distribution of *trans*-2,3-enoyl-CoA reductase mirrors that of KAR. The substrate specificity has not been described.

2.2. Mitochondrial fatty acid elongation

Although less active than the microsomal system, mitochondrial chain elongation has been extensively investigated, particularly in liver and brain. The two-carbon elongation donor in mitochondria is acetyl-CoA. Generally, a monounsaturated fatty acyl-CoA substrate is more active than saturated CoA and both support higher activity than PUFA, particularly in brain.

Mitochondrial elongation occurs by successive addition and reduction of acetyl units in a reversal of fatty acid oxidation. Although fatty acid β-oxidation and mitochondrial chain elongation have the same organelle location, reversal of a *trans*-2-enoyl-CoA reductase β-oxidation is not feasible; the FAD-dependent acyl-CoA dehydrogenase of β-oxidation is substituted by a more thermodynamically favorable enzyme reaction, catalyzed by NADPH-dependent enoyl-CoA reductase, to produce overall negative free-energy for the sequence. Enoyl-CoA reductase from liver mitochondria is distinct from

the ER reductase, based on pH optima and specificities for saturated and unsaturated acyl-CoAs. Kinetic studies suggest that enoyl-CoA reductase is rate limiting in mitochondrial elongation. Maximal rates of mitochondrial elongation in liver, brain, kidney, and adipose tissue seems to require both NADPH and NADH as electron donors, whereas heart, aorta, and muscle require only NADH (W. Seubert, 1973). The function of the mitochondrial elongation system is unclear but has been proposed to participate in the biogenesis of mitochondrial membranes and/or in the transfer of reducing equivalents between carbohydrates and lipids.

2.3. Peroxisomal fatty acid elongation

Peroxisomal fatty acid elongation is tightly coupled to fatty acid oxidation in peroxisomes, where generated acetyl-CoAs are primarily used as precursors in the de novo synthesis of alcohols. These alkyl chains can subsequently be incorporated into glycerol ether lipids (Chapter 9). Specific roles for fatty acid elongation in peroxisomes have not been well defined but this organelle might produce very-long-chain SFA and PUFA of 24–36 carbons. Import of very-long-chain fatty acids into peroxisomes is defective in patients with the inherited disease X-linked adrenoleukodystrophy (M. Slawecki, 1995). This condition results in increased cellular and serum levels of very-long-chain fatty acids.

3. Desaturation of long-chain fatty acid in mammals

3.1. Δ9 Desaturase

Stearoyl-CoA desaturase (SCD) resides in the ER where it catalyzes the biosynthesis of MUFA from SFA that are either synthesized de novo or derived from the diet (Fig. 2). In conjunction with NADH, the flavoprotein cytochrome b_5 reductase, the electron acceptor cytochrome b_5, and molecular oxygen, SCD introduces a single double bond into a spectrum of methylene-interrupted fatty acyl-CoA substrates (Fig. 3).

Based on recent kinetic isotope data of the plant desaturase, the current hypothesis for the desaturation reaction is that the enzyme removes hydrogen atoms starting with that at the C-9 position, followed by the removal of the second hydrogen atom from the C-10 position. This stepwise mechanism is highly specific for the position at which the double bond is introduced, implying that the C-9 and C-10 bond is accurately positioned with respect to the diiron center of the enzyme. The desaturation of a fatty acid is an oxidation reaction that requires molecular oxygen and two electrons. However, oxygen itself is not incorporated into the fatty acid chain but is released in the form of water (Fig. 3). Although the insertion of a double bond occurs in a spectrum of methylene-interrupted fatty acyl-CoA substrates including *trans*-11 octadecenoic acid, the preferred substrates are palmitoyl- and stearoyl-CoA, which are converted into palmitoleoyl- and oleoyl-CoA, respectively. The MUFA synthesized by SCD are then used as substrates for the synthesis of various lipid classes including PLs, TGs, and cholesteryl esters.

In 1960, Bloomfield and Bloch first reported the presence of a Δ9 desaturation system in mammals. The rat Δ9 desaturase (SCD) was first purified by Strittmatter et al. [9].

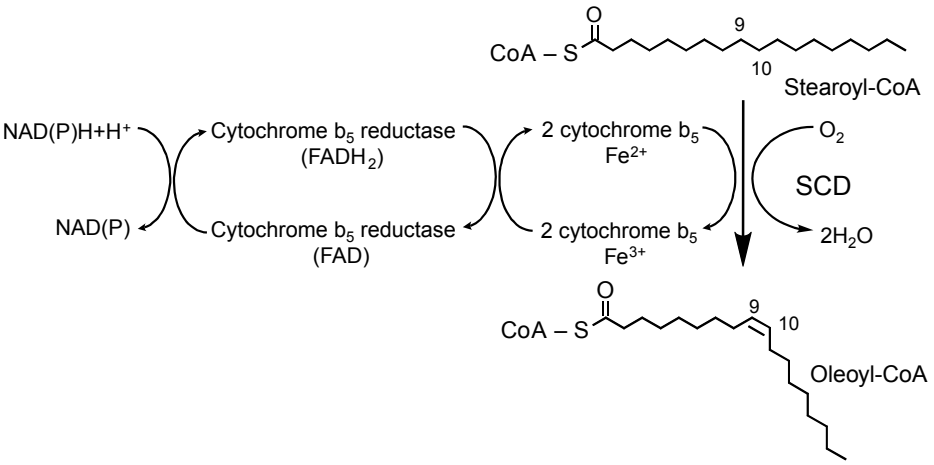

Fig. 3. Desaturation of stearoyl-CoA by stearoyl-CoA desaturase (SCD). The reaction is dependent on O_2 and a short electron chain consisting of NAD(P)H, cytochrome b_5 reductase and cytochrome b_5.

Subsequently, its cDNA cloned as *Scd1* was identified from amino acid sequence. Four genes encoding Δ9 desaturases (*Scd1*, *Scd2*, *Scd4*, and *Pcd*) have been cloned in mice [10–14], whereas two isoforms (*SCD1* and *SCD5*) are present in humans [15,16]. Δ9 Desaturase genes share a very similar organization consisting of six exons and five introns except for the human *SCD5* gene, which contains five exons. All mouse Δ9 desaturase genes are localized on chromosome 19 and form a cluster within the 200-kb region, whereas human *SCD1* and *SCD5* are localized on chromosomes 10 and 4, respectively. In addition, there is a pseudo-gene on human chromosome 17. All mammalian Δ9 desaturases contain four transmembrane domains and three regions of conserved histidine boxes, which are essential for the enzymatic reaction. In contrast to their structural similarity, the substrate specificity, tissue distribution, and physiological importance of the desaturases are different, as described below.

Mouse *Scd1* gene was first isolated from differentiated 3T3-L1 adipocytes and is ubiquitously expressed with the highest expression in lipogenic tissues including liver, adipose tissue, and sebaceous glands. Scd1 desaturates saturated fatty acyl-CoA with 12–19 carbons with the highest preference for the conversion of stearoyl-CoA (C18:0) into oleoyl-CoA (C18:1) [11].

Many developmental, dietary, hormonal, and environmental factors regulate mouse *Scd1* gene expression. High-carbohydrate diets, insulin, glucose and fructose, cholesterol, cold temperatures, light, some drugs (fibrates, peroxisome proliferators, and LXRα agonists), and retinoic acid induce hepatic *Scd1* gene expression. PUFA, especially those of the n – 6 and n – 3 families, cAMP (and drugs that increase its intracellular levels), tumor necrosis factor-α, and thyroid hormone, however, inhibit *Scd1* gene transcription in the liver. Previous studies in 3T3-L1 adipocytes have shown that thiazolidinediones and some steroid hormones decrease *Scd1* mRNA levels as well as SCD1-specific activity. Sulfur-substituted (thia) fatty acids inhibit *Scd1* either at the transcriptional level or at the level of

enzyme activity, while cyclopropene fatty acids (e.g., sterculic acid) directly inhibit SCD activity in vivo and in vitro. The SCD protein also undergoes rapid degradation producing transient reduction of enzyme activity in response to physiological demands. A high level of *Scd1* gene expression occurs in skeletal muscles of very obese humans. The regulation of SCD is therefore very diverse and its expression appears to affect a variety of key physiological variables, including insulin sensitivity, metabolic rate, adiposity, atherosclerosis, and cancer [17].

Scd2 is the second SCD isoform that was cloned from differentiated mouse 3T3-L1 preadipocytes. Similar to *Scd1*, *Scd2* mainly converts palmitoyl-CoA and stearoyl-CoA into palmitoleoyl-CoA and oleoyl-CoA, respectively. Although the tissue distribution of *Scd2* is distinct from that of Scd1, Scd2 is predominantly expressed in the brain and is developmentally induced during the neonatal myelination period. *Scd2* mRNA is expressed to a lesser extent in kidney, spleen, and lung where it is induced in response to a high-carbohydrate diet. In addition, *Scd2* mRNA is expressed in B cells and is downregulated during lymphocyte development. *Scd2* expression in liver is high in the embryo and neonate but decreases in adult mice, whereas *Scd1* expression is induced after weaning. Consistent with the expression of *Scd2* occurring in the early stages of life, in neonatal $Scd2^{-/-}$ mice MUFA (including oleate and palmitoleate) and TG contents in liver and skin are lower but these are not altered in adults. In addition, $Scd2^{-/-}$ neonatal mice have a skin permeability barrier defect with decreased levels of ceramides including acylceramides. It is now clear that *Scd1* expression is crucial in adult mice, whereas *Scd2* is important in the synthesis of MUFA that are required for maintaining normal epidermal barrier function and lipid biosynthesis during early liver and skin development [18].

Pcd (formally denoted Scd3) expression is restricted to mature sebocytes present in exocrine glands such as sebaceous, meibomian, preputial, and Harderian glands. However, SREBP-1a over-expression induces expression of *Scd3* in liver (J. Horton, 2003). Compared to other mammalian Δ9 desaturases, *Pcd* mainly utilizes saturated fatty acyl-CoAs with 14–16 carbons. Pcd is not able to desaturate fatty acyl-CoAs with more than 17 carbons including stearoyl-CoA. Although the reason for the existence of Pcd and its ability to produce shorter chain MUFA is unknown, it could be due to the unique tissue-specific expression of the *Scd* genes and differences in the melting point of their MUFA products. The most abundant MUFA in mouse sebum is 16:1n – 9. Since skin is poikilothermal (i.e., the temperature varies), the environmental temperature affects the composition of sebum. The melting point of 16:1n – 9 (0.5 °C) is lower than that of 18:1n – 9 (16.2 °C). Thus, 16:1n – 9 can be stored in liquid form in cold temperature and could be preferentially utilized by acyl-CoA wax alcohol acyltransferases (AWAT1 and AWAT2) in the synthesis of skin waxes. The skin of $Scd1^{-/-}$ mice exhibits alopecia, atrophy of the sebaceous gland, and a decrease in sebum production. Interestingly, *Pcd* gene expression is lost or decreased in sebocytes of $Scd1^{-/-}$ mice and its expression is normalized by testosterone administration (M. Miyazaki, 2002). Mouse skin expresses three *Scd* gene isoforms (*Scd1*, *Scd2*, and *Pcd*). Although expression of *Scd1* and *Pcd* in skin is limited to the sebaceous gland, the distribution within the gland is different. In situ hybridization shows that the transcript of *Scd1* is located within undifferentiated sebocytes, while *Pcd* mRNA resides in differentiated sebocytes. In $Scd1^{-/-}$ mice, the expression of *Pcd* is decreased, while that

of *Scd2* in the hair follicles is still evident suggesting that *Scd2* expression cannot compensate for the lack of *Scd1* or *Pcd*. Hence, for proper differentiation of sebocytes and hair follicle development, *Scd1* gene expression is a prerequisite. The presence of three genes of the *Scd* gene family in mouse skin raises the question of the specific function of each gene.

The *Scd4* isoform is predominantly expressed in the heart and meibomian gland in mice. *Scd4* expression is increased in the hearts of leptin-deficient *ob/ob* mice and reduced by leptin administration. The liver X receptor (LXR) and a high-carbohydrate diet also induce *Scd4* expression whereas PUFA have no effect [12]. *Scd4* is able to desaturate 16:0-CoA and 18:0-CoA to 16:1n – 9 and 18:1n – 9, respectively; however, the desaturase activity is significantly lower than that of other *Scd* isoforms. Therefore, *Scd4* might utilize fatty acyl-CoAs other than 16:0-CoA and 18:0-CoA as major substrates.

Mutations in human *Scd5* (formerly cloned as *ACOD4*) were identified as being responsible for human cleft lip (S. Beiraghi, 2003). *Scd5* is highly expressed in the human brain and pancreas. Interestingly, *Scd5* orthologs have not been found in genomes of rodents but have been identified in other primates. The *Scd5* gene encodes a truncated SCD protein consisting of 330 amino acids [19]. The main substrate for *Scd5* is stearoyl-CoA.

3.2. Δ5 and Δ6 desaturases

Δ5 Desaturase (*D5D*) and Δ6 desaturase (*D6D*) are membrane-bound desaturases catalyzing the synthesis of n – 3 and n – 6 series of HUFA [20,21] from dietary α-linolenic acid (18:3n – 3) and linoleic acid (18:2n – 6) (Fig. 4). The two enzymes share structural

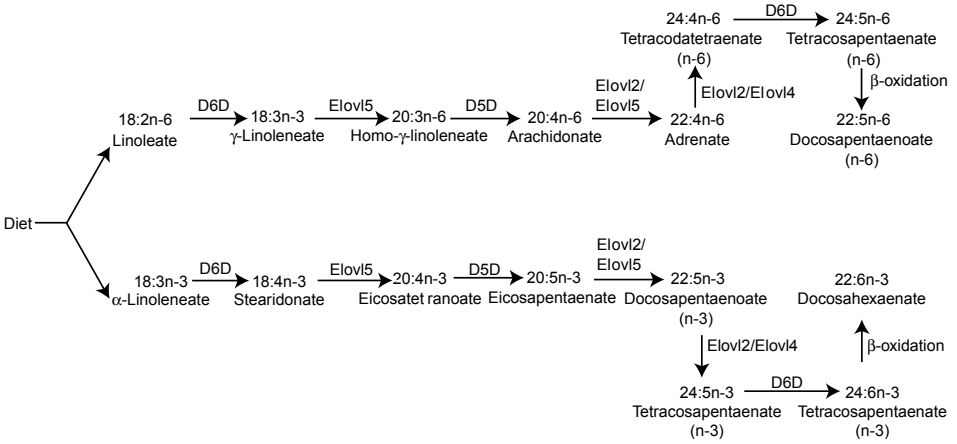

Fig. 4. Synthesis of n – 6 and n – 3 series of PUFA from dietary essential fatty acids linoleic and α-linolenic acids, respectively, by elongation and desaturation.

characteristics. Both enzymes have a N-terminal cytochrome b_5 domain carrying heme-binding motifs, two membrane-spanning domains and three histidine boxes characteristic of membrane desaturases. However, the first His residue of the third His-box is replaced with Glutamine (QXXHH instead of HXXHH). The glutamine residue is essential for desaturase activity because mutations of this amino acid residue to histidine or isoleucine abolish the activity of the enzyme. *D5D* and *D6D* are widely expressed in human tissues with the highest levels in liver. D6D catalyzes the Δ6 desaturation of PUFA of both 18 and 24 carbons. This desaturase converts linoleic acid (18:2n – 6) and α-linolenic acid (18:3n – 3) to γ-linolenic acid (18:3n – 6) and stearidonic acid (18:4n – 3), respectively. Elovl-5 then elongates these fatty acids by two carbons to synthesize 20:3n – 6 and 20:4n – 3. After desaturation and elongation by D6D and the appropriate elongase, respectively, D5D introduces another double bond at the 5-position of 20-carbon fatty acids 20:3n – 6 and 20:4n – 3 (Fig. 4). In addition, D6D is able to desaturate palmitate (16:0) to sapienoic acid (16:1n – 10), a major fatty acid of human sebum. In fact, D6D is highly expressed in the sebaceous gland in human skin. Thus, mammalian D6D, unlike other desaturases, can desaturate a wide range of fatty acids with 16–22 carbons. Although neither *D5D*- nor *D6D*-deficient mice have been reported, a nucleotide insertion in 5′-flanking region of human *D6D* was recently reported to cause human D6D deficiency. The *D6D*-deficient female patient who had decreased synthesis of arachidonic acid and DHA developed clinical characteristics of essential fatty acid deficiency including corneal ulceration, feeding intolerance, growth failure, photophobia, and skin abnormalities. Supplementation of the patient's diet with arachidonic acid and DHA ameliorated all of the symptoms (J. Nwankwo, 2003).

Human *D5D* and *D6D* genes are localized on chromosome 11 (11q12–q13.1) and form a cluster within the 100-kb region of the chromosome. The mouse homologs on chromosome 19 also occur as a cluster with a similar exon/intron organization, suggesting that these desaturases arose evolutionarily by gene duplication. The *D5D* and *D6D* genes both consist of 12 exons and 11 introns spanning 17.2 and 39.1 kb regions, respectively. The proximity of their promoters suggests that the transcription of the *D5D* and *D6D* genes is coordinately controlled by common regulatory sequences within the 11-kb region.

3.3. FADS3

Fatty acid desaturase 3 (*FADS3*) is a recently cloned desaturase gene. Like *D5D* and *D6D*, *FADS3* has 12 exons and 11 introns and is located in the 6.0-kb telomeric side from the *D6D* gene. The human *FADS3* gene has an open reading frame of 1338 bp encoding 445 amino acids, while the mouse *fads3* gene possesses a 1350-bp open reading frame encoding 449 amino acids. The predicted amino acid sequence contains all of the conserved structural features of D6D. Alignment of the putative amino acid sequence of human FADS3 demonstrates that FADS3 has a high degree of identity with D5D (52%) and D6D (62%) [22]. However, the function of FADS3 is unknown. A search of the human expressed sequence tag databases reveals that *FADS3* mRNA is expressed in brain, placenta, ovary, β-cell, and skin as well as in fetal brain.

4. Functions of fatty acids synthesized by Δ9, Δ6, and Δ5 desaturases

4.1. Monounsaturated fatty acids (n – 9)

As shown in Fig. 2, mammals have all enzymes required for the synthesis of MUFA from acetyl-CoA. The roles of MUFA are diverse and crucial in living organisms. Palmitoleic acid and oleic acid are the major MUFA of TGs, cholesteryl esters, wax esters, and membrane PLs. The ratio of SFA to MUFA contributes to membrane fluidity, while changes in the acyl chain composition of cholesteryl esters and TGs affects lipoprotein metabolism. Apart from being the components of more complex lipids, MUFA have also been implicated as mediators of signal transduction, cellular differentiation, and metabolic homeostasis. Therefore, this diverse role of MUFA, despite their dietary abundance, suggests that the SCDs that make these MUFA are of considerable physiological importance. Regulation of the amount of MUFA has the potential to affect a variety of key physiological variables, which include insulin sensitivity, metabolic rate, adiposity, atherosclerosis, cancer, and obesity.

Understanding the physiological role of MUFA has been advanced by recent studies in a mouse model with a targeted disruption of the *Scd1* gene. *Scd1*$^{-/-}$ mice are deficient in TGs, cholesteryl esters, and wax esters whose major acyl chains are MUFA. In addition to a decrease in tissue neutral lipids, *Scd1* deficiency results in reduced body adiposity, increased insulin sensitivity, and resistance to diet-induced obesity. The expression of several genes of lipid oxidation is upregulated in *Scd1*$^{-/-}$ mice, whereas lipid synthetic genes are downregulated. *Scd1* was also found to participate in a novel metabolic response to the hormone leptin (Chapter 10).

Similar to mouse models that lack the *acyl-CoA:cholesterol acyltransferase-1*, *diacylglycerol acyltransferase-1*, and *Elovl3* genes, *Scd1*$^{-/-}$ mice exhibit cutaneous abnormalities with atrophic sebaceous glands and narrow eye fissure with atrophic meibomian glands, suggesting an important role of MUFA in skin and eyelid homeostasis. The major function of sebaceous and meibomian glands is to secrete lipid complex lubricants, termed sebum and mebum, respectively. These lubricants contain wax esters, TGs, and cholesteryl esters, and prevent the evaporation of moisture from the skin and the eyeball.

In the mouse, Harderian gland SCD is involved in the biosynthesis of another class of lipids: the 1-alkyl-2,3-diacylglycerols [23]. The Harderian gland is located in the orbit of the eye. The major products of the gland vary among different species of mammals. In rodents, the gland synthesizes lipids, indoles, and porphyrins, which are secreted by an exocytotic mechanism. The major lipid synthesized by the mouse Harderian gland is 1-alkyl-2,3-diacylglycerol. 1-Alkyl-2,3-diacylglycerol is a lubricant of the eyeball and, along with mebum from the meibomian gland, is crucial in facilitating the movement of the eyelid. *Scd1*$^{-/-}$ mice exhibit a deficiency in 1-alkyl-2,3-diacylglycerols and gondoate (20:1n – 9), which is the main MUFA of 1-alkyl-2,3-diacylglycerol and an elongation product of 18:1n – 9. Feeding the mice diets containing high levels of either oleate or gondoate does not increase 20:1n – 9 and fails to restore the deficiency in 1-alkyl-2,3-diacylglycerol.

Very low levels of n – 3 and n – 6 PUFA are present in the Harderian gland, indicating that the lipids of this gland are mainly composed of SFA and MUFA of the n – 9 series.

C18:1 is a major component of the Harderian gland membranes and a decrease in the level of C18:1 would be expected to reduce membrane fluidity of the Harderian gland. Consistent with this notion, the Harderian gland isolated from the *Scd1*$^{-/-}$ mice is very rigid, perhaps due to decreased membrane fluidity. The Harderian gland might, therefore, be a useful model for studying the metabolism of MUFA of the n – 9 series and the roles they play in physiological processes.

4.2. Polyunsaturated fatty acids (n – 3 and n – 6)

Arachidonic acid (20:4n – 6) is one of two major PUFA synthesized by the D6D/D5D pathway (Fig. 4). In many tissues and cell types, 20:4n – 6 is esterified to the *sn*-2 position of membrane PL, and is used for the eicosanoid-mediated signaling to perform specialized cell functions. Arachidonic acid esterified in PL is a storage form of this fatty acid and is hydrolyzed from the PL by phospholipases prior to enzymatic conversion into eicosanoids (Chapter 13). Eicosanoids are autocrine/paracrine hormones that mediate a variety of localized reactions, such as inflammation, homeostasis, and protection of digestive tract epithelium. D6D deficiency in humans leads to severe food intolerance and growth retardation (J. Nwankwo, 2003). These symptoms are reversed by arachidonic acid supplementation to the diet, which supports the essential role of eicosanoids in the protection of digestive tract mucosa in humans.

Requirements for PUFA cannot be met by de novo metabolic processes in mammalian tissues. Animals are absolutely dependent on plants (or insects) for providing double bonds in the Δ12 and Δ15 positions of the two major precursors of the n – 6 and n – 3 fatty acids, linoleic and α-linolenic acids (Fig. 5). These two fatty acids therefore are called 'essential' fatty acids. Classical studies of essential fatty acid deficiency in rodents demonstrated that the main symptoms are dry skin, dermatitis, and massive water

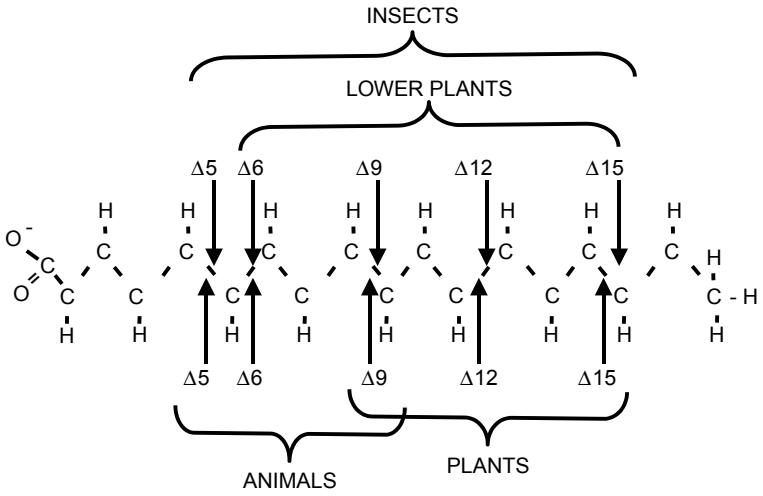

Fig. 5. Positions of fatty acyl chain desaturation by enzymes of animals, plants, insects, and lower plants.

loss through the skin. These symptoms are reversed by dietary n – 6 or n – 3 PUFA, although α-linolenic acid is less effective than linoleic acid. Subsequent studies have shown that 18:2n – 6 is required in skin ceramides to prevent water loss (P. Wertz, 1983). However, in a human case of D6D deficiency, severe abnormalities have been also reported in skin, hair, and nails, indicating that desaturation of PUFA substrates by D6D is required for skin functions. Moreover, dietary supplementation of PUFA does not reverse the PUFA deficiency symptoms completely, highlighting the importance of the endogenous D6D pathway for skin functions.

DHA is very abundant in excitable membranes in the retina and brain, particularly in PL of the rod outer segment of retina and of synaptic vesicles, and is important in vision. However, the mechanism by which DHA functions in retina is not well understood. Chen et al. (Y. Chen, 1993) suggest that DHA in retina might be involved in shuttling 11-*cis*-retinal to photoreceptors, whereas Niu et al. (S. Niu, 2004) propose that DHA in PL increases the efficiency of G-protein-mediated signal transduction of rhodopsin. In humans, supplementation of infant formula with DHA accelerates the development of visual functions in pre-term infants. The novel protective lipid mediator docosanoids, namely, Protectin D1 (C. Serhan, 2002) and 17S-hydroperoxy-DHA (V. Marcheselli, 2003), have been suggested to mediate the beneficial effects of DHA.

DHA plays a major part in learning spatial tasks and olfactory-based learning and has been reported to improve symptoms of human patients with dementia including Alzheimer's disease. DHA improves neuronal cell survival and differentiation and is involved in neurotransmitter recycling. By slowing the ion channel activity, n – 3 HUFA may also alleviate the symptoms of convulsive seizures, which are characterized by uncontrolled firings of neurons in the central nervous system.

n – 3 HUFA prevent cardiac arrhythmia during reperfusion after ischemia. In the heart, upon release from the membrane PL in response to hypoxia, the non-esterified HUFA facilitate the synchronization of contraction of cardiac myocytes upon reperfusion by increasing the recovery time of ion channels. This effect is the likely mechanism by which dietary fish oils appear to reduce deaths from myocardial infarction (S. Grundy, 2003). DHA is also used as a preventive supplement against many inflammatory diseases such as rheumatoid arthritis, cystic fibrosis, ulcerative colitis, asthma, cancer, and cardiovascular disease.

PUFA regulate gene expression by affecting the activities of a number of transcription factors both in the cytoplasm and the nucleus. PUFA released from membrane PL by agonist-induced stimulation of phospholipases are involved in signal transduction through modulation of activities of cellular proteins including isoforms of protein kinase C and in translocation of specific enzymes to membranes. Specific types of fatty acid modulate many of these diverse functions. Thus, a large variety of fatty acyl chains are required in the lipids of biological membranes and storage depots.

5. Transcriptional regulation of desaturases and elongases

The expression of the three desaturase genes as well as that of the *Elovl-5* and *Elovl-6* genes is highly regulated by many nutrients including fatty acids and hormones at the transcriptional level. While the PUFA are the best characterized for their ability to activate

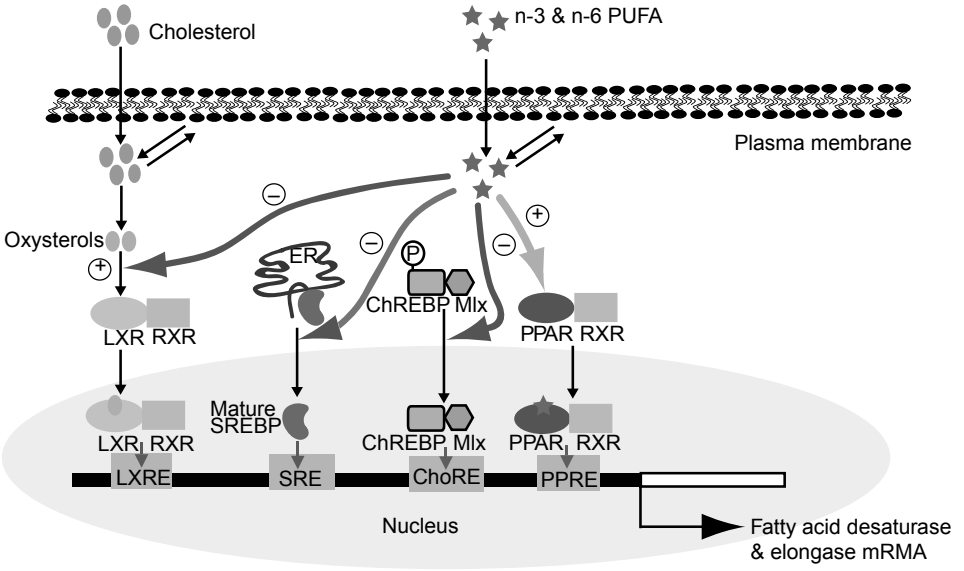

Fig. 6. Coordinated transcriptional regulation of fatty acid desaturases and elongases in mammals. PUFA, polyunsaturated fatty acids; +, stimulation; –, inhibition; LXR, liver X receptor; RXR; retinoid X receptor; SREBP, sterol regulatory element binding protein; ChREBP, carbohydrate response element binding protein; Mlx, Max-like receptor; PPAR-α, peroxisome proliferator activated receptor alpha; LXRE, liver X receptor response element; SRE, sterol response element; ChoRE, carbohydrate response element; PPRE, peroxisome proliferator response element. (See color plate section, plate no. 6.)

genes of fatty acid oxidation and repress genes of lipogenesis, it is now evident that MUFA as well as SFA and *trans*-fatty acids can influence gene expression. Understanding the molecular mechanisms by which nutrients and hormones control transcription of desaturases and elongases has been advanced in the last few years by identification of several key transcription factors that regulate lipid metabolism. For example, the transcription factors, SREBP-1c, PPAR-α, LXR, and carbohydrate response element binding protein (ChREBP) play key roles in the regulation of desaturases and elongases (Fig. 6).

5.1. Sterol regulatory element binding proteins

SREBPs belong to the basic helix-loop-helix leucine zipper (bHLH) family of transcription factors [24]. Three isoforms (SREBP-1a, SREBP-1c, and SREBP-2) have been identified in mammalian cells (Chapter 15). SREBPs are synthesized as precursor proteins that are anchored to the ER. After two proteolytic events in the Golgi apparatus, the N-terminal bHLH domain of the SREBPs is released as a mature protein and translocated to nucleus where it activates transcription by binding to a sterol regulatory element in the 5′-flanking region of target genes, particularly those responsible for lipid metabolism. SREBP-1a activates all SREBP target genes involved in cholesterol and fatty acid metabolism, whereas SREBP-2 mainly activates transcription of genes involved in cholesterol synthesis. SREBP-1c is the main isoform in liver and preferentially activates

transcription of genes of fatty acid metabolism, including the three desaturases and Elovl-6. One or two sterol response elements exist in promoters of the *Scd1*, *Scd2*, *D6D*, *D5D*, and *Elovl-6* genes. Mice over-expressing SREBPs have a significantly increased expression of all the desaturases and *Elovl-6* in the liver. A series of in vitro and in vivo studies demonstrated that SREBP-1c is involved in the PUFA-mediated repression of the transcription of the desaturases and *Elovl-6* in liver. Several mechanisms underlying PUFA repression of SREBP-1c have been proposed: (i) blocking the proteolytic processing of SREBP-1c [25], (ii) reducing the stability of SREBP-1c mRNA [26], and (iii) acting as antagonists of the LXR, which activates SREBP-1c transcription [25]. The precise mechanisms by which PUFA suppress the proteolytic maturation, transcription, and stability of SREBP-1c are currently unknown.

5.2. Liver X receptors

LXR belongs to the nuclear hormone receptor superfamily that is involved in the regulation of cholesterol and lipid homeostasis (Chapter 15). Two LXR isoforms have been identified: LXRα, which is most abundant in liver, and LXRβ, which is expressed ubiquitously. LXR forms a heterodimer with the retinoid X receptor and binds a direct repeat-4 element (LXR response element) in target genes. Cholesterol metabolites, the oxysterols, are thought to be the natural ligands that activate LXR. A synthetic LXR agonist T0901317 was developed as an antiatherogenic agent due its ability to increase the level of high-density lipoprotein (J. Schultz, 2000). Undesirably, however, administration of T0901317 causes hypertriglyceridemia and liver steatosis concomitant with increased expression of lipogenic genes, including *Scd1*. The accumulation of lipids and increased lipogenesis by T0901317 were primarily explained by the increased expression of SREBP-1c, whose promoter contains two LXR response elements. However, cholesterol also induces *Scd1* gene expression despite the fact that cholesterol decreases nuclear SREBP-1c protein. T0901317 induces the expression of *Scd1* and other desaturases in livers of SREBP-1c-deficient mice, suggesting the presence of a direct LXR response element in the promoters of these genes. Cholesterol induction of *Scd1* is eliminated in livers of *Lxrα/β*-deficient mice suggesting that LXR directly activates the transcription of *Scd1* gene. An LXR response element has been identified in the mouse *Scd1* promoter [27] and is probably also present in the *D6D* and *D5D* promoters. *Elovl-6*, like the desaturases, is a target of LXR (Y. Wang, 2006).

5.3. Peroxisome proliferator activated receptor alpha

PPAR-α also belongs to the nuclear hormone receptor superfamily and is involved in the regulation of lipid and glucose homeostasis. *Scd1*, *D6D*, *D5D*, and *Elovl-5* are targets of PPAR-α. A peroxisome proliferator response element (PPRE) is present in the promoters of *Scd1* and *D6D*. Induction of *Scd1* by the synthetic PPAR-α agonist Wy14643 was masked in liver of *Ppar-α* null mice. PUFA are considered to be one of the endogenous PPAR-α ligands. However, PUFA repression of lipogenic genes including *Scd1* still occurs in the livers of *Ppar-α* null mice, indicating that repression of desaturases by PUFA is not mediated through PPAR-α.

The induction of desaturases by both PPAR-α and SREBP-1c is paradoxical because, except for the desaturases, these two transcription factors induce mutually exclusive sets of genes. In general, PPAR-α induces fatty acid oxidation genes, whereas SREBP-1 induces genes of fatty acid synthesis. PPAR-α also directly activates the *D6D* gene and plays a crucial role in the feedback regulation of the synthesis of HUFA. The *D6D* mRNA is not increased in Ppar-α null mice fed essential fatty acid-deficient diets, although nuclear SREBP-1c is elevated in both Ppar-α null and wild-type mice. Peroxisome proliferators might induce desaturases in rodents partly by indirect mechanisms. First, administration of peroxisome proliferators is likely to increase the degradation of unsaturated fatty acids by inducing enzymes for fatty acid oxidation in both peroxisomes and mitochondria. Second, the requirement of unsaturated fatty acids for membrane PL would be increased by administration of peroxisome proliferators, which induce proliferation of peroxisomes and enlargement of the liver in rodents. These changes would be expected to increase the demand of unsaturated fatty acids resulting in induction of the desaturases. Indeed, despite a strong induction of the synthesis of HUFA by peroxisome proliferators, little change is observed in the composition of HUFA in PL. Moreover, contrary to the rapid induction of PPAR-α-responsive genes involved in fatty acid oxidation, such as acyl-CoA oxidase and carnitine palmitoyl transferase-1, the maximal induction of *D6D* and *D5D* mRNAs takes much longer. Induction of the *Scd1* gene is also delayed by clofibrate in mouse liver, indicating that indirect mechanisms contribute to the strong induction of desaturases by peroxisome proliferators. Thus, both SREBP-1c and PPAR-α are required to mediate the feedback induction of *D6D* when HUFA are low. The role that PPAR-α plays in the regulation of elongases has not been well established (Y. Wang, 2005).

5.4. Carbohydrate response element binding protein

ChREBP is another transcription factor of the bHLH family that is involved in glucose and lipid metabolism [28]. Glucose itself, independent of insulin, increases expression of desaturases and elongases. Although a carbohydrate response element (ChoRE) has not been identified in the promoter of any desaturase or elongase, a study of mice lacking ChREBP indicated that *Scd1* is a target of ChREBP. In addition, glucose still activates *Scd1* expression in the livers of *Srebp-1c*$^{-/-}$ mice. ChREBP and Max-like receptor protein bind as heterodimers onto ChoRE in the promoters of their target genes. The regulation of ChREBP is controlled by phosphorylation-dependent mechanisms. When excess glucose enters tissues, glucose is converted to xylulose-5-phosphate via the hexose monophosphate pathway. Xylulose-5-phosphate serves as an activator of protein phosphatase 2A that activates ChREBP by dephosphorylation. The dephosphorylated ChREBP translocates to the nucleus, where it activates transcription of target genes. Conversely, under fasting (low energy) conditions, cAMP and AMP are elevated and activate protein kinase A and AMP-activated protein kinase, respectively. The phosphorylation of ChREBP inhibits its nuclear translocation and DNA-biding capacity. ChREBP might also be involved in PUFA repression of desaturases. A recent study demonstrated that PUFA suppress ChREBP activity in parallel with reducing the expression of liver-type pyruvate kinase and fatty acid synthase by decreasing ChREBP mRNA stability and inhibiting ChREBP nuclear translocation. In addition, feeding mice a diet enriched in PUFA decreased the hepatic

content of xylulose-5-phosphate [29]. PUFA also decreased Max-like receptor expression without affecting the nuclear level of ChREBP (J. Xu, 2006). Taken together, these data suggest that PUFA-mediated repression of desaturases and elongases can be accounted for by reduced nuclear abundance of the ChREBP/Max-like receptor complex.

6. Summary and future directions

Lipids are no longer considered to be just a static store of energy, but are widely recognized as modulators of metabolism in the whole body. A common function of fatty acid desaturases and elongases is the production of long-chain fatty acids. These fatty acids maintain the physical attributes of membrane PL and stored TGs. In addition, fatty acids and their metabolites are used by cells for other functions, especially cell signaling. Although eicosanoid functions are well characterized, mechanisms of many other functions of PUFA remain to be elucidated. The regulation of the three human desaturases and specific elongases demonstrates that they have shared, as well as distinct, properties. Recent progress in elucidating regulatory mechanisms of gene expression of desaturases and elongase presents exciting examples of the sophisticated control of mammalian gene transcription that is achieved by a combination of multiple transcription factors such as SREBP-1c, LXR, PPAR-α, and ChREBP. With new knowledge of the regulation of transcription by a variety of transactivation factors, combined with the increasing prevalence of disorders of lipid metabolism, there is renewed interest in understanding the role of long-chain fatty acids in gene regulation and metabolism. Further research into this topic will be key in understanding mechanisms of disease progression and thereby for devising methods of disease prevention.

Abbreviations

ChREBP	carbohydrate response element binding protein
DHA	docosahexaenoic acid
D5D	Δ5 desaturase
D6D	Δ6 desaturase
ER	endoplasmic reticulum
HUFA	highly unsaturated fatty acids
KAR	ketoacyl-CoA reductase
LXR	liver X receptor
MUFA	monounsaturated fatty acids
PCD	palmitoyl-CoA desaturase
PL	phospholipid
PPAR	peroxisome proliferator activated receptor
PUFA	polyunsaturated fatty acids
SCD	stearoyl-CoA desaturase
SFA	saturated fatty acids
SREBP	sterol regulatory element binding protein

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CHAPTER 8

Phospholipid biosynthesis in eukaryotes

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Contents

1. Introduction

This chapter provides an overview of eukaryotic phospholipid biosynthesis. A discussion of phospholipid synthesis in mammalian cells is emphasized but the synthesis of phospholipids in yeast is also discussed. Phospholipid synthesis in plants is considered in Chapter 4 and in bacteria in Chapter 3. Phospholipids make up the essential milieu of cellular membranes and act as a barrier for entry of compounds into cells. Phospholipids also function as precursors of second messengers such as diacylglycerol (DG) and inositol-1,4,5-P_3. A third, and usually overlooked function of phospholipids, is storage of energy in the form of fatty acyl components. This function is probably quantitatively important only under extreme conditions such as starvation.

2. Phosphatidic acid biosynthesis and conversion to diacylglycerol

Phosphatidic acid (PA) is an intermediate that occurs at a branchpoint in glycerolipid biosynthesis (Fig. 1). Significant developments in elucidation of the biosynthetic pathway for PA occurred in the 1950s when Kornberg and Pricer demonstrated that fatty acids are activated to acyl-CoA prior to reaction with glycerol-3-P. Subsequent studies from the laboratories of Kennedy, Shapiro, Hübscher, and others delineated the biosynthetic pathway for PA. An important step in PA biosynthesis is the activation of fatty acids by acyl-CoA synthetases to yield acyl-CoA (Fig. 2). Five different forms of rat acyl-CoA synthetase have been identified, each encoded by a separate gene (R.A. Coleman, 2001). Distinct forms of the enzyme have been found on the endoplasmic reticulum (ER), mitochondria and mitochondria-associated membranes (MAM), a sub-fraction of the ER (J.E. Vance, 1990). Hence, these synthetases might provide distinct pools of acyl-CoA substrates for the biosynthesis of phospholipids and triacyglcyerols (TGs).

2.1. Glycerol-3-P acyltransferase

This enzyme catalyzes the first committed reaction in the biosynthesis of PA, i.e., the production of lyso-PA. The importance of this acyltransferase in regulation of phospholipid biosynthesis has not been clearly established. In mammals, two glycerol-3-P acyltransferases (GPATs) have been identified, one associated with mitochondria, the other with the ER (Fig. 2). The ER acyltransferase (GPAT3) is inhibited by *N*-ethylmaleimide whereas the mitochondrial enzyme (GPAT1) is not. GPAT1 prefers palmitoyl-CoA as an acyl donor compared to oleoyl-CoA, whereas the ER enzyme does not show a preference for saturated versus unsaturated acyl-CoAs. For this and other reasons the mitochondrial enzyme is thought to be primarily responsible for the abundance of saturated fatty acids in the *sn*-1 position of glycerophospholipids (D. Halder, 1994). GPAT1 has been localized to the

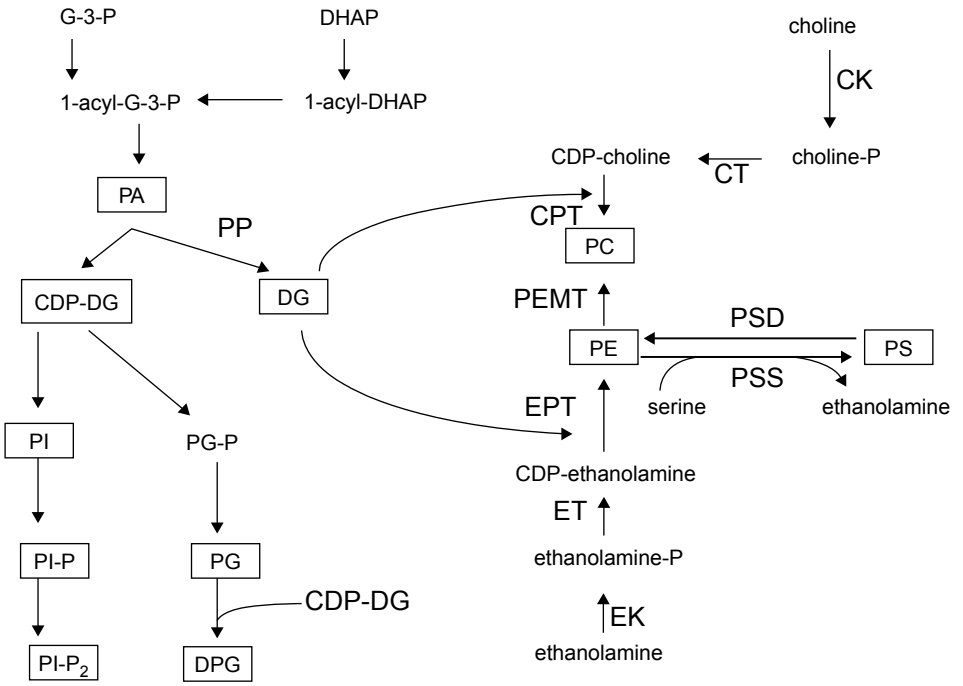


Fig. 1. Phospholipid biosynthetic pathways in animal cells. The abbreviations are: DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; PA, phosphatidic acid; DG, diacylglycerol; CDP-DG, cytidine diphosphodiacylglycerol; PI, phosphatidylinositol; PG, phosphatidylglycerol; PG-P, phosphatidylglycerol phosphate; DPG, diphosphatidylglycerol; PP, phosphatidic acid phosphatase; PE, phosphatidylethanolamine; PC phosphatidylcholine; PEMT, phosphatidylethanolamine *N*-methyltransferase; CT, CTP:phosphocholine cytidylyltransferase; PS, phosphatidylserine; CK/EK, choline/ethanolamine kinase; CPT, CDP-choline:1, 2-diacylglycerol cholinephosphotransferase; EPT, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; ET, CTP:phosphoethanolamine cytidylyltransferase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase.

mitochondrial outer membrane with the active site facing the cytosol (R.A. Coleman, 2001). Targeted deletion of the gene encoding GPAT1 results in a decrease in the TG and cholesterol content of liver and striking changes in glycerolipid fatty acid composition (Table 1) [1].

Transcription of the *GPAT1* gene is decreased by starvation and glucagon, and increased by a high carbohydrate diet (H.S. Sul, 1997). The GPAT activity on the ER is not significantly altered by these treatments. These responses make physiological sense since animals do not need to make TG or, to a lesser extent, phospholipids when energy supply is limited. The 5′ flanking region of the murine gene encoding GPAT1 was linked to a luciferase reporter plasmid and expressed in 3T3-L1 pre-adipocytes. Experiments in these cells indicated that sterol response element-binding protein (SREBP) and NF-Y transcription factors were important in stimulating GPAT activity (P.A. Edwards, 1997). As indicated in Chapters 6, 7, and 14, SREBP is a key regulator of the synthesis of cholesterol and fatty acids, as well as of fatty acid desaturation. Thus, it is biologically reasonable that SREBP would also enhance glycerolipid, particularly TG, biosynthesis by increasing the expression of GPAT.

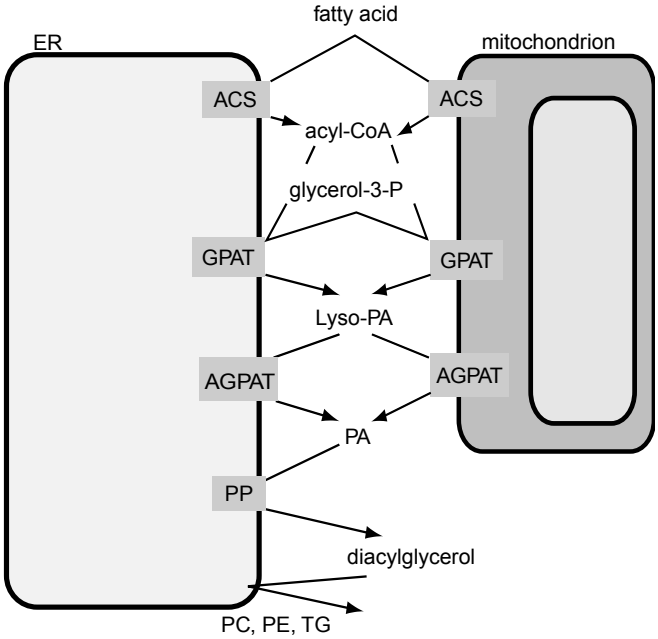


Fig. 2. Biosynthesis of phosphatidic acid (PA) occurs on both the endoplasmic reticulum (ER) and the outer membrane of mitochondria. The abbreviations are: ACS, acyl-CoA synthetase, GPAT, glycerol-3-P acyltransferase; AGPAT, 1-acylglycerol-3-P acyltransferase; PP, phosphatidic acid phosphatase (lipin); PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol.

Table 1
Natural and induced mutations in mammalian phospholipid biosynthesis

Source/gene	Encoded enzyme	Phenotype
Mouse *Gpat1*	Glycerol-3-phosphate acyltransferase 1	Lower weight gain, decreased hepatic TG
Human *AGPAT2*	1-Acylglcyerol-3-P acyltransferase 2	Decreased adipose, hypertriglyceridemia insulin resistance, hepatic steatosis
Mouse *Fld*	Phosphatidic acid phosphatase 1 (lipin-1)	Impaired adipose development, insulin resistance, increased β-oxidation
Mouse *Chka*	Choline kinase α	Embryonic lethality ~day E4
Mouse *Chkb* (*rmd)*	Choline kinase β	Hindlimb muscular dystrophy, neonatal bone deformity
Mouse *Pcyt1a*	CTP:phosphocholine cytidylyltransferase α	Embryonic lethal ~day 3
Mouse *Pcyt1b*	CTP:phosphocholine cytidylyltransferase β 2	Gonad degeneration, reproductive deficiency
Mouse *Pemt*	Phosphatidylethanolamine *N*-methyltransferase	Liver failure after 3 days of choline-deficient diet
Mouse *Pisd*	Phosphatidylserine decarboxylase	Embryonic lethal ~day 8
Mouse *Pss1*	Phosphatidylserine synthase-1	Viable
Mouse *Pss2*	Phosphatidylserine synthase-2	Partial male infertility
Mouse *Pcyt2*	CTP:phosphoethanolamine cytidylyltransferase	Embryonic lethality prior to day 8
Mouse *Etnk2*	Ethanolamine kinase	Placental thrombosis, neonatal lethality

2.2. 1-Acylglycerol-3-P acyltransferase

Far less is known about the second step in the PA biosynthetic pathway (Fig. 2) although multiple genes encoding this activity have been identified. The activity of this acyltransferase is much lower in mitochondria than in ER. It is presumed that much of the lyso-PA formed in mitochondria is transferred to ER for the second acylation step. In vitro studies indicate that a carrier protein is not required for the transfer (A.K. Hajra, 1992). The esterification at the *sn*-2 position utilizes mainly unsaturated fatty acids. However, the types of fatty acyl-CoAs available also influence the acyl-CoA species selected for transfer to lyso-PA. In congenital generalized lipodystrophy — a disease characterized by decreased adipose tissue, hypertriglyceridemia, hepatic steatosis, and insulin resistance — several different mutations have been found in 1-acylgycerol-3-P acyltransferase 2 (A. Garg, 2002).

2.3. Dihydroxyacetone-P acyltransferase

This enzyme is an integral membrane protein exclusively localized to the lumenal side of peroxisomes (A. Poulos, 1993). Reports on the presence of this enzyme in other organelles are likely a result of peroxisomal contamination. This acyltransferase is important in ether lipid biosynthesis (Chapter 9). Once 1-acyldihydroxyacetone-P has been formed it can be used as a substrate for 1-alkyldihydroxyacetone-P synthesis (Chapter 9) or can be reduced to lyso-PA by a peroxisomal acyldihydroxyacetone-P reductase (Fig. 1) that also utilizes 1-alkyldihydroxyacetone-P as a substrate.

2.4. Phosphatidic acid phosphatase

This enzyme hydrolyzes PA to DG which can be converted to TG, phosphatidylcholine (PC), or phosphatidylethanolamine (PE) (Figs. 1 and 2). Two types of the phosphatase have been identified. The activity of the cytosolic-ER form is dependent on Mg^{2+} and is inhibited by thiol-reactive reagents such as *N*-ethylmaleimide. This enzyme activity can be regulated by reversible translocation between cytosol and ER. The cytosolic form of the enzyme is inactive and is translocated to the ER membrane in the presence of fatty acids, fatty acyl-CoAs, and PA. Since the substrate, PA, is found on the ER it is logical to expect that the ER is where the enzyme functions in the cell. Many attempts to purify the mammalian enzyme and clone the cDNA were unsuccessful. However, the yeast ortholog was purified and the cDNA was cloned and expressed [2]. Unexpectedly, it was discovered that the mammalian ortholog of this phosphatase is lipin-1, a lack of which in mice causes fatty liver dystrophy, a deficiency of adipose tissue (lipodystrophy), insulin resistance, and increased β-oxidation [3]. Two additional lipins have been described that have PA phosphatase activity (K. Reue, 2007). In lipin-1 deficient mice, hepatic PA phosphatase activity is normal suggesting that lipin-2 and/or -3 account for most of the PA phosphatase activity in liver. Hence, the phenotype of fatty liver dystrophy does not appear to be due to a lack of lipin-1 in liver but rather due to a lack of the enzyme in muscle and adipose tissues. Since fatty acids cannot be utilized normally for glycerolipid synthesis in these tissues, they may be directed to the liver where they are converted to

TG via lipin-2, -3. In addition, lipin-1 has a putative nuclear localization signal and lipin-1 expression is induced by fasting and glucocorticoids via a mechanism that depends on PGC1-α, a co-activator of the transcription factor peroxisome proliferator activated receptor-α (Chapter 10). Evidence suggests that lipin cooperates with this transcription factor and its co-activator PGC1α to activate expression of many genes involved in fatty acid oxidation (D.P. Kelly, 2006).

In contrast to lipin-1, the second type of PA phosphatase is neither inhibited by *N*-ethylmaleimide nor stimulated by Mg^{2+}. The cDNA encoding this phosphatase was cloned and expressed [3] and appears to be a glycosylated protein of the plasma membrane with six putative transmembrane regions. This lipid phosphatase also hydrolyzes ceramide-1-phosphate, sphingosine-1-phosphate, and lyso-PA. The active site is predicted to face outside the cell and has activity with lyso-PA but low activity with PA (H. Kanoh, 2000). Thus, the plasma membrane phosphatase probably plays no role in intracellular phospholipid biosynthesis. Consistent with a signaling role, however, the plasma membrane phosphatase shares 34% sequence identity with the Wunen protein that also has phosphatase activity and functions in germ cell migration in *Drosophila* embryos. Furthermore, both of these proteins are similar to Dri42, a protein whose expression is upregulated during epithelial differentiation of rat intestinal mucosa.

A novel DG pyrophosphate phosphatase has been identified in yeast (G.M. Carman, 1997). The enzyme has PA phosphatase activity but prefers DG pyrophosphate as substrate. A yeast mutant defective in the pyrophosphatase gene is viable and accumulates DG pyrophosphate (G.M. Carman, 1998). The enzyme is similar to the mammalian plasma membrane PA phosphatase that also hydrolyzes DG pyrophosphate. The biological function of DG pyrophosphate is unknown. Since this lipid is present at a very low level in yeast (0.18 mol% of total phospholipids), DG pyrophosphatase might have a signaling function.

3. Phosphatidylcholine biosynthesis

3.1. Historical background

PC was first described by Gobley in 1847 as a component of egg yolk and was named 'lecithin' after the Greek equivalent for egg yolk (*lekithos*). In the 1860s Diakonow and Strecker demonstrated that lecithin contained two fatty acids linked to glycerol and that choline was attached to the third hydroxyl by a phosphodiester linkage. The first significant advance in understanding PC biosynthesis occurred in 1932 with the discovery by Charles Best that animals have a dietary requirement for choline. In the 1950s, the CDP-choline pathway for PC biosynthesis (Fig. 3) was described by Eugene Kennedy and co-workers. A key observation was that CTP, rather than ATP, was the activating nucleotide for PC biosynthesis [4]. CTP is required not only for PC biosynthesis but also for the de novo synthesis of all phospholipids (prokaryotic and eukaryotic) excluding PA which can be considered as an intermediate in glycerolipid biosynthesis).

An alternative pathway for PC biosynthesis, of quantitative significance only in liver, is the conversion of PE to PC via PE methylation (Fig. 4). This pathway was first observed

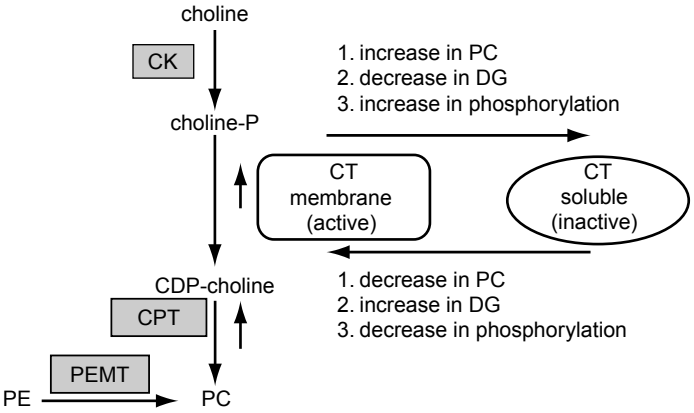


Fig. 3. Regulation of PC biosynthesis via the CDP-choline pathway by modulation of the binding of CTP: phosphocholine cytidylyltransferase (CT) to membranes. Three different modes of regulation of CT activity are indicated. The abbreviations are: CK, choline kinase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; PEMT, phosphatidylethanolamine *N*-methyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol.

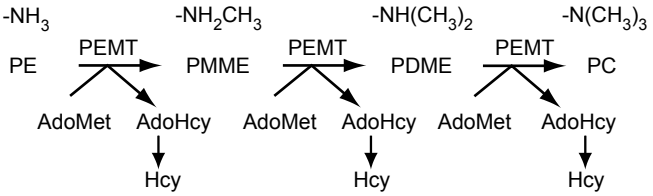


Fig. 4. Reactions catalyzed by phosphatidylethanolamine *N*-methyltransferse (PEMT). AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine: Hcy, homocysteine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine.

in 1941 when Stetten fed [^{15}N]ethanolamine to rats and isolated [^{15}N]choline. Two decades later Bremer and Greenberg detected a microsomal enzyme activity that converted PE to PC via transfer of methyl groups from *S*-adenosylmethionine.

3.2. Choline transport and oxidation

Choline is not made de novo in animal cells except by the methylation of PE to PC and subsequent hydrolysis of the choline moiety from PC. Therefore, choline is imported from extracellular sources. Choline is an important nutrient in the diet of animals and is required in the medium of animal cells in culture (H. Eagle, 1955). Choline is essential because of the cell's requirement for PC for growth and division. There are two distinct transport mechanisms for entry of choline into cells [5]; a high affinity (K_m or $K_t < 5$ μM), Na^+-dependent transporter and a lower affinity ($K_t > 30$ μM), Na^+-independent transporter. Several cDNAs encoding proteins that show high affinity transport of choline have been reported.

Once choline has entered the cell, its normal fate is rapid phosphorylation by choline kinase (Fig. 3). In neurons choline is also converted to the neurotransmitter, acetylcholine. Choline can also be oxidized to betaine [-00C-CH_2–$N^+(CH_3)_3$] in the liver and kidney. In liver, betaine is an important donor of methyl groups for methionine biosynthesis. Betaine is produced in mitochondria into which choline is transported by a specific transporter on the inner membranes. Next, choline is oxidized to betaine aldehyde by choline dehydrogenase on the inner leaflet of the mitochondrial inner membranes and the subsequent conversion to betaine is catalyzed by betaine-aldehyde dehydrogenase in the mitochondrial matrix. Betaine can be transported into kidney medulla by a betaine transporter. In renal medulla and many plants and organisms, betaine accumulates as an osmolyte (a small organic solute that accumulates in response to hypertonicity without adverse effects to the cell or organism) (J.S. Handler, 1992). Hypertonicity of the renal medulla is important for the kidney's ability to concentrate urine.

3.3. Choline kinase

The conversion of choline to choline-P was first demonstrated in yeast extracts by Wittenberg and Kornberg (more famous for his contributions to DNA replication) in 1953. The enzyme was purified (K. Ishidate, 1984) from rat kidney and shown also to phosphorylate ethanolamine [5]. This kinase is now referred to as choline kinase β. Three isoforms of choline kinase have been identified (α1 α2, and β). Northern analyses indicate that the mRNA encoding choline kinase α1 is most abundant in testis. Choline kinase α2 is a splice variant of choline kinase α1. The choline kinase α and β genes (*Chka* and *Chkb*, respectively) have been characterized. The length of *Chka* is 40 kb whereas *Chkb* is only 3.5 kb in length (K. Ishidate, 2000).

Induced mutations in both choline kinase genes have been reported (Table 1). Targeted deletion of *Chka* results in embryonic lethality around day 4 (G. Wu, 2008). A natural mutation in choline kinase β was described in the *rmd* mouse that has hindlimb muscular dystrophy and neonatal bone deformity [6]. Although choline kinase β appears to be the major isoform in muscle, the levels of PC are only slightly decreased in the hindlimb muscles of *rmd* mice.

3.4. CTP:phosphocholine cytidylyltransferase (CT)

CT activity was first described by Kennedy and Weiss in 1955. Over three decades later CT was finally purified to homogeneity (P.A. Weinhold, 1987). The *CT* gene was cloned from *Saccharomyces cerevisiae* (S. Yamashita, 1987) by complementation of a yeast mutant defective in CT activity and rat liver CT cDNA was subsequently cloned (R.B. Cornell, 1990). CT exists as a homodimer in soluble extracts of rat liver and is also associated with membranes. In most cells CT is thought to exist in an inactive reservoir in its soluble form and to be active when associated with membranes (Fig. 3).

Two genes (*Pcyt1a* and *Pcyt1b*) encode distinct isoforms of CT, α, and β, respectively. The CTα gene spans approximately 26 kb. Exon 1 is untranslated, exon 2 encodes the translation start site and a nuclear localization signal, exons 4–7 encode the catalytic domain, exon 8 encodes the alpha helical membrane-binding domain, and exon 9 encodes

a C-terminal phosphorylation domain (I. Tabas, 1997). The *CTβ* gene is located on the X chromosome of humans and mice and encodes two isoforms in mice, CTβ2 and CTβ3. The CTβ isoforms differ from CTα at the amino terminus because they lack the nuclear localization signal and are therefore found in the cytoplasm of animal cells. CTβ2 differs from CTβ3 at the N-terminus due to alternative splicing of exons 1 and 2 of the CTβ gene (S. Jackowski, 2003). There are significant differences between the sequences of the phosphorylation domains of CTα and CTβ2/3.

CT has classically been considered a cytoplasmic enzyme since its activity is found in the cytosol and on microsomal membranes in cellular homogenates. However, Kent and co-workers demonstrated that CT is present in the nuclear matrix and is associated with the nuclear membrane (C. Kent, 1997). To explore the role of the nuclear localization signal in CTα, experiments were performed in a CHO mutant (MT-58) that was temperature-sensitive for CT activity. In MT-58 cells, CT activity is present at low levels and the cells grow at 33°C (C. Raetz, 1980). At the restrictive temperature of 40°C, CT activity is abolished and the cells die via apoptosis (F. Tercé, 1996). CTα in which residues 8–28 (the nuclear localization signal) were deleted, was expressed in MT-58 cells and the result was that CT was largely, but not exclusively, present in the cytoplasm. These cells survived at the restrictive temperature. However, since some CTα was expressed in the nucleus, these experiments did not unambiguously demonstrate that cells can grow and divide when CTα is present only in the cytoplasm. Intriguing evidence indicates that CTα migrates into the cytoplasm during the G1 phase of the cell cycle, at a time when PC biosynthesis is activated (R.B. Cornell, 1999). CTα is not exclusively nuclear in all cell types but appears to be largely cytoplasmic in pulmonary epithelial cells and tissues (M. Post, 2001). Thus, the role the nuclear localization signal of CT in cellular PC biosynthesis remains an intriguing question. The nucleus contains a network of tubular invaginations of the nuclear envelope, the nucleoplasmic reticulum, the precise function of which is unknown. Recent studies suggest that proliferation of the nucleoplasmic reticulum is regulated by CTα (N.D. Ridgway, 2005).

The lipid-binding domain and the phosphorylated domains of CT are involved in the regulation of CT activity. To determine the function of these regions of CT, these domains of CTα were deleted by either proteolysis with chymotrypsin or by construction of CTα truncation mutants [7]. For example, CTα truncated at amino acid residue 314, lacks the phosphorylation segment, and CTs truncated at residues 236, 231, or 228 lack both the phosphorylation and lipid-binding domains When both the lipid-binding and phosphorylation domains were deleted, CT was a soluble, active enzyme that did not bind to membranes. Thus, the lipid-binding domain regulates the binding to membranes and the activation of CT. The binding of phospholipids to CT appears to activate the enzyme by decreasing the apparent K_m for CTP (S.L. Pelech, 1982; Jackowski, 1995).

3.5. CDP-choline:1,2-diacylglycerol cholinephosphotransferase

This enzyme was also discovered by Kennedy and co-workers and is considered to be located on the ER but is also found on the Golgi, MAM, and nuclear membranes (C.R. McMaster, 1997). Although the enzyme has been known for more than four decades, and despite intense efforts in many laboratories, the cholinephosphotransferase

has never been purified. Purification of the enzyme has been difficult because it is an intrinsic, membrane-bound protein that requires detergents for solubilization. Detergents complicate purification procedures commonly used, such as gel filtration, because the protein binds to micelles. The purification of membrane-bound enzymes has been described as 'masochistic enzymology' (D.E. Vance, 1990).

Yeast genetics has, however, permitted the cloning of cholinephosphotransferase. Two genes, *CPT1* and *EPT1*, each account for 50% of the cholinephosphotransferase activity in yeast extracts (C.R. McMaster, 1997). By construction of null mutations in these two genes, *CPT1* was shown to be responsible for 95% of the PC made, whereas the *EPT1* gene product accounts for 5%. The *EPT1* gene product utilizes both CDP-choline and CDP-ethanolamine whereas CPT1 catalyzes only the reaction with CDP-choline. A human dual specificity choline/ethanolaminephosphotransferase cDNA (hCEPT1) was cloned and expressed (C.R. McMaster, 1999). The open reading frame predicts a protein with seven membrane-spanning domains. Subsequently, a human cDNA was cloned that encoded a CDP-choline-specific enzyme (hCPT1) that had 60% sequence identity to hCEPT1. hCEPT1 mRNA is expressed ubiquitously in all tissues tested whereas the expression of hCPT1 is highest in heart, testis, intestine, and colon.

Cholinephosphotransferase acts at a branchpoint in the metabolism of DG (Fig. 1) that can be converted into PE, TG, and PA. Most studies indicate that cells and tissues contain an excess of cholinephosphotransferase activity. Hence, the amount of active enzyme does not normally limit PC biosynthesis. However, it is clear that the in vivo activity of cholinephosphotransferase is regulated by substrate supply. The supply of one of the substrates (CDP-choline) is regulated by the activity of CT (Section 3.4), and the supply of DG seems to be controlled by the availability of fatty acids. Excess DG that is not utilized for PC or PE biosynthesis is stored in the liver as TG.

3.6. Phosphatidylethanolamine N-methyltransferase

All nucleated cells contain PC and utilize the CDP-choline pathway. Thus, it was not obvious why the alternative pathway for PC synthesis, the PE methylation pathway (Fig. 4), survived during evolution. Nor was it obvious why PE methyltransferase (PEMT) activity is essentially a liver-specific enzyme whereas 2% or less of the hepatic PEMT activity is found in other tissues. In addition to producing PC, PEMT generates three *S*-adenosylhomocysteine molecules for each PC synthesized (Fig. 4). The *S*-adenosylhomocysteine is catabolized to homocysteine that can be recycled to form methionine, converted to cystathionine, or secreted into plasma. The PEMT reaction accounts for ~50% of plasma homocysteine (A. Noga, 2003) which is an independent risk factor for cardiovascular disease.

PEMT, an intrinsic membrane protein, was purified from rat liver microsomes (N.R. Ridgway, 1987). Sequence of the amino terminus enabled the cloning of the cDNA encoding rat PEMT (Z. Cui, 1993). However, immunoblotting experiments with an antibody directed against the carboxyl terminal peptide showed immunoreactivity only with a protein that was exclusively localized to MAM (Z. Cui, 1993). This isoenzyme of PEMT is referred to as PEMT2 whereas the activity on the ER is designated PEMT1. Both PEMTs catalyze all three transmethylation reactions that convert PE to PC (Fig. 4). The

difference between PEMT1 and PEMT2 has not been established but only one *PEMT* gene has been identified in mice and humans. The human gene encoding PEMT has been cloned and characterized. Human and mouse liver have three mRNAs encoding PEMT that differ only at the 5′ end, in a non-coding region (D.J. Shields, 2001). Thus, the three transcripts encode the same protein.

The first knockout mouse in phospholipid biosynthesis was one in which the *Pemt* gene was disrupted, resulting in a complete lack of PEMT activity [8]. $Pemt^{-/-}$ mice are fertile and appear outwardly normal. As compensation for the lack of PEMT, CT activity in the liver is increased by 50%. Since the mice retain the CDP-choline pathway, the lack of an obvious phenotype is not surprising. However, when the $Pemt^{-/-}$ mice were fed a choline-deficient diet for 3 days (which attenuates PC synthesis via the CDP-choline pathway) the mice exhibit liver failure (C.J. Walkey, 1998) whereas $Pemt^{+/+}$ mice that were fed the choline-deficient diet were normal with no obvious liver pathology. Thus, it appears that the PEMT pathway has survived during evolution to provide PC at times when the CDP-choline pathway is less active (e.g., during starvation, pregnancy, or in mothers during suckling) (S.H. Zeisel, 2000). Consequently, the PEMT pathway might provide an evolutionary advantage. The structurally related compound, dimethylethanolamine [$HOCH_2$-CH_2-$N^+(CH_3)_2$] does not substitute for choline in $Pemt^{-/-}$ mice even though it is converted to the closely related PC analog phosphatidyldimethylethanolamine (K.A. Waite, 2002). Thus, the third methyl group on PC has a critical function in mice. Propanolamine can substitute for choline in yeast that lack the methylation pathway for PC synthesis (D.R. Voelker, 2004) whereas propanolamine does not substitute for choline in $Pemt^{-/-}$ mice (Z. Li, 2007).

The mechanism responsible for the rapid liver failure in $Pemt^{-/-}$ mice fed the choline-deficient diet has been determined. A mouse liver secretes the equivalent of its entire pool of PC (~22 mg) into bile each day. To determine if the drain of PC into bile were responsible for the liver failure, $Pemt^{-/-}$ mice were bred with mice that lacked ABCB4 (MDR2), a protein responsible for flipping PC across the membrane of the bile duct (canalicular membrane) so PC can be secreted into bile. The $Pemt^{-/-}/Mdr2^{-/-}$ mice did not secrete PC into bile (Z. Li, 2005). Dramatically, however, $Pemt^{-/-}/Mdr2^{-/-}$ mice fed the choline-deficient diet survived for at least 90 days whereas $Pemt^{-/-}$ mice fed this diet survived for only 3–4 days. A variety of experimental approaches strongly suggest that choline-deficient $Pemt^{-/-}$ mice die from liver failure because the ratio of PC to PE in the plasma membrane and other membranes of hepatocytes is decreased [9]. PC is a cylindrical shaped molecule whereas PE has an inverted cone shape (Chapter 1). When the concentration of PC decreases in choline-deficient $Pemt^{-/-}$ mice, PC on the cell surface is replaced with PE and the integrity of the cell membrane is compromised. Consequently, the liver leaks hepatic enzymes leading to inflammation (steatohepatitis) and rapid liver failure.

Yeast also use both the PE methylation pathway and the CDP-choline pathway. In yeast two enzymes convert PE to PC (S.A. Henry, 1997). The methylation of PE to phosphatidylmonomethylethanolamine is catalyzed by the *PEM1*/*CHO2* gene product whereas the subsequent two methylations are catalyzed by the *PEM2*/*OPI3* gene product. Deletion of both *PEM1* and *PEM2* genes is lethal unless the growth medium contains choline. Thus, the CDP-choline pathway and the PE methylation pathway can compensate for each other in yeast.

Bacteria generally do not contain PC but *Rhodobacter sphaeroides* and some other bacteria make PC by the methylation of PE. Interestingly, this methyltransferase is a soluble protein that has virtually no homology to PEMT or the yeast enzymes (V. Arondel, 1993). Also, in some bacteria, such as *Sinorhizobium meliloti*, a novel choline-dependent pathway was discovered in which choline reacts with CDP-DG to form PC (O. Geiger, 2003).

4. Regulation of phosphatidylcholine biosynthesis

4.1. The rate-limiting reaction

The CT reaction usually limits the rate of PC biosynthesis. The first evidence in support of this conclusion was drawn from the relative pool sizes of the aqueous precursors (in rat liver, choline = 0.23 mM, phosphocholine = 1.3 mM, CDP-choline = 0.03 mM). Calculation of these values assumes that 1 g wet tissue is equivalent to 1 ml and that there is no compartmentation of the pools. The second assumption may not be valid as there is evidence for compartmentation of PC precursors (M.W. Spence, 1989). The concentration of phosphocholine is 40-fold higher than that of CDP-choline, consistent with a 'bottleneck' in the pathway at the reaction catalyzed by CT. Pulse-chase experiments illustrate this bottleneck more vividly. After a 0.5 h pulse of hepatocytes with [methyl-^{3}H]choline, more than 95% of radioactivity in the precursors of PC was in phosphocholine, with the remainder in choline and CDP-choline. When the radioactivity was chased with unlabeled choline, labeled phosphocholine was quantitatively converted to PC (Fig. 5). The radioactivity in CDP-choline remained low during the chase and CDP-choline was rapidly converted to PC. There was minimal radioactivity in choline which suggests that choline is immediately phosphorylated after it enters the cell. It is important to note that if a cell or tissue is in a steady state, pool sizes and reaction rates do not change. Thus, although the rate of PC synthesis is determined by the CT reaction, the rates of the reactions catalyzed by choline kinase and cholinephosphotransferase are the same as that of the reaction catalyzed by CT, otherwise, the pool sizes of precursors would change. For example, if the choline kinase reaction were faster than the CT reaction, the amount of phosphocholine would increase. Thus, CT sets the pace of the pathway.

4.2. The translocation hypothesis

CT is recovered from cells and tissues in both the cytosol and microsomal membranes. However, in the early 1980s evidence from several laboratories suggested a close correlation between CT activity on membranes and the rate of PC biosynthesis. The proposed explanation for this observation was that the active form of CT was membrane-bound whereas CT in the cytosol acted as an inactive reservoir (Fig. 3). In agreement with this proposal, cytosolic fractions contain essentially no phospholipid and CT requires phospholipids for activity. Thus, cells have a facile mechanism for altering the rate of PC biosynthesis by reversibly translocating CT between a soluble, inactive reservoir, and

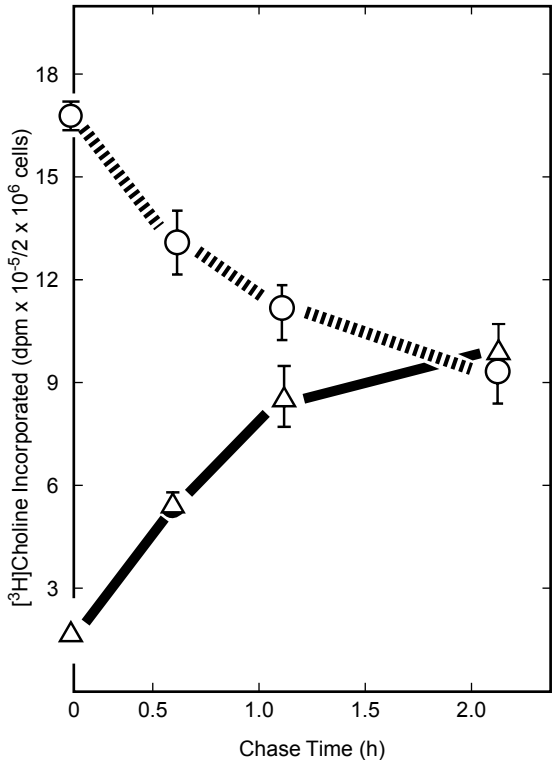


Fig. 5. Incorporation of [^{3}H-methyl]choline into phosphocholine and PC as a function of time. Hepatocytes from rat liver were incubated with [^{3}H]choline for 30 min. Subsequently, the cells were washed and incubated (chased) for indicated times with unlabeled choline. The disappearance of radioactivity from phosphocholine (dashed line) and its appearance in PC (solid line) are shown. Adapted from Fig. 1 of Ref. [20], with permission.

cellular membranes. This mechanism for activation applies to CTα and both CTβ2 and CTβ3.

Binding of CT to membranes begins by electrostatic adsorption followed by hydrophobic interactions that involve intercalation of the protein into the non-polar core of the membrane [7] (Fig. 6). When insertion of CT into the membrane lipids is blocked in the presence of viscous gel phase lipids, CT binds electrostatically to the membrane but is not activated. Four properties of membranes promote CT insertion [7]: (i) interfacial packing defects as might occur when lipids with small head groups such as DG are in the membrane; (ii) low lateral surface pressure (loose packing) as observed in highly curved compared to planar bilayers; (iii) acyl chain disorder that can be caused by oxidation of the fatty acyl chains; (iv) curvature strain that occurs when membranes are enriched in hexagonal phase-preferring lipids such as PE and DG. Synthesis of PC would reverse these properties of membranes resulting in a more stable bilayer. See Chapter 1 for more information on CT translocation.

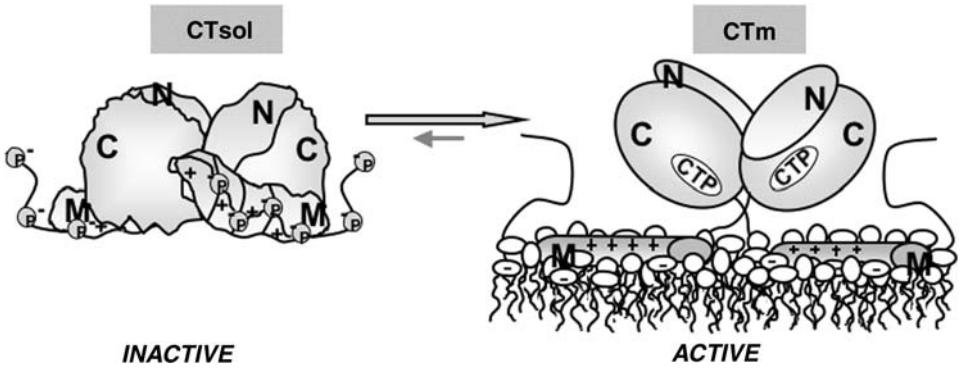


Fig. 6. Translocation of CTP:phosphocholine cytidylyltransferase (CT) from an inactive soluble form (CTsol) to a membrane-associated activated form (CTm). The reversible interaction of CT with membranes involves the amphipathic helical region lying on the surface of the membrane so that the hydrophilic region interacts with the negatively charged lipid head groups and the hydrophobic side intercalates into the membrane core. N, amino terminal domain; C, carboxyl terminal domain; M, membrane-binding domain. Figure kindly supplied by Prof. R. Cornell, Simon Fraser University. (See color plate section, plate no. 7.)

4.3. Regulation of phosphatidylcholine biosynthesis by lipids

As indicated in Fig. 3, the association of CT with membranes and CT activation can be modulated by lipids. Both feedforward and feedback mechanisms for regulation of CT activity have been identified. DG can alter the rate of PC biosynthesis both as a substrate and as a modulator of CT binding to membranes. In vitro, an increase in the DG content of membranes enhances the binding of CT. Feedback regulation of CT and PC biosynthesis by PC has also been described (H. Jamil, 1990). An elegant feedback regulation of CT has been shown in the yeast *S. cerevisiae* (V.A. Bankaitis, 1995). Sec14p is an essential gene product that when assayed in vitro transfers phosphatidylinositol (PI) or PC between membranes with a preference for PI (Chapter 16). PC-bound Sec14p inhibits the CDP-choline pathway via CT. In contrast, when PI is bound CT is minimally inhibited. Thus, in yeast under conditions where PC is abundant, there is a feedback inhibition of CT and the CDP-choline pathway.

CTP has also been implicated as a regulator of PC synthesis in animals and yeast. For example, over-expression of CTP synthetase in yeast stimulates the biosynthesis of PC via the CDP-choline pathway (G.M. Carman, 1995).

4.4. Phosphorylation of cytidylyltransferase

CTα contains a domain that is extensively phosphorylated and the state of phosphorylation can affect CT activity (S.L. Pelech, 1982). CT that is bound to membranes is less phosphorylated than soluble CT. Incubation of hepatocytes with oleic acid demonstrated that CT associates with membranes in an active, phosphorylated form and that is subsequently dephosphorylated (M. Houweling, 1994). Thus, a change in the lipid composition of membranes mediates the initial binding of CT to membranes and subsequently CT is

dephosphorylated. Deletion of the phosphorylation domain of CT does not impair the ability of CT to make enough PC for cell survival. Stable transfection of mutant CHO cells (MT-58) that have a temperature-sensitive defect in CT (C. Kent, 1995) with a cDNA encoding CTα lacking the phosphorylation domain allowed the MT-58 cells to grow at the restrictive temperature. Interestingly, transfection of CT that lacked both the lipid-binding domain and the phosphorylation domain also rescued these cells (C. Kent, 1999). Thus, neither of these domains is essential for CT activity. Nevertheless, the phosphorylation domain of CT does appear to be important for regulating CT activity because CT activity is inhibited when Ser 315 is phosphorylated by ERK (p42 kinase) (R.K. Mallampalli, 2005). Thus, the current view is that in physiologically relevant cell models, the state of phosphorylation can regulate CT activity.

4.5. Transcriptional and post-transcriptional regulation of CTα

Most studies on CT activity and PC biosynthesis have not indicated regulation at the level of gene expression. The ability of a cell to translocate and activate the soluble form of CT would normally satisfy the cell's requirement for PC. Nevertheless, some control over the expression of the genes encoding CT does occur. The proximal promoter of the CTα gene contains numerous potential regulatory elements (I. Tabas, 1997). Sp1, the first mammalian transcription factor to be purified and cloned (R. Tjian, 1986, 1987), plays an important role in regulating the expression of the *CTα* gene (M. Bakovic, 1999) and the related nuclear factor, Sp3, also activates CTα transcription (M. Bakovic, 2000). In addition to Sp1 and Sp3, transcription factors important in cell division and growth (Rb, Ets-1, Tef4, and E2F) activate CTα transcription [10] whereas a relative of Ets-1, Net, inhibits CTα transcription and binds at the same element on the CTα promoter. As discussed in Chapters 6, 10, and 14, SREBP is a master regulator of fatty acid and cholesterol biosynthesis. Results from several labs suggest that SREBP does not play a direct role in regulation of CTα transcription (N.D. Ridgway, 2003). Thus, it seems that transcriptional regulation of CTα is mainly linked to cell cycle, growth, and differentiation rather than cholesterol homeostasis or energy metabolism.

The level of CTα mRNA can also be regulated by alterations in mRNA stability. When a macrophage cell line was depleted of colony stimulating factor and then repleted, CTα mRNA increased 4-fold (S. Jackowski, 1991) because the stability of CTα mRNA increased. An increase in CTα mRNA in fetal lung type II cells has also been ascribed to enhanced mRNA stability (M. Post, 1996).

Relationships between proliferation of the ER and PC biosynthesis have been described. A spliced form of the transcription factor X-box binding protein 1, XBP1(S), stimulated proliferation of the ER and PC biosynthesis (J.W. Brewer, 2004). Subsequently, it was demonstrated that there was an increase in CTα activity and protein when XBP1(S) was over-expressed in fibroblasts (J.W. Brewer, 2007). The increase in CTα expression was due to elevated translation of the enzyme and not to enhanced transcription. Thus, the authors speculated that XBP1(S) indirectly stimulates PC biosynthesis by altering the synthesis of an unknown protein that enhances CTα translation. The level of CTα can also be modulated by proteolysis by caspases (N.D. Ridgway, 2002) and via the ubiquitin-proteosome pathway (R.K. Mallampalli, 2000).

4.6. Transgenic and gene-disrupted mouse models of CTα and β

Targeted mutations in the *Pcyt1a* and *Pcyt1b* genes (encoding *CTα* and CTβ2, respectively) have been reported (Table 1). Elimination of CTα results in early embryonic lethality around day 3 (S. Jackowski, 2004). In contrast CTβ2 knockout mice survive with gonadal degeneration and reproductive deficiency [11].

However, using the Cre-lox method for selective disruption of a gene in specific cell type, it is possible to explore the function a particular gene in a single tissue. Thus, the *CTα* gene was selectively disrupted in hepatocytes at birth when Cre recombinase was expressed behind the albumin promoter. These mice survived without CTα in the liver although CT activity was only ~15% of that in wild-type mice (R.L. Jacobs, 2004). The remaining CT activity was attributed to CTβ2 in hepatocytes and non-hepatic cells in liver that still expressed CTα. The plasma levels of PC, cholesterol, TG, and apo B100 were decreased in these mice and a defect in very low density lipoprotein secretion was demonstrated (Chapter 18). Thus, CTα in the liver is required for normal plasma lipid homeostasis. Although PEMT activity was increased by 2-fold in these mice, this did not compensate for the deficiency in CTα.

Similarly, the role of CTα in lung development was studied in an inducible, epithelial cell-specific CTα knockout mouse (S. Jackowski, 2007). Elimination of CTα starting at embryonic day 7.5 did not adversely limit lung development but resulted in respiratory failure at birth. Thus, CTα is required for secretion of PC and formation of lamellar bodies and surfactant protein homeostasis.

To determine if enhanced PC biosynthesis protected macrophages from excess cholesterol-induced toxicity, a truncated version of CTα lacking the phosphorylation domain was expressed specifically in macrophages of mice under control of the scavenger receptor (Chapter 21) promoter (I. Tabas, 1999). These cells were protected from cholesterol-induced toxicity. Expansion of the amount of PC probably entraps cholesterol, thereby decreasing its toxicity. CTα expression was also eliminated in macrophages using the Cre-lox method for selective gene disruption in specific cells (I. Tabas, 2000). The lack of CTα and the resulting decrease in PC biosynthesis enhanced the sensitivity of macrophages to apoptosis in response to cholesterol loading. In the absence of cholesterol loading, the macrophages without CTα appeared normal, possibly due to increased expression of CTβ2.

5. Phosphatidylethanolamine biosynthesis

5.1. Historical background and biosynthetic pathways

PE was first described in 1884 by Thudichum who reported that ‘kephalin’, a nitrogen- and phosphorus-containing lipid, was different from lecithin. In 1930 Rudy and Page isolated the first pure preparation of PE and its structure was elucidated in 1952 by Baer and colleagues.

The biosynthesis of PE in eukaryotic cells can occur by four pathways (Figs. 1 and 7). The CDP-ethanolamine pathway represents the de novo synthesis of PE as first described by Kennedy and Weiss in 1956. The other pathways for PE biosynthesis involve

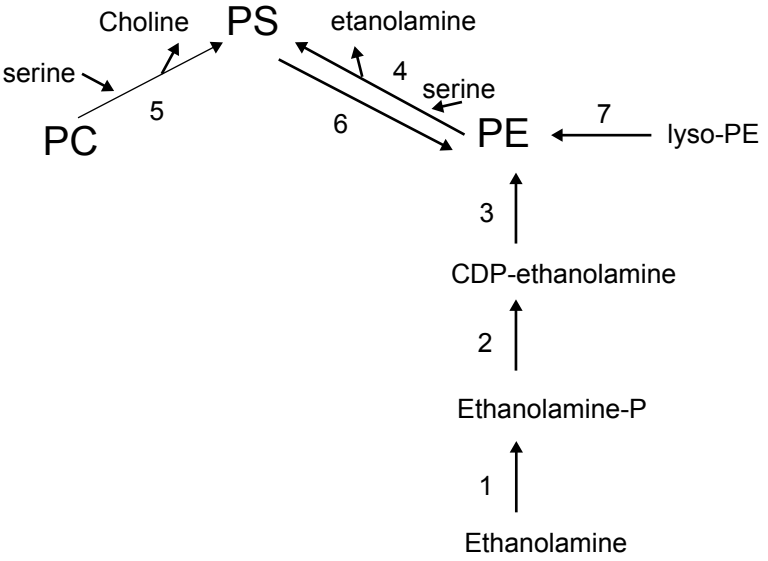


Fig. 7. Pathways for the biosynthesis of phosphatidylethanolamine (PE) and phosphatidylserine (PS) in animals. The numbers indicate the enzymes. 1, ethanolamine kinase; 2, CTP:phosphoethanolamine cytidylyltransferase; 3, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; 4, PS synthase-2; 5, PS synthase-1; 6, PS decarboxylase; 7, acyl-CoA:lyso-PE acyltransferase. PC, phosphatidylcholine.

modification of a pre-existing phospholipid. For example, one major pathway for PE synthesis in eukaryotes is the decarboxylation of PS (E.P. Kennedy, 1964). The two other (quantitatively minor) pathways for PE synthesis in eukaryotic cells (Figs. 1 and 7) are the acylation of lyso-PE and a base-exchange reaction in which ethanolamine is exchanged for serine of PS (Section 6). Interestingly, the decarboxylation of PE occurs on the external leaflet of mitochondrial inner membranes (J. Zborowski, 1983) whereas the final reaction of the CDP-ethanolamine pathway occurs on the ER. Thus, it is possible that (at least) two spatially distinct pools of PE exist — one made in mitochondria, the other in the ER. The quantitative contribution of the two major pathways for PE synthesis in mammalian cells is not clear but appears to depend on the cell type. In rat liver/hepatocytes and hamster heart the CDP-ethanolamine pathway was reported to produce the majority of PE whereas in many types of cells PS decarboxylase makes >80% of PE even when ethanolamine is abundant (D.R. Voelker, 1984). It should be noted, however, that in all studies in which the relative contribution of the two PE biosynthetic pathways has been evaluated, the pool of the immediate precursor of PE was assumed to be homogeneously radiolabeled, an assumption that is not necessarily valid (K.S. Bjerve, 1985).

5.2. Enzymes of the CDP-ethanolamine pathway

The reactions of the CDP-ethanolamine pathway (Figs. 1 and 7) parallel those of the CDP-choline pathway for PC synthesis (Section 3). Ethanolamine is an obligatory precursor of PE synthesis by this route and is derived from the diet with smaller amounts

being produced from PE degradation. Another endogenous source of ethanolamine results from the degradation of sphingosine-P by sphingosine-P lyase (Chapter 13). The ethanolamine generated by these reactions can subsequently be utilized for PE biosynthesis via the CDP-ethanolamine pathway. The direct decarboxylation of serine to ethanolamine has not been demonstrated in mammalian cells although this reaction is the major source of ethanolamine in the plant *Arabidopsis thaliana* (A.D. Hanson, 2003).

In the CDP-ethanolamine pathway, ethanolamine is first phosphorylated by ethanolamine kinase. Four related genes encode proteins with ethanolamine kinase activity. Two of these primarily phosphorylate choline (Section 3.3) but also phosphorylate ethanolamine to a lesser extent. In addition, higher eukaryotes contain ethanolamine-specific kinases. For example, a *Drosophila* ethanolamine kinase, encoded by the *eas* gene, possesses negligible choline kinase activity (P. Pavlidis, 1994). A defect in this gene results in the neurological 'bang-sensitive' phenotype (transient paralysis following a mechanical shock). Mammals express two ethanolamine-specific kinases, ETNK1 (S. Jackowski, 2001) and ETNK2 (S. Jackowski, 2006), both of which are soluble cytosolic proteins but their tissue distribution is different. Ethanolamine kinase-2 is most highly expressed in liver and reproductive tissues whereas ETNK1 is more widely expressed, with highest expression in liver and kidney. Homozygous disruption of the *Etnk2* gene in mice did not affect liver phospholipid metabolism, neural development, or testicular function. However, female *Etnk2*$^{-/-}$ mice exhibited a marked increase in placental thrombosis leading to a reduction in litter size and perinatal death of ~20% of the pups (S. Jackowski, 2006).

The second step in the CDP-ethanolamine pathway is catalyzed by CTP:phosphoethanolamine cytidylyltransferase (ET) which converts phosphoethanolamine to CDP-ethanolamine (Figs. 1 and 7). The enzyme is located primarily in the cytosol but has also been detected in rough ER membranes by immunoelectron microscopy. ET is distinct from CT and is not activated by lipids. There is no report of ET, unlike CTα (Section 3.4), being present in the nucleus. Disruption of the *Pcyt2* gene that encodes ET in mice is lethal prior to embryonic day 8.5, illustrating the absolute requirement of ET for mouse development (M. Bakovic, 2007).

In the final reaction of the CDP-ethanolamine pathway, PE is made by reaction between CDP-ethanolamine and DG via CDP-ethanolamine:1,2-DG ethanolaminephosphotransferase (Figs. 1 and 7), an integral membrane protein that resides primarily on ER membranes and to a lesser extent on membranes of the Golgi and MAM. The enzyme has a distinct preference for DG species that contain 1-palmitoyl-2-docosahexaenoyl (22:6) acyl chains. In cultured hepatocytes, nearly 50% of PE made by the CDP-ethanolamine pathway is this species; the function of this striking selectivity is unknown. Mutants of *S. cerevisiae* have been generated that are deficient in an ethanolaminephosphotransferase activity that has dual specificity for both CDP-ethanolamine and CDP-choline and generates PE and PC, respectively (R.M. Bell, 1991). The human ortholog of this gene was cloned and encodes a choline/ethanolaminephosphotransferase activity that was predicted to be solely responsible of the final reaction of PE synthesis via the CDP-ethanolamime pathway (C.R. McMaster, 1999). However, recently, another human cDNA was identified that encodes ethanolaminephosphotransferase activity. This enzyme appears to be specific for CDP-ethanolamine and is likely involved in PE synthesis via the CDP-ethanolamine pathway (Y. Hirabayashi, 2007).

5.3. Regulation of the CDP-ethanolamine pathway

Very little information is available on the mechanisms that control the production of PE via the CDP-ethanolamine pathway. Factors that regulate this pathway for PE production at the level of gene expression have not yet been elucidated. In the 1970s Akesson and Sundler reported that ET catalyzes the rate-limiting step of this pathway (R. Sundler, 1975). In addition, under some metabolic conditions the supply of the substrate, DG, can limit the rate of PE biosynthesis from ethanolamine (L.M.G. van Golde, 1989). Two studies have implicated a channeling of intermediates of the pathway in the biosynthesis of PE in mammalian cells (M.W. Spence, 1989).

5.4. Phosphatidylserine decarboxylase

The second major pathway for PE biosynthesis in eukaryotes is the PS decarboxylation pathway. PS decarboxylase activity was first described by Kanfer and Kennedy in 1964. PS decarboxylase belongs to a small family of decarboxylases that contain a pyruvoyl prosthetic group (E.E. Snell, 1977). The active form of PS decarboxylase is generated by autocatalytic cleavage of a precursor protein (W. Dowhan, 1988). PS decarboxylase is restricted to the outer leaflet of the mitochondrial inner membrane (J. Zborowski, 1983). Consequently, for synthesis of PE by this pathway, PS must be transferred from its site of synthesis in the ER and MAM to mitochondrial inner membranes; this transfer step is rate-limiting for the conversion of PS to PE (D.R. Voelker, 1989). The majority of PE in mitochondria is made in situ in mitochondria via PS decarboxylase whereas only small amounts of PE made by the CDP-ethanolamine pathway in the ER are imported into mitochondria (J.E. Vance, 1995). Mice lacking PS decarboxylase do not survive beyond embryonic day 9 probably because of a mitochondrial defect. Mouse embryonic fibroblasts lacking PS decarboxylase contain fragmented mitochondria that are reminiscent of a defect in mitochondrial fusion (J.E. Vance, 2006). It is likely that elimination of PS decarboxylase reduces the mitochondrial PE content thereby causing mitochondrial abnormalities and embryonic lethality. These studies clearly demonstrate that the CDP-ethanolamine pathway cannot substitute for PS decarboxylase. Thus, both major pathways of PE synthesis are essential during mouse embryonic development.

In contrast to mammalian cells that appear to express only one isoform of PS decarboxylase, yeast have two PS decarboxylase genes. One of these, *PSD1*, is similar to the mammalian PS decarboxylase gene and encodes a mitochondrial protein. Disruption of the *PSD1* gene in yeast revealed the existence of the second PS decarboxylase gene, *PSD2* that encodes a vacuolar/Golgi PS decarboxylase (Chapter 16) (D.R. Voelker, 1995). Yeast lacking both *PSD1* and *PSD2* genes are ethanolamine auxotrophs.

5.5. Functions of PE

All eukaryotic and prokaryotic cell membranes contain PS and PE. In mammalian cells PE is typically the second most abundant phospholipid, comprising ~20% of total phospholipids. Different organelles — plasma membrane, ER, lysosomes, and mitochondria — have distinct phospholipid compositions. The PE content of mitochondria is higher than

that of other organelles. Even within mitochondria the PE content of inner membranes is higher than that of the outer membranes [12]. Not only is PE presumed to serve a structural role in membranes but PE performs numerous additional functions. For example, in *E. coli*, PE has been described as a 'lipid chaperone' that regulates the folding and topology of integral membrane proteins (W. Dowhan, 1998) (Chapter 1). PE is clearly implicated in membrane fusion and fission events (P.R. Cullis, 1983). In addition, PE is required for contractile ring disassembly at the cleavage furrow of mammalian cells during cytokinesis (M. Umeda, 1997). A novel function of PE was revealed in *Drosophila* in which PE is the major membrane phospholipid. Unlike mammalian cells, *Drosophila* do not synthesize cholesterol but the processing of sterol regulatory element-binding protein (Chapter 14) is controlled by PE instead of cholesterol (R.B. Rawson, 2002). PE is also the donor of the ethanolamine moiety of glycosylphosphatidylinositol anchors of many signaling proteins that are located in lipid rafts (Chapters 1 and 2). Furthermore, PE is the precursor of *N*-acylethanolamine that functions as a neurotransmitter in the brain (H.S. Hansen, 1995). The metabolism of PE appears to be particularly important in the heart because the asymmetrical transbilayer distribution of PE in sarcolemmal membranes is altered during ischemia, leading to sarcolemmal disruption and irreversible cell damage (J.A. Post, 1995).

6. Phosphatidylserine biosynthesis

6.1. Historical background and biosynthetic pathways

PS accounts for 5–15% of the phospholipids of eukaryotic cells. In 1941 Folch identified PS as a component of 'kephalin' which was, until that time, thought to consist of PE alone. The correct structure of PS was proposed by Folch in 1948 and confirmed by chemical synthesis in 1955 by Baer and Maurukas. PS is made in prokaryotes (Chapter 3), and yeast via the CDP-DG pathway (Fig. 12). This route for PS synthesis has not been detected in mammalian cells. Instead, in these cells PS is made by a calcium-dependent base-exchange reaction in which the head group of a PC or PE molecule is exchanged for L-serine in a reaction catalyzed by a PS synthase (originally named the 'base-exchange enzyme') (Fig. 7) (H.G. Hubscher, 1959). PS synthesis in plants was, until recently, thought to occur exclusively via a serine-exchange reaction. However, a PS synthase that uses CDP-DG and serine, and is encoded by a cDNA that is 54% identical to the yeast PS synthase, has been identified in wheat (R.C. Gardner, 1999).

6.2. PS synthases

Mammalian cells contain two distinct serine-exchange activities: PS synthase-1 uses PC for the exchange reaction whereas PS synthase-2 uses PE (Fig. 7) [12]. An enzyme that catalyzed the synthesis of PS via serine-exchange with PE was partially purified from rat brain (J.N. Kanfer, 1985) and an epitope-tagged version of this protein was recently purified to near homogeneity (O. Kuge, 2003). The existence of two mammalian PS synthase genes was firmly established by the generation of mutant CHO cells in which

PS was synthesized from PE but not PC (i.e., the cells lacked PS synthase-1 activity) (O. Kuge, 1985; D.R. Voelker, 1986). In these cells the rate of PS synthesis is ~50% lower than in parental CHO-K1 cells and the mass of PS and PE was correspondingly reduced. Growth of the cells was severely impaired unless the culture medium was supplemented with PS, PE, or ethanolamine. The residual serine-exchange activity (contributed by PS synthase-2) in the mutant cells used PE but not PC as substrate (Fig. 7). Further mutagenesis of the CHO cells that lacked PS synthase-1 generated a cell line in which total serine-exchange activity was reduced by 95%, and the level of PS synthase-2 mRNA was reduced by 80%, compared to parental CHO-K1 cells. These cells required supplementation with PS for viability but did not survive when supplemented with PE, suggesting that CHO cells have a requirement for PS that is distinct from its requirement as a precursor of PE.

Mammalian cDNAs encoding the two isoforms of PS synthase were subsequently cloned. The amino acid sequences of PS synthase-1 and PS synthase-2 are ~30% identical and each protein is predicted to contain multiple membrane-spanning domains. As is the case for many lipid biosynthetic enzymes, the serine-exchange activity in mammalian cells is located on microsomal membranes. However, the majority of this activity is not in the bulk of the ER but is highly enriched in MAM, a domain of the ER that has been implicated in the import of PS into mitochondria (J.E. Vance, 2000) (Chapter 16). Although little is known about the active site of either PS synthase, several amino acids that are crucial for serine-exchange activity have been identified (M. Nishijima, 2004). Over-expression of PS synthase-2 in PS synthase-1-deficient CHO cells eliminated the requirement for exogenously added PS. Thus, in CHO cells PS synthase-2 can substitute for PS synthase-1.

Intriguing questions arise from the discovery of two mammalian PS synthases. Why do mammalian cell possess two distinct enzymes that make the same product, PS? Is each PS synthase required for a specific function or does the duplication of enzymatic activities represent merely a back-up mechanism? In phospholipid metabolism many examples are known in which the same, or a similar, enzymatic activity is encoded by more than one gene. Furthermore, as has been clearly illustrated in this chapter, more than one biosynthetic pathway can often be used to synthesize the same phospholipid. In mice, PS synthase-1 and PS synthase-2 are expressed in a broad and partially overlapping set of tissues (J.E. Vance, 2001, 2002). Whereas PS synthase-1 is widely distributed throughout many tissues, PS synthase-2 is most highly expressed in Sertoli cells of the testis. The gene encoding the PS synthase-1 gene was recently disrupted in mice (J.E. Vance, unpublished data). PS synthase-1-deficient mice are viable and appear outwardly normal but the phenotype of these mice has not yet been characterized. Mice lacking PS synthase-2 activity have also been generated (J.E. Vance, 2002). The testis size of male PS synthase-2 knockout mice is smaller than that of wild-type littermates and some males are infertile. Consistent with a defect in Sertoli cells, the principal site of PS synthase-2 expression, follicle-stimulating hormone levels are increased by elimination of PS synthase-2 in mice. Nevertheless, the PS and PE content of all tissues is normal, probably because the activity of PS synthase-1 is compensatorily increased. Thus, neither PS synthase-1 nor PS synthase-2 is uniquely required for viability of mice. By future inter-breeding of these two strains of PS synthase knockout mice one should be able to determine if, and how much, PS synthase activity is essential for mouse viability.

6.3. Regulation of PS synthesis

Mechanisms that regulate PS synthesis in mammalian cells are, for the most part, unknown. The transcriptional regulation of expression of neither of the PS synthase genes has been reported. Preliminary experiments in rat brain suggest that PS synthesis is regulated by protein kinase C-mediated phosphorylation (J.N. Kanfer, 1988). Consistent with the high proportion of docosahexaenoic acid in PS in the brain, a dietary deficiency of docosahexaenoic acid decreases the PS content of neuronal membranes (L. Bittova, 2001). The major mechanism that has been identified for the regulation of PS synthesis in mammalian cells is a feedback mechanism. In a series of elegant studies, Nishijima and co-workers demonstrated that when CHO cells were incubated with PS, the rate of PS synthesis was strikingly reduced (M. Nishijima 1998, 1999). Subsequently, mutant CHO cells were isolated in which the rate of PS synthesis, and the level of PS, were higher than in wild-type CHO cells. In addition, PS synthesis in the mutant cells was resistant to inhibition by PS; a point mutation at Arg-95 PS synthase-1 was shown to confer the aberrant regulation of PS synthesis (M. Nishijima, 1989). A parallel regulation of PS synthesis by end-product inhibition of PS synthase-2 has also been demonstrated. This feedback inhibition of the PS synthases appears to be the result of a direct interaction between PS and the PS synthase protein. There also appears to be 'cross-talk' between PE biosynthesis via the CDP-ethanolamine pathway and the PS synthase-1/PS decarboxylation pathway because over-expression of PS synthase-1 (but not PS synthase-2) increased PE synthesis via PS decarboxylation and reciprocally decreased PE synthesis via the CDP-ethanolamine pathway (S. Stone, 1999).

6.4. Functions of PS

PS is quantitatively a minor phospholipid in mammalian cells (3–10% of total phospholipids) and is not equally abundant in all organelle membranes. For example, PS is less abundant in mitochondrial and lysosomal membranes than in the ER or plasma membrane. As discussed in Section 5.4, PS is the precursor of PE in the reaction catalyzed by PS decarboxylase (Figs. 1 and 7). PS is also required for activation of several signaling proteins including protein kinase C (L. Bittova, 2001), neutral sphingomyelinase (W. Stoffel, 2000) and cRaf1 kinase (K. Inoue, 1999), as well as for the activity of the Na^+/K^+ ATPase and dynamin-1. In addition, a highly specific interaction of PS with some Hsp70 heat shock proteins induces formation of ion channels in the plasma membrane (N. Arispe, 2004). Interest in understanding the regulation of PS metabolism has increased recently because PS exposure on cell surfaces is the signal by which apoptotic cells are recognized and subsequently engulfed by a receptor on macrophages (V.A. Fadok, 1992) although the identity of the PS receptor remains under debate. PS is normally highly enriched on the inner leaflet of the plasma membrane bilayer, but during the early stages of apoptosis PS becomes exposed on the outside of the plasma membrane. However, neither PS synthase is uniquely required for PS externalization during apoptosis of CHO cells (P.A. Grandmaison, 2004). In neuron-like cells, docosahexaenoic acid (22:6 n-3), which is highly enriched in the brain, increases the cellular PS content and

prevents apoptosis (H.-Y. Kim, 2005). Another important role for PS is in blood clotting. Exposure of PS on the surface of activated platelets is required for initiation of the blood-clotting cascade (R. Zwaal, 1982). Furthermore, increased exposure of PS on the surface of sperm cells occurs during sperm maturation (B.M. Gadella, 2001). Thus, although PS is quantitatively a minor phospholipid in mammalian cells, it contributes to many diverse and biologically important functions.

7. Inositol phospholipids

7.1. Historical developments

A major fate of PA is conversion to DG that can be metabolized to PC, PE, and TG (Fig. 1). Alternatively, PA can react with CTP to form CDP-DG that is utilized for biosynthesis of the inositol phospholipids as well as phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (Fig. 1). Inositol is a cyclohexane derivative in which all six carbons contain hydroxyl groups. The most common inositol isoform is *myo*-inositol but other less abundant inositols with different structures also occur. The first report of an inositol-containing lipid was in 1930 in *Mycobacteria* which is ironic since inositol lipids are rarely found in bacteria. Brain is the richest source of inositol-containing lipids, as first discovered by Folch and Wooley in 1942. In 1949, Folch described a PI phosphate (PI-P) that was later found to include PI and PI bisphosphate (PI-P_2). The chemical structures of PI, PI-P, and PI-P_2 were determined by Ballou and co-workers between 1959 and 1961. PI (1.7 μmol/g liver) constitutes ~10% of the phospholipids in cells and tissues. PI-P and PI-P_2 are present at much lower concentrations (1–3% of PI). In 1958, Agranoff and co-workers first reported the incorporation of [^{3}H]inositol into PI. Subsequently, Paulus and Kennedy showed that CTP was the preferred nucleotide donor.

7.2. CDP-diacylglycerol synthase

Regulation of the conversion of PA to CDP-DG is not well understood. The enzyme, CDP-DG synthase, is largely microsomal but is also found in mitochondrial inner membranes. A cDNA encoding an isoform of CDP-DG synthase-1 that is specifically located in photoreceptor cells was cloned from *Drosophila* (C.S. Zuker, 1995). Mutations in this isoform lead to a defect in PI-P_2 biosynthesis. As a result, mutant photoreceptor cells show severe defects in phospholipase C-mediated signal transduction since PI-P_2 is a key lipid second messenger. cDNAs encoding human and murine CDP-DG synthases-1 and 2 have also been cloned and expressed (S. Jackowski, 1997; B. Franco, 1999). CDP-DG synthase-2 is expressed during embryogenesis in the central nervous system whereas CDP-DG synthase-1 is present at high levels in adult retina (S. Halford, 2005). Curiously, in *S. cerevisiae*, CDP-DG synthase activity is found in both microsomes and mitochondrial inner membranes although only one gene encodes this activity and only a single mRNA species was found. The yeast CDP-DG synthase gene is essential for cell viability as well as germination of spores.

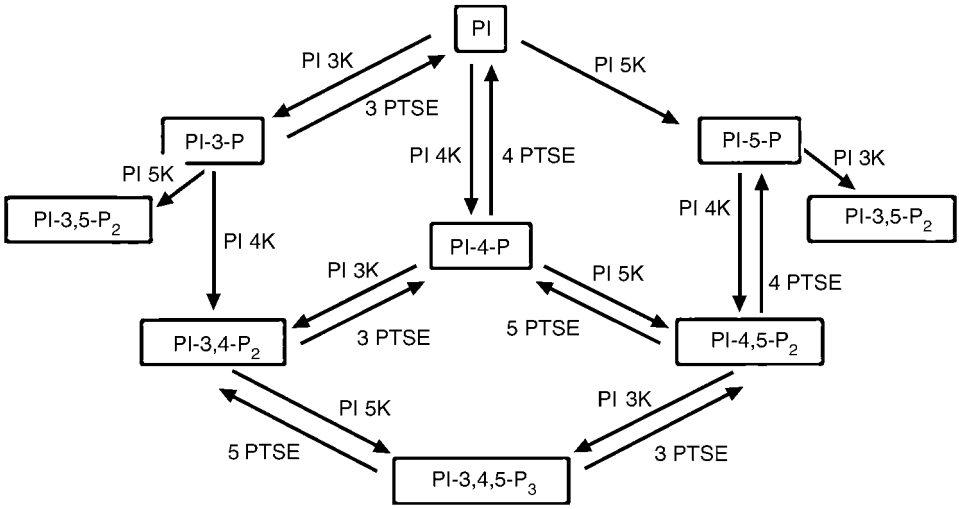


Fig. 8. Interconversions of phosphatidylinositides. PI, phosphatidylinositol; PI-4-P, PI-4-phosphate; PI-4,5-P_2, PI-4,5-bisphosphate; PI-3,4,5-P_3, PI-3,4,5-trisphosphate; PI 3K, PI-3-kinase; PTSE, PI phosphate phosphatase; PI 5K, PI 5 kinase; PI 4K, PI 4 kinase.

7.3. Phosphatidylinositol synthase

Three potential sources for cellular inositol are: diet, de novo biosynthesis, and recycling of inositol. Inositol is biosynthesized from glucose in the brain and testes, and other tissues to a lesser extent. The rate-limiting step in the inositol biosynthetic pathway appears to be the synthesis of inositol-3-phosphate from glucose-6-phosphate (C.P. Downes, 1990). Inositol-3-phosphate is hydrolyzed to inositol by a phosphatase.

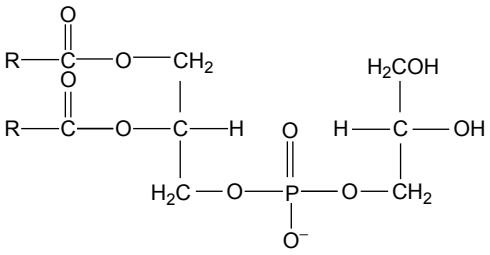
PI synthase was purified from human placenta (B. Antonsson, 1997). When the cDNAs encoding either CDP-DG synthase-1 or PI synthase, or both, were over-expressed in COS 7 cells, the rate of PI biosynthesis did not change indicating that the level of these enzymes was not limiting for PI biosynthesis (S. Jackowski, 1997). Disruption of the PI synthase gene in yeast is lethal indicating that PI is essential (S. Yamashita, 1997).

Many phosphorylated derivatives of PI are important in cell signaling (Fig. 8). A complex set of PI kinases and phosphatases is involved in the interconversions of these signaling molecules. The activities of PI-metabolizing enzymes are regulated by important receptor-triggered signaling processes that result in highly localized changes in the levels of specific PI derivatives that alter cellular metabolism and function [13].

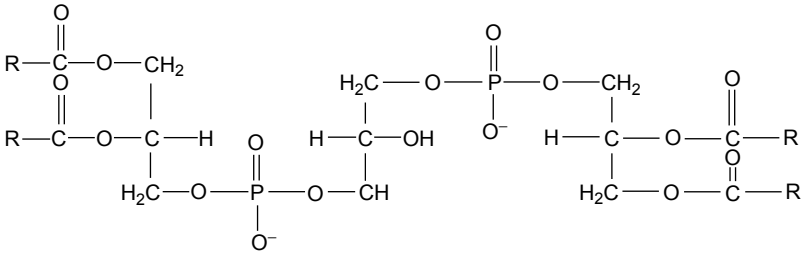
8. Polyglycerophospholipids

8.1. Historical developments and biosynthetic pathways

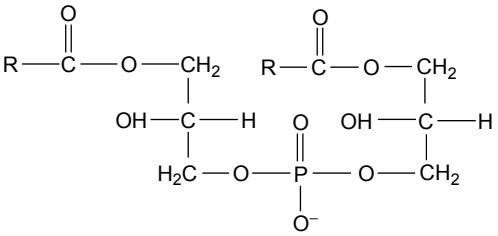
Diphosphatidylglycerol (DPG), commonly known as cardiolipin, was discovered in beef heart in 1942 by Pangborn. The structure (Fig. 9) was proposed in 1956–1957 and



Phosphatidylglycerol



Diphosphatidylglycerol



Bis(monoacylglycero)phosphate

Fig. 9. Structures of polyglycerophospholipids.

confirmed by chemical synthesis in 1965–1966 by de Haas and van Deenen. PG was first isolated in 1958 from algae and the structure was confirmed by Haverkate and van Deenen in 1964–1965. The third lipid in this class, bis(monoacylglycerol)phosphate was recovered from pig lung by Body and Gray in 1967. The stereochemistry differs from PG and DPG since bis(monoacylglycerol)phosphate contains *sn*-(monoacyl)glycerol-1-phospho-*sn*-1′-(monoacyl)glycerol rather than a *sn*-glycerol-3-phospho linkage. These three lipids (Fig. 9) are widely distributed in animals, plants, and microorganisms. In animals, DPG is found in highest concentration in cardiac muscle (9–15% of phospholipid), hence the name cardiolipin, and is exclusively localized to mitochondria. PG is generally present at a concentration of less than 1% of total cellular phospholipids, except in the lung, where it comprises 2–5% of the phospholipid. In pulmonary surfactant and alveolar type II cells, 7–11% of the total lipid phosphorous is in PG. Bis(monoacylglycerol)phosphate comprises $<1\%$ of total phospholipids in animal tissues, except in alveolar (lung) macrophages where it is 14–18% of total phospholipid.

The biosynthetic pathway for PG was elucidated by Kennedy and co-workers in 1963 (Fig. 1). For DPG biosynthesis, PA is transferred from CDP-DG to PG to yield DPG. DPG synthesis in *E. coli* differs from that in eukaryotes and involves the condensation of two molecules of PG (Chapter 3). Understanding the biosynthesis of bis(monoacylglycerol)phosphate was a particular challenge because the carbon linked to the phosphate residue is the *sn*-1, rather than *sn*-3, configuration (M. Waite, 1995). An intermediate in the biosynthesis of bis(monoacylglycerol)phosphate is 1-acyl-lyso-PG, also known as lyso-bis-PA. Compared to other organelle membranes, the inner membranes of late endosomes are enriched in lyso-bis-PA (J. Gruenberg, 1998). The presence of lyso-bis-PA is thought to promote the export of cholesterol out of this organelle (J. Storch, 2006).

8.2. Enzymes and subcellular location

PG is made in mitochondria and microsomes of animal cells and appears to be primarily converted to DPG. DPG is biosynthesized exclusively on the matrix side of the mitochondrial inner membrane and is found only in this organelle. There is evidence that the rate-limiting step in DPG biosynthesis is the conversion of PA into CDP-DG (G.M. Hatch, 1994). Consistent with this idea, the levels of CTP regulate DPG biosynthesis in cardiac myoblasts (G.M. Hatch, 1996). Using techniques developed by Raetz and co-workers [14], a temperature-sensitive mutant of PG-P synthase in CHO cells was isolated (M. Nishijima 1993). The mutant had only 1% of wild-type PG-P synthase activity at 40°C and exhibited a temperature-sensitive defect in PG and DPG biosynthesis. This mutant was used to show that DPG is required for the NADH-ubiquinone reductase (complex I) activity of the respiratory chain.

In yeast, DPG synthesis has been genetically interrupted (Schlame, 2000). The yeast grow without DPG at temperatures of between 16 and 30°C but fail to grow at 37°C on fermentable carbon sources such as glucose, a condition for which mitochondria are not required for ATP synthesis. These data support the idea that mitochondria perform a necessary function in yeast survival other than the generation of energy. The fatty acyl chain content of phospholipids can impact mitochondrial function. Incubation of cardiomyocytes with palmitic acid increased the palmitic acid content of PA and PG and decreased DPG levels in mitochondria with a concomitant release of cytochrome c leading to apoptosis (W. Dowhan, 2001).

9. Remodeling of acyl substituents of phospholipids

The fatty acid components of phospholipids made de novo are those of the precursors DG and CDP-DG. Once the phospholipid is made, the fatty acid substituents can be remodeled via deacylation–reacylation reactions (Fig. 10) [15]. Remodeling can occur at either the *sn*-1 or *sn*-2 position of the glycerolipid. For example, a major molecular species of PC formed from the conversion of PE to PC is 16:0–22:6 (R.W. Samborski, 1990). However, this species of PC has a half-life of less than 6 h and is converted to other molecular species, particularly those containing 18:0 at the *sn*-1 position and 20:4, 18:2, or 22:6 at the *sn*-2 position. Other studies have suggested that the main products of de novo

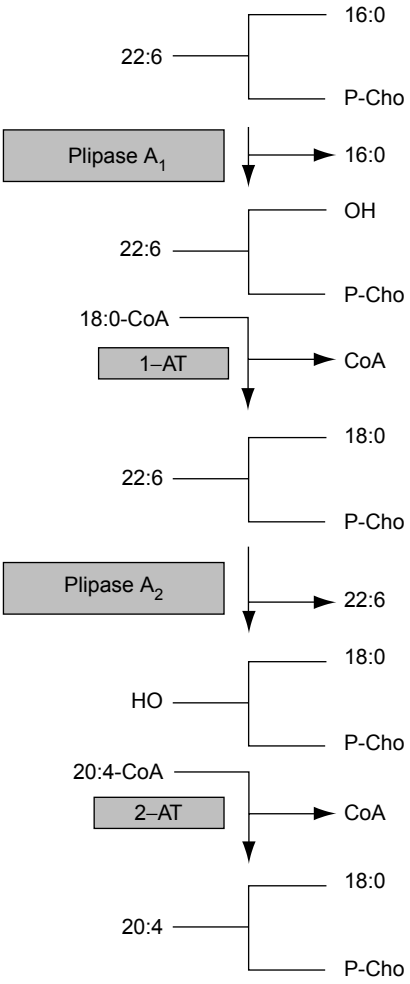


Fig. 10. Fatty acids at both *sn*-1 and *sn*-2 positions of PC can be deacylated by phospholipases and used for reacylation by acyltransferases. For example, palmitic acid (16:0) can be removed from the *sn*-1 position and replaced with stearic acid (18:0). The fatty acid at the *sn*-2 position is depicted as docosahexaenoic acid (22:6) that can be replaced with 20:4 or 18:2. If the fatty acid at the *sn*-2 position were oleic acid, it could also be deacylated and reacylated. Alternatively, deacylation/reacylation could occur initially at the *sn*-2 position. Plipase, phospholipase; 1-AT, acyl-CoA:lyso-phosphatidylcholine 1-acyltransferase; 2-AT, acyl-CoA:lyso-phosphatidylcholine 2-acyltransferase; cho, choline.

PC and PE biosynthesis by the CDP-base pathway are 16:0–18:2, 16:0–18:1, 16:0–22:6, and 18:1–18:2. The major remodeled product is 18:0–20:4 for both PC and PE (H.H.O. Schmid, 1995). Why 18:0–20:4-PC and -PE are made by this circuitous route, rather than directly, is not known.

Tafazzin is a CoA-independent phospholipid transacylase with substrate preference for monolyso-DPG and PC (Fig. 11). Linoleate is the preferred fatty acid that is transferred

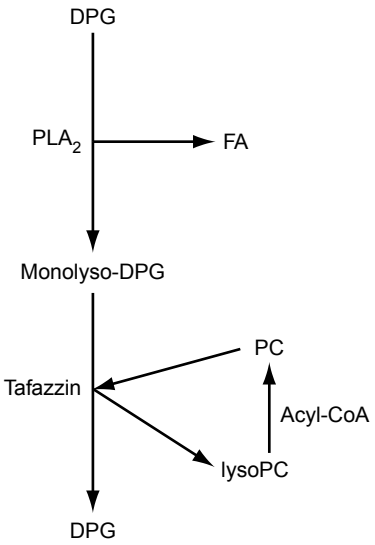


Fig. 11. Proposed pathway for remodeling of diphosphatidylglycerol (DPG) (cardiolipin). PLA_2, phospholipase A_2; PC, phosphatidylcholine; FA, fatty acid. Tafazzin catalyzes the reacylation of monolyso-DPG with linoleoyl-CoA being the preferred acyl-CoA.

from PC to monolyso-DPG (M. Schlame, 2006). Mutations in the human tafazzin gene, carried on the X chromosome, cause Barth syndrome that results in cardiomyopathy, skeletal muscle myopathy, and growth retardation [16]. Mutations in the tafazzin gene decrease the levels of DPG and increase the amounts of monolyso-DPG, consistent with the in vitro activity of tafazzin.

10. Regulation of gene expression in yeast

The pathways for the biosynthesis of phospholipids in yeast were largely elucidated by Lester and co-workers in the late 1960s (Fig. 12). These pathways are similar to those in other eukaryotes except that PS in yeast is made via the same pathway as in *E. coli* where CDP-DG reacts with serine to yield PS and CMP (Chapter 3). Interest in yeast as a model system for studying phospholipid metabolism has developed over the past two decades because of the availability of a large knowledge base in classical genetics, the ease of making mutant strains, and the ability to grow yeast in large quantities. Whereas understanding the regulation of expression of phospholipid biosynthetic enzymes in animal cells is still in its infancy, considerable progress has been made in the yeast system [17–19]. When yeast cells are grown in the presence of choline and inositol, the expression of the enzymes involved in the conversion of PA and glucose-6-P to PI, PC, PS, and PE is depressed (Fig. 12). Both positive and negative regulatory factors are involved in the regulation of expression of phospholipid biosynthetic enzymes in yeast.

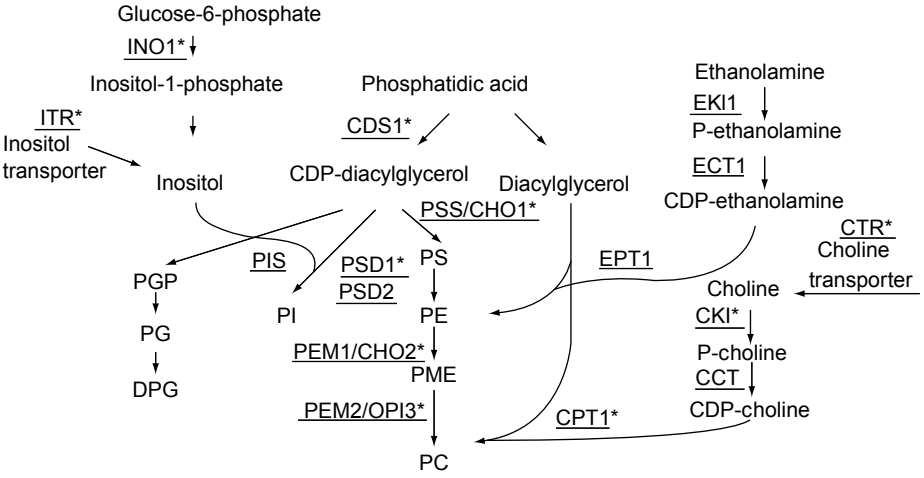


Fig. 12. The pathway for phospholipid biosynthesis in yeast and designation of the genes (italics, underlined) encoding the enzymes that catalyze the reactions. The abbreviations are: PE, phosphatidyethanolamine; PME, phosphatidylmonomethylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PGP, PG phosphate; DPG, diphosphatidylglycerol. The genes encode the following enzymes: *INO1*, inositol-1-P synthase; *PIS*, PI synthase; *PSS* (also known as *CHO1*), PS synthase; *EPT1*, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; *PEM1* (*CHO2*), PE methyltransferase; *PEM2* (*OPI3*), phospholipid methyltransferase; *CK1*, choline kinase; *CCT*, CTP: phosphocholine cytidylyltransferase (abbreviated as CT elsewhere in this chapter); *CPT1*, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; *PSD*, PS decarboxylase. CDS, CDP-diacylglycerol synthetase; EK, ethanolamine kinase; ECT, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase. * Indicates genes that are regulated by inositol-choline in the growth medium.

The *INO2* and *INO4* genes encode transcription factors that are required for expression of inositol-1-P synthase (*INO1*). In vitro transcribed/translated proteins derived from the *INO2* and *INO4* genes form a heterodimer that binds a specific DNA fragment of the *INO1* gene referred to as UAS_{INO} (also known as inositol-choline response element) (S. Yamashita, 1991; S.A. Henry, 1994). Ino4p (the protein encoded by *INO4*) and Ino2p contain basic helix-loop-helix domains. The *OPI1* gene encodes a protein that negatively regulates phospholipid biosynthesis in yeast (S.A. Henry, 1991). Opi1p contains a leucine zipper, a motif implicated in protein–DNA interactions and transcriptional control. *Opi1* mutants exhibit a 2-fold increase in the constitutive expression of inositol-1-P synthase and other enzymes involved in PI, PC, PS, and PE biosynthesis. Opi1p does not interact directly with UAS_{INO} or with Ino2p or Ino4p. The mechanism of translocation of Opi1p to the nucleus has been clarified [17]. A chimera of green fluorescent protein and Opi1p was constructed and the cellular localization was examined. Opi1p was shown to bind to PA and the Scs2p protein on the ER membrane. When the amount of the phospholipid precursor, PA, decreased and PI increased shortly after the addition of inositol, Opi1p dissociated from the ER, translocated to the nucleus, and suppressed the transcription of *INO1* and PI synthesis by binding to the DNA element UAS_{INO}.

Phospholipid biosynthesis in yeast is also regulated by the availability of zinc in response to nutrient depletion [19]. In zinc-depleted cells, the expression of enzymes of the CDP-DG pathway and PS synthase decreases whereas the activity of PI synthase increases. Regulation of the CDP-DG pathway and PS synthase by zinc availability is mediated by the transcription factors Ino2p, Ino4p, and Op1p acting through the UAS_{INO} element in the *CDS1* (*CHO1*) promoter. Under conditions of zinc depletion, Opi1p is released from its interaction with PA and Scs2p in the ER, migrates into the nucleus, and associates with Ino4p and Ino2p on the UAS_{INO} element of the promoter, thereby repressing transcription of the *CDS1* (*CHO1*) gene. In contrast, when zinc is present, the transcription factor Zap1p is induced and binds to the UAS_{ZRE} element in the promoter of the *PIS* gene inducing the transcription of PI synthase.

11. Future directions

Since the first edition of this book was published in 1985 there has been astonishing progress in understanding phospholipid metabolism. The purification of some enzymes involved and the use of genetic screens has allowed the use of molecular biological techniques to clone and express cDNAs and genes for eukaryotic phospholipid biosynthetic enzymes. In addition, genetically modified mouse models are being developed. The following advances in the field are anticipated in the near future.

(i) Crystal structures of some of the soluble proteins will be reported.
(ii) Genes that encode additional phospholipid biosynthetic enzymes will be cloned and characterized. Elements of the genes involved in regulation of transcription will be mapped and positive and negative transcription factors will be identified.
(iii) More transgenic mice that over-express some of these enzymes, as well as mice in which phospholipid biosynthetic genes have been disrupted, will be generated. Studies with these animals should provide valuable insight into the role of these enzymes in whole animal physiology.
(iv) The yeast system will continue to be exploited for studies on gene function and expression as well as regulation of phospholipid biosynthesis.
(v) Progress is expected in understanding the regulation of the biosynthesis of PE, PI, PS, and DPG.

In the process of testing hypotheses and asking fundamental questions about phospholipid biosynthesis, we can continue to expect the unexpected.

Abbreviations

CHO	Chinese hamster ovary
CT	CTP:phosphocholine cytidylyltransferase
DG	diacylglycerol
DPG	diphosphatidylglycerol (cardiolipin)
ER	endoplasmic reticulum
GPAT	glycerol-3-phosphate acyltransferase

MAM	mitochondria-associated membranes
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine *N*-methyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
SREBP	sterol response element-binding protein
TG	triacylglycerol

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CHAPTER 9

Ether-linked lipids and their bioactive species

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Contents

1. Introduction

Ether lipids account for up to one-fifth of the human phospholipid pool, and inborn errors of metabolism that abolish ether lipid synthesis result in severe pathologies. However, the multifarious defects in the absence of ether lipid synthesis obfuscate a precise definition of the specific physiological roles of this class of complex lipids. Cellular and circulating phospholipids are primarily, but not exclusively, diacyl lipids consisting of long-chain fatty acyl residues esterified at the *sn*-1 and *sn*-2 positions of the glycero-3-phosphoryl backbone. However, select inflammatory cells, electrically excitable cells, and tumor cells contain significant amounts — up to 70% of the ethanolamine-linked phosphoglyceride or nearly half of the pool of choline-linked phosphoglyceride pool — of phospholipids that contain an *sn*-1 ether bond. These occur with (plasmalogen) or without (plasmanyl) a unique 1′ *cis* double bond. Whether such specialized phospholipids have a corresponding unique role had been unknown, but identification of mutations in enzymes catalyzing the first two steps of complex ether phospholipid synthesis as causal events in human genetic disorders has now shown that, indeed, these alkyl phospholipids have a unique and irreplaceable role in how cells and tissues organize themselves in complex organisms. The inability to synthesize alkyl phospholipids is not lethal to individual cells, but the defect in membrane trafficking in the absence of ether phospholipids instead manifests as defects in neurological and eye development in mice [1], and usually death in the human genetic disorders types 2 and 3 rhizomelic chondrodysplasia punctata [2,3]. The unique role fulfilled by ether phospholipids is also evident in other genetic abnormalities, such as the paradigmatic Zellweger syndrome, that prevent peroxisome maturation and thereby abolish the first two steps in ether phospholipid bond formation [4]. Ether phospholipids display distinct physical properties compared to their diacyl homologs, the vinyl ether of plasmalogens functions as a chain-breaking antioxidant, and ether phospholipids are precursors of highly potent signaling molecules. The task now is to understand how these distinctive properties alter cell and animal physiology.

The focus of this chapter is on the pathways, properties, and functions that distinguish ether phospholipids from their diacylglycerolipid homologs. A number of books [5,6] and review articles [7–11] on ether lipids, some specifically emphasizing the ether phospholipid platelet-activating factor [12–14], summarize the biochemistry and biosynthesis of *O*-linked glycerolipids and plasmalogens, and provide a comprehensive listing of published papers.

2. Structure

Diacyl phospholipids, the paradigmatic phospholipid of biochemical and medical texts, contain two long-chain fatty acyl residues esterified at the *sn*-1 and *sn*-2 positions of the glycero-3-phosphoryl backbone. This class of glycerolipid accounts for 95% of the phospholipids of organs such as liver. However, in other tissues and cells, e.g., heart, neurons, white blood cells, and hepatic Kupffer cells, abundant pools of ether phospholipids (Fig. 1) are present [15]. Ether phospholipids are classified by the presence or absence of a 1′ double bond. Plasmalogens contain a *cis* double bond adjacent to the *sn*-1 ether bond, while *sn*-1-alkyl-2-acyl phospholipids lack this *O*-alk-1′-enyl function. These alkylacyl phospholipids, in accordance with the plasmalogen nomenclature, are defined as plasmanyl phospholipids, but this term, in contrast to plasmalogen, is not commonly employed. Alkylacyl glycerophosphocholine and ethanolamine plasmalogens have non-overlapping distribution and functions, but because they are derived from a common, and non-redundant, biosynthetic pathway, they are properly considered ether phospholipids.

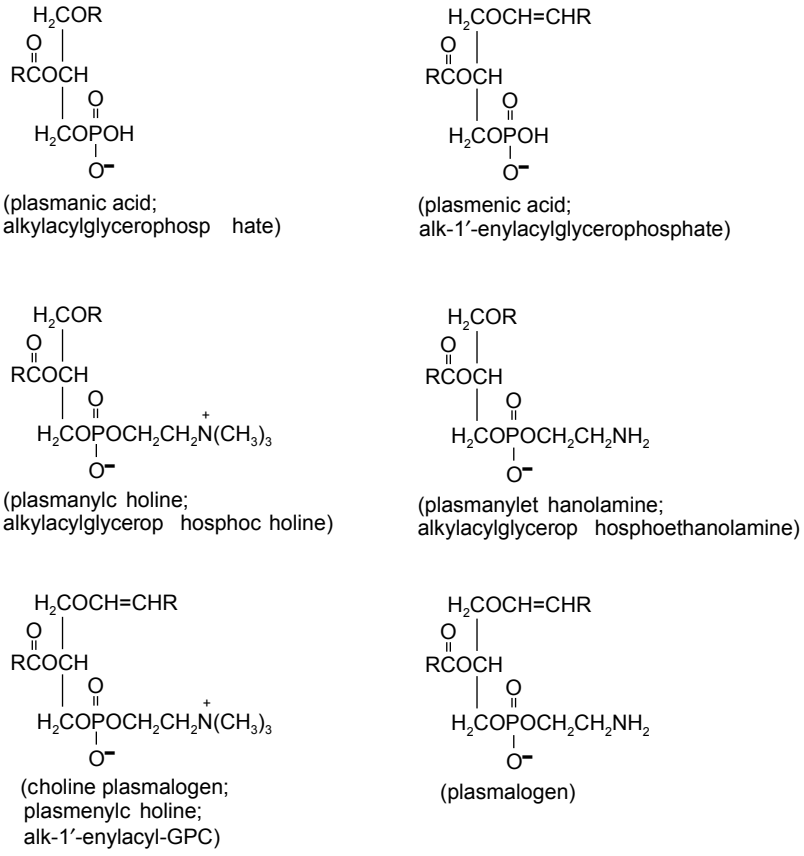


Fig. 1. Chemical structures of biologically significant ether-linked lipids of mammalian cells.

Naturally occurring ether lipids primarily contain these *sn*-1 *O*-alkyl or *O*-alk-1′-enyl functions, although di- and tetra-*O*-alkylglycerolipids have been described in some cells.

Diacylglycerolipids are marked by a huge diversity of esterified fatty acyl residues that generates thousands of isomers, while *sn*-1 *O*-alkyl and *O*-alk-1′-enyl ether-linked chains generally consist of 16:0, 18:0, and 18:1 aliphatic species. Other types of chain lengths, degrees of unsaturation, and occasional branched chains exist, but only as minor components. The composition of plasmalogens is notable for the marked enrichment of the polyunsaturated fatty acid arachidonate ($C_{20:4}$) and the n-3 fatty acid docosahexanoate ($C_{22:6}$) at the *sn*-2 position. Plasmalogens are a primary source of arachidonic acid for eicosanoid synthesis by stimulated inflammatory cells, and docosahexanoic acid is so prevalent in ether phospholipids that its total level is reduced in cells derived from plasmalogen-deficient patients. Except for intermediary metabolites and certain structural or bioactive lipids, ether linkages in phospholipids of mammalian cells exist almost exclusively in the choline and ethanolamine glycerolipid classes. The majority of the *O*-alkyl moieties normally occur as plasmanylcholines, whereas the *O*-alk-1′-enyl function is mainly associated with the ethanolamine plasmalogens, with the exception of heart where plasmenylcholines are prominent. Some neutral lipids such as alkyldiacylglycerols (glyceryl ether diesters) and alkylacylglycerols, analogs of triacylglycerols and diacylglycerols, respectively, are also found in cells. Fig. 1 illustrates the chemical structures of the most common ether lipids found in mammals.

3. Historical highlights

The early literature concerning ether-linked lipids has been covered in detail [5,16,17]. Perhaps the first evidence, albeit circumstantial, to suggest the existence of *O*-alkyl lipids in nature was reported in publications by C. Dorée (1909) in England and A. Kossel and S. Edlbacher (1915) in Germany. These workers isolated an unsaponifiable fraction of lipids from starfish that was referred to as ‘astrol’, which was subsequently shown to have similar properties to batyl alcohol, an alkylglycerol possessing an 18-carbon aliphatic chain at the *sn*-1 position of the glycerol moiety. During the same period, the presence of alkyl ether lipids in liver oils of various saltwater fish was described by M. Tsujimoto and Y. Toyama (1922). The common names of the alkylglycerols, *chimyl* [16:0 alkyl], *batyl* [18:0 alkyl], and *selachyl* [18:1 alk-9′-enyl] alcohols, are based on the fish species from which they were originally isolated. Complete proof of the precise chemical nature of the alkyl linkage at the *sn*-1 position in these glycerolipids was provided by W.H. Davies, I.M. Heilbron, and W.E. Jones (1933) from England.

The German scientists R. Feulgen and K. Voit (1924) originally described plasmalogens in a variety of fresh tissue slices preserved in a $HgCl_2$ solution after being erroneously treated with a fuchsin-sulfurous acid reagent without the normal fixation and related histological processing with organic solvents. Only the cytoplasm of cells, but not the nuclei, was stained a red-violet color, which led to the conclusion that an aldehyde was present in the cell plasma. This substance was called ‘plasmal’. If the histological preparations were treated with a lipid-extracting solvent before exposure to the dye, no colored stain appeared in the cytoplasm. This unknown precursor of the cytosolic aldehyde that reacted

with the dye was called plasmalogen, a name still retained as the generic term for all alk-1′-enyl-containing glycerolipid classes that are susceptible to Hg^+ hydrolysis.

It was not until the 1950s that the precise chemical structure of the alk-1′-enyl linkage in ethanolamine plasmalogens was proven, primarily through the combined efforts of M.M. Rapport and G.V. Marinetti in the United States, G.M. Gray in England, E. Klenk and H. Debuch in Germany, and coworkers. The first cell-free systems to synthesize the alkyl ether bond were described independently in 1969 by F. Snyder, R.L. Wykle, and B. Malone, and A. Hajra. Shortly thereafter, studies by R.L. Wykle, M.L. Blank, B. Malone, and F. Snyder and by F. Paltauf and A. Holasek demonstrated that the *O*-alkyl moiety of an intact phospholipid could be enzymatically desaturated to the alk-1′-enyl function (Section 7.2.5). One significant development in the ether-lipid field occurred in 1979 when one of the most potent bioactive molecules known, an acetylated form of a choline-containing alkylglycerolipid named platelet-activating factor or PAF, was identified independently by three groups: D.J. Hanahan, C.A. Demopoulas, and R. N. Pinkard (1979); M.L. Blank, F. Snyder, L.W. Byers, B. Brooks, and E.E. Muirhead (1979); and J. Benveniste, M. Tencé, P. Varenne, J. Bidault, C. Boullet, and J. Polonsky (1979).

4. Natural occurrence of ether lipids

Chemical, chromatographic [18], and mass-spectral methods for analyzing phospholipids [19] and ether-linked glycerolipids have been reviewed [20]. Ether-linked phospholipids are isolated as a mixture with their ester-linked counterparts and it is important to understand the various analytical techniques used for complex biological materials. In particular, the very high affinity of the receptor for PAF and its selectivity for the *sn*-1 ether bond, coupled with contamination of many commercial sources of diacyl phosphatidylcholines and lysophosphatidylcholine (these are all derived from processed natural sources) with ether-linked choline phospholipids — often at levels that are difficult to detect by chemical or physical methods — means that techniques that distinguish between diacyl and ether phospholipids can be critical to properly interpret experimental results.

Ether-linked lipids occur throughout the animal kingdom and are even found as minor components in several higher plants. Some mammalian tissues, and avian, marine, molluscan, protozoan and bacterial lipid extracts, contain significant proportions of ether-linked lipids. Harderian glands, located on the posterior side of the eyeball in animals possessing a nictitating membrane, secrete an oily substance to facilitate the movement of the third eyelid. These glands have been of particular use in metabolic studies. The highest levels of ether lipids in mammals occur in nervous tissue, heart muscle, testes, kidney, preputial glands, tumor cells, erythrocytes, bone marrow, spleen, skeletal tissue, neutrophils, eosinophils, macrophages, platelets, and lipoproteins. Heart tissue is unique with respect to its plasmalogen content where, in some mammals, it is the only tissue known to contain significant amounts of choline plasmalogens instead of the usually encountered ethanolamine plasmalogens [5–7]. Ethanolamine phosphatides constitute only 10% of the phospholipids that coat the surface of human lipoproteins, but plasmalogens account for half of this pool. This observation is of particular interest because the liver contains relatively low amounts of ether lipids and the plasmalogens are not acquired by the lipoproteins after their secretion. Although the dietary consumption of ether lipids

by humans has largely been ignored by nutritionists, it is clear that certain meats and seafoods can contain relatively high amounts of these lipids.

Waxes are long-chain fatty alcohols esterified to long-chain fatty acids and constitute the second anabolic use of long-chain fatty alcohols. Waxes coat environmentally exposed surfaces of mammals, insects, and plants forming a slippery, water impervious barrier. A wax synthase gene has now been cloned from a mouse preputial gland and is present in other glands associated with wax production (Section 7.1.1). The enzyme has a broad fatty alcohol and fatty acid substrate preference, yet does not employ acceptors other than fatty acids.

Ether analogs of triacylglycerols have been described. 1-Alkyl-2,3-diacyl-*sn*-glycerols are characteristically elevated in tumor lipids and 1-alk-1′-enyl-2,3-diacyl-*sn*-glycerols (neutral plasmalogens) have also been detected in tumors, in adipose tissue of mammals, and in fish liver oil. Alkylacetylacylglycerols, PAF precursors, also have been described in human leukemic cells.

1-Alkyl-2-acyl-*sn*-glycero-3-phosphocholines are a significant component of platelets, neutrophils, macrophages, eosinophils, basophils, monocytes, and endothelial cells, mast cells, and HL-60 cells (a human promyelocytic leukemic cell line). This phospholipid class is a precursor of PAF (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; Fig. 2) and is

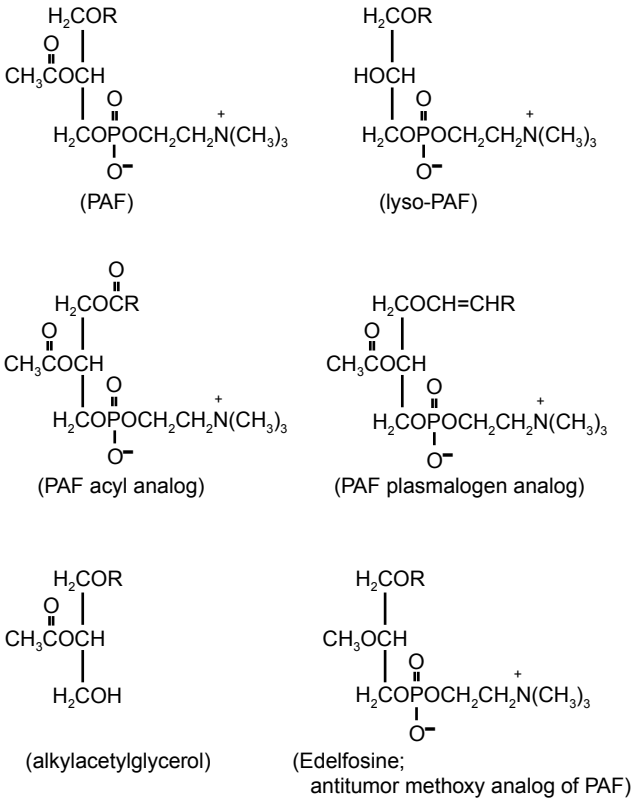


Fig. 2. Chemical structures of platelet-activating factor (PAF) and structurally related ether-linked glycerolipids possessing biological activities.

a constituent of all cells known to produce PAF by the remodeling pathway. In human neutrophils and eosinophils, the alkyl subclass comprises 45 and 70 mol% of the choline-linked phosphoglycerides, respectively, while the ethanolamine-linked class contains 60–65 mol% plasmalogen. Ether phospholipids, both alkylacyl and plasmalogen, have long been known [21] to be present at high abundance in tumor cells, especially in cell lines that display a strong metastatic potential [22].

Dialkylglycerophosphocholines have been reported as minor constituents of bovine heart and spermatozoa. Halophilic bacteria contain an unusual dialkyl type of glycerolipid (a diphytanyl ether analog of phosphatidylglycerophosphate) that has an opposite stereochemical configuration from all other known ether-linked lipids, i.e., the ether linkages are located at the *sn*-2 and *sn*-3 positions. The biosynthetic pathway for the formation of the ether bond in halophiles is still unknown. Acidophilic thermophiles contain tetra-alkyl glycerolipids with their two glycerol moieties linked across their membranes, which prevents the bilayer from being freeze-fractured.

Many anaerobic bacteria are highly enriched in plasmalogens. For example, *Clostridium butyricum* contains significant amounts of ethanolamine plasmalogens and *Megasphaera elsdenii* has been reported to contain very large quantities of plasmenylethanolamine and serine. However, despite the large pool of plasmalogens in such anaerobes, the mechanism for the synthesis of the alk-1′-enyl ether bond is unknown.

5. Physical properties

Replacement of ester linkages in glycerolipids with ether bonds mainly affects hydrophobic– hydrophilic interactions. Nevertheless, the closer linear packing arrangement attainable with ether-linked moieties also is capable of influencing the polar head-group region of phospholipids. The novel placement of the 1′ double bond in plasmalogens can also exert effects on stereochemical relationships and therefore the presence of an ether linkage in phospholipids can modify both the configuration and the functional properties of membranes. In particular, ethanolamine plasmalogen has a propensity to form a non-bilayer structure, an inverse hexagonal phase, and it is postulated that this non-lamellar structure aids membrane reorganization and vesicle fusion (Chapter 1). The vinyl ether function of plasmalogens is acid- and oxidant-sensitive, and plasmalogens have been proposed as sacrificial scavengers of reactive oxygen species (R.A. Zoeller, 1999).

In model membranes, ether-linked lipids decrease ion permeability and surface potential, and lower the phase temperature of membrane bilayers when compared to their diacyl counterparts. Ethanolamine plasmalogen is enriched, along with cholesterol, in membrane lipid rafts. These domains have a distinct hydrophobic environment with ordered lipid packing, and plasmalogen can serve to stabilize the interaction of specific raft proteins (Chapter 1).

C. butyricum appears to regulate the stability of the bilayer arrangement of membranes by altering the ratio of ether versus acyl ethanolamine phospholipids in response to changes in the degree of lipid unsaturation of the membranes. Experiments with bacteria indicate that substitution of plasmenylethanolamine for phosphatidylethanolamine in biomembranes would have only small effects on lipid melting transitions, whereas the tendency to form non-lamellar lipid structures would be significantly increased.

6. Biologically active ether lipids

6.1. Platelet-activating factor

In 1979, the chemical structure of PAF was identified as 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Fig. 2) (D.J. Hanahan, 1979; M.L. Blank, 1979; J. Benveniste, 1979). The semi-synthetic preparation tested in these initial experiments aggregated platelets at concentrations as low as 10^{-11} M and induced an anti-hypertensive response when as little as 60 ng were administered intravenously to hypertensive or normotensive rats. Threshold concentrations vary by cell type and organism, but PAF can activate human inflammatory cells at concentrations as low as 10^{-14} M. PAF induces diverse biological responses (Table 1) and has been implicated as a contributing factor in the pathogenesis of such diverse disease processes as asthma, hypertension, allergies, inflammation, and anaphylaxis.

Table 1
Biological activities associated with platelet-activating factor

I. In vivo responses
   1. Bronchoconstriction ↑
   2. Systemic blood pressure ↓
   3. Pulmonary resistance ↑
   4. Dynamic lung compliance ↓
   5. Pulmonary hypertension and edema ↑
   6. Heart rate ↑
   7. Hypersensitivity responses ↑
   8. Vascular permeability ↑

II. Cellular responses
   1. Aggregation of neutrophils, monocytes, and platelets ↑
   2. Degranulation of platelets, neutrophils, and mast cells ↑
   3. Shape changes in platelets, neutrophils, and endothelial cells ↑
   4. Chemotaxis and chemokinesis in neutrophils ↑

III. Biochemical responses
   1. Ca^{2+} uptake ↑
   2. Respiratory burst and superoxide production ↑
   3. Protein phosphorylation ↑
   4. Arachidonate turnover ↑
   5. Phosphoinositide turnover ↑
   6. Protein kinase ↑
      (a) Protein kinase C
      (b) Mitogen-activated protein kinase
      (c) G-protein receptor kinase
      (d) Protein tyrosine kinase
   7. Glycogenolysis ↑
   8. Tumor necrosis factor production ↑
   9. Interleukin-2 production ↓
   10. Activation of immediate-early genes, e.g., c-*fos* and c-*jun*, and zif/268 ↑
   11. Induction of early-response genes (IL-1β, IL-6R, and RXRα) ↑

PAF has been isolated and characterized from a number of cellular sources. Basophils, neutrophils, macrophages, monocytes, and mast, endothelial, and HL-60 cells are among the highest producers of PAF when stimulated by agonists such as chemotactic peptides, zymosan, thrombin, calcium ionophores, antigens, bradykinin, ATP, leukotrienes, C_{5a}, or collagen. The amount of PAF produced by various stimuli is dependent on the cell type and the specific agonist used, but PAF is primarily a locally acting hormone (autacoid) that controls the actions of adjacent cells, and total plasma concentration is not the critical parameter. Rather, PAF is made by agonist-stimulated endothelial cells and is displayed only on the surface of the activated cells that produced it. This spatially restricted display of PAF, and relevant adhesion molecules, localizes the inflammatory response [13] to areas where endothelial cell agonists are present.

Acetylated glycerolipids structurally related to PAF include plasmalogen, and acyl analogs of PAF that possess choline or ethanolamine moieties. The choline plasmalogen analog of PAF (Fig. 2) mimics the actions of PAF, although it is one-fifth as potent, through interaction with the PAF receptor. A single G-protein-linked receptor recognizes PAF, and PAF receptor antagonists block platelet aggregation by PAF and its plasmalogen homolog. The ethanolamine *O*-alkyl ether and plasmalogen analogs of PAF are more than a thousand times less potent than PAF but their potency, at least for neutrophils, is enhanced 100-fold by a co-stimulus. Little is known about the biological significance of these analogs. The acyl analog of PAF is produced by activated endothelial and other cells at a relative abundance that can be 10 times that of PAF, but the potency of these PAF analogs ranges from a ten to several thousand times less that of PAF, depending on the response being assayed [23].

6.1.1. PAF receptor

The PAF receptor has been cloned and sequenced from a number of organisms [13,14]. Two transcripts derive from the intronless gene driven by differentially regulated promoters, and PAF can regulate the synthesis of its receptor through one of these promoters. The human and guinea pig receptors consist of 342 amino acids with a C-terminal cytoplasmic tail possessing serine and threonine residues that are phosphorylation sites. The PAF receptor structure is typical of other G-protein-coupled receptors that span the membrane seven times. Based on modeling and alanine-scanning mutagenesis of the receptor, it is proposed that the central portion of the receptor and the histidine residues 188, 248, and 249 form the PAF-binding pocket (I. Ishii, 1997). Mutations variously increase or decrease PAF affinity, create constitutively active or inactive receptors, or form a dominant negative receptor affecting other G-protein receptors. A single nucleotide polymorphism (A224D) that reduces cellular signaling occurs at a frequency of 7.8% in the Japanese population. The fate of the receptor-bound PAF is not clear, but this molecule can be slowly internalized by activated neutrophils.

The role of PAF in vivo has been examined by overexpressing the guinea pig receptor in mice, although expression of the transgene was heterologous and many tissues and cells, including neutrophils and other inflammatory cells, did not express the transgene [14]. Nevertheless, these mice had an increased death rate in response to endotoxin, bronchial hyperreactivity, and, surprisingly, developed melanocytic tumors of the skin. Takao Shimizu and coworkers (1998) also have developed PAF receptor knockout mice. These mice

have less severe anaphylactic responses, including diminished cardiovascular instability, alveolar edema, airway constriction, and they display delayed responses to ischemia followed by reperfusion. The animals are less sensitive to folate-induced renal damage, and have impaired immunomodulatory responses to cutaneous inflammation. However, these animals reproduce normally and remain susceptible to endotoxin without a receptor for PAF. Several review articles [7,8,13,14] discuss the cloning, sequencing, and role of PAF receptors in signal transduction.

6.1.2. Receptor antagonists

A number of PAF antagonists are available that block binding of PAF to its receptor and specifically prevent PAF-induced responses. Some of these antagonists are derived from plants such as *Ginkgo biloba*, while others are structural analogs of PAF, and yet others are chemically synthesized compounds found through screening. Although the inhibitors effectively block PAF responses in vitro, for unknown reasons they generally have not proven effective as anti-inflammatory drugs. Genetic deletion of the PAF receptor shows that its ligands have a role in acute inflammatory responses, so it is possible that the bioavailability of the inhibitors limits their effectiveness, or that the extreme affinity of the receptor for PAF, and the lower affinity of these competitive inhibitors compared to PAF, is sufficiently unfavorable to preclude continuous long-term suppression of PAF signaling.

6.2. Oxidized phospholipids

Chemical oxidation of plasma lipoprotein or cell membrane phospholipid generates numerous oxidation products, and when alkyl polyunsaturated phosphatidylcholines are the oxidation target, some of the resulting fragmented phospholipids with very short *sn*-2 remnants display PAF-like activity [13]. A key difference between formation of PAF receptor agonists by chemical oxidation and the biochemical pathway is that the latter process is tightly controlled such that PAF is present only after cell-appropriate stimulation. In contrast, chemically oxidized phospholipids are the result of uncontrolled free radical reactions. Other phospholipid oxidation products are cytotoxic, activate peroxisome proliferator-activated receptors, or alter normal cellular function, but the selectivity for the *sn*-1 ether bond is not as great as that displayed by the PAF receptor. The enzyme PAF acetylhydrolase selectively acts on these oxidatively generated phospholipids to remove the oxidized chains, inactivating these responses. These oxidized phospholipids may participate in inflammation, development of atherosclerotic plaques, and other cardiovascular disorders where reactive oxygen species are created.

6.3. Other ether-linked mediators

Phospholipase D hydrolyzes the phosphoester bond, primarily of phosphatidylcholine, to form phosphatidic acid or 1-alkyl-2-acyl-*sn*-glycero-3-phosphate when the substrate is an ether phospholipid (Chapter 11). In human neutrophils, the phosphatidic acids produced upon stimulation of the cells by chemotactic peptides and other stimuli are approximately 50% alkylacyl-linked species and 50% diacyl-linked species, reflecting the cellular

composition of choline-containing phosphoglycerides. Phosphatidic acid activates raf protein kinases and the NADPH oxidase of neutrophils, but few studies have compared the relative activities of alkylacyl- and diacyl-*sn*-glycero-3-phosphates. Metabolism of 1-alkyl-2-acyl-*sn*-glycero-3-phosphate by a phosphohydrolase forms 1-alkyl-2-acyl-*sn*-glycerol. Alkylacylglycerols, in sharp contrast to diacylglycerols, do not activate protein kinase C, and in fact may inhibit its activity. Both alkylacylglycerols and diacylglycerols increase responses of neutrophils to other stimuli (priming), leading to arachidonic acid release and an oxidative burst. However, only diacylglycerol primes for the formation of lipoxygenase products (Chapter 12). Both alkyl- and acyl-linked lysophosphatidic acid can be derived from choline-containing phosphoglycerides.

Three high-affinity receptors for lysophosphatidic acid have been described, and synthesis of alkyl lysophosphatidic acid and a series of homologs (G.D. Prestwich, 2005) shows that these receptors — LPA1, LPA2, and LPA3 — are all stimulated by alkyl lysophosphatidic acids. However, the way this latter species is recognized differs from that of acyl lysophosphatidic acid, and this property can be exploited by using stabilized homologs that show receptor selectivity. Alkyl lysophosphatidic acid, in some individuals, is far more effective in stimulating platelet function than its oleoyl homolog. The pharmacokinetics of acyl and alkyl lysophosphatidic acid have yet to be compared, but the ether bond is not hydrolyzed by phospholipases, thus the half-life of the ether-linked species could be much longer than that of the acyl-linked species. 1-Alkyl-2-lyso-*sn*-glycero-3-phosphate is formed by the action of phospholipase A_2 (PLA_2) on 1-alkyl-acyl-*sn*-glycero-3-phosphate or by lysophospholipase D acting on 1-alkyl-2-lyso-glycerophosphocholine. There is robust formation of lysophosphatidic acid after platelet activation where lysophospholipase D (autotaxin) is released to act on the abundant (~150 μM) circulating pool of lysophosphatidylcholine. The extent to which the alkyl species accompanies acyl lysophosphatidic acid accumulation is unknown.

Natural ligands for the cannabinoid receptors CB_1 and CB_2, so named because they bind the active ingredient delta^9-tetrahydrocannabinol in *Cannabis sativa*, include arachidonoyl glycerol and also 2-arachidonoyl glycerol ether. The metabolic steps that form this unusual ether lipid are unknown since the synthesis of *sn*-2 glycerol ethers has not been studied to date.

7. Enzymes involved in ether lipid synthesis and regulatory controls

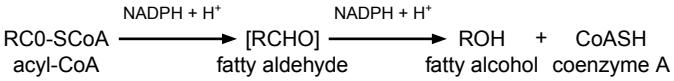
A significant literature describing the enzymes involved in the metabolism of ether-linked lipids [8–10,24,25] and PAF [13,14] is available.

7.1. Ether lipid precursors

7.1.1. Acyl-CoA reductase

Fatty alcohol precursors of ether lipids and waxes are derived from acyl-CoAs via a fatty aldehyde intermediate in a reaction sequence catalyzed by a membrane-associated acyl-CoA reductase (Fig. 3). The genes encoding two enzymes — FAR1 and FAR2 — with this activity have been cloned (J.B. Cheng and D.W. Russell, 2004). These proteins are 58%

I)



II)

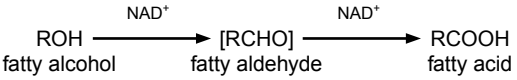


Fig. 3. Enzymatic synthesis and oxidation of long-chain fatty alcohols. These reactions are catalyzed by (I) acyl-CoA reductase and (II) fatty alcohol oxidoreductase, respectively.

identical, but retain only about 30% identity with plant and insect orthologs. These enzymes preferentially employ C_{16} and C_{18} unsaturated fatty acyl-CoA esters (FAR1) and saturated ones (FAR2). Both are peroxisomal enzymes and are located in glands with high wax production, although FAR2 distribution is more limited than FAR1. The reductant for both enzymes is NADPH. The enzyme mechanism is not defined but traces of fatty aldehydes can normally be detected in these reactions with the use of trapping agents such as semi-carbazide.

7.1.2. Dihydroxyacetone phosphate acyltransferase

The first step in complex ether lipid synthesis (Fig. 4A) is the acylation of dihydroxyacetone phosphate (DHAP) by dihydroxyacetone phosphate acyltransferase (DHAPAT). This is an obligatory step in the biosynthesis of the ether bond in glycerolipids because targeted deletion of this gene in mice ablates in vitro enzymatic activity and abolishes ethanolamine plasmalogen accumulation [1]. This outcome establishes that the acylation of DHAP by the cytosolic *sn*-glycerol-3-phosphate acyltransferase, which unlike DHAPAT can use either DHAP or glycerol-3-phosphate as acceptors, cannot substitute for the peroxisomal enzyme activity. The peroxisomal enzyme is found in all animal cells, although it is not required for cell viability, and is not present in plants or bacteria. DHAPAT forms a complex with, and is unstable in the absence of, its peroxisomal partner, alkyl-DHAP synthase (Fig. 4) (Section 7.2.1). Purification and cloning show that DHAPAT contains a C-terminal type 1 peroxisomal targeting sequence (PST1), while alkyl-DHAP synthase contains a PST2 element. These elements are recognized by Pex receptors and transporters responsible for peroxisomal protein targeting. Mutations in these import proteins cause defects in peroxisome biogenesis that underlay the disorders Zellweger syndrome and a type of rhizomelic chondrodysplasia [11].

7.2. Ether lipids

7.2.1. O*-alkyl bond: mechanism of formation*

Formation of the alkyl ether bond in glycerolipids is catalyzed by alkyl-DHAP synthase (Fig. 4B). This reaction, which forms alkyl-DHAP as the first committed intermediate

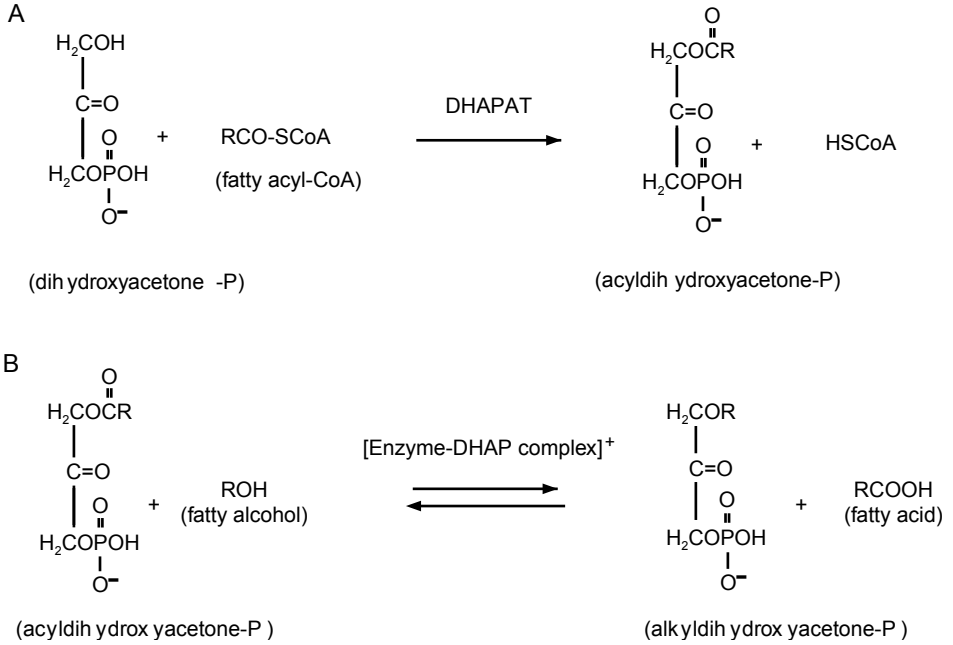


Fig. 4. Ether phospholipid synthesis from dihydroxyacetone-phosphate. (A) Dihydroxyacetone-P acyl transferase (DHAPAT). The first step of ether phospholipid synthesis is catalyzed by peroxisomal DHAPAT. This enzyme is a required component of complex ether lipid biosynthesis and its role cannot be assumed by a cytosolic enzyme that also forms acyldihydroxyacetone-P. (B) Ether bond formation by alkyl-DHAP synthase. The reaction that forms the *O*-alkyl bond is catalyzed by alkyl-DHAP synthase and is thought to proceed via a ping-pong mechanism. Upon binding of acyl-DHAP to the enzyme alkyl-DHAP synthase, the pro-*R* hydrogen at carbon atom 1 is exchanged by enolization of the ketone, followed by release of the acyl moiety to form an activated enzyme–DHAP complex. The carbon atom at the 1-position of DHAP in the enzyme complex is thought to carry a positive charge that may be stabilized by an essential sulfhydryl group of the enzyme; thus, the incoming alkoxide ion reacts with carbon atom 1 to form the ether bond of alkyl-DHAP. It has been proposed that a nucleophilic cofactor at the active site covalently binds the DHAP portion of the substrate.

in the biosynthetic pathway for ether-linked glycerolipids, is unique because it is the only known reaction where a fatty alcohol is directly substituted for a covalently linked acyl ester. This enzyme is widely distributed and is present in mammals, *Caenorhabditis elegans*, and protozoa (*Tetrahymena pyriformis* and *Trypanosoma bruci*). Alkyl-DHAP synthase exhibits a very broad specificity for fatty alcohols of different carbon chain lengths, while the specificity of the synthase for acyl-DHAP possessing different acyl chains has been less studied.

Kinetic experiments with a partially purified enzyme from Ehrlich ascites cells and with recombinant protein suggest that the reaction catalyzed by alkyl-DHAP synthase involves a ping-pong mechanism, with an activated enzyme–DHAP intermediary complex playing a central role [26]. The existence of this intermediate would explain the reversibility of the reaction since the enzyme–DHAP complex can react with either fatty alcohols (forward reaction) or fatty acids (back reaction) (Fig. 4). Acyl-DHAP acylhydrolase does not

participate in this mechanism because recombinant alkyl-DHAP synthase carries out the entire reaction. A number of novel features distinguish the reaction that forms alkyl-DHAP. The pro-R hydrogen at C-1 of DHAP exchanges with water without any change in the configuration, one H derived from H_2O is incorporated into the alkyl product, and cleavage of the acyl group of acyl-DHAP occurs before the addition of the fatty alcohol. There is no evidence for a Schiff's base being formed, but a ketone function is an essential feature of the DHAP substrate. Finally, mass spectrometry has clearly shown that the oxygen of the ether bond is donated by the fatty alcohol and that both oxygens of the acyl linkage of acyl-DHAP are recovered in the released fatty acid (A.J. Brown, 1985).

The cDNAs encoding human and guinea pig alkyl-DHAP synthase have been cloned and expressed [26] with the surprising findings that the enzyme contains a FAD-binding domain, that FAD is present, and that, even though the reaction gives no net change in oxidation potential, the FAD cofactor is required for activity of the enzyme. FAD participates directly in catalysis and becomes reduced upon incubation with acyl-DHAP, suggesting that the DHAP moiety is oxidized as the acyl chain is removed. Addition of fatty alcohol and synthesis of alkyl-DHAP result in re-oxidation of the $FADH_2$. Normally, acylhydrolase reactions proceed by acyl oxygen fission where only one of the oxygens of the ester bond remains with the acyl chain. In contrast, alkyl oxygen fission catalyzed by the alkyl synthase is unusual because both oxygens of the ester bond remain with the released acyl chain.

7.2.2. NADPH:alkyl-DHAP oxidoreductase

The third step in ether lipid synthesis is the reduction of the keto function of alkyl-DHAP to the hydroxyl of alkyl-*sn*-glycero-3-phosphate by an NADPH: alkyl-DHAP oxidoreductase activity (Fig. 5, Reaction I). Activity is present both in peroxisomes, where alkyl-DHAP is exclusively present, and in endoplasmic reticulum. *Saccharomyces cerevisiae* contains both DHAP (and glycerol-3-phosphate) acyltransferase activity and an acyl/alkyl-DHAP reductase activity, but *S. cerevisiae* contains little ether phospholipid. Acyl/alkyl-DHAP reductase activity in this yeast primarily associates with lipid particles, the major site of lipid synthesis, and is found in the endoplasmic reticulum. The *S. cerevisiae* acyl/alkyl-DHAP reductase gene has been identified as Ayr1p (open reading frame YIL124w) because deletion of the gene abolishes reductase activity of lipid bodies, and it abolishes sporulation (K. Athenstaedt, 2000). Since this mutation did not completely abolish microsomal activity, an additional enzyme(s) catalyzes this reaction.

The human genome does not contain a sequence homologous to Ayr1p and the molecular identity of a mammalian reductase catalyzing the final step of ether phospholipid synthesis remains undefined. NADPH-dependent oxidoreductase appears to reduce the ketone group of both alkyl-DHAP and acyl-DHAP, and cells deficient in this activity display reduced synthesis of both ethanolamine plasmalogen and diacyl phosphatidylcholine. This result is consistent with the competition between palmitoyl-DHAP and hexadecyl-DHAP for reduction using partially purified preparations from guinea pig liver. In contrast to the apparent dual acyl/alkyl-DHAP reductase of guinea pig and *S. cerevisiae*, it appears that in *Leishmania* distinct enzymes with distinct localization may separately reduce acyl-DHAP and alkyl-DHAP.

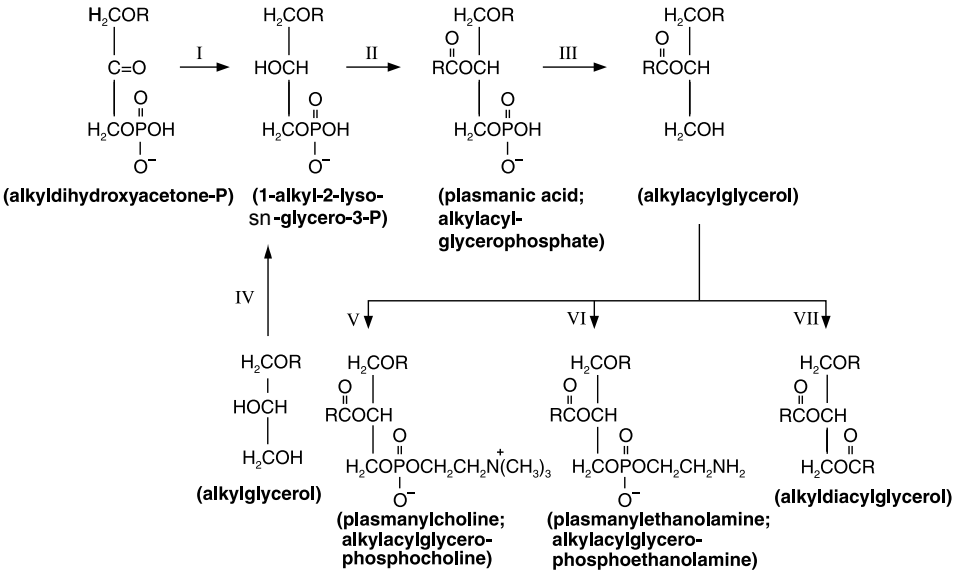


Fig. 5. Biosynthesis of membrane phospholipids from alkyldihydroxyacetone-P (alkyl-DHAP), the first detectable intermediate formed in the biosynthetic pathway for ether-linked glycerolipids. Enzymes catalyzing the reactions are: (I) NADPH: alkyl-DHAP oxidoreductase, (II) acyl-CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphate acyltransferase, (III) 1-alkyl-2-acyl-*sn*-glycero-3-phosphate phosphohydrolase, (IV) ATP:1-alkyl-*sn*-glycerol phosphotransferase, (V) CDP-choline:1-alkyl-2-acyl-*sn*-glycerol cholinephosphotransferase (dithiothreitol-sensitive), (VI) CDP-ethanolamine:1-alkyl-2-acyl-*sn*-glycerol ethanolaminephosphotransferase, and (VII) acyl-CoA:1-alkyl-2-acyl-*sn*-glycerol acyltransferase.

7.2.3. O-*alkyl analogs of phosphatidic acid and alkylacylglycerols*

The 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate product of the alkyl-DHAP reductase is converted to plasmanic acid (Fig. 5, Reaction II), the *O*-alkyl analog of phosphatidic acid, by acyl-CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphate acyltransferase. Dietary ether lipids can also enter this pathway, since alkylglycerols formed by catabolism of dietary ether-linked lipids during absorption are phosphorylated by an ATP:alkyl-glycerol phosphotransferase to form 1-alkyl-2-lyso-*sn*-glycero-3-phosphate (Fig. 5, Reaction IV). Plasmanic acid can then be dephosphorylated to alkylacylglycerols (Fig. 5, Reaction III), a class of lipids that occupies an important branch point in the ether lipid pathway in a manner analogous to the diacylglycerols in glycerolipid synthesis.

7.2.4. O-*alkyl choline- and ethanolamine-containing phospholipids*

1-Alkyl-2-acyl-*sn*-glycerols are utilized as substrates by cholinephosphotransferase (Fig. 5, Reaction V) and ethanolaminephosphotransferase (Fig. 5, Reaction VI) to form plasmanylcholines and plasmanylethanolamines, respectively, the alkyl analogs of phosphatidylcholine and phosphatidylethanolamine. These reactions are thought to be catalyzed by the same enzymes involved in the pathways originally established by Kennedy and coworkers in the late 1950s for diacylglycerolipids (Chapter 8). Both of these major classes of ether-linked phospholipids serve as precursors for two other important classes

of lipids containing ether bonds. Plasmanylcholine is the membrane source of lyso-PAF, the ether lipid precursor of the potent biologically active phospholipid PAF, whereas plasmanylethanolamine is the direct precursor of ethanolamine plasmalogens. Choline phosphate and ethanolamine phosphate transfer to alkylacylglycerol and diacylglycerol acceptors are considered to be indistinguishable in the remodeling of cellular glycerolipids; however, inhibitor, divalent ion sensitivity, and thermal denaturation in myocardial membranes indicates that separate ethanolaminephosphotransferases for diacyl- and alk-1′-enyl-acyl glycerols may exist in heart (D.A. Ford, 2003). Moreover, human cholinephosphotransferase, hCPT1, does not use ether-linked precursor lipids (A.L. Henneberry, 2000).

7.2.5. Ethanolamine plasmalogens

The 1′-alkyl desaturase system, a microsomal mixed-function oxidase responsible for the biosynthesis of ethanolamine plasmalogens from alkyl lipids (Fig. 6), was initially characterized in the early 1970s (F. Snyder, 1971; A. Paltauf, 1973). The reverse of this reaction (i.e., conversion of an alk-1′-enyl moiety to an alkyl chain via a reductase) has not been observed. The alkyl desaturase is a unique activity since it can specifically and stereospecifically abstract hydrogen atoms from C-1′ and C-2′ of the *O*-alkyl chain of an intact phospholipid molecule to form the *cis* double bond of the *O*-alk-1′-enyl moiety. Only intact 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine is known to serve as a substrate for the alkyl desaturase. As with other reactions in complex ether phospholipid synthesis, the molecular identity of the responsible enzyme is unknown.

Like the acyl-CoA desaturases (Chapter 7), the 1′-alkyl desaturase exhibits the typical requirements of a microsomal mixed-function oxidase. Molecular oxygen, a reduced pyridine nucleotide, cytochrome b_5, cytochrome b_5 reductase, and a terminal desaturase protein that is sensitive to cyanide are all required. The precise reaction mechanism responsible for the biosynthesis of ethanolamine plasmalogens is unknown, but it is clear from an investigation with a tritiated fatty alcohol that only the 1*S* and 2*S* (erythro)-labeled hydrogens are lost during the formation of the alk-1′-enyl moiety of ethanolamine plasmalogens.

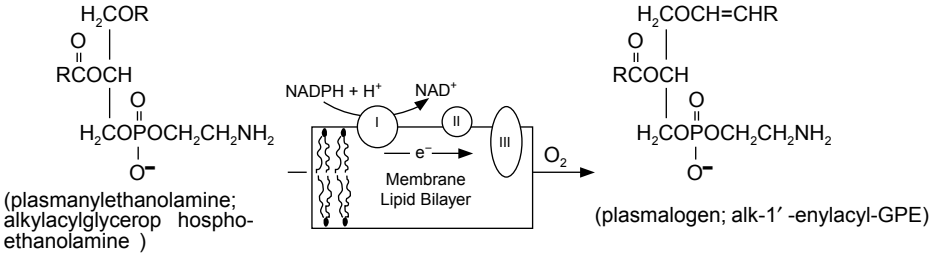


Fig. 6. Biosynthesis of ethanolamine plasmalogens by 1′-alkyl desaturase. Components of the enzyme complex responsible for this unusual desaturation between carbons 1 and 2 of the *O*-alkyl chain are: (I) NADH cytochrome b_5 reductase, (II) cytochrome b_5, and (III) Δ1′-alkyl desaturase, which is cyanide-sensitive. GPE, *sn*-glycero-3-phosphoethanolamine.

7.2.6. Choline plasmalogens

1′-Alkyl desaturase does not utilize 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine as a substrate and the available data strongly imply that the significant quantities of choline plasmalogens, present in some heart tissues, are derived from the ethanolamine plasmalogens [9]. Direct base exchange or coupled phospholipase C/cholinephosphotransferase reactions likely contribute to the synthesis of plasmenylcholine. The conversion of plasmenylcholine to plasmenylethanolamine was examined in Madin–Darby canine kidney cells (J.C. Strum, 1992). These studies indicated that reversal of the choline and ethanolamine phosphotransferase reactions may be responsible for the exchange of head groups in some cells. Choline plasmalogens can therefore arise from direct polar head-group remodeling mechanisms (Fig. 7) or a combined enzymatic modification of the *sn*-2 and *sn*-3 positions of ethanolamine plasmalogens (Fig. 8).

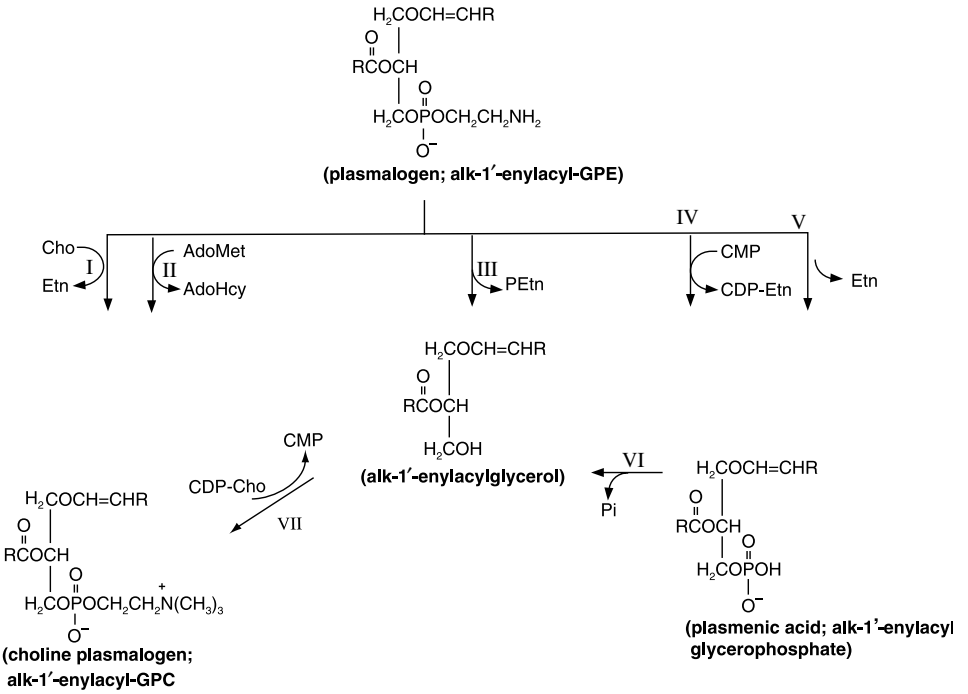


Fig. 7. Biosynthesis of choline plasmalogens (plasmenylcholines) via modification of the *sn*-3 polar head group of ethanolamine plasmalogens (plasmenylethanolamines). These reactions are proposed to be catalyzed directly by (I) a base exchange enzyme or (II) *N*-methyltransferase. A combination of other enzymatic reactions could also result in replacement of the ethanolamine moiety of plasmenylethanolamine to produce plasmenylcholines; the enzymes responsible include (III) phospholipase C, (IV) the reverse reaction of ethanolamine phosphotransferase, (V) phospholipase D, (VI) phosphohydrolase, and (VII) cholinephosphotransferase. AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; Etn, ethanolamine; GPE, *sn*-glycero-3-phosphoethanolamine.

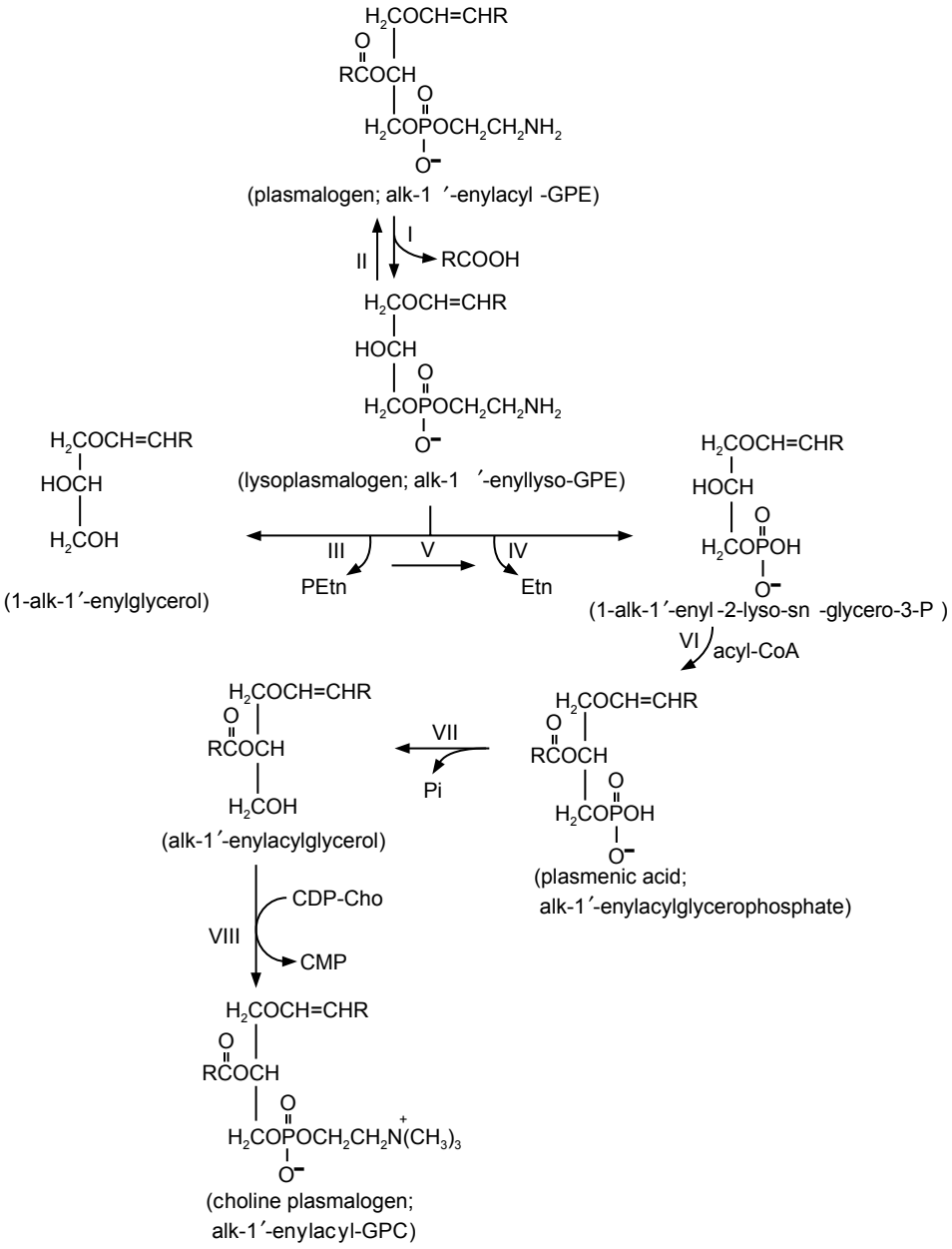


Fig. 8. Biosynthesis of plasmenylcholine via the modification of the *sn*-2 acyl and *sn*-3 head-group moieties of plasmenylethanolamine. The reactions are catalyzed by the following enzymes: (I) PLA_2, (II) CoA-independent transacylase, (III) lysophospholipase C, (IV) lysophospholipase D, (V) phosphotransferase, (VI) acyl-CoA acyl-transferase, (VII) phosphohydrolase, and/or (VIII) cholinephosphotransferase. Etn, ethanolamine; Cho, choline; GPE, *sn*-glycero-3-phosphoethanolamine; GPC, *sn*-glycero-3-phosphocholine.

7.2.7. Neutral ether-linked glycerolipids

Alkyldiacylglycerols, the *O*-alkyl analog of triacylglycerols, are produced by acylation of 1-alkyl-2-acyl-*sn*-glycerols in a reaction catalyzed by an acyl-CoA acyltransferase (Fig. 5, Reaction VII). The acyltransferase can also acylate 1-alk-1′-enyl-2-acyl-*sn*-glycerols to form the 'neutral plasmalogen' analog of triacylglycerols. In addition, an acetylated *O*-alkyl analog of triacylglycerols has been shown to be synthesized from 1-alkyl-2-acetyl-*sn*-glycerols in HL-60 cells. Whether these ether-linked neutral lipids have a distinct biological role is unknown, but the skin of hairless mice and skin keratinocytes express alkyldiacylglycerols that are more hydrophobic than triacylglycerols. In addition, alkyldiacylglycerol is a primary product of the harderian glands of golden hamsters where its fatty acyl composition, and hence physical properties, varies by gender.

7.3. Platelet-activating factor

7.3.1. Remodeling route

The remodeling pathway of PAF synthesis (Fig. 9) is thought to be the primary contributor to hypersensitivity reactions and for this reason this route has been implicated in most pathological responses involving PAF. Biosynthesis of PAF during inflammatory cellular

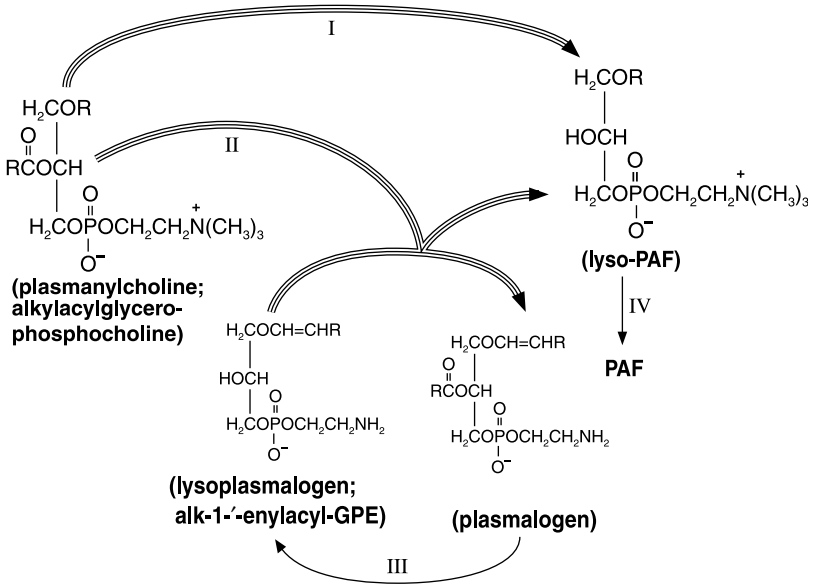


Fig. 9. Biosynthesis of platelet-activating factor (PAF) via the remodeling pathway. Lyso-PAF, the immediate precursor of PAF, can be formed from 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine through the direct action of (I) PLA_2 or (II) CoA-*independent* transacylase. The lysoplasmenylethanolamine (or other potential ethanolamine- and choline-containing lysoglycerophospholipids) is thought to be generated by (III) a PLA_2 that exhibits a high degree of selectivity for substrates with an arachidonoyl moiety at the *sn*-2 position. The transacylase (II) appears to possess both acyl transfer and PLA_2 hydrolytic activities. Lyso-PAF produced by either the transacylation (II) or direct PLA_2 (I) action can be acetylated to form PAF by (IV) an acetyl-CoA acetyltransferase.

responses or following stimulation by various agonists occurs via the enzymatic remodeling of alkylacyl glycerophosphocholines, phospholipids that are highly enriched in *sn*-2 arachidonoyl residues. Structural modification of this ether-linked phospholipid consists of replacing the *sn*-2 acyl moiety with an acetyl residue. The enzymes responsible for catalyzing the hydrolytic deacylation step appear to be highly specific for the molecular species of alkylacyl (or diacyl) glycerophosphocholines possessing an arachidonoyl moiety at the *sn*-2 position (T.G. Tessner, 1990). The initial reaction that produces lyso-PAF requires either the combined actions of a CoA-independent transacylase or PLA_2 (Fig. 9, Reaction II; indirect route) or a single direct hydrolytic step catalyzed by a PLA_2 (Fig. 9, Reaction I). A CoA-dependent transacylase (reversal of an acyl-CoA acyltransferase reaction) is also capable of generating lyso-PAF (Fig. 10, Reaction I).

A number of studies indicate that the 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) (Chapter 11) is likely the phospholipase responsible for release of arachidonic acid, and therefore PAF synthesis, in stimulated cells (B.B. Rubin, 2005). This $cPLA_2$ is highly selective for the arachidonoyl residue, as is the CoA-independent transacylase. One of the most convincing findings showing that $cPLA_2$ is responsible for initiating the remodeling pathway is that macrophages from $cPLA_2$ knockout mice almost completely lose their ability to synthesize both PAF and eicosanoids [13,14]. What is not clear is whether the enzyme acts on plasmanylcholine directly to produce lyso-PAF or acts on arachidonate-rich

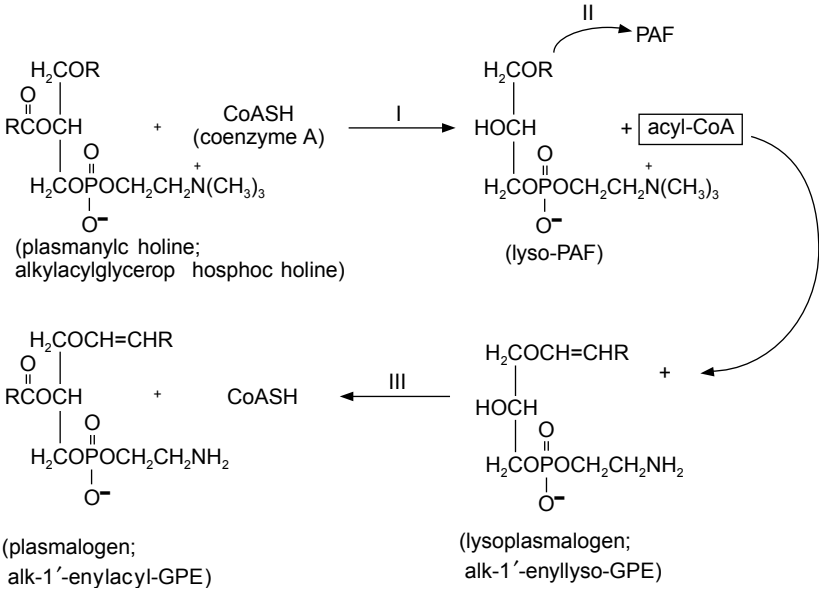


Fig. 10. Involvement of a CoA-*dependent* transacylase in the production of lyso-platelet-activating factor (lyso-PAF) for the synthesis of PAF and the remodeling of the *sn*-2 acyl group of membrane phospholipids. The enzymes catalyzing these reactions are (I) CoA-*dependent* transacylase (with CoA as the acyl acceptor), (II) acetyl CoA:lyso-PAF acetyltransferase, and (III) acyl-CoA:lysophospholipid acyltransferase. The reaction depicted for the CoA-*dependent* transacylase represents the reversal of the reaction catalyzed by acyl-CoA:lysophospholipid acyltransferase. GPE, *sn*-glycero-3-phosphoethanolamine.

ethanolamine plasmalogen, e.g., to form a lysoplasmalogen acceptor for the transacylation reaction that then forms lyso-PAF. Since the $cPLA_2$ does not distinguish between the ester and ether linkage in the *sn*-1 position, both PAF and acyl-PAF are recovered from stimulated cells. The $cPLA_2$ activity is regulated by phosphorylation of the enzyme and by a translocation from the cytosol to membranes that requires micromolar levels of Ca^{2+}.

Production of lyso-PAF via the transacylation step can occur in either a CoA-*independent* (Fig. 9) or CoA-*dependent* (Fig. 10) manner. With the CoA-*independent* transacylase, ethanolamine lysoplasmalogens, as well as other ethanolamine- or choline-containing lysoglycerophosphatides, serve as the acyl acceptor for the selective transfer of an arachidonoyl moiety from alkylarachidonoyl glycerophosphocholine. CoA itself, instead of a lysophospholipid, is the acyl acceptor in the reaction catalyzed by the CoA-dependent transacylase. This type of transacylation is thought to represent the reverse of the reaction catalyzed by an acyl-CoA:lyso-PAF acyltransferase and is not as selective for arachidonate. In addition to participating in the formation of lyso-PAF, transacylase activity also serves an important role in the remodeling of acyl moieties located at the *sn*-2 position of the choline- and ethanolamine-containing phospholipids.

The lysoplasmalogen and other lysophospholipid acceptors that are substrates for transacylase activity appear to be formed by the direct action of a PLA_2 on the appropriate membrane-associated phospholipid, thereby simultaneously releasing arachidonic acid for its subsequent metabolism to bioactive eicosanoid products (Chapter 12). In both the direct and indirect routes of lyso-PAF production, the action of a PLA_2 is required; it is plausible that both routes participate in PAF synthesis to varying degrees. It is notable that both eicosanoid and PAF mediators can be formed via the remodeling pathway and that these mediators can act synergistically.

Acetylation of lyso-PAF, the final step in the remodeling pathway, is catalyzed by an acetyl-CoA:lyso-PAF acetyltransferase (Fig. 9, Reaction IV). This membrane-bound enzyme acetylates both the alk-1′-enyl and the acyl analogs of lyso-PAF, and utilizes short-chain acyl-CoAs ranging from C_2–C_6 as substrates (T.-C. Lee, 1985), although PAF is by far the prominent cellular product. The acetyltransferase responsible for the final step in PAF synthesis is a membrane-bound activity whose identity is opaque. Recently however, homology cloning has identified an acyltransferase that transfers acetyl residues to lyso-PAF, and its activity is enhanced in cells stimulated by lipopolysaccharide [27]. The enzyme displays the anticipated specificity for a choline head group of the acceptor lipid. This enzyme, however, also transfers arachidonoyl residues from arachidonoyl-CoA and does so with a lower K_m and higher V_{max} than the reaction-forming PAF from acetyl-CoA, so definition of its role in inflammatory physiology awaits results from genetically targeted animals. Enzymatic activity of the enzyme in transfected cells is suppressed by a p38 kinase inhibitor, and previous work indicates that agonist-stimulated activity is aided by the p38 stress-activated MAP kinase, but not by p42 and p44 ERKs. In contrast, $cPLA_2$ is activated in these cells by both ERKs and p38 kinases.

7.3.2. De novo route

PAF biosynthesis via the de novo pathway is thought to be the primary source of the endogenous PAF in unstimulated cells and blood (Fig. 11). Physiological factors such as fatty acids and neurotransmitters stimulate PAF synthesis by this pathway, but, unlike the

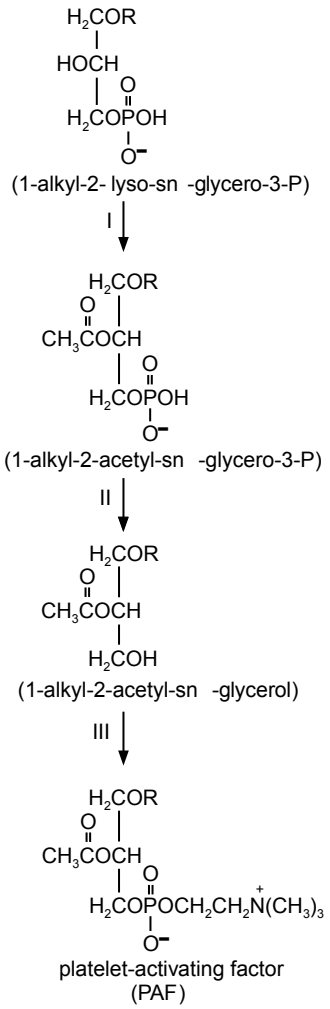


Fig. 11. Biosynthesis of platelet-activating factor (PAF) via the de novo pathway. The three-step reaction sequence, beginning with 1-alkyl-2-lyso-*sn*-glycero-3-phosphate as the precursor, is catalyzed by (I) acetyl-CoA:alkyllysoglycerophosphate acetyltransferase, (II) alkylacetylglycero-phosphate phosphohydrolase, and (III) dithiothreitol-insensitive CDP–choline:alkylacetylglycerol cholinephosphotransferase.

remodeling pathway, the de novo pathway does not generate free arachidonic acid for eicosanoid synthesis.

The sequence of enzymatic reactions (Fig. 11) involved in the de novo route include (a) acetylation of 1-alkyl-2-lyso-*sn*-glycero-3-phosphate by an acetyl-CoA-dependent acetyl-transferase (Fig. 11, Reaction I), (b) dephosphorylation of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphate (Fig. 11, Reaction II), and (c) the transfer of phosphocholine from CDP-choline to 1-alkyl-2-acetyl-*sn*-glycerol by a dithiothreitol-insensitive cholinephosphotransferase (Fig. 11, Reaction III) to form PAF. The acetyltransferases associated with the remodeling

(Fig. 9) and de novo routes (Fig. 11) possess distinctly different properties and substrate specificities (F. Snyder, 1997). Also, the dithiothreitol-insensitivity of this cholinephosphotransferase contrasts with the inhibitory effect of dithiothreitol on the cholinephosphotransferase that synthesizes phosphatidylcholine and plasmanylcholine from diacylglycerols and alkylacylglycerols, respectively. In addition, the two dissimilar cholinephosphotransferase activities that synthesize PAF and phosphatidylcholine exhibit different pH optima and respond differently to detergents, ethanol, temperature, and substrates. Although the enzymes in the de novo pathway exhibit a relatively high degree of substrate specificity, the *sn*-1 acyl analogs of the corresponding *O*-alkyl equivalents can also be utilized as substrates by the acetyltransferase, phosphohydrolase, and the dithiothreitol-insensitive cholinephosphotransferase.

8. Catabolic enzymes

8.1. Ether lipid precursors

8.1.1. Fatty alcohols

Fatty alcohols are oxidized to fatty acids by an NAD^+:fatty alcohol oxidoreductase, a microsomal enzyme found in most mammalian cells (Fig. 3). The high activity of this enzyme probably accounts for the extremely low levels of unesterified fatty alcohols generally found in tissues or blood. Detection of fatty aldehydes, by trapping them as semi-carbazide derivatives during oxidation of the alcohol, suggests that the fatty alcohol oxidoreductase catalyzes a two-step reaction that involves an aldehyde intermediate.

8.1.2. Dihydroxyacetone phosphate and acyldihydroxyacetone phosphate

DHAP can be diverted from its precursor role in ether lipid synthesis by conversion to *sn*-glycerol-3-phosphate by NADH:glycerol-3-phosphate dehydrogenase. Another bypass that prevents the formation of alkyl-DHAP occurs if the ketone function of acyl-DHAP is first reduced by an NADPH-dependent oxidoreductase, since the product, 1-acyl-2-lyso-*sn*-glycerol-3-phosphate, can then be converted only to diacylglycerolipids. The metabolic removal and/or formation of fatty alcohols, DHAP, or acyl-DHAP from the ether lipid precursor pool may control ether lipid synthesis and accumulation.

8.2. Ether-linked lipids

8.2.1. O*-alkyl-cleavage enzyme*

Oxidative cleavage of the *O*-alkyl linkage in glycerolipids is catalyzed by a microsomal tetrahydropteridine ($Pte{\cdot}H_4$)-dependent alkyl monooxygenase (Fig. 12) (T.-C. Lee, 1981). The required cofactor, $Pte{\cdot}H_4$, is regenerated from $Pte{\cdot}H_2$ by an NADPH-linked pteridine reductase, a cytosolic enzyme. Oxidative attack on the ether bond in lipids is similar to the enzymatic mechanism described for the hydroxylation of phenylalanine. Fatty aldehydes produced via the cleavage reaction can be either oxidized to the corresponding acid or reduced to the alcohol by appropriate enzymes.

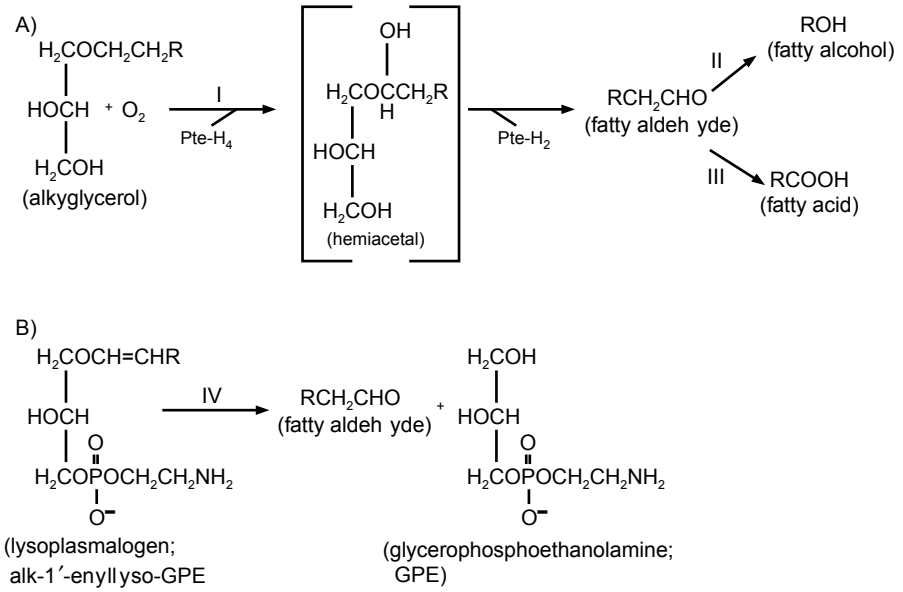


Fig. 12. Cleavage of the *O*-alkyl linkage in glycerolipids (A) is catalyzed by (I) a Pte·H_4-dependent alkyl monooxygenase, a microsomal enzyme found primarily in liver and intestinal tissues. The hemiacetal shown in this reaction has not been isolated because of its instability. The fatty aldehyde product can be either reduced to a long-chain fatty alcohol by (II) a reductase or oxidized to a fatty acid by (III) an oxidoreductase. Removal of the *O*-alk-1′-enyl moiety from plasmalogens (B) is catalyzed by plasmalogenase. As with the *O*-alkyl monooxygenase, the fatty aldehyde can be converted to either the corresponding fatty alcohol or the fatty acid. GPE, *sn*-glycero-3-phosphoethanolamine.

Alkyl-cleavage enzyme activities are highest in liver and intestinal tissue, whereas most other cells and tissues possess very low activities. Tumors and other tissues that contain significant quantities of alkyl lipids generally have very low alkyl-cleavage enzyme activities, so the level of ether-linked glycerolipids is inversely proportional to the activity of the alkyl-cleavage enzyme.

Structural features of glycerolipid substrates utilized by the alkyl-cleavage enzyme are (a) an *O*-alkyl moiety at the *sn*-1 position, (b) a free hydroxyl group at the *sn*-2 position, and (c) a free hydroxyl or phosphobase group at the *sn*-3 position. If the hydroxyl group at the *sn*-2 position is replaced by a ketone or acyl function, or when a free phosphate is at the *sn*-3 position, the *O*-alkyl moiety at the *sn*-1 position is not cleaved by the Pte·H_4-dependent monooxygenase. Thus, 1-alkyl-2-lysophospholipids (e.g., lyso-PAF) are substrates for the cleavage enzyme, but they are attacked at much slower rates than are alkylglycerols.

8.2.2. Plasmalogenases

Plasmalogenases (Fig. 12) hydrolyze the *O*-alk-1′-enyl function of plasmalogens or lysoplasmalogens. The products of this reaction are a fatty aldehyde and either 1-lyso-2-acyl-*sn*-glycero-3-phosphoethanolamine (or choline) or *sn*-glycero-3-phosphoethanolamine

(or choline), depending on the structure of the parent substrate. Plasmalogenase activities have been described in microsomal preparations from liver and brain of rats, cattle, and dogs, but their biological significance is poorly understood.

8.2.3. Phospholipases and lipases

In general, the *sn*-2 and *sn*-3 esters of either alkyl or alk-1′-enyl glycerolipids are hydrolyzed by lipolytic enzymes with the same degree of substrate specificity as their acyl counterparts (Fig. 13). One exception is a recently described Ca^{2+}-independent $PLA_2\gamma$ that effectively hydrolyzes *sn*-2 arachidonoyl residues from plasmenylcholine but not

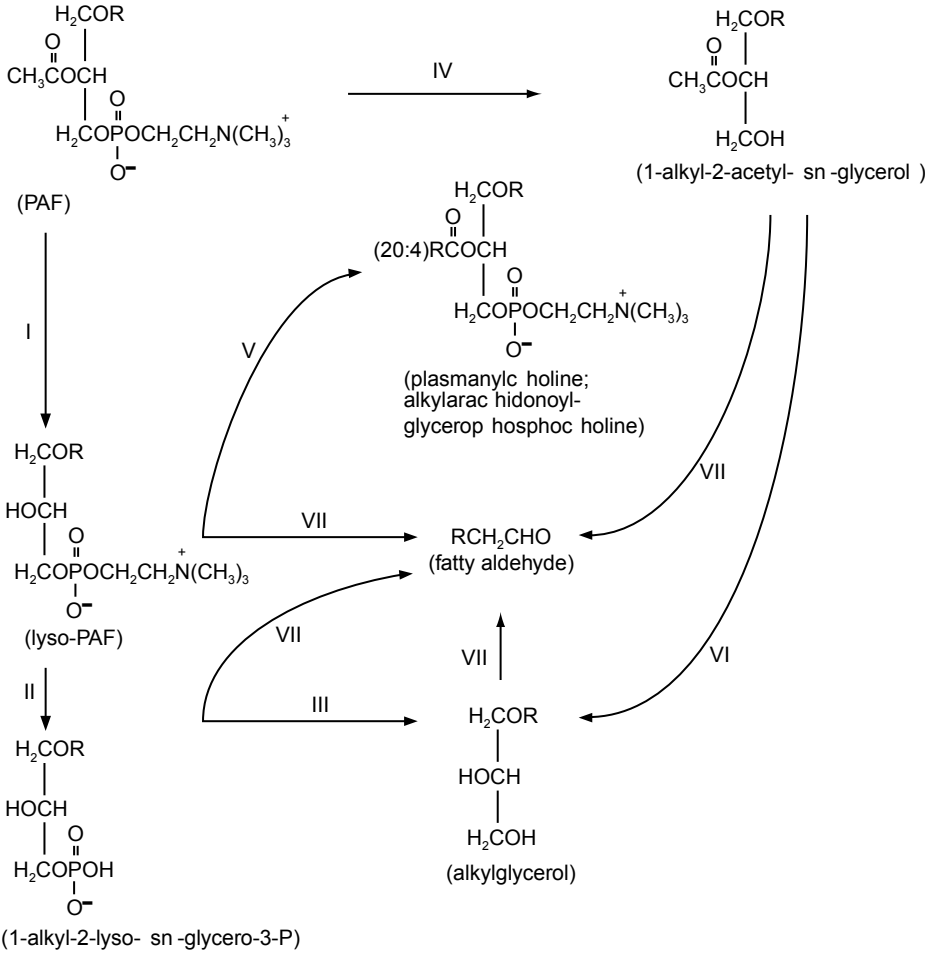


Fig. 13. Catabolism of platelet-activating factor (PAF) and its metabolites by (I) PAF-AH, (II) lysophospholipase D, (III) phosphohydrolase, (IV) phospholipase C, (V) CoA-*independent* or CoA-*dependent* transacylase, and/or (VI) alkylacetylglycerol acetylhydrolase. The *O*-alkyl linkage in products that contain free hydroxyl groups can be cleaved by (VII) the *O*-alkyl Pte·H_4-dependent monooxygenase.

palmitoyl arachidonoyl phosphatidylcholine (W. Yan, 2005). However, the ether linkages themselves are not hydrolyzed by lipases or phospholipases and the presence of an ether linkage at the *sn*-1 position of the glycerol moiety generally reduces the overall metabolic rate. The only other lipolytic activity (other than those that cleave the ether linkages) known to display specificity for ether-linked lipids is a microsomal lysophospholipase D (R.L. Wykle, 1980).

8.3. PAF and related bioactive species

8.3.1. Acetylhydrolases

PAF acetylhydrolases (PAF-AHs) (Fig. 13) are a group of Ca^{2+}-independent PLA_2s that remove the acetyl moiety from the *sn*-2 position of PAF [13]. These enzymes, in contrast to other PLA_2s, are highly selective and do not attack phospholipids with unmodified long-chain *sn*-2 residues. Mammalian PAF-AHs have been classified into type VIII (PAF-AH-Ib) that consists of two family members that homo- or heterodimerize, and two type VII enzymes (liver and plasma isoforms).

PAF-AH-Ib is enriched in brain where it is exclusively located in cytosol, and is also the form present in erythrocytes. The enzyme is an unusual G-protein-like heterotrimeric complex composed of 45(beta)-, 30($alpha_2$)-, and 29($alpha_1$)-kDa subunits. The 45-kDa subunit is not catalytically active and appears to be a scaffolding protein. The catalytic 30($alpha_2$)-kDa subunit is highly homologous (63.2% identity) to that of the 29($alpha_1$)-kDa subunit, especially (86%) in the catalytic and PAF receptor homologous domains. When these subunits are purified or overexpressed, they form homodimers or heterodimers that specifically hydrolyze PAF. Genetic ablation of the $alpha_1$ murine gene did not cause an overt phenotype, while deletion of the $alpha_2$ gene altered only testicular size.

PAF-AH type II (PAF-AH II), most abundant in liver and kidney, is a monomeric 40-kDa protein [14]. A region surrounding the active serine residue in the GXSXG lipase motif exhibits high homology with the active sites of other lipases and esterases. This enzyme exhibits broader substrate specificity than PAF-AH-Ib, and PAF-AH II hydrolyzes oxidized phospholipids with fragmented, shortened *sn*-2 residues as effectively as PAF. Furthermore, unlike PAF-AH I, PAF-AH II is distributed in both the membrane and soluble fractions. Indeed, PAF-AH II is an *N*-myristoylated enzyme (Chapter 2) that translocates between cytosol and membranes in response to the redox state of the cell. This enzyme protects cells against oxidative stress-induced death when overexpressed, suggesting that PAF-AH II hydrolyzes oxidized phospholipids produced during reactive oxygen species-induced apoptosis. The enzyme forms an acetyl enzyme intermediate and transfers the acetyl group from PAF to water, but also may transfer it to a variety of lysophospholipids and sphingosine to form a series of PAF analogs (K. Bae, 2000). Among all the lysophospholipids tested, acyl lyso-glycero-3-phosphocholine is the most active acetyl group acceptor. This CoA-independent transacetylase activity differs from the CoA-independent transacylase that transfers long-chain acyl moieties, reflecting the substrate specificity of the enzyme.

Plasma PAF-AH is unique in that it is primarily associated with circulating high-density and low-density lipoprotein particles and rapidly transfers between these particles (D.M. Stafforini, 1987). The cDNA for this enzyme encodes a 44-kDa secretory protein

that contains a typical signal sequence and a serine esterase/neutral lipase consensus motif GXSXG. Serine-273 (of the GXSXG motif), Asp-296, and His-351, which form a catalytic triad, are required for catalysis. The rate of PAF degradation under conditions likely to be present in vivo, i.e., at low PAF concentrations, is strongly influenced by the lipoprotein environment because the enzyme associated with low-density lipoprotein particles is a much more effective catalyst than when the same enzyme is associated with high-density lipoproteins. Plasma PAF-AH displays 40% homology with intracellular PAF-AH II, but not with PAF-AH I, over the whole amino acid sequence. Thus, plasma PAF-AH also hydrolyzes PAF and structurally related oxidized phospholipids with fragmented *sn*-2 residues of any length if, and only if, the *sn-2* residue contains a newly introduced oxy function. In the absence of such polar additions, the enzyme will effectively hydrolyze *sn*-2 fragments of oxidized phospholipids only up to five carbon atoms in length. Pretreatment of animals with recombinant plasma PAF-AH blocks PAF-induced inflammation. PAF-AH has been tested clinically, but the phase III trial showed no decrease in all-cause mortality in a population of patients with sepsis syndrome (S. Opal, 2004).

9. Metabolic regulation

Regulatory mechanisms that control the metabolism of ether-linked lipids are still poorly understood, although recent progress in molecularly identifying ether phospholipid synthetic enzymes demonstrates that the relative abundance of this class of complex lipid is regulated. Regulatory controls that must be considered in the metabolism of ether-linked lipids are those that influence (a) the enzymes responsible for catalyzing the biosynthesis and catabolism of the ether lipid precursors (fatty alcohols and DHAP), (b) alkyl-DHAP synthase which is responsible for the synthesis of the committed precursor, and (c) branch point enzymes, e.g., those that utilize diradylglycerols. Recent data show that a cell line defective in DHAPAT activity, which initiates ether lipid synthesis, recovers half of its plasmalogen pool when the enzyme is expressed at just 10% of its wild-type level, but that full recovery requires six times the level of enzyme expressed by control cells (D. Liu, 2005). Plasmalogen synthesis is therefore independently regulated, but by factors yet to be defined.

Glycolysis plays an important role in controlling the levels of ether lipids at the precursor level. For example, the high glycolytic rate of tumors generates significant quantities of DHAP, which could explain the relatively high levels of ether lipids found in cancerous cells. Such a correlation has been reported for several transplantable hepatomas that possess high rates of glycolysis, low glycerol phosphate dehydrogenase activities, and high levels of ether-linked lipids. Elevated levels of long-chain fatty alcohols, the precursors of the *O*-alkyl chains, also occur in conjunction with the higher concentrations of DHAP in tumor cells.

Arachidonic acid influences PAF formation by the remodeling pathway, and cells depleted of alkylarachidonoyl glycerophosphocholines lose their ability to synthesize PAF. Therefore, the transacylase/PLA_2 step (Fig. 9, Reaction II) as well as a specific PLA_2 (Fig. 9, Reaction I or III) can be rate-limiting. Regulation of the acetyl-CoA:lyso-PAF acetyltransferase in the remodeling pathway (Fig. 9, Reaction IV) appears to be controlled by a p38 kinase phosphorylation or phosphohydrolase system.

10. Functions

10.1. Insights from genetic diseases with impaired ether phospholipid synthesis

The initial two steps of ether phospholipid synthesis are unique to the pathway and are exclusively localized in the peroxisomal compartment. Peroxisomal disorders where either mutation of DHAP acyltransferase or alkyl-DHAP synthase, or mutation of the importer that localizes these enzymes to peroxisomes, results in a similar phenotype, a syndrome of rhizomelic chondrodysplasia punctata [4,10]. Mutations in *Pex7*, which recognizes and imports the type 2 peroxisomal localization signal of alkyl-DHAP synthase into peroxisomes, is defective in type 1 rhizomelic chondrodysplasia punctata. Type 2 rhizomelic chondrodysplasia punctata results from mutation of the gene encoding DHAP acyltransferase, while type 3 results from mutations in the alkyl DHAP synthase gene. The prototypical peroxisomal disease Zellweger syndrome also lacks functional peroxisomes and ether phospholipid synthesis. Mice with these characteristics are phenocopied by defects in Pex peroxisomal transporters. The similarity of the outcomes of these varied mutations suggests that the key defect resulting from the absence of functional peroxisomes is the accompanying loss of plasmalogen and alkyl phospholipid synthesis. The developmental defects in humans, in DHAP acyltransferase knockout mice [1], and cultured cells with defective ether phospholipid synthesis [9] all show that ether phospholipids have roles that cannot be assumed by diacyl lipids. Among the defects at the cellular level resulting from the loss of ether phospholipid synthesis are structural alterations in caveolae, clathrin-coated pits, Golgi, and endoplasmic reticulum leading to defective membrane flux and organelle trafficking [28]. Plasmalogens are components of membrane lipid rafts and disorganization of these specialized domains that sequester signaling molecules may contribute to the phenotypes displayed when ether phospholipid synthetic capacity is lost. Direct manipulation of ether phospholipid synthesis by knocking out murine genes for the DHAP acyltransferase, DHAP synthase or genes involved in correctly localizing peroxisomal genes, confirms that ether phospholipids have unique roles in development and cell function consistent with events arising when the corresponding human genes are mutated [11].

10.2. Insights from genetic alterations in PAF signaling and metabolism

PAF is primarily a specialized signaling molecule acting on closely opposed cells and has been the focus of the bulk of the current literature on ether phospholipids because of its participation in acute inflammation. Critical observations by Shimizu and colleagues [14] after the single receptor for PAF was deleted in mice were that these mice were unable to respond to PAF, that the mice developed normally, and that these mice did not recapitulate the phenotype of any of the mice where ether phospholipid synthesis was abolished. These observations show that PAF is not the only relevant ether phospholipid, and that either other processes can substitute for PAF signaling — although none are known — or PAF can serve to modulate, rather than initiate, physiological processes. Indeed, subjecting mice that either lack the PAF receptor or have been engineered to overexpress the

receptor show that differences do arise when the animals are appropriately stressed. For instance, mice lacking the PAF receptor have a sharply curtailed mortality in a model of systemic anaphylaxis induced by antigen challenge (S. Ishii, 1998). Conversely, transgenic overexpression of the PAF receptor yields mice that display more severe bronchopulmonary response to methacholine challenge, a model of airway hyperreactivity and asthma, than control mice, and are more susceptible to endotoxic shock (S. Ishii, 1997). However, experiments with either the knockout or the currently derived transgenic animals have yet to fully reveal the physiological role of the PAF receptor because not all experiments used inbred knockout strains, while transgenic overexpression was confined to non-inflammatory cells. It is possible that PAF receptor overexpression in its normal distribution is lethal, suggesting that transgenic animals with an inducible PAF receptor would offer new insights.

A single human PAF receptor variant with an allelic frequency of 7.8% in the Japanese population, giving ~14% heterozygotes, has been identified that partially reduces PAF signaling (K. Fukunaga, 2001). To date, this polymorphism has not been associated with a phenotype or syndrome.

The role of PAF-AHs in pathophysiology is yet to be fully defined because murine knockouts of neither the plasma nor the liver PAF-AH has been reported. However, single and double knockouts of the type Ib alpha$_1$ and alpha$_2$ PAF-AH genes reveal a role in spermatogenesis. A human deficiency of plasma PAF-AH has been reported that results from an inactivating point mutation in exon 9 (D.M. Stafforini, 1996). This mutation is infrequent in the Caucasian population, but 4% of the Japanese population are homozygotic nulls for the plasma PAF-AH, with 27% being heterozygotes that have half the normal PAF hydrolytic activity in their circulation. These individuals display a propensity for inflammatory complications, but the loss of this circulating activity confers no overt phenotype. An increase in plasma PAF-AH activity has been reported in humans or rats with hypertension, and in the plasma of patients with atherosclerosis, showing that the level of PAF-AH is modulated. In fact, the enzyme acts as an acute-phase reactant during sepsis, and the gene is transcriptionally regulated by endotoxin. Differentiated macrophages, but not the monocytes from which they derive, are robust sources of secreted enzyme. The human exon 9 mutation has shown that circulating plasma PAF-AH is primarily derived from Kupffer cells, resident liver macrophages, because the phenotype after bone marrow transplantation reflects that of the donor.

The plasma and liver isoforms of PAF-AH additionally hydrolyze phospholipid *sn*-2 residues that have been oxidatively modified in any of several ways. These enzymes therefore act as oxidized phospholipid phospholipases and this role may have been an evolutionarily early one because a homologous gene in the distantly related yeast *Schizosaccharomyces pombe* encodes a functional enzyme that reduces oxidative cell death. Overexpression of plasma or liver PAF-AH blocks apoptosis in response to a variety of insults and excess plasma PAF-AH reduces atherogenesis in a murine model of the disease (R. Quarck, 2001). Presumably, this beneficial effect results from hydrolysis of the pro-inflammatory mediator PAF or oxidatively fragmented phospholipids that, depending on the nature of the fragment left at the *sn*-2 position, also are PAF receptor agonists or act as potent pro-apoptotic agents.

10.3. Tumor ether phospholipids and anti-tumor ether phospholipids

Myeloid cells, neutrophils, monocytes, and macrophages are enriched in ether phospholipids, while chronic granulocytic leukemic cells have a lower content that correlates with cellular differentiation. An unnatural synthetic analog of PAF (Fig. 2), 1-alkyl-2-methoxy-*sn*-glycero-3-phosphocholine (edelfosine, ET-18-OCH_3) and related derivatives, displays highly selective anti-tumor activity (C. Gajat, 2002). The mechanism of action of this class of PLA_1- and PLA_2-resistant di-ether phospholipids has been difficult to ascertain, but for some cell lineages the critical difference between their effect on tumor cells compared to normal cells is the selective modification of the composition of plasma membrane lipid rafts that organize critical mitogenic signaling systems. Interestingly, the susceptibility of related tumor cells to the cytotoxic effect of anti-tumor ether phospholipids depends on the size of the cellular pool of ether phospholipids.

10.4. Membrane components

Cellular functions of ether-linked glycerolipids are especially poorly understood, but their ability to serve as both membrane components and as cellular mediators is now well established. The physical property of plasmalogens alters membrane packing and phase behavior that may participate in membrane trafficking [10]. Plasmalogens are enriched in polyunsaturated fatty acyl residues and may serve as reservoirs during essential fatty acid deficiency, while their unique *sn*-1 vinyl ether bond makes them particularly susceptible to oxidative attack. The latter property, it is proposed, may make this class of phospholipids effective scavengers of reactive oxygen species.

11. Future directions

There are many unanswered questions about the dual role that ether lipids serve as membrane components and as cellular signaling molecules. Although it is clear that arachidonic acid is closely associated and tenaciously retained by ether lipids in membranes, even in essential fatty acid deficiency, much remains to be elucidated about the enzymatic systems and regulatory controls that affect the release of this sequestered pool of arachidonic acid for its subsequent conversion to bioactive eicosanoid metabolites. The significance of ether lipids as a dietary nutrient has received little attention even though they occur in a variety of foods and it is known that ether lipid supplements are readily incorporated into cellular lipids.

Work continues on the subcellular distribution of the ether lipids and the enzymes responsible for their synthesis and enrichment in polyunsaturated fatty acids. The role of ether lipids in peroxisome-deficiency disorders has been highly informative, but the pleiotropic changes that occur with the loss of peroxisomal targeting proteins make it difficult to fully discern cause from effect. It is noteworthy that organisms living in harsh environments of high temperatures or high salt and low pH contain only ether lipids, suggesting these serve as the Teflon of lipids. Migratory cells such as neutrophils and eosinophils also have high levels of ether lipids, leading to the speculation that the high levels of ether lipids in most tumor cells may contribute to their motility.

A major enigma is the function of plasmalogens. Despite the large quantities of ethanolamine plasmalogens found in nervous tissue and other cells, neither their cellular role nor the regulatory controls for their formation are known. The alkyl desaturase has yet to be purified, cloned, and carefully compared to fatty acid desaturases. Likewise, the biosynthesis of choline plasmalogens is still not fully understood, although compelling evidence indicates that they originate from ethanolamine plasmalogens via remodeling mechanisms. Much remains to be discovered.

Abbreviations

CoA	coenzyme A
DHAP	dihydroxyacetone phosphate
DHAPAT	dihydroxyacetone phosphate acyltransferase
$FADH_2$	reduced flavin adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PAF	platelet-activating factor
PAF-AH	PAF acetylhydrolase
PLA_2	phospholipase A_2
PST	peroxisomal targeting sequence
$Pte{\cdot}H_4$	tetrahydropteridine

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CHAPTER 10

Lipid metabolism in adipose tissue

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Contents

1. Introduction

The development of adipose tissue and the biochemistry of the adipocyte are research areas that have intrigued investigators for decades. Originally considered as simply a storage organ for triacylglycerol, interest in the biology of adipose tissue has increased substantially within the last decade, coming to the forefront in areas such as molecular

genetics, endocrinology, and neurobiology. Recent advances have demonstrated that the adipocyte is not a passive lipid storage depot but a dynamic cell that plays a fundamental role in energy balance and overall body homeostasis. Moreover, the fat cell functions as a sensor of lipid levels, transmitting information to a neural circuit affecting major biological processes including hunger, sleep, and reproduction.

This chapter will focus on the biochemistry of lipid metabolism in the adipocyte. Adipocytes make up approximately one-half of the cells in adipose tissue, the remainder being blood and endothelial cells, adipose precursor cells of varying degrees of differentiation, macrophages, and fibroblasts. The reader is referred to an excellent review by Rosen and MacDougald (2006) that focuses exclusively on the differentiation process. While touching on adipose cell biology, this chapter will emphasize the biochemistry of triacylglycerol metabolism and lipid signaling in the adipocyte.

2. Adipose development

2.1. Development of white and brown adipose tissue

The study of white adipose tissue (WAT) development in mammals has been facilitated by the use of experimental animal models. Rodents, guinea pigs, rabbits, pigs, as well as humans, have all been evaluated for the development of WAT. In general, WAT is not detected at all in mice or rats during embryogenesis but in pigs and humans is evident during the last third of gestation. In humans, small clusters of adipocytes are present that increase in size during gestation. Larger clusters of fat cells are associated with tissue vascularization and a general increase in cluster size is positively correlated with larger blood vessels. Paracrine/autocrine factors play a significant role in both capillary growth and adipose conversion.

After birth, sex- and site-dependent differences in fat deposits are well known in humans and several animal species. The diet plays a critical role in the degree of lipid filling within an adipocyte. However, controversy surrounds the mechanism of new fat cell development following a long-term fast. In general, starvation conditions lead to a loss of adiposity and some apparent diminution in the number of fat cells. Refeeding restores lipid levels and the apparent number of adipocytes. It is generally accepted that adipose precursor cells are present throughout life and that removal of adipocytes, either by diet or surgical methods, will only temporarily reduce adiposity.

In contrast to WAT, brown adipose tissue (BAT) develops during fetal life and is morphologically and biochemically identifiable at birth. Using the uncoupling protein-1 (UCP1) as a BAT marker (specific for BAT mitochondria, Section 3.6), brown fat development has been shown to occur maximally during the last third of gestation. Two conditions enhance the development of brown fat hyperplasia in rodents: cold-acclimation and hyperphagia (overeating). Both conditions result in a metabolic demand for high-energy expenditure, either in the form of increased requirement for heat generation or increased metabolism. BAT is common in rodents, camels, and hibernating animals such as bears and marmots. The oxidation of triacylglycerol stores in BAT depots during hibernation or fasting provides certain animals with a source of water and energy during nutrient

deprivation. In humans, although still somewhat controversial, it is generally accepted that brown fat is found in only small amounts and that WAT carries out the body's energy storage functions.

In general it is assumed that WAT and BAT develop from different precursor cells. However, a common precursor for both cannot be ruled out and the possible transformation of BAT into WAT has been considered. The conversion from BAT to WAT would be correlated with a decrease in BAT-specific gene products such as UCP. Recently, experiments have suggested that under certain conditions WAT might take on a BAT-like phenotype, with increased mitochondria function.

2.2. In situ models of adipose conversion

Rosen, MacDougald, and colleagues [1] have described the adipoblast to adipocyte conversion as a multi-step process initiating with the commitment of pluripotent proliferative cells to the adipocyte pathway, Table 1. To better characterize the differentiation process and examine the molecular basis of adipose development, a number of murine, hamster, and rat model cell lines (3T3-L1, 3T3-F442A, $10T^1/_2$, Ob1771) have been established. In general, committed preadipocytes express few markers associated with mature fat cells and are capable of DNA replication and cell division. Terminal differentiation of cultured cells is frequently induced through the addition of insulin-like growth factor-1 (or high concentrations of insulin), glucocorticoids (e.g., dexamethasone), and a phosphodiesterase inhibitor (isobutylmethylxanthine). Following differentiation, the mature adipocytes exhibit a massive triacylglycerol accumulation and are responsive to hormonal stimulation.

For decades, brown fat metabolism has been studied with tissue explants. While WAT is important for the storage of energy in the form of triacylglycerol, BAT functions to dissipate energy in the form of heat through the action of a specific mitochondrial proton transporter, UCP1. While the 3T3-L1 or 3T3-F442A cells lines provided a convenient

Table 1
Stages of adipose conversion

Stages	Cell type	Characteristics
Stage 1	Mesenchymal/pluripotent	Multipotential — ability to differentiate into muscle, cartilage, or fat
	Determination	
Stage 2	Adipoblasts	Unipotential — can differentiate only into adipocytes
	Commitment	
Stage 3	Preadipocytes	No lipid accumulation, early transcription factors, e.g., C/EBPβ and early markers of differentiation expressed, e.g., lipoprotein lipase
	Terminal differentiation	
Stage 4	Adipocytes	Lipid accumulation and expression of late transcription factors, e.g., PPARγ and C/EBPα and late markers of differentiation, e.g., PEPCK, AFABP/aP2, FATP

system to study WAT metabolism, similar BAT models were, until recently, lacking. However, by expressing the simian virus 40 early genes under control of the strong fat cell-specific adipocyte fatty acid binding protein/aP2 (FABP4) promoter in transgenic animals, brown fat tumors developed due to t-antigen induced oncogenesis. Such tumors were used to derive hibernoma cell lines (rapidly growing brown fat cells) exhibiting the properties of BAT. The BAT hibernomas express the UCP mRNA upon stimulation with cAMP, cAMP analogs, or a variety of β_2 and β_3 receptor agonists. Such cell lines have been extremely useful for the study of BAT gene expression and metabolism.

2.3. Transcriptional control during development

To characterize the molecular basis for differential gene expression, a number of laboratories have identified transcription factors regulating genes expressed in adipocytes. Of those genes most actively studied, the adipocyte fatty acid binding protein gene (FABP4) and the insulin-stimulated glucose transporter gene (GLUT4) have proven particularly useful. The FABP4 gene is expressed in an adipose-specific manner and is upregulated at least 50-fold as a consequence of adipose conversion [2]. The FABP4 gene is regulated by glucocorticoids, insulin, and polyunsaturated fatty acids while GLUT4 is regulated primarily by insulin, cAMP, and fatty acids. Using these and other adipose genes as templates, four different transcription factor families have been identified as critical components affecting either the adipocyte differentiation program and/or the lipid storage pathway.

2.3.1. C/EBP family of transcription factors

The CCAAT/enhancer-binding proteins (C/EBP) comprise a family of transcription factors strongly implicated in the control of genes involved in intermediary metabolism [3]. Originally cloned by McKnight and colleagues (S.L. McKnight, 1989), the C/EBPs are leucine-zipper transcription factors whose sequences are characterized by the presence of a basic region followed by a leucine-rich motif. Leucine-zipper proteins are capable of forming coiled–coil interactions with other similar types of factors. As such, the C/EBPs form homo- and hetero-dimers with other family members, thereby allowing their binding to *cis*-regulatory elements within the promoter/enhancers of genes regulated by C/EBPs.

A number of genes involved in adipose lipid metabolism are regulated by the C/EBP family of transcription factors including FABP4, stearoyl-coenzyme A (CoA) desaturase, and insulin-stimulated glucose transporter genes. Expression of antisense C/EBPα RNA in 3T3-L1 preadipocytes blocked the expression of C/EBPα and concomitant expression of several adipocyte genes including GLUT4 and the adipocyte fatty acid binding protein (aP2). Moreover, in such antisense C/EBPα-expressing cells the accumulation of cytoplasmic triacylglycerol was blocked, suggesting that a global inhibition of genes expressing proteins of adipose lipid metabolism was occurring. Consistent with a central role for C/EBPα in lipid metabolism, mice bearing a targeted disruption in the C/EBPα allele (J. Yang, 1995) fail to accumulate triacylglycerol in both adipose and liver.

Three members of the C/EBP family of transcription factors are expressed in adipocytes: α, β, and δ. The temporal expression of the three isoforms during 3T3-L1 differentiation suggests that the C/EBP genes are subject to exquisite regulatory controls. Numerous studies have shown that C/EBPβ and C/EBPδ regulate the expression of

C/EBPα (M.D. Lane, 1994). In addition, insulin regulates the transcription of the C/EBPα, β, and δ genes in fully differentiated 3T3-L1 adipocytes. Furthermore, glucocorticoids reciprocally regulate expression of the C/EBPα and δ genes in 3T3-L1 adipocytes and WAT.

2.3.2. PPAR/RXR family of transcription factors

While the C/EBP family of transcription factors has been implicated as central to the control of gene expression in the differentiated adipocyte, a member of another family of DNA binding proteins, peroxisome proliferator-activated receptor (PPAR) γ, is instrumental in regulating the differentiation of preadipocytes into mature fat cells. Spiegelman and colleagues employed transgenic animal technology to map the region of the gene necessary and sufficient to direct a reporter transgene of the aP2 in a fat cell-specific manner [4]. Surprisingly, they found that while the region of DNA necessary for C/EBP action was essential for regulation in the mature adipocyte, a distinct 518-bp enhancer region, some 5.4-kb upstream of the start of transcription was required for fat cell-specific expression and subsequently showed that PPARγ bound to this region.

PPARs belong to a family of nuclear transcription factors that heterodimerize with retinoid X receptors (RXR) and function in a ligand-dependent manner [5]. They can activate transcription through binding peroxisome proliferator activated receptor response elements (direct repeat of AGGTCA spaced by one nucleotide). To date, three different PPAR isoforms α, δ/β, and γ (and splice variants) have been identified that are encoded by separate genes. The tissue-specific expression pattern of these transcription factors is indicative of their function in those tissues [6].

Unliganded PPARγ associates with co-repressors, thereby suppressing expression of target genes. In the presence of ligands, the co-repressors are exchanged with co-activators, such as peroxisome proliferator-activated receptor gamma co-activator-1 (PGC-1), p300, and SRC-1 [7]. While PGC-1 was originally believed to function only in white and brown adipocytes, it is also expressed in hepatocytes where it plays a primary role in controlling the expression of genes involved in gluconeogenesis. Adipocyte differentiation relies on the activation of the master regulator PPARγ. Consistent with this, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, a ligand for PPARγ, has been shown to stimulate the differentiation of 3T3-L1 and 3T3-F422A preadipocytes into adipocytes at micromolar concentrations.

PPARα targets genes involved in fatty acid catabolism (β and ω oxidation pathways) and is most abundantly expressed in liver, although it is also found in kidney, heart, and BAT [5]. The δ isoform is most widely distributed and is found in a variety of tissues including heart, kidney, brain, intestine, muscle, spleen, lung, and adrenal. The γ isoform is the most highly restricted in its expression pattern with primary sources being adipose, macrophage, and mammary tissue. Alternate promoter usage coupled with differential mRNA splicing result in two closely related PPARγ isoforms that differ only by the amino terminal 30 amino acid residues. The γ_1 isoform is found in adipose tissue and to a lesser extent in liver, kidney, and heart. The γ_2 isoform is found almost exclusively in WAT. During the adipocyte differentiation program, the level of expression of the three different PPAR isoforms is temporally regulated. The low levels of PPARα in WAT compared to BAT suggest that the role of PPARα in adipocyte differentiation is minor.

In cultured cell lines, PPARγ, which is expressed during the late stages of adipocyte differentiation, has been shown to be the master regulator of adipogenesis. Some of the

genes regulated by PPARγ in adipose tissue include aP2, lipoprotein lipase, fatty acid transport protein-1 (FATP1), acyl-CoA synthetase, stearoyl-CoA desaturase, and phosphoenolpyruvate carboxykinase. The expression of PPARγ in adipose tissue is upregulated by hormones like insulin and glucocorticoids while cytokines, tumor necrosis factor α (TNFα) in particular, have been shown to decrease the expression of PPARγ and C/EBPα [6]. Overall, PPARγ activation leads to beneficial effects arising from the change in expression and secretion of a range of factors including adipokines such as adiponectin, resistin, interleukin-6, TNFα, plasminogen activator inhibitor-1, and monocyte chemoattractant protein-1, as well as a reduction in plasma fatty acid levels.

2.3.3. SREBP family of transcription factors

Sterol regulatory element binding proteins (SREBPs) belong to a family of transcription factors that regulate genes involved in cholesterol and fatty acid metabolism [8] (Chapter 14). SREBPs consist of three major isoforms, SREBP1a, 1c, and 2. SREBP1a has a longer acidic amino terminal transactivation domain and an increased capacity to induce a wider range of target genes. SREBP2 is encoded by a separate gene. SREBPs are basic helix-loop-helix leucine-zipper DNA binding proteins. These proteins have dual DNA specificity. They can recognize and bind to inverted repeat sequences known as the E box motif (5′-CANNTG-3′) as well as direct repeat sterol regulatory elements.

SREBPs are unique transcription factors because they are initially synthesized as precursor, membrane-bound proteins present in the endoplasmic reticulum (ER) and the nuclear envelope [9]. When sterol levels are high, SREBP is complexed to the membrane-bound escort, SREBP-cleavage activating protein, and is retained on the ER membrane through an interaction of SREBP-cleavage activating protein with the insulin-induced gene, Insig. Upon reduction of sterol levels, Insig is released for the complex and is subject to ubiqitin-dependent degradation (M.S. Brown, 2006). The SREBP/SREBP-cleavage activating protein complex can then be incorporated into COPII-coated vesicles that bud from the ER and are transported to the Golgi complex. In the Golgi, two proteases cleave SREBP, releasing a soluble form which is capable of entering the nucleus and activating transcription (J.L. Goldstein, 1998; Chapter 15).

Over-expression of SREBP1a in cultured cells and animal livers results in increased expression of genes of cholesterol and fatty acid metabolism. In WAT, this resulted in a decrease in the mass of triacylglycerol. Over-expression of SREBP1c in adipose cells led to the development of hyperglycemia, fatty liver, and increased levels of serum triacylglycerol, while over-expression of SREBP2 led to a significant increase in the accumulation of cholesterol in liver and adipose (J.L. Goldstein, 1998). In mature adipocytes, insulin has been shown to upregulate the expression of SREBP1c which induces the expression of genes involved in lipogenesis, e.g., lipoprotein lipase and fatty acid synthase and possibly enzymes necessary for synthesis of an endogenous PPARγ ligand. The phenotype of the SREBP1 knockout mice includes partial embryonic lethality, with the surviving animals having a 2–3-fold increase in active SREBP2 in the liver (J.L. Goldstein, 1997). Due to the increase in SREBP2 activity, cholesterol synthesis in the liver increased.

2.3.4. LXR family of transcription factors

Another family of nuclear hormone receptors intimately involved in adipose tissue lipid metabolism is the liver X receptors (LXR), which also heterodimerize with RXRα. Within

adipose tissue, these receptors are particularly important in macrophages, but play a role in adipocytes. Two members of this family, LXRα and LXRβ, function as cholesterol sensors and regulate the expression of genes required for sterol homeostasis [10]. Although originally identified as an orphan receptor, the physiological ligands have been determined to be cholesterol metabolites, such as oxysterols (Chapter 14). Additionally, recent data suggests that LXR binds glucose directly, resulting in increased LXR transcriptional activity (E. Saez, 2007).

In adipocytes, both LXRα and PPARγ regulate the expression of each other as well as themselves. Thus, PPARγ agonists as well as LXR agonists result in increased levels of both LXRα and PPARγ. Similar to PPARγ, LXR receptors use PGC-1 as a co-activator. Treatment of adipocytes with a LXRα agonist leads to increased lipid accumulation. This is thought to be due to an increased expression of SREBP-1c, fatty acid synthase, and GLUT4 genes, as well as indirectly through the actions of PPARγ. The two LXRs can compensate for each other in mediating ligand-activated regulation of LXR target genes involved in lipid homeostasis in adipose tissue, but the dual LXRα/β null mice have smaller adipose depots [11]. In animal models of obesity and diabetes, LXRα is downregulated which is thought to exacerbate the obesity-induced insulin resistance.

3. Triacylglycerol biosynthesis and mobilization

3.1. Insulin and lipogenesis

A primary function of adipose tissue is to serve as a storage site for the excess energy derived from food consumption. Energy stored in the form of triacylglycerol can be utilized by the organism to fulfill subsequent metabolic requirements during times of little or no consumption (Fig. 1). In the case of WAT, these requirements entail efficient storage of large amounts of energy in a form that can be readily mobilized to supply the needs of peripheral organs and tissues. Lipids, particularly fatty acids, are exceptionally efficient fuel storage species. The highly reduced hydrocarbon tail of triacylglycerol can be readily oxidized to produce large quantities of NADH and $FADH_2$ and subsequently ATP. At the same time, the very hydrophobic nature of the hydrocarbon tail precludes concomitant storage of excess water that would increase the mass and spatial requirements of the organism. Also, the relatively straight, chain-like structure of the fatty acid permits dense packing of many molecules into each cell, maximizing the use of storage space available. BAT also stores energy in lipid form, but more frequently produces heat by oxidizing fatty acids within the adipocyte, rather than by supplying free fatty acids for use by other cell types.

During feeding, pancreatic β cells secrete insulin in response to both elevated blood glucose levels and elevated blood lipid levels. Insulin is the most important physiological stimulus for energy storage. The effect of insulin directly counteracts the effects of glucagon and the catecholamines. The insulin receptor is found in many diverse cell and tissue types, not the least significant of which is adipose.

The insulin receptor is an integral membrane protein and a receptor tyrosine kinase that functions as a tetramer composed of two α and two β subunits. The β subunits each span the plasma membrane once, and the α subunits are covalently attached to the β subunit

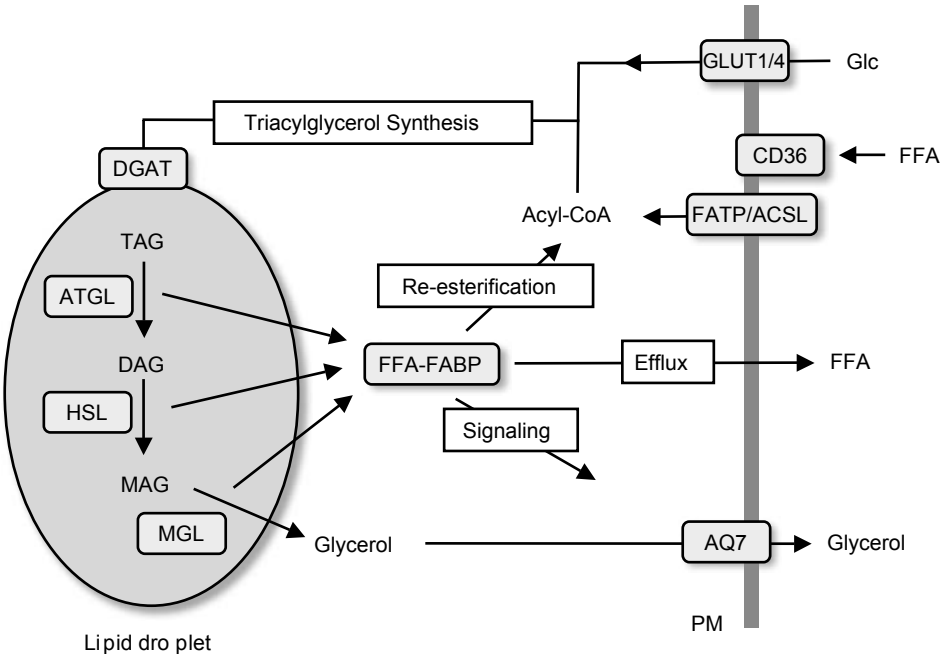

Fig. 1. Lipid cycling in adipocytes. Adipocytes transport glucose (Glc via GLUT 1 or 4) and fatty acids (FFA via CD36/ACSL) into the cell in response to insulin stimulation leading to the synthesis of triacylglycerol, the terminal step of which is catalyzed by diacylglycerol acyltransferase (DGAT). Triacylglycerol synthesis is balanced by hydrolysis catalyzed by a series of triacylglycerol (adipose triacylglycerol lipase; ATGL), diacylglycerol (hormone-sensitive lipase, HSL), and monoacylglycerol (MGL) lipases. Glycerol produced by complete TAG hydrolysis is exported from the adipocyte via an aquaporin protein (AQ7) while FFA are bound by intracellular fatty acid binding proteins (FABPs) and subjected to re-esterification, efflux, or metabolized to signaling molecules. TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; ACSL, long chain acyl-CoA synthetase.

extracellularly by disulfide bonds. The insulin-binding site is extracellular and the intracellular domains contain many tyrosine phosphorylation sites. Ligand binding induces autophosphorylation of several intracellular domains, activating the kinase activity of each β subunit. A complex series of interactions follows in which the insulin receptor phosphorylates some of its substrates directly, such as insulin receptor substrate-1 (IRS-1) and IRS-2, or recruits various adaptor proteins, such as Shc and Grb2 that transmit the insulin signal [12].

Insulin binding to the adipocyte insulin receptor simultaneously stimulates lipogenesis and inhibits lipolysis. Insulin action effectively clears fatty acids and glucose from the blood both by increasing uptake and storage, and by decreasing mobilization of stored energy. The mechanisms by which these effects are accomplished are highly complex and involve an integrated series of regulated events (P. Cohen, 2006). In regards to insulin-stimulated glucose uptake, the insulin receptor acts mainly through the phosphatidylinositol-3-kinase (PI3 kinase) pathway. Activated insulin receptor binds and phosphorylates IRS-1, thereby allowing PI3 kinase to bind IRS-1. PI3 kinase subsequently phosphorylates

its substrate, phosphatidylinositol-4,5-bisphosphate, forming phosphatidylinositol-3,4, 5-trisphosphate at the plasma membrane. The kinases AKT/protein kinase B (PKB) and PDK1 are recruited to the plasma membrane by binding phosphatidylinositol-3,4, 5-trisphosphate. PDK1 phosphorylates and activates AKT/PKB. AKT/PKB then transmits the signal to increase glucose uptake through the phosphorylation of the AKT substrate of 160 kDa, which is a Rab-GTPase activating protein. This inactivation of AKT substrate of 160 kDa through an unknown mechanism, facilitates the translocation of GLUT4, to the plasma membrane and stimulates glucose transport (Fig. 2). AKT/PKB is also

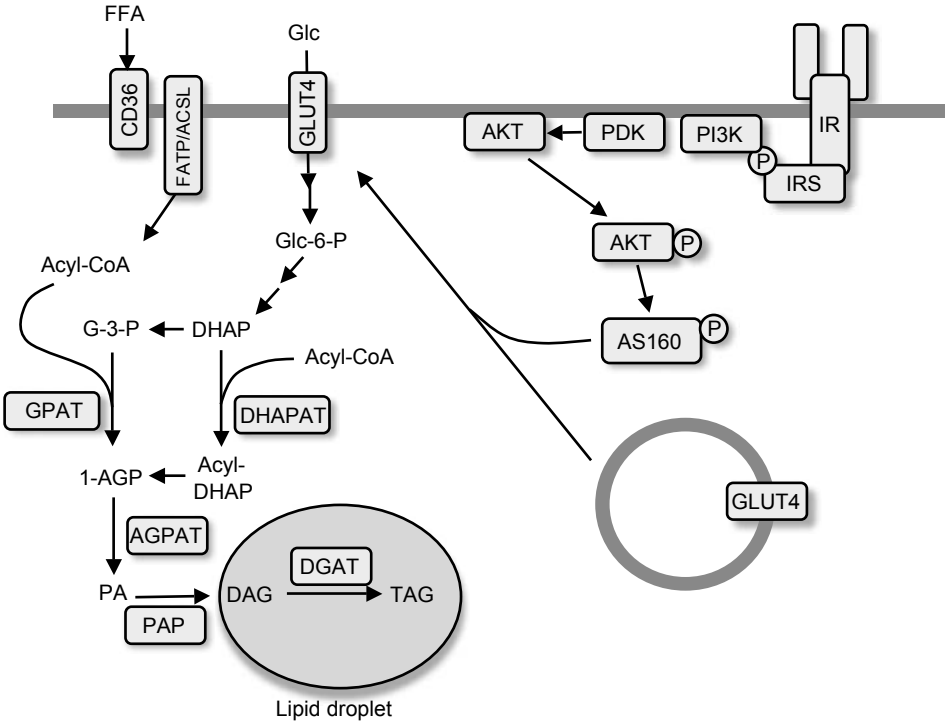

Fig. 2. Insulin-stimulated glucose uptake and triacylglycerol biosynthesis. Activation of the insulin receptor (IR) upon insulin binding results in the recruitment of IRS to the insulin receptor and subsequent tyrosine phosphorylation of IRS. This allows the subsequent recruitment and activation of phosphatidylinositol-3-phosphate kinase (PI3K), phosphatidyl inositol-dependent protein kinase (PDK), and AKT/PKB. PDK phosphorylates and activates AKT at the plasma membrane. AKT dissociates from the plasma membrane and phosphorylates AS160. Phosphorylated AS160 facilitates the translocation of GLUT4 containing vesicles to the plasma membrane, resulting in an increase in glucose transport. Glucose (Glc) transported into the adipocyte is phosphorylated (Glc-6-P) and converted to dihydroxyacetone phosphate (DHAP) via glycolysis and, subsequently, glycerol-3-phosphate (G-6-P). DHAP is converted to acyl-dihydroxyacetone phosphate (Acyl-DHAP) via DHAPAT and, subsequently, to 1-acyl-glycerolphosphate (1-AGP). 1-AGP is also directly produced from G-3-P via GPAT. AGPAT, then, adds a second acyl-CoA to 1-AGP to form phosphatidic acid (PA). PA is dephosphorylated by PAP to form diacylglycerol (DAG) and DGAT esterifies a third acyl-CoA with DAG to create triacylglycerol (TAG). IRS, insulin receptor substrate; AS160, AKT substrate of 160 kDa; DHAPAT, dihydroxyacetone phosphate:acyltransferase; GPAT, glycerolphosphate:acyltransferase; AGPAT, acyl-glycerolphosphate:acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol:acyltransferase.

capable of phosphorylating and activating the cGMP-inhibited phosphodiesterase (PDE-3B), and several protein serine phosphatases (most likely protein phosphatases 1, 2A, and 2C), leading to decreased lipolysis. Acetyl-CoA carboxylase, the enzyme that catalyzes the first committed step in de novo fatty acid synthesis, is also activated upon dephosphorylation. In addition, insulin enhances lipoprotein lipase expression and secretion to the nearby capillary endothelium, thereby enhancing fatty acid uptake, however the mechanism that leads to increased lipoprotein lipase secretion is unclear.

3.2. Fatty acid uptake and acyl-CoA production

At the adipose tissue beds, fatty acids are liberated from triacylglycerol rich lipoproteins through the action of lipoprotein lipase (Chapter 19). Released fatty acids are bound by albumin and are the donors of lipid for fatty acid uptake. The mechanism of fatty acid influx into adipocytes is complex, involving multiple steps. While not completely defined, several general concepts dominate the field. CD36 (also called FAT) might serve as a fatty acid receptor, accepting lipids from albumin thereby producing a localized high concentration. CD36 is a 88-kDa highly glycosylated integral membrane protein, located in caveolae (Chapter 1), a region of the plasma membrane rich in sphingolipids containing saturated very long chain fatty acyl groups, and belongs to the family of class B scavenger receptors. CD36 is largely expressed in adipocytes, macrophages, heart, and skeletal muscle, and deficiencies have been genetically linked to hyperlipidemia and hypertension (N.A. Abumrad, 2001). Several studies in skeletal muscle have shown the important role of CD36 in facilitating fatty acid influx, though it has not been as well studied in the context of adipocytes. Protonation of fatty acids due to the relative acidity of the plasma membrane, coupled with the low aqueous solubility of fatty acids at neutral pH and high permeability of fatty acids in the membrane creates a sufficient driving force for diffusion across the outer and inner leaflets of the membrane. The actual site of diffusion across the membrane is unknown but may be localized to caveolae.

For fatty acids on the inner membrane, a variety of acyl-CoA synthetases (ACS) catalyze the ATP- and CoA-dependent esterification thereby providing a thermodynamic driving force for further diffusion. Using expression cloning, Lodish and co-workers identified the long chain acyl-CoA synthetase 1 (ACSL1) as well as a novel homologue of ACSL1 termed fatty acid transport protein-1 (FATP1) as facilitators of fatty acid influx [13]. ACSL1, as well as other members of the acyl-CoA synthetase family, facilitate the esterification of fatty acids with CoA via an ATP-dependent mechanism forming fatty acyl-CoA. FATP1 is now known to belong to the larger FATP multigene family (FATP1-6) and possesses both long chain and very long chain acyl-CoA synthetase activity. Adipocytes predominately express ACSL1, FATP1, and FATP4. Over-expression of FATP1 and ACSL1 in fibroblastic cell lines results in increased fatty acid influx, which is not seen with over-expressed catalytically inactive forms. This finding suggests that esterification of long chain and very long chain fatty acids is coupled to fatty acid influx in a process termed vectoral acylation (Fig. 2). Similar to translocation of GLUT4, ACSL1 and FATP1 translocate from intracellular vesicles to the plasma membrane in response to insulin stimulation. However, the magnitude of insulin-stimulated fatty acid influx is modest (2–3-fold) relative to hexose uptake. Since fatty acyl-CoAs are the biologically active

form of fatty acids, coupling fatty acid esterification with fatty acid influx into the adipocyte would allow fatty acids to be redirected and utilized for phospholipid synthesis, oxidation, or triacylglycerol synthesis. Indeed, triacylglycerol synthesis may occur on or around the plasma membrane and be functionally coupled to fatty acid influx [14].

3.3. Glucose transport and synthesis of the triacylglycerol backbone

The immediate backbone precursor for acylglycerol formation is primarily glycerol-3-phosphate, derived from glycolysis or glycerolgenesis within adipocytes. Fat cells express specific glucose transporters on the plasma membrane to ensure a ready supply of glycolytic intermediates for triacylglycerol synthesis. There are two types of glucose transport proteins in adipose: GLUT1 and GLUT4. Both are structurally similar with 12 membrane-spanning α-helices and intracellular amino and carboxyl termini. Both proteins are expected to have a large, hydrophilic intracellular loop separating transmembrane domains six and seven, as well as an extracellular loop containing *N*-glycosylation site(s) demarcated by transmembrane domains one and two. The majority of GLUT1 has been shown to be present in the plasma membrane (cells unstimulated by insulin), constitutively facilitating transport of glucose down a concentration gradient (S.W. Cushman, 1998). However, the bulk of insulin-stimulated glucose transport results from the activity of GLUT4. In the basal state, GLUT4 is largely found in small, intracellular vesicles but rapidly translocates to the plasma membrane following insulin stimulation. In addition, insulin promotes a change in the rate of intracellular GLUT4 recycling and trafficking which results in a net 10- to 15-fold stimulation of hexose transport in response to insulin [15]. Once inside the cell, facilitative transport of glucose by GLUT1 and GLUT4 is rendered unidirectional by the action of hexokinase. Glucose-6-phosphate can only proceed to the glycolytic pathway because adipocytes do not express significant levels of glucose-6-phosphatase. Subsequent steps in glycolysis result in the production of dihydroxyacetone phosphate and glycerol-3-phosphate, the precursors of triacylglycerol synthesis (Fig. 2).

3.4. Fatty acid and triacylglycerol biosynthesis

Adipocytes readily convert the products of glycolysis into fatty acids via the de novo biosynthetic pathway (Chapter 6). Briefly, surplus citrate is transported from the mitochondrion and cleaved to produce cytosolic acetyl-CoA. This acetyl-CoA is acted upon by acetyl-CoA carboxylase producing malonyl-CoA. The next steps of the fatty acid biosynthetic pathway are carried out by the multifunctional fatty acid synthase that utilizes NADPH to catalyze multiple condensations of malonyl-CoA with acetyl-CoA or the elongating lipid, eventually generating palmitate.

De novo fatty acid synthesis is, in part, negatively regulated by the AMP-activated protein kinase (AMPK). When the adipocyte is in an energy deficient state due to the lack of available glucose or other cellular stresses, ATP levels decrease and AMP levels increase. This causes the AMP:ATP ratio in the adipocyte to increase and thus AMP binds and activates AMPK kinase, LKB. AMP also binds to AMPK, which allows LKB to recognize AMPK as a substrate and results in the phosphorylation and activation of AMPK.

Active AMPK phosphorylates acetyl-CoA carboxylase, deactivating it and, hence, decreasing fatty acid synthesis [16].

Triacylglycerol synthesis combines the products of glycolysis, glycerolphosphate, and dihydroxyacetone phosphate with acyl-CoAs (Fig. 2). In adipocytes, two pathways exist for the production of phosphatidic acid. In one, glycerol-3-phosphate is sequentially esterified with two acyl-CoAs to produce 1-acylglycerolphosphate and 1,2-diacylglycerolphosphate. In the second pathway, dihydroxyacetone phosphate is esterified with acyl-CoA to produce acyl-dihydroxyacetone phosphate. An acyl-dihydroxyacetone phosphate reductase subsequently produces 1-acylglycerolphosphate that leads to the production of 1,2-diacylglycerolphosphate. The resultant phosphatidic acid is dephosphorylated generating 1,2-diacylglycerol and triacylglycerol is formed through the activity of diacylglycerol acyltransferase (DGAT).

The DGAT catalyzed reaction is crucial for it represents a branch point for hydrocarbon flow towards either the triacylglycerol or phospholipid pathways. However, this view has been modified through the production of DGAT null mice. DGAT deficient mice are viable, are resistant to diet-induced obesity, and have reduced triacylglycerol synthesis, though they still have significant amounts of triacylglycerol. This implies that either an alternate triacylglycerol biosynthetic pathway is utilized or additional DGAT isoforms are present in adipose cells. To address this question, Farese and colleagues identified a second DGAT (DGAT2) with kinetic properties distinct from the original DGAT (now termed DGAT1; R.V. Farese, 2001). DGAT2 null mice have lipopenia and skin barrier abnormalities. The DGAT2 null mice also have severely reduced triacylglycerol synthesis and triacylglycerol content in their tissues, suggesting that DGAT2 is responsible for the majority of triacylglycerol biosynthesis (R.V. Farese, 2004). Moreover, additional DGAT-like sequences are present in the murine and human genome suggesting an unappreciated complexity in diacylglycerol metabolism.

3.5. Triacylglycerol mobilization

3.5.1. Lipases and perilipin

Lipolysis refers to the process by which triacylglycerol molecules are hydrolyzed to fatty acids and glycerol. During times of metabolic stress (i.e., during fasting or prolonged strenuous exercise when the body's energy needs exceed the circulating nutrient levels), the triacylglycerol droplet within an adipocyte is degraded producing fatty acids to be used as an energy source by other tissues. Numerous stimuli are capable of eliciting the lipolytic response in adipocytes [17]. A family of lipases, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase catalyzes the hydrolysis of the triacylglycerol ester bonds producing glycerol and three moles of fatty acids.

The process of triacylglycerol hydrolysis is a complex phenomenon that involves at least three lipases, lipid droplet associated proteins, and FABPs, although other adipocyte lipases (i.e., triacylglycerol hydrolase) may play a role in basal lipolysis. The data at this time support the model that three lipases are the major contributors to adipocyte lipolysis. Complete hydrolysis of triacylglycerol involves the hydrolysis of three ester bonds to liberate three fatty acids and a glycerol moiety. ATGL catalyzes hydrolysis of the first

ester bond to release one fatty acid and results in diacylglycerol formation (R. Zechner, 2004). HSL can catalyze the hydrolysis of triacylglycerol, but has 10-fold greater activity towards diacylglycerol, hydrolyzing the second ester bond at either the *sn*-1 or *sn*-3 position. A third enzyme, monoacylglycerol lipase, catalyzes hydrolysis of the remaining ester to yield a third fatty acid and glycerol (Fig. 1). Since adipocytes do not express glycerol kinase to any great extent (although they might when PPARγ is activated), they are generally unable to reuse glycerol. Therefore, glycerol is transported out of adipocytes via aquaporin 7 and must be shuttled back to the liver for oxidation or gluconeogenesis. Mono- and diacylglycerols can be re-esterified by the ER acyltransferases. During a lipolytic stimulus, re-esterification is minimized so that the net direction of these reactions is toward fatty acid efflux from the cell.

Free fatty acids are minimally soluble in the aqueous cytoplasm. The charged carboxylate group provides enough electrostatic hindrance to prevent association with the neutral triacylglycerols whereas the hydrocarbon tail reduces solubility in water. At sufficiently high concentrations fatty acids exert a detergent-like effect that would disrupt membranes and/or they could cluster together in micelles in the crowded cytoplasm. To alleviate this problem, the adipocyte and other lipid-metabolizing cell types have evolved intracellular FABPs, a family of small, soluble, highly abundant proteins that bind and sequester fatty acids. Adipocytes express two FABPs, the adipocyte and epithelial FABPs. Once outside the adipocyte, fatty acids are immediately bound to serum albumin and carried in the bloodstream to the liver, muscle, and other tissues for oxidation.

During lipolysis, changes in plasma fatty acids occur in an oscillatory rather than constant manner (R.N. Bergman, 2000). This oscillation is intrinsic to the adipocyte, is dependent on glucose metabolism, and is regulated by fatty acids. Synchronization of this intrinsic oscillation is mediated by the sympathetic nervous system. Thus, through β-adrenergic receptors, catecholamines coordinate this oscillation that gives rise to large bursts of free fatty acids and glycerol every 8 min.

HSL has long been thought of as the rate-limiting enzyme in lipolysis. This lipase is regulated through the stimulation of β-adrenergic receptors by catecholamines or more recently discovered, by natriuretic peptides, and the resulting activation of cAMP-dependent protein kinase (PKA). HSL is an 84-kDa protein modeled to be organized into N-terminal (1–300) and C-terminal (300–767) domains. The regulatory and catalytic activity of the enzyme lies within the predicted α/β hydrolase fold of the C-terminal domain. Three residues, Asp703, His733, and Ser423, form the catalytic triad. Phosphorylation of Ser659 and Ser660 in the C-terminal domain results in a modest increase in specific activity of the enzyme and is correlated with a translocation of the protein from the cytoplasm to the surface of the lipid droplet [18]. Regulation of HSL by phosphorylation/dephosphorylation makes it unique among lipases.

Adipocytes of HSL null mice retain 40% of the triacylglycerol lipase activity compared to wild type animals (N. Yamada, 2000). In addition, they accumulate diacylglycerol, while showing no difference in triacylglycerol hydrolysis compared to wild type animals. These data led to the consideration that HSL is rate-limiting for hydrolysis of diacylglycerol rather than triacylglycerol and to the discovery of ATGL (R. Zechner, 2004).

ATGL is a 54-kDa protein which contains a patatin-like domain in its N-terminal region, a domain commonly found in lipases. In addition, ATGL has a conserved glycine-rich

GXGXXG nucleotide binding motif, a GXSXG serine hydrolase motif, and a DX(G/A) motif with a conserved aspartate residue. The serine and aspartate residues form a catalytic dyad that is necessary for lipase activity in this family of proteins. In vitro hydrolase activity assays showed that ATGL has triacylglycerol hydrolase activity but no diacylglycerol, cholesteryl ester, or retinyl ester hydrolase activity. ATGL knockout mice have defective lipolysis in adipose tissue resulting in increased adipose tissue mass confirming its importance as a major adipose tissue lipase (R. Zechner, 2006). ATGL is located at the lipid droplet surface under basal and stimulated conditions making it likely that ATGL is responsible for the majority of basal lipolysis although other triglyceride lipases might also play a role.

The process of lipolysis involves structural proteins in addition to hydrolases. The most significant contribution is by the lipid droplet associated protein perilipin. Perilipin constitutively interacts with lipid droplets through three hydrophobic domains. Overexpression of perilipin results in increased triacylglycerol deposition and inhibition of lipolysis, whereas perilipin null mice are lean under high fat conditions with increased basal lipolysis (C. Londos, 2001). It is thought that perilipin blocks access of lipases to their substrate under basal conditions. PKA stimulation results in the phosphorylation of perilipin at six serine residues. The three C-terminal phosphorylation sites are necessary for stimulated lipolysis in adipocytes. These serine residues have been implicated in HSL translocation to the lipid droplet and HSL interaction with perilipin at the droplet surface. Ablation of one of these, Ser517, results in decreased ATGL activity and decreased lipolysis under stimulated conditions [19]. The mechanism by which perilipin affects ATGL activity is unknown but may be mediated through a cofactor CGI-58. CGI-58 is an α/β hydrolase fold-containing protein that resembles a lipase, but lacks lipase activity since the active site serine is missing (T. Osumi, 2004). Perilipin interacts with CGI-58 under basal conditions and dissociates under stimulated conditions. Recently it has been shown that CGI-58 interacts with ATGL under stimulated conditions resulting in increased activity.

The fatty acid binding protein (AFABP/aP2) facilitates the intracellular solubilization and diffusion of fatty acids produced by lipolysis. The N-terminal domain of HSL is a docking site for interaction with AFABP/aP2. AFABP/aP2 stimulates the activity of HSL in vitro by relieving product inhibition by fatty acids, however, this activity seems to be independent of the interaction between AFABP/aP2 and HSL. The AFABP/aP2 may also shuttle fatty acids produced by ATGL or the monoacylglycerol lipase as well, but there is no evidence for physical association with these enzymes. Consistent with the model of AFABP/aP2 as a fatty acid shuttle facilitating efflux, AFABP/aP2 null mice exhibit reduced lipolysis while fatty acids accumulate intracellularly (D.A. Bernlohr, 1999).

3.5.2. Cyclic AMP-dependent lipolysis

Although many signals and signaling pathways stimulate or inhibit lipolysis, the vast majority converge on PKA. The generic route for PKA activation is that a stimulatory G-protein coupled receptor receives a signal that results in the activation of adenylyl cyclase. Adenylyl cyclase catalyzes the conversion of ATP to the second messenger cAMP which binds the regulatory subunit of PKA, resulting in the dissociation and activation of the catalytic subunit. PKA phosphorylates two major lipolytic proteins: HSL and

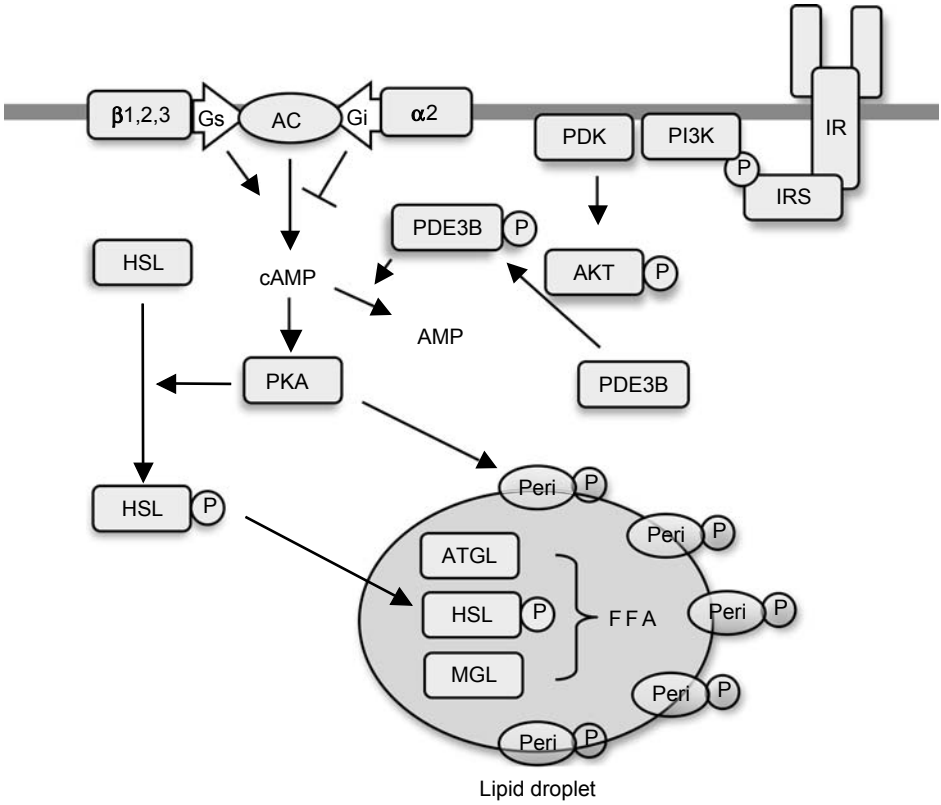

Fig. 3. Regulation of lipolysis via adrenoreceptor-coupled systems and insulin. Binding of lipolytic agonists to β-adrenoreceptors (β_1, β_2, and β_3) couples to G_s protein that in turn activates adenylyl cyclase (AC) thereby producing cAMP. cAMP activation of protein kinase A (PKA) results in phosphorylation and activation of hormone-sensitive lipase (HSL) and perilipin (peri) and subsequent translocation of HSL to the lipid droplet. Lipolytic activation stimulates the catalytic activity and access of regulatory lipases (ATGL, HSL, and MGL) to the droplet surface resulting in generation of fatty acids (FFA). Ligand binding to α_2-adrenoreceptors results in coupling with G_i and a decrease in adenylyl cyclase activity. Insulin binds to its cell surface receptor (IR) and initiates a signaling cascade linking phosphorylation of insulin receptor substrate (IRS) to the activation of phosphatidylinositol-3-phosphate kinase (PI3K), phosphatidyl inositol-dependent protein kinase (PDK), and AKT/PKB. AKT phosphorylates and activates the cGMP-inhibited phosphodiesterase (PDE-3B) increasing cAMP hydrolysis. The resulting decrease in PKA activity thus provides the basis for the anti-lipolytic effects of insulin.

perilipin. These phosphorylations allow HSL translocation to the lipid droplet where it interacts with perilipin and increases lipolysis (Fig. 3). Under PKA stimulation CGI-58 interacts with ATGL and activates its lipase activity. It is unclear how this interaction and activation come about as neither of these proteins is a substrate for PKA. Recent studies suggest that the dissociation of CGI-58 from perilipin results in CGI-58 being available to interact with ATGL resulting in its activation. One of the strongest physiological stimulants of this pathway is catecholamines [17].

The catecholamines epinephrine and norepinephrine (adrenaline and noradrenaline) originate in the inner medullar region of the adrenal glands. Stimulation of the adrenal by the sympathetic nervous system leads to secretion of catecholamines into the bloodstream. In addition, adipose tissue is itself directly innervated by the sympathetic nervous system. Various types of metabolic stress trigger the sympathetic nervous system to release its neurotransmitter, norepinephrine, directly into adipose where its effects on the adipocyte are mediated by specific plasma membrane adrenoreceptors. Rapid reflex responses are primarily stimulated by the sympathetic nervous system, whereas more long-term (i.e., on the scale of hours, days, and weeks) and/or basal effects are subject to regulation by catecholamine secretion.

The effects of catecholamines and the mechanisms that mediate them have been extensively studied in adipocytes. Adipocytes express a combination of five different adrenoreceptor isoforms: α_1, α_2, β_1, β_2, and β_3 [20]. Lipolysis is signaled by β-adrenergics. An anti-lipolytic signal is transduced by the α_2-adrenergics, and the α_1-adrenergics are involved in a separate pathway. In short, although lipolysis is the observed outcome of catecholamine stimulation, it is the steady-state result of competition between two opposing pathways triggered by the same signal.

The mechanisms of signal transduction are reasonably well known. Binding of catecholamines to the β-adrenoreceptors activates adenylyl cyclase via a stimulatory G-protein (G_s) (Fig. 3). The same signal bound to the α_2-adrenoreceptor affects an inhibitory G-protein (G_i), which inhibits the activity of adenylyl cyclase. Reduction in the cAMP level leads to dissociation of cAMP from the regulatory subunit of PKA, which then inactivates the catalytic subunit by reassociation. In the absence of continued phosphorylation, HSL returns to the basal state. With simultaneous activation of opposing pathways, the relative contribution of each receptor type becomes very important. Small mammals, such as rats and hamsters, express mainly β_1 and β_3 while rats express very little of the α_2 isotype. Large mammals (e.g., humans and monkeys) express almost exclusively β_1 and β_2 and a significant amount of α_2 receptor. It appears that the β_3 receptor is expressed to a greater extent in brown adipocytes than in white adipocytes.

A second observed pattern of receptor regulation was demonstrated by the use of agonists and antagonists for each receptor isotype. At very low agonist concentrations, only α_2 receptor activity is observed (anti-lipolysis). As the agonist concentration is increased, β_1 becomes active and initiates lipolysis. Only under much more stimulatory agonist conditions do β_3 receptors become active. β_2, in animals that express it, seems to be active under conditions more similar to β_1. Affinity for ligands and the level of expression of receptors are two methods utilized by adipocytes to regulate catecholamine effects. The interplay between the various isotypes is responsible for the adrenergic balance of lipolysis and anti-lipolysis. In general, α_2 mediated anti-lipolysis modulates resting adipocyte activity, whereas during stress-induced norepinephrine release, increased binding to the β-adrenergics overcomes the α_2 inhibitory effect and β-mediated lipolysis prevails.

Many other hormones alter lipolysis through their effect on catecholamine signaling, including alteration of β-adrenergic receptor levels, adenylyl cyclase activity, or inhibitory G-protein levels. All of these effects result in increased cAMP generation in the presence of catecholamines. The main hormones involved are glucocorticoids, chronic growth

hormone, thyroid hormone, and the sex steroids estrogen and testosterone. These hormones may be important in the maintenance of catecholamine-induced lipolysis by enhancing transcription of the genes involved in that signal cascade.

Although catecholamines are perhaps the strongest physiological lipolytic stimulus, other hormones also play an important role in mediating energy balance. One such hormone is glucagon, one of three polypeptide hormones secreted by endocrine cells located within the pancreas. Glucagon is secreted into the circulation in response to low blood glucose levels and results in mobilization of stored energy. Stimulation by glucagon takes place by a virtually identical pathway to stimulation by catecholamines. Glucagon binds extracellularly to a specific seven-transmembrane-domain receptor, activating adenylyl cyclase via a stimulatory G-protein resulting in PKA activation and HSL and perilipin phosphorylation. Because the same regulatory pathway as that initiated by catecholamine is activated, the same feedback mechanisms used to modulate chronic catecholamine effects are equally significant for prolonged glucagon stimulation. PKA phosphorylation of the cell surface receptors leads to uncoupled G-protein activity and heterologous desensitization to both the glucagon and catecholamine signals. It is also relevant that PKA can phosphorylate and thereby activate PDE-3B, which hydrolyzes cAMP to 5′-AMP as a negative feedback mechanism.

3.5.3. Cyclic AMP-independent lipolysis

An additional mechanism controlling lipolysis specifically in humans has recently come to light (J. Galitsky, 2000). Atrial natriuretic peptide has a potent stimulatory effect on lipolysis in human adipocytes, where administration of the peptide was associated with increased phosphorylation of HSL and perilipin. This effect was blocked by inhibition of cGMP-dependent protein kinase I. At this point it is unclear whether this kinase phosphorylates HSL and perilipin directly or if it has an indirect effect mediated through PKA.

TNFα is an inflammatory cytokine secreted by adipocytes and macrophages. Obesity is associated with increased TNFα levels which are correlated with insulin resistance and uncontrolled lipolysis. There are three mechanisms that contribute to TNFα stimulated lipolysis [21]. One is inhibition of insulin receptor signaling, thus maintaining a low PDE-3B activity and high cAMP levels. The second is a decrease in the expression of G_i subunits leading to increased adenylyl cyclase activity. The third is a novel mechanism, which involves increased phosphorylation and decreased levels of perilipin. The phosphorylation event is indirect through the downregulation of PDE-3B protein levels which increases cAMP. TNFα directly downregulates perilipin mRNA by the action of p42/44 MAP kinase and c-Jun N-terminal kinase. After 6 h of TNFα treatment, decreased protein levels of perilipin are seen along with increased lipolysis. This chronic treatment mimics obesity-linked inflammation, thus TNFα may be involved in the increased basal lipolysis associated with obesity.

3.5.4. Insulin and anti-lipolysis

Insulin action in the adipocyte results in inhibition of lipolysis. Insulin signals activate AKT/PKB, which phosphorylates and activates PDE-3B [12]. This results in cleavage of cAMP by PDE-3B, thus decreasing stimulated lipolysis (Fig. 3). Insulin signaling also activates protein serine phosphatases 1, 2A, and 2C which contribute to the dephosphorylation

of HSL and perilipin, thereby turning off lipolysis. The two processes block catecholamine-induced lipolysis during insulin stimulation, contributing to insulin's anabolic action. During insulin resistant states, such as obesity, this mechanism is no longer active resulting in increased basal lipolysis, which coincides with an increase in plasma fatty acids. These fatty acids can act on resident macrophages in adipose tissue (Section 4.4) to increase TNFα production, which can then act on adipocytes. As mentioned above, TNFα causes insulin resistance and increase lipolysis. In the obese state, this feedforward mechanism gives rise to uncontrolled lipolysis and severe insulin resistance.

Anti-lipolysis effects are seen by other factors such as adenosine and prostaglandin E_2. The mechanisms of anti-lipolysis originate from any and all ways of reducing cAMP. The signals are coupled to G_i-proteins, activate PDE-3B, activate protein serine phosphatases, or decrease β-receptor levels. One alternative mechanism that seems to inhibit lipolysis is the activation of AMPK (E.M. Berry, 2006). AMPK is sensitive to AMP levels and historically signals an increase in cellular ATP levels. AMPK phosphorylates HSL at Ser565, which has been suggested to inhibit HSL translocation and activity. This hypothesis has been challenged by data suggesting that this site is necessary for full activation of HSL. The majority of data sides with an anti-lipoytic effect but it cannot be ruled out that the phosphorylation may have unanticipated roles in regulating HSL.

3.6. Brown fat lipid metabolism

Brown fat derives its color from extensive vascularization and the presence of many densely packed mitochondria (due to the heme cofactors in the mitochondrial enzyme cytochrome oxidase) (Table 2). BAT is traversed by many more blood vessels than is white fat. These blood vessels assist in delivering fuel for storage and oxidation, and in dispersing heat generated by the numerous mitochondria to other parts of the body. Brown adipocytes differ in appearance from white adipocytes by the presence of many small triacylglycerol droplets, as opposed to a single large droplet (i.e., multilocular, rather than unilocular). Regulation of brown fat activity is accomplished primarily through the action of the sympathetic nervous system. The blood vessels and each individual brown adipocyte

Table 2
Comparison of major features of white and brown adipose tissue

Major feature	White adipose	Brown adipose
Vascularization	Some, limited	Extensive
Distribution	Extensive, many sites	Restricted
Sympathetic innervation	Some, limited	Extensive
Fatty acid role(s)	Synthesis, storage, signaling	Storage, oxidation, signaling
Uncoupling protein	Low expression level	Highly expressed
Thermogenesis	Negligible	Highly developed
Insulin effects	Extensive	Extensive
Adrenoreceptors	Primarily α_2, β_1, β_2, β_3	Primarily α_1, β_1, β_3
Droplet size	Large, single	Small, multiple
Mitochondria	Few	Many, densely packed

are directly innervated by sympathetic nervous system nerve endings that exert control by release of norepinephrine. Stimulation by the sympathetic nervous system in response to a decrease in external temperature is essential for maintenance of BAT function, and atrophy occurs when regular sympathetic nervous system activity declines [22].

Brown adipocytes also differ from white adipocytes at the molecular level. The major adrenergic receptor subtype expressed by brown adipocytes is the β_3, but α_1 and β_1 are also found. The most notable difference between brown and white adipocytes is the production of UCP by the former and its relative absence in the latter [20,22]. Brown adipocytes also express a type II 5′-deiodinase enzyme which converts the thyroid hormone thyroxine to its more potent form, triiodothyronine. Brown adipocytes are capable of secreting triiodothyronine into circulation.

Uncoupling proteins confer to the brown adipocyte the ability to catabolize fatty acids without the usual concomitant ATP production and dissipate the heat generated by this excessive catabolic activity to other tissues via the bloodstream (Fig. 4). This process is known as thermogenesis and is characterized on two levels (M. Klingensport, 2003).

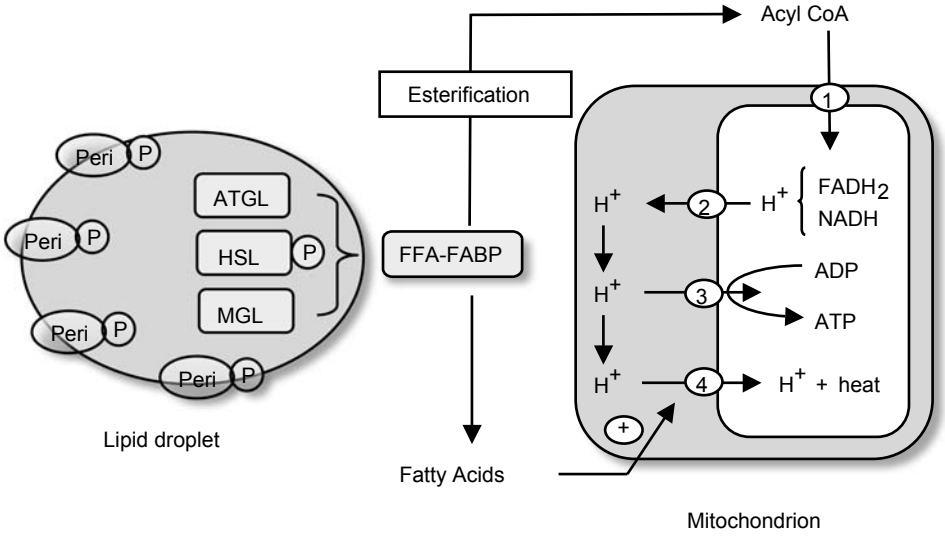

Fig. 4. Thermogenesis in the brown fat mitochondrion. The major fuel oxidation pathway of brown fat is represented. When excess fuel is present, or when heat is needed, fatty acids (FFA) produced by lipolysis via adipocyte triacylglycerol lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL) in the lipid droplet (left) are bound by fatty acid binding protein (FABP), activated with CoA, and transported into the mitochondrion (right). Via β-oxidation (1), the long-chain fatty acids are degraded to acetyl-CoA and generate reduced coenzymes (NADH and $FADH_2$). The coenzymes transfer their reducing equivalents across the mitochondrial inner membrane (2) producing a proton concentration gradient. Typically the proton gradient is dissipated by the action of the proton-ATPase (3) that uses the energy to drive ATP synthesis. However, brown fat mitochondria possess the uncoupling protein (4), which allows proton transport across the membrane down the concentration gradient with the change in free energy lost as heat. The uncoupling protein is positively regulated by fatty acids. Peri, perilipin.

Obligatory thermogenesis occurs in all cell types as the result of ubiquitous nominal inefficiencies in metabolism. Facultative thermogenesis occurs specifically in response to stimuli such as cold (non-shivering thermogenesis in adipose, shivering thermogenesis in muscle) or overfeeding (diet-induced thermogenesis). Facultative thermogenesis, particularly non-shivering thermogenesis, is a specific role of brown adipose. It should be noted that UCP isoforms are expressed ubiquitously throughout the body suggesting that thermogenesis and/or proton dissipation are common regulatory themes.

BAT, in its cold/epinephrine-activated state compared to an atrophied or quiescent state, demonstrates increases in blood flow, lipoprotein lipase activity, triacylglycerol synthesis, 5′-deiodinase activity, and triiodothyronine-enhanced UCP gene expression. The processes of fatty acid uptake and triacylglycerol synthesis are essentially the same in both BAT and WAT. However, norepinephrine release by the sympathetic nervous system in acute cold exposure stimulates BAT to enhance expression and secretion of lipoprotein lipase to its sites in the vascular epithelium. Lipoprotein lipase releases fatty acids from passing chylomicrons and very low-density lipoproteins (Chapter 20), causing an influx of fatty acids into the brown adipocytes.

Fatty acids and norepinephrine inhibit acetyl-CoA carboxylase. Increased fatty acid uptake, lipolysis and esterification occur simultaneously in BAT. BAT fatty acyl-CoA synthetase and acyltransferases, associated primarily with the ER, catalyze triacylglycerol formation as in WAT. Triacylglycerol synthesis is decreased during fasting, and increases sharply in an insulin- and norepinephrine-dependent fashion, upon refeeding. Probably the increased triacylglycerol synthesis is required by active brown adipocytes to accommodate the enhanced fuel influx, which is in turn required for thermogenesis. Concomitant synthesis of triacylglycerol and degradation of fatty acids probably constitutes a futile process that is itself thermogenic.

Fatty acids utilized by BAT for thermogenesis are derived from several sources including dietary triacylglycerol (via chylomicrons), very low-density lipoprotein triacylglycerol from the liver, fatty acids from WAT bound to circulating albumin, hydrolysis of internal acyl-CoA molecules, and hydrolysis of internal triacylglycerol stores by HSL. In fact, the capacity of BAT for lipolysis actually exceeds its capacity for thermogenesis, such that BAT becomes an exporter of fatty acids at very high norepinephrine concentrations. Norepinephrine stimulates HSL via β_1 and β_3 adrenoreceptors as described for white adipose (Section 3.5.2). Increased synthesis of thyroxine 5′-deiodinase, responsible for increased levels of triiodothyronine and UCP, is mediated by α_1 adrenoreceptors [20,22].

Non-shivering thermogenesis is induced by heat loss when the temperature of the environment is significantly below the temperature of the organism. Thermogenesis can be suppressed by fever, exercise, and environmental temperatures similar to body temperature. Heat generation occurs through the dissipative relaxation of the proton gradient independent of ATP production (Fig. 4). The uncoupling protein, UCP, facilitates proton movement down a concentration gradient across the mitochondrial membrane without simultaneous production of ATP by the proton-dependent ATP synthetase. Since the rate of ATP synthesis is usually the limiting factor of respiration and is dependent on utilization of energy from proton movement along the gradient, dissipation of the gradient by UCP uncouples oxidation from its rate limitations. Unlimited oxidation produces the large amounts of heat that are distributed by BAT during thermal distress.

The original uncoupling protein (termed UCP1) was discovered nearly 30 years ago, and is now recognized as one member of a burgeoning UCP multigene family. The proteins are found in several tissues and across species. UCP1 is expressed predominantly in BAT and is a ~306 amino acid (~33 kDa) protein predicted to span the inner mitochondrial membrane several times, projecting its C-terminus into the intermembrane space [23]. The activity of UCP1 is increased by fatty acids, which interact with the protein in the membrane and probably lower the membrane potential for proton translocation, facilitating gradient dissipation. UCP also has a highly pH-dependent C-terminal purine nucleotide-binding site that may serve as a regulator of the protein's activity. Small changes in pH drastically affect ADP and ATP binding to this site, and ADP/ATP binding inhibits proton translocation in reconstituted phospholipid vesicles.

The oxidative fuel for thermogenesis is exclusively fatty acids even if glucose is available. This finding is interesting because insulin facilitates uptake of large amounts of glucose during thermogenesis, much more than the cell requires for synthesis of glycerol backbones. During thermogenesis, norepinephrine activates key regulated glycolytic enzymes such as phosphofructokinase and pyruvate dehydrogenase, thus upregulating glycolysis as well as fatty acid oxidation. It has been postulated that upregulation of glycolysis may be essential for ATP production through increased levels of phosphoenolpyruvate and the direct transfer of the phosphoryl group to ADP, in a process referred to as substrate-level phosphorylation. Since ATP synthesis is uncoupled from oxidation, the cell's ATP requirements must be met in another way. In addition, the cell continues to utilize large amounts of reduced cofactors to produce heat. These can be replenished by an elevated glycolytic rate.

The transcription of UCP mRNA is upregulated by norepinephrine activation of adrenoreceptors and by increased cAMP. This upregulation can be enhanced by the presence of triiodothyronine and abolished if the deiodinase activity of the cell is inhibited. BAT has a nuclear receptor for triiodothyronine that functions as a transcription factor and probably binds upstream of the UCP gene to activate transcription. The presence of both thyroid response elements and cAMP response elements is likely to be required.

4. Lipid-mediated signal transduction

4.1. Fatty acids and acyl-CoAs

Fatty acids and acyl-CoAs are not only substrates for metabolic enzymes, but also serve as regulatory molecules influencing the activity of several metabolic enzymes. For example, the lipolytic activity of HSL is inhibited by non-esterified fatty acids, resulting in feedback inhibition of the enzyme. Thus, if there is a surplus of fatty acids, HSL activity is reduced resulting in a potential accumulation of diacylglycerol that in turn would lead to insulin resistance. Additionally, fatty acids inhibit de novo lipogenesis via regulation of fatty acid synthase as well as cholesterol synthesis through the inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase.

Acyl-CoAs are also key regulators of enzymatic activity and particularly affect de novo lipogenesis [24]. Acetyl-CoA carboxylase utilizes acetyl-CoA as a substrate to

catalyze the formation of malonyl-CoA. This product is an essential substrate for lipogenesis via fatty acid synthase, as well as being an inhibitory factor for fatty acid oxidation. Acetyl-CoA carboxylase is inhibited by acyl-CoAs resulting in feedback inhibition of de novo lipogenesis, reduced malonyl-CoA production, and concomitant activation of the oxidative pathways. Acyl-CoA, like fatty acids, also inhibits HSL, fatty acid synthase, and 3-hydroxy-3-methylglutaryl-CoA reductase. Additionally, acyl-CoAs, as well as the acyl-CoA metabolite diacylglycerol, are key regulators of protein kinase C (PKC) which when activated in adipocytes results in serine phosphorylation of IRS-1 and the development of insulin resistance (Section 4.3).

Fatty acids have also been implicated in the inflammatory response in adipose tissue (Section 4.4), although the mechanisms are complex and likely to be indirect. Addition of supraphysiological levels of fatty acids, particularly palmitate but to a lesser extent oleate, to cultured adipocytes results in the activation of the c-Jun N-terminal kinase. This kinase is a critical regulator of the inflammatory response and regulates cell proliferation, survival, cell death, ER stress response, DNA repair, and metabolism. Importantly, c-Jun N-terminal kinase is also activated by TNFα which results in serine phosphorylation of insulin receptor substrate, thereby reducing insulin signaling [25]. However, with the discovery of vectoral acylation (Section 3.2), it is now apparent that fatty acid influx results in esterification of the fatty acids at the plasma membrane and direct channeling of the products into the triacylglycerol biosynthetic pathway. Thus the actual molecular mechanism of c-Jun N-terminal kinase activation by fatty acids is unclear and is not likely to be due to an increase in intracellular fatty acids.

Fatty acids and its metabolites also function as ligands for transcription factors [5]. Most notably, the PPAR receptors have been shown to directly bind fatty acids, although with lower affinity than some other potential ligands. However, fatty acids are relatively abundant in adipose tissue and may still be very important for endogenous PPAR activity. Additionally, nitroalkenes have also been suggested to be PPARγ ligands. Although present at much lower concentrations than free fatty acids, nitrated fatty acids have a much higher affinity for PPARγ (B.A. Freeman, 2005). Besides fatty acids, acyl-CoAs have been implicated in transcriptional control. The crystal structure of bacterially expressed hepatocyte nuclear factor-4 reveals the presence of a tightly bound acyl-CoA implying that such lipids are physiological regulators of protein structure and function.

4.2. Eicosanoids

While many investigators have focused on the study of PPAR receptors, the identity of a biologically relevant ligand has remained elusive. Extensive studies have demonstrated the ability of a variety of hydrophobic ligands to bind and activate PPARγ. Two classes of enzymes that might be involved in the generation of a natural ligand include the cyclooxygenase enzymes, which catalyze the rate-limiting step of prostanoid biosynthesis, as well as the lipoxygenase enzymes (Chapter 12). Phospholipase A_2 may be important in cleaving a fatty acid from phospholipids generating the main source of arachidonic acid, the substrate for the cyclooxygenases.

The search for potential natural ligands has focused on polyunsaturated and oxidized fatty acids. Some arachidonic acid metabolites, in particular, 15-deoxy-$\Delta^{12,14}$-prostaglandin

J_2 and 15*S*-hydroxyeicosatetraenoic acid, produced by 15-lipoxygenase, have been suggested as direct ligands of PPARγ. Other arachidonic acid metabolites derived from the lipoxygenase pathway, namely 8*S*-hydroxyeicosatetraenoic acid and leukotriene B4 have been suggested as direct ligands for PPARs [6]. Additionally, derivatives of linoleic acid, including 9- and 13-hydroxyoctadecadienoic acids, 13-oxooctadecadienoic acid, and nitroalkenes have been identified as potential natural ligands for PPARγ. While many of these molecules have been suggested as biologically relevant ligands, there also have been concerns over the actual endogenous concentration of these that is sufficient to support PPARγ activity. This factor is particularly important when considering that fatty acids themselves are weak activators of PPARγ suggesting that within the cellular context there are a variety of biologically relevant ligands with differing affinities and abundances. Since different ligands produce subtly different structures when bound to PPARγ, the ability to bind to co-activator complexes varies among lipids. Hence, different ligands have the capacity to activate different cassettes of genes, implying that lipids have the ability to selectively regulate adipocyte metabolism.

Cyclopentenone prostaglandins of the A- and J-type contain a reactive α,β-unsaturated carbonyl group in a cyclopentane ring which allows covalent modification of nucleophiles through Micheal addition reactions. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and prostaglandin A_1 can covalently modify redox-sensitive transcription factors, typically through a cysteine residue [26]. For IKKα and β, covalent modification of a cysteine within the activation loop inhibits its activity. NF-κB can also be modified by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, resulting in decreased DNA binding and transcriptional activity.

Increased activity through covalent modification occurs with PPARγ, Keap1 (increases Nrf2 activity), and p53. Transcription factors that have decreased activity include c-Jun (decrease AP-1 activity) and hypoxia inducible factor 1.

Other metabolites of arachidonic acid exhibit profound effects on lipid metabolism in adipose tissue. Prostaglandin E_1 and E_2 (PGE_1 and PGE_2) acutely inhibit catecholamine-induced lipolysis [27]. PGE_2 exerts its action through binding to EP receptors, which contain seven-transmembrane regions and are members of the G-protein coupled receptor family. The receptors mediating prostanoid-induced inhibition of lipolysis in adipocytes are thought to be of the EP3 sub-type, which couples to the inhibitory G_i subunit. Mechanistically, EP3 receptors have been associated with inhibition of adenylyl cyclase, thus reducing cAMP levels. Thus it is through this mechanism that only stimulated (and not basal) lipolysis is inhibited. Contrary to acute PGE treatment, prolonged treatment of adipocytes with PGE results in downregulation of G_i subunits and increases lipolysis.

4.3. Diacylglycerol and ceramide

PKC is a family of serine/threonine kinases with varying tissue-specific expression patterns and whose activity can affect a multitude of cellular functions. The family is divided into three subgroups: conventional isozymes, novel isozymes, and atypical isozymes. The first two are activated by diacylglycerol. Diacylglycerol activation of PKC leads to serine phosphorylation of IRS-1, resulting in reduced insulin stimulated tyrosine phosphorylation as well as increased degradation of insulin receptor substrate-1 [28]. There is evidence for direct phosphorylation of insulin receptor substrate-1 by PKCθ, however,

PKCθ also activates IκB kinase and c-Jun N-terminal kinase in adipocytes. Both of these protein kinases have been shown to phosphorylate a serine residue of IRS-1. In contrast to PKCθ, PKCδ leads to phosphorylation of p47, activating NADPH oxidase. These molecular mechanisms that result from increased diacylglycerol accumulation in adipocytes may at least in part, explain the insulin resistance associated with obesity.

Ceramide (*N*-acyl sphingosine) is a serine-based lipid containing saturated acyl chain typically of 16 and 24 carbons in length (Chapter 13). By virtue of their hydrophobicity, ceramides function in a variety of signaling pathways, particularly those affecting membrane structure and permeability [29]. Ceramide is an important signal effector molecule in stress pathways, such as those activated by TNFα, resulting in activation of protein kinases including c-Jun N-terminal kinase and the atypical PKCζ/λ. TNFα through increases in ceramide and subsequent activation of the atypical PKC$\tilde{\zeta}$/λ, has been shown to decrease PPARγ in adipose tissue. Ceramide also activates protein phosphatase 2A, thereby dephosphorylating proteins such as PKB/AKT in the insulin-signaling cascade and preventing efficient insulin signaling and/or promoting growth arrest.

4.4. Lipids as mediators of inflammation

Adipose tissue is composed not only of adipocytes, but also other cell types, including adipocyte progenitor cells, endothelial cells, and blood cells. More recently, several studies have indicated the presence of infiltrated macrophages in adipose tissue, with the degree of adiposity correlating with the quantity of macrophages. In the extremely obese individual, it is thought that up to 40% of the mass of adipose tissue is comprised of resident inflammatory cells. This correlation also extends to the degree of insulin resistance. Of the paracrine factors thought to play key roles in cellular regulation, fatty acids produced by adipocyte lipolysis have been considered central. Accordingly, a number of studies have focused on the local adipocyte fatty acid–macrophage signaling system [30].

Both adipocytes and macrophages secrete a number of paracrine and endocrine factors, some specific to only one cell type, while others are secreted by both types. The initiating factor is thought to be fatty acids effluxed from the adipocyte. The fatty acids liberated from the adipocyte are thought to bind to TLR4 receptors (classically studied with respect to binding a range of microbial products) and signal an inflammatory response in the macrophage. Such activated macrophages are thought to be the major source of locally produced proinflammatory cytokines, such as TNFα and interleukin-6, which can bind to receptors on adjacent adipocytes and regulate lipid metabolism and insulin action. Thus a paracrine loop exists between adipocytes that release fatty acids and macrophages that secrete TNFα and interleukin-6, creating a feedforward cycle that potentiates inflammatory conditions in WAT. Additionally, the adipocyte expresses and secretes a diverse range of additional factors (Table 3) that have a direct impact on adipocyte metabolism, as well as on whole body energy homeostasis and insulin action.

Adipocytes have profound sensitivity to TNFα, interferons α, β, and γ, and interleukins 1, 6, and 11. In general, such cytokines inhibit lipogenesis and triacylglycerol storage by adipocytes, activate lipolysis, and antagonize insulin action. Additionally, many cytokines interfere with proliferation of preadipocytes in 3T3-L1 or 3T3-F442A cell lines and/or

Table 3
Summary of several adipocyte secreted factors

Adipokine	Expression in the obese state	Function in vivo
Acylation stimulating protein (ASP)	Decreased	Stimulates triacylglycerol synthesis
Adiponectin/AdipoQ/Acrp 30	Decreased	Associated with insulin sensitivity
Adipsin	Decreased	Activation of alternative complement pathway
Angiotensinogen	Increased	Regulates blood pressure
Insulin-like growth factor 1	Increased	Proliferation/mediates effects of growth hormone
Interleukin-6	Increased	Immune response/glucose and lipid metabolism
Leptin	Increased	Energy expenditure; reproduction satiety factor
MCP-1	Increased	Recruits monocytes
Plasminogen activator inhibitor	Increased	Cardiovascular function/wound healing
Prostaglandin E_2	Increased	Anti-lipolytic, suppresses cAMP production
Prostaglandin F_2	Increased	Inhibits adipogenesis
Prostaglandin I	Increased	Adipogenic in preadipocytes
Resistin	Increased	Associated with insulin resistance
Transforming growth factor β	Increased	Involved in proliferation, differentiation, and apoptosis
Tumor necrosis factor α	Increased	Contributes to insulin resistance and type 2 diabetes

diminish adipogenesis in vivo. TNFα is expressed at high levels in adipose tissue as seen in several genetically defined rodent models of obesity and insulin resistance. In leptin receptor deficient mice, TNFα expression is elevated but expression of other cytokines such as interleukin-1 or interleukin-6 is not increased. Measurements of insulin receptor tyrosine kinase activity in obese leptin-deficient rats show a reduced function, which could be restored by administering soluble TNFα receptor to sequester the secreted TNFα protein. TNFα interferes with insulin signaling by affecting the phosphorylation of the insulin receptor, thus contributing to the development of insulin resistance in these animals. In an adipocyte cell culture model, 3T3-L1, TNFα inhibits the expression of C/EBPα and PPARγ, thus leading to reduced expression of genes that are involved in triacylglycerol accumulation and metabolism. Interleukin-6 is secreted by WAT under basal conditions and its expression is increased by TNFα and by other conditions linked to wasting disorders (cancer, cachexia, HIV).

TNFα and the interferons decrease lipogenesis, by downregulating the mRNA levels of the key lipogenic enzymes acetyl-CoA carboxylase and fatty acid synthase; the interferons diminish mRNA levels of fatty acid synthase but not of acetyl-CoA carboxylase. TNF-α, interleukin-1, and some interferons also increase lipolysis, although the mechanism is probably post-transcriptional, since Northern blot analysis shows that TNF-α and the interferons decrease the level of perilipin mRNA.

4.5. Leptin and adiponectin

Adipose tissue secretes a large number of proteins, many of which affect overall whole body energy homeostasis (Table 3). Leptin is synthesized and secreted from adipocytes and circulates at levels that are proportional to the body fat content. Consequently, leptin is capable of signaling the brain as to the degree of whole body adiposity (J.M. Friedman, 1997). Although the brain expresses the highest number of leptin receptors, leptin receptors are ubiquitously expressed in peripheral tissues. Leptin receptors are members of the class 1 cytokine receptor family and are linked to JAK-STAT signaling systems (J.S. Flier, 1997). This signal affects overall energy homeostasis by affecting processes such as food intake, satiety, and energy expenditure. Consistent with these observations, the lack of leptin in mice results in hyperphagia and severe obesity.

Adiponectin is a protein exclusively expressed and secreted from adipocytes. PPARγ ligands have been shown to upregulate adiponectin expression. Circulating levels of adiponectin correlate positively with insulin sensitivity and negatively with body mass index. Adiponectin exerts its affect by binding two receptors. The oligomeric state of adiponectin determines which receptor is bound and activated. Signaling through the adiponectin receptors activates AMPK (T. Kadowaki, 2002) and results in suppression of inflammatory cascades such as TNFα, as well as suppression of macrophage foam cell formation. Several groups have generated adiponectin-null mice with somewhat varying results. The phenotypes found range from severe insulin resistance on a high fat diet to no change in insulin sensitivity, but an increase in fatty acid oxidation in skeletal muscle. More recently (P.E. Scherer, 2007), showed that a lack of adiponectin causes a defect in insulin-dependent suppression of hepatic glucose output, with minor effects on muscle glucose uptake or oxidation. Although the exact mechanism of action is still being elucidated, overall it is clear that adiponectin functions as an important insulin sensitizer.

5. Future directions

Adipocytes synthesize and secrete over 100 different polypeptides and nearly as many different lipid species. As the lipid droplet enlarges due to increased metabolic load, not only does the flux through various metabolic pathways change but also a number of control systems are coordinately affected. With increased triacylglycerol deposition, multiple kinases are activated and signal to selectively regulate target pathways. Transcriptional control circuits respond leading to altered profiles of gene expression and protein synthesis, and secretion pathways are controlled to affect adipokine expression. Added onto this framework is the consideration that different fat depots fulfill different regulatory niches demonstrating that the adipose system is complex and requires the integration of genomic, proteomic, and lipidomic information. Acquiring such systems-level information on a tissue like adipose is a daunting task but will likely provide key insights as to how the human responds to energy excess and how adipose lipid metabolism functions in vivo. The next decade will bring the systems biology approach to adipose lipid metabolism and, in all likelihood, will reveal new levels of complexity that are now just beginning to be appreciated.

Abbreviations

ACS	acyl-CoA synthetase
AKT/PKB	protein kinase B
AMPK	AMP-activated protein kinase
aP2	adipocyte fatty acid binding protein
ATGL	adipose triacylglycerol lipase
BAT	brown adipose tissue
C/EBP	CAAT/enhancer-binding protein
DGAT	diacylglycerol acyltransferase
FABP	fatty acid binding protein
FATP	fatty acid transport protein
GLUT	glucose transporter
HSL	hormone-sensitive lipase
LXR	liver X receptor
PDE	phosphodiesterase
PGC-1	peroxisome proliferator-activated receptor gamma coactivator-1
PI3-kinase	phosphatidylinositol-3-kinase
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
RXR	retinoid X receptor
SREBP	sterol regulatory element binding protein
TNFα	tumor necrosis factor alpha
UCP	uncoupling protein
WAT	white adipose tissue

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CHAPTER 11

Phospholipases

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Contents

1. Overview

1.1. Definition of phospholipases

Phospholipases (PLs) are a ubiquitous group of enzymes that share the property of hydrolyzing a common substrate, phospholipid. Nearly all share another property: they are more active on aggregated substrate above the phospholipid's critical micellar concentration (cmc). As shown in Fig. 1, phospholipases have very low activity on monomeric substrate but become activated when the substrate concentration exceeds the cmc. The properties of phospholipids that define the aggregation state (micelle, bilayer vesicle, hexagonal array, etc.) are described in Chapter 1.

The phospholipases are diverse in the site of action on the phospholipid molecule, their function and mode of action, and their regulation. The diversity of function suggests that

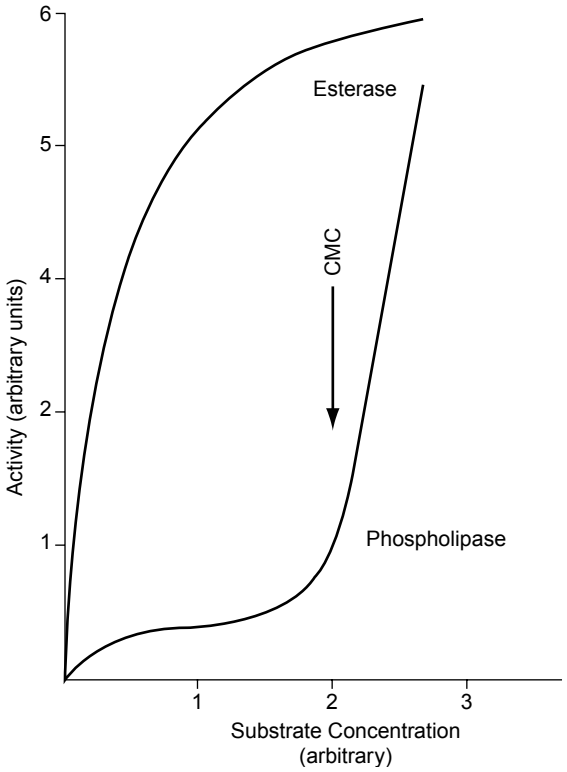

Fig. 1. Dependence of phospholipase and non-specific esterase activity on substrate concentration. Esterase exhibits Michaelis–Menten kinetics on soluble substrates, whereas a phospholipase becomes fully active above the cmc of the substrate.

phospholipases are critical to life since the continual remodeling of cellular membranes requires the action of one or more phospholipases. Their functions go beyond their role in membrane homeostasis; they also function in such diverse roles as the digestion of nutrients and the formation of bioactive molecules involved in cell regulation. There are indications that a few phospholipases may carry out a biological function independent of their catalytic activity by binding to a regulatory membrane receptor. Phospholipase-like proteins with toxic properties, yet which lack a functional catalytic site, are found in venoms. It is of interest that most, but not all, phospholipases studied in detail thus far are soluble proteins. The soluble nature of many phospholipases suggests that their interaction with cellular membranes is one of the regulatory mechanisms that exist to prevent membrane degradation or to precisely control the formation of phospholipid-derived signaling molecules.

The classification of the phospholipases, based on their site of attack, is given in Fig. 2. The phospholipases A (PLAs) are acyl hydrolases classified according to their hydrolysis of the 1-acyl ester (PLA_1) or the 2-acyl ester (PLA_2). Some phospholipases will hydrolyze both acyl groups and are called phospholipase B (PLB). In addition, lysophospholipases

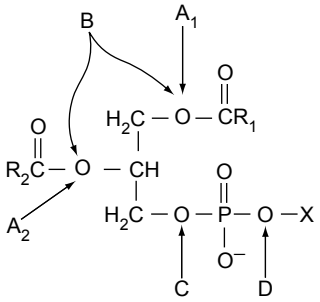

Fig. 2. Sites of hydrolysis by phospholipases.

remove the remaining acyl groups from monoacyl(lyso)phospholipids. Cleavage of the glycerophosphate bond is catalyzed by phospholipase C (PLC), while the removal of the base group is catalyzed by phospholipase D (PLD). The PLC and PLD are therefore phosphodiesterases.

1.2. Assay of phospholipases

The subject of phospholipase assays has been concisely reviewed [1–5]. A standard approach employs phospholipids with radioisotopes incorporated into specific positions in the molecule. By the appropriate choice of labeling, the specificity of the enzymes can readily be established and as little as a few picomoles of product can be detected. The use of isotopes has been helpful in the measurement of phospholipase activity using the membranes of whole cells or isolated subcellular fractions previously labeled with radioactive phospholipid precursors.

A second commonly used approach employs synthetic substrates for spectrophotometric or fluorometric assays [3,4]. These substrates permit a continual assay well suited for kinetic studies and provide reasonable sensitivity. The major drawback is that the substrates are not natural substrates and as such, their use should be considered as a model that may or may not reflect the enzyme's kinetic properties in biological systems. The thioacylester analogs of phospholipids provide a sensitive spectrophotometric assay for some PLA_1 or PLA_2 assays based on the reaction of released thiol with Ellmann's reagent.

Fluorescent assays require that the product has different fluorescent characteristics from those of the substrate, normally involving physical separation of the two species as part of the assay. Fluorescent substrates are now being used that incorporate both a fluorophore and a quenching group in the same molecule. Hydrolysis will separate the two groups and the loss of intramolecular quenching will result in an enhanced fluorescence signal. It is possible to incorporate such substrates into cells and to visualize phospholipase activity using fluorescence microscopy (G.T. Wijewickrama, 2006). An alternative approach that does not involve synthetic fluorescent substrates is to use a fluorescent displacement assay where the normal product of hydrolysis, such as a long-chain fatty acid, displaces a fluorescent probe from a protein that binds long-chain fatty acids. The change in fluorescence is monitored and the assay has the advantage that any

source of natural phospholipid substrate can be used including cell membranes and lipoproteins (D.C. Wilton, 1990, 1991; G.V. Richieri, 1995).

1.3. Interaction of phospholipases with interfaces

The catalytic turnover of phospholipases at the interface distinguishes them from the general class of esterases (Fig. 1). Therefore, the study of phospholipases must include an understanding of their interaction with the lipid interface. The increased enzyme activity seen when phospholipids aggregate at a concentration above their cmc (Fig. 1) implies that phospholipases have an interfacial binding surface and that interfacial binding will precede normal catalysis. Interfacial binding is crucial because the cmc of normal cellular phospholipids is very low ($<<10^{-9}$ M) and hence the concentration of monomeric substrate in the aqueous phase is sub-nanomolar. Moreover, the half-time for desorption of a long-chain phospholipid from the bilayer interface is of the order of hours. What this means in practice is that the phospholipase must first bind to the interface and that the interfacial binding step has a profound effect on overall catalysis. This concept is illustrated in Fig. 3. Interfacial binding ($E \rightarrow E^*$) often provides the basis for the physiological regulation of these types of enzyme. Unless the phospholipase can bind productively to the phospholipid surface, it cannot access substrate and express activity.

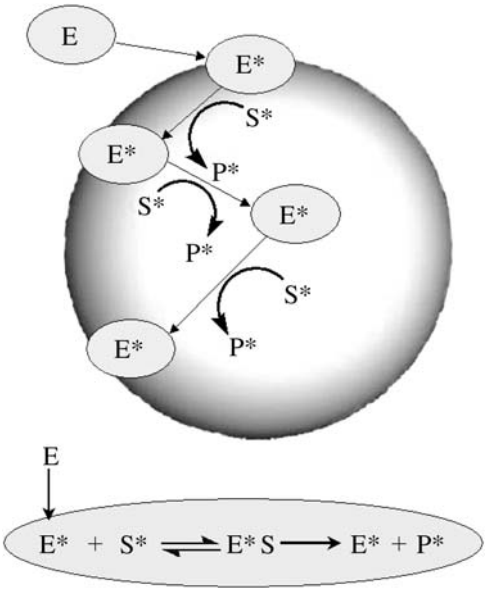

Fig. 3. Schematic illustration of interfacial catalysis with vesicles. The interface-free form of the enzyme, E, binds to the interface to produce the E* form. The complex E*S is the interfacially activated form that reacts to form the E*P complex. Following product release, the enzyme remains bound to the interface surface (scooting mode) and continues through multiple catalytic cycles. Adapted from Ref. [9].

Moreover, conformational changes in the enzyme that result from interfacial binding will allow the enzyme to express optimum activity (interfacial activation).

The nature of interfacial binding and the rate enhancements that are achieved are controversial areas. However, it is clear that both polar and non-polar interactions are involved [6] and the precise contribution of each must depend on the nature of the phospholipid interface and the interfacial binding surface of the phospholipase. A number of factors can be considered that could make a major contribution to the enhanced hydrolysis at interfaces and these relate primarily either to the *substrate* or to the *enzyme*.

1.3.1. Substrate effects

Phospholipids in aqueous solution form aggregates whose nature is determined by the structure of the phospholipid (Chapter 1). Long-chain phospholipids normally form structures containing bilayers, whereas shorter chain phospholipids form micelles. Once the phospholipid is present as an aggregate, it is the concentration of substrate on the surface of the aggregate that is critical and is defined as mole fraction, being unity with a pure phospholipid. Therefore, provided the enzyme can bind to the interface, the effective concentration of substrate will rise dramatically producing a corresponding rate enhancement.

It is apparent that the interfacial binding step ($E \rightarrow E^*$) will have a dramatic effect on classical enzyme kinetics and methods of assay should ideally not be affected by this step. An elegant kinetic analysis of secreted PLA_2s ($sPLA_2$s) has been developed by Jain and coworkers (reviewed in Refs. [7,8]). This group of PLA_2s has, in general, a high affinity for anionic phospholipids such as the model substrate, phosphatidylmethanol. The high dissociation constant for the phosphatidylmethanol interface, $<<10^{-10}$ M, allowed the identification of the scooting mode of action of the porcine pancreatic phospholipase (Fig. 3). Under these conditions, the enzyme remains attached to the vesicle interface, thus promoting hydrolysis of the outer phospholipid monolayer without loss of vesicle integrity or release of enzyme from the vesicle. Hydrolysis can be measured in the absence of interfacial effects demonstrating that interfacial binding is separate from catalysis. Care must be taken, however, to demonstrate that the integrity of the vesicle is maintained and that the number of enzyme molecules is substantially less than the number of vesicles present. This is the preferred assay method for the assessment of potential enzyme inhibitors [7,8]. The inhibitor or other factors will not affect interfacial binding of the enzyme and only the effect of the inhibitor on classical catalysis ($E^* + S \rightarrow E^*S \rightarrow E^* + P$) will be measured.

Important factors when considering the enhanced hydrolysis at interfaces are the substrate environment in the monolayer and the need to transfer a substrate molecule from this monolayer to the active site. Interfacial disorder may provide an important parameter that facilitates such transfer of substrate to the active site. Phospholipase activity is enhanced under conditions that affect phospholipid fluidity, packing density of the phospholipids, and polymorphism of the aggregate. A highly ordered structure seen with phosphatidylcholine either above or below the transition temperature tends to give low rates of hydrolysis. Discontinuities in such ordered structures occur at temperatures close to the transition temperatures and the presence of other lipids such as anionic lipids or non-bilayer-forming phospholipids promote catalysis by perturbing the interface.

1.3.2. Enzyme effects

An important question is whether or not binding of the phospholipase to the interface promotes a conformational change in the structure of the enzyme that facilitates catalysis compared with the enzyme structure in free solution. This is clearly the case with many lipases where lid opening is seen on binding to the aggregate. In the case of the porcine pancreatic $sPLA_2$, NMR analysis of the enzyme when bound to anionic micelles compared to the enzyme in solution has revealed significant differences compared with the X-ray structure. In particular, the N-terminal region of the enzyme is disordered in free solution but is condensed as an α-helix when bound to an anionic micelle (B. van den Berg, 1995), the conformation seen in crystal structures.

The effect of specific phospholipid molecules on overall protein conformation and hence activity may be considered as an example of allostericity. Such allosteric behavior has been discussed in terms of a second phospholipid-binding site or as discrete interactions between the head groups of phospholipids and specific residues on the interfacial binding surface of the enzyme. The emerging evidence for $sPLA_2$s supports a model involving multiple interactions between enzyme and interfacial lipid [8].

Crystallographic support for the nature of the interfacial binding surface and possible conformational changes upon interfacial binding has come from examination of a crystal structure for the pancreatic PLA_2 known as the anion-assisted dimer (Y.H. Pan, 2001). In order to mimic binding to an anionic phospholipid interface, crystals were obtained in the presence of sulfate or phosphate. This dimer shared five anions between the two subunits while a single molecule of a phospholipid analog was bound at one active site with the alkyl chain extending into the active-site slot of the second subunit. It is argued that the plane of contact between subunits reflects the interfacial binding surface of the enzyme and has allowed detailed analysis of structural differences with normal monomeric crystal structures [9] that support the concept of interfacial activation.

In other attempts to define how a $sPLA_2$ sits on the membrane surface, the technique of electrostatic potential-modulated spin relaxation magnetic resonance has been used. Briefly, the method involves site-specific labeling of the protein with a nitroxide group and the use of an aqueous or membrane-soluble spin relaxant to measure the distance of the spin-label from the membrane–aqueous interface. Studies using this method showed that interfacial binding of the bee venom $sPLA_2$ is driven by the shallow penetration into the anionic membrane by a collar of hydrophobic residues that surround the opening to the active-site slot (Y. Lin, 1998). This technique has been extended to the study of the human group IIA $sPLA_2$ (S. Canaan, 2002), while the study of C2 domains, a ubiquitous phospholipid-binding motif, has allowed investigations of other phospholipases [10].

2. The phospholipases

Many types of phospholipases have now been purified and characterized and full crystal structures have been elucidated. Further, the cellular function of many phospholipases has been examined using various techniques of molecular biology including gene

transfection, gene knockouts, and antisense strategies. Since it is impossible to cover all phospholipases that have been characterized, only examples where significant information relating structure to function is available will be discussed in detail [11]. The roles of phospholipases in signal transduction including detailed discussions of PLC and PLD are not dealt with in this Chapter.

2.1. Phospholipase A_1

The phospholipases A_1 (PLA_1s) comprise a large group of 1-acyl hydrolases, some of which also degrade neutral lipids (lipases) or remove the acyl group at position 2 in addition to that at position 1 (PLB), and thus must have lysophospholipase activity. Where the enzyme appears to show low selectivity for the *sn*-1 or *sn*-2 position, the term phospholipase A is used. The term phospholipase B should be restricted to those enzymes where the mechanism involves minimal accumulation of lysophospholipid product. In this section, we consider various enzymes of the PLA type that do not fit a more precise definition in terms of acyl chain selectivity.

2.1.1. Escherichia coli phospholipases A

Two PLAs have been purified from *Escherichia coli* based on their differential sensitivity to treatment with detergents [2]. A detergent-insensitive enzyme is localized in the outer membrane, whereas a detergent-sensitive enzyme is found on the cytoplasmic membrane and in soluble fractions. The outer membrane enzyme, known as outer membrane phospholipase A, has broad substrate specificity and demonstrates PLA_1, PLA_2, lysophospholipase A_1, and lysophospholipase A_2 activity as well as activity for hydrolyzing monoacylglycerols and diacylglycerols. The crystal structure allows a more detailed discussion of an integral membrane phospholipase [12].

The protein is a 12-stranded anti-parallel β-barrel with amphipathic β-strands traversing the membrane (Fig. 4). The active-site catalytic residues are similar to a classical serine hydrolase triad except that in addition to the serine (Ser-144) and histidine (His-142), there is an asparagine (Asn-156) in place of the expected aspartic acid. Calcium at the active site is predicted to be involved in the reaction mechanism facilitating hydrolysis of the ester.

The active site is located at the exterior of the β-barrel at the outer leaflet side of the membrane (Fig. 4). This lipopolysaccharide outer leaflet does not normally contain phospholipid and this structural isolation of active site from substrate present in the inner leaflet is believed to be important in preventing uncontrolled phospholipase activity. It is proposed that appropriate bacterial stimulation results in phospholipid moving to the outer monolayer and enzyme activation, including dimerization (Fig. 4), thereby allowing phospholipid hydrolysis. This hydrolysis enhances the permeability of the outer cell membrane producing the appropriate adaptations such as the release of bacteriocins. The fact that *E. coli* mutants deficient in PLA have normal growth characteristics and phospholipid turnover is consistent with the specialized role of this enzyme in bacterial adaptation. The role of this enzyme as a virulence factor in pathogenesis has been reviewed (T.S. Istivan, 2006).

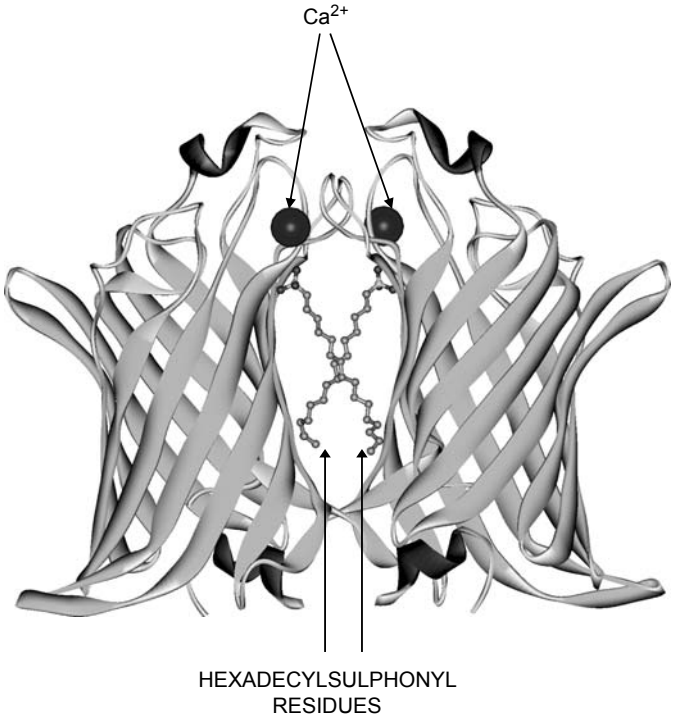

Fig. 4. Crystal structure of the outer membrane phospholipase A dimer from *E. coli* shown in the plane of the membrane. The top half of the molecule is located in the lipopolysaccharide monolayer facing the exterior of the cell. The phospholipid monolayer of the outer cell membrane would be located around the bottom half of the protein. Two calcium ions are shown at the active sites while Ser-144 of each active site is covalently modified with a hexadecylsulfonyl moiety represented in a ball and stick format. Structure is adapted from Ref. [12]. (See color plate section, plate no. 8.)

2.1.2. Lipases with phospholipase A_1 activity

Lipoprotein lipase and hepatic lipase are two lipases that degrade triacylglycerols in lipoproteins (Chapter 19) and also demonstrate significant PLA_1 activity. The enzymes have 50% sequence identity and are members of a superfamily of lipases and phospholipases that share the G-X-S-X-G motif at the active site and an Asp–His–Ser triad that is required for catalysis. Hepatic lipase is about 2–3-fold more efficient at hydrolyzing phospholipids than is lipoprotein lipase and has lysophospholipase activity. Another lipase, intestinal lipase, has lysophospholipase activity and is also referred to as a PLB.

2.2. Phospholipase B and lysophospholipases

The distinction between PLB and lysophospholipases is not clear [2] since both diacyl- and monoacyl-phospholipids are substrates. As discussed above (Section 2.1), a working definition of these enzymes can be based on the extent to which the lysophospholipid

accumulates upon hydrolysis of the diacyl substrate. Those PLBs for which there is significant structural and functional information are discussed below. In addition, mammalian lysophospholipases are reviewed.

2.2.1. Phospholipase B from microorganisms

The amino acid sequence of the PLB from *Penicillium notatum* was deduced from its cDNA (N. Masuda, 1991). The details of the overall catalytic mechanism are unknown, but intriguing, as substrate specificity is affected by the presence or absence of detergent as well as the glycosylation state of the enzyme. PLB activity has subsequently been identified in other fungi including *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and *Candida albicans*. In the case of *C. albicans*, gene disruption has identified this PLB as a major factor in host cell penetration and hence pathogenecity (S.D. Leidich, 1998). It is likely that this secreted enzyme has a similar role in other fungi. PLB activity has also been isolated from *Mycobacterium phlei* and *Mycobacterium lepraemurium* where product analysis indicates that the initial activity is that of a PLA_1 (S. Maeda, 1996). The roles of PLB in fungi have recently been reviewed (G.A. Kohler, 2006).

2.2.2. Mammalian lysophospholipases

Lysophospholipids [13] are generally found in very low concentrations (0.5–6% of total lipid membrane weight) in biological membranes. High concentrations of lysophospholipids affect membrane properties and membrane enzymes and even lead to cell lysis. Increased lysophospholipid levels are associated with atherosclerosis, while lysophosphatidylcholine is highly abundant in atherogenic lipoproteins, such as oxidatively modified low-density lipoprotein, where it constitutes up to 40% of the total lipid [13]. Other diseases where there are links with lysophospholipid levels include inflammation, hyperlipidemia, and lethal dysrhythmias in myocardial ischemia. At low concentrations, lysophospholipids such as lysophosphatidylcholine and lysophosphatidic acid have the properties of signaling molecules [14]. Against this background, the role of lysophospholipases in mammalian tissues assumes a considerable significance.

There are two small mammalian lysophospholipases (I and II) that despite their similar size appear to be the products of separate genes rather than splice variants or post-translational modifications [13]. The enzymes, which are calcium independent, have been purified from a variety of tissues and lack PLA_1, PLA_2, or acyltransferase activity. Their mechanism of action appears to involve the catalytic triad characteristic of serine proteases and lipases. Historically, lysophospholipase activity has been associated with acyl chain hydrolysis. However, the purification of a lysophospholipase D from serum and its identification as autotaxin (M. Umezu-Goto, 2002; A. Tokumura, 2002), a multifunctional phosphodiesterase originally found as a tumor cell motility-stimulating factor, provides a mechanism for the synthesis of lysophosphatidic acid. This compound is an important signaling molecule involved in cell proliferation and migration.

2.3. Phospholipase A_2

The phospholipases A_2 (PLA_2s) were the first of the phospholipases to be recognized. Over a century ago, Bokay (1877–1878) observed that phosphatidylcholine was degraded

by some component in pancreatic fluid that is now known to be the pancreatic PLA_2. At the turn of the century, cobra venom was shown to have hemolytic activity directed toward the membranes of erythrocytes (P. Keyes, 1902). The lytic compound produced by the venom phospholipase was identified a decade later and termed lysocithin (later, lysolecithin). These studies spurred further investigation of this intriguing class of enzymes and their mechanism of attack on water-insoluble substrates.

A large number of PLA_2s have now been identified within this expanding superfamily and this has required a continual re-evaluation of classification criteria. Recently, the classification of these enzymes has been updated [15]. They are now categorized into five distinct types, namely, the secreted PLA_2s ($sPLA_2$s), the cytosolic PLA_2s ($cPLA_2$s), the Ca^{2+} independent PLA_2s ($iPLA_2$s), the platelet-activating factor acetylhydrolases (PAF-AHs), and the lysosomal PLA_2s. These enzymes can also be categorized into two types based on catalytic mechanism with the $sPLA_2$s utilizing a catalytic histidine as the primary catalytic residue, whereas the other group involves a catalytic serine and normally an acyl-serine intermediate.

2.3.1. The 14-kDa secreted phospholipases A_2

These enzymes now include $sPLA_2$s from such diverse sources as venoms and mammalian and plant tissues. They are characterized by requiring millimolar concentrations of calcium for optimum activity and involve an active-site histidine and aspartate pair. They are typically extracellular enzymes with a large number of disulfide bonds that provide structural stability. The enzymes have been subdivided into 10 groups [15]. The enzymes in groups I–III have provided the foundation for our understanding of these secreted enzymes and sufficient quantities of natural and mutant enzymes from these groups have been obtained for X-ray crystallographic analysis. The sequences of a very large number of these phospholipases are now known and have been used to demonstrate their structural, functional, and evolutionary relatedness [16].

The conserved active-site residues, His-48 and Asp-99 (pancreatic enzyme numbering), provide the catalytic dyad and, with the availability of the crystal structure of the pancreatic enzyme, have been shown to produce the proton-relay mechanism (Fig. 5) [17]. In this mechanism, a water molecule acts instead of the serine found in the classical protease/lipase catalytic triad mechanisms. More recently, an alternative mechanism of catalysis has been proposed that involves two water molecules (W5 and W6) seen at the active site of the crystal structure (Fig. 6). In this mechanism, proposed by Jain (J. Rogers, 1996) and referred to as a 'calcium-coordinated oxyanion' mechanism, the attacking nucleophile (W5) is coordinated to the calcium thus enhancing its nucleophilicity. This water is connected to His-48 by a second water molecule (W6). Thus, the major formal difference between the two mechanisms is that the latter mechanism involves a second water molecule, while the first water molecule is activated by coordination to the calcium. It is argued that the rate-limiting step occurs during the decomposition of the tetrahedral intermediate, whereas in the originally proposed mechanism the formation of this tetrahedral intermediate is rate limiting [8].

2.3.1.1. Group I secreted PLA_2s. This group of phospholipases contains group IA enzymes such as those from cobra and krait venom, while the IB enzymes are the

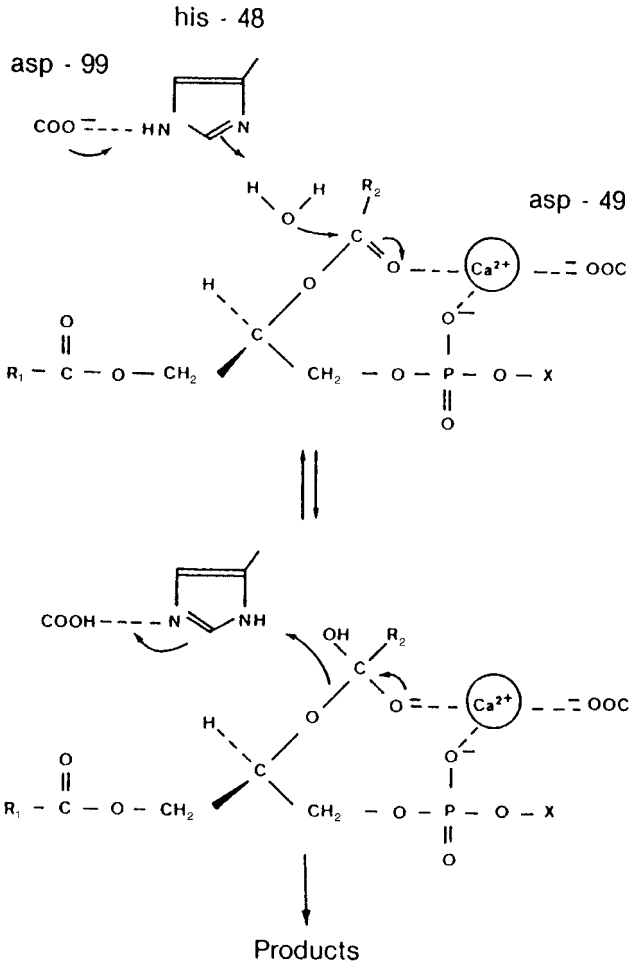

Fig. 5. Proton-relay mechanism of hydrolysis proposed by Verheij for secreted phospholipases A_2 [17].

mammalian pancreatic enzymes. Both the cobra and pancreatic enzymes were early models for structure–function analysis [18] including crystal structures of both apo-enzyme and enzyme with bound phospholipid inhibitors. However, at this time there are no crystal structures of group I enzymes bound to a lipid interface or with a phospholipid substrate at the active site.

A space-fitting model of substrate bound to the active site of the cobra venom enzyme (Fig. 7) gives insight into how the enzyme functions even though bulk interactions with the lipid interface are missing (E.A. Dennis, 1994). The most obvious feature is the existence of the active-site tunnel into which the substrate enters. However, the enzyme interacts loosely with the first 9–10 carbons of the acyl group at the *sn*-2 position that may account for the enzyme's lack of acyl specificity. This model also suggests that the

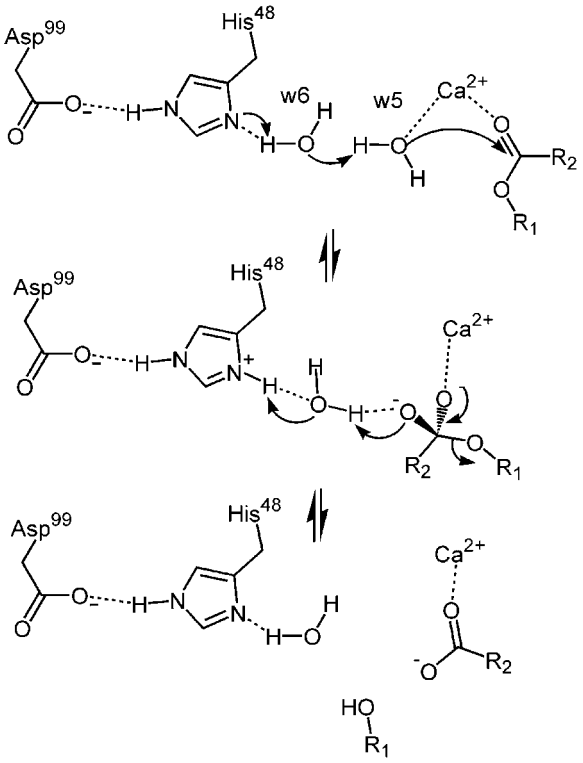

Fig. 6. Calcium-coordinated oxanion mechanism of hydrolysis proposed by Jain for secreted phospholipases A_2 (J. Rogers, 1996). The two water molecules at the active site that are implicated in catalysis are shown as W5 and W6.

substrate molecule is not completely withdrawn from the bilayer and significant hydrophobic interactions of the molecule undergoing hydrolysis and the interface are maintained. A particular feature is the ability of the cobra venom enzyme to hydrolyze phosphatidylcholine in vesicles and cell membranes, a feature that may in part reflect the presence of tryptophans and other aromatic residues on the interfacial surface. Such residues, particularly tryptophan, are able to partition into the interfacial region of a phosphatidylcholine interface (W.M. Yau, 1998) promoting interfacial binding and catalysis [19]. The presence of such residues in the presumptive interface region can be clearly seen in Fig. 7.

The mammalian pancreatic enzymes (group IB) have primarily a digestive role and are secreted as the pro-enzyme (zymogen) that requires subsequent proteolytic cleavage to remove a hexapeptide at the N-terminus. The pro-enzyme is unable to bind to the phospholipid interface. Unlike the cobra venom enzyme, the pancreatic enzyme expresses low activity with a zwitterionic interface such as that provided by phosphatidylcholine. The enzyme has a considerable preference for an anionic interface that can be provided by anionic phospholipid per se or by the inclusion of other anionic lipids in the

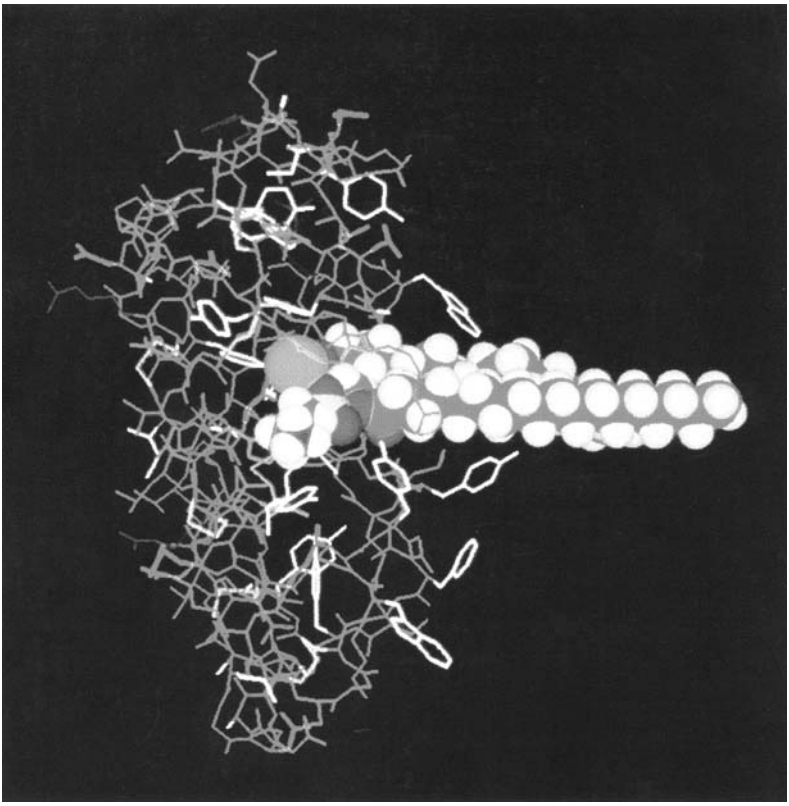

Fig. 7. X-ray crystal structure of cobra venom (*Naja naja naja*) phospholipase A_2 with bound Ca^{2+} showing a space-filling model of dimyristoyl phosphatidylethanolamine bound in the catalytic site. The ends of the fatty acid chains stick out of the enzyme and are presumably associated with the micelle or membrane (E.A. Dennis, 1994).

phosphatidylcholine interface. Presumably, the anionic bile salts provide such negative charge in the mixed micelles produced in the intestine to allow lipid digestion. The expression of the pancreatic enzyme in tissues other than the pancreas, and the presence of cell surface receptors for this enzyme (as well as for other $sPLA_2$s) suggest additional physiological roles [16,20].

2.3.1.2. Group IIA secreted PLA_2s. The group IIA $sPLA_2$s include venom enzymes from rattlesnakes and vipers. This group is characterized by a C-terminal extension while they lack a surface loop region (elapid loop) present in the group I enzymes. However, the most interesting member of this group IIA is the mammalian enzyme that was isolated from the synovial fluid of patients with rheumatoid arthritis and from platelets. This was the first mammalian non-pancreatic PLA_2 to be identified and was implicated as a key enzyme in arachidonic acid release from phospholipids, the first step in the production of the inflammatory eicosanoids. However, it has subsequently been established that the

intracellular group IV enzyme plays the dominant role in eicosanoid production (see Section 2.3.2.1) and hence the precise physiological role of this IIA enzyme in the inflammatory response remains to be clarified.

It is clear that the human IIA enzyme behaves as an acute phase protein (R.M. Crowl, 1991) and extracellular levels increase dramatically in acute inflammatory conditions such as septicemia where blood levels can rise over 100-fold. The detailed structural properties of the enzyme are unusual and appear to reflect at least some of its physiological roles. The enzyme is highly cationic (p*I* 9.4) and has a net positive charge of +19 due mostly to cationic residues globally distributed across the surface of the protein, a feature that is linked to the high affinity of the protein for heparin. An unusual characteristic of the enzyme is its marked preference for anionic phospholipid interfaces and very low affinity for a zwitterionic interface as provided by phosphatidylcholine. This preference is expressed at the stage of interfacial binding, while active-site substrate preference for anionic phospholipids is modest, favoring phospholipids such as phosphatidylglycerol over phosphatidylcholine (A.G. Singer, 2002). These surface properties are consistent with one particular physiological role for the enzyme, namely, as an anti-bacterial protein that shows specificity towards gram-positive bacteria [21]. The highly cationic nature of the protein allows the enzyme (but not other more neutral $sPLA_2$s) to penetrate the highly anionic bacterial cell wall (S.A. Beers, 2002). Moreover, the anionic bacterial cell membrane that is rich in phosphatidylglycerol provides an optimum substrate for this enzyme (Fig. 8). In contrast, this enzyme is essentially inactive against the host cell membranes that are normally zwitterionic being rich in phosphatidylcholine and sphingomyelin. This lack of activity against host membranes, which is partly due to lack of an interfacial tryptophan residue (S.F. Baker, 1998), is important as the serum levels of the enzyme can rise to above 1 μg/ml.

The enzyme is released from cells that possess anti-bacterial activity (macrophages and Paneth cells) and is present, along with lysozyme, at very high concentrations (~30 μg/ml) in human tears (X.D. Qu, 1998). The increased sensitivity of mice deficient in the IIA enzyme to gram-positive bacteria and increased bacterial resistance of mice expressing the human group IIA enzyme provides further support for the anti-bacterial role of the enzyme (V.J.O. Laine, 2000). The preference of this enzyme for anionic interfaces also means that this enzyme may be active in helping to destroy apoptotic and damaged cells [20].

The role of the group IIA enzyme in the regulation of host cell function remains to be clarified. The involvement of this enzyme in the inflammatory response has been demonstrated, where it is linked to the delayed release of prostaglandins. Clearly, the responsiveness of the plasma membrane to the extracellular enzyme must vary dramatically with cell type, which in extremis allows cells of the corneal epithelium to be bathed by fluid containing 30 μg/ml of the enzyme, over 1000-fold higher than normal serum levels, without adverse effects. The concept of cells having to be activated before being affected by the external enzyme has emerged. Activation may reflect the surface exposure of phosphatidylserine in apoptotic cells that would promote interfacial binding and catalysis or binding to heparan sulfate proteoglycans, the expression of which is a function of cell status. It has been proposed that the enzyme binds to one particular type of proteoglycan, namely, the glypicans and that a continual translocation of glypicans from the cell surface to the nuclear region occurs during cell activation [20]. It remains to be established what other effects the human group IIA enzyme has on host cell function

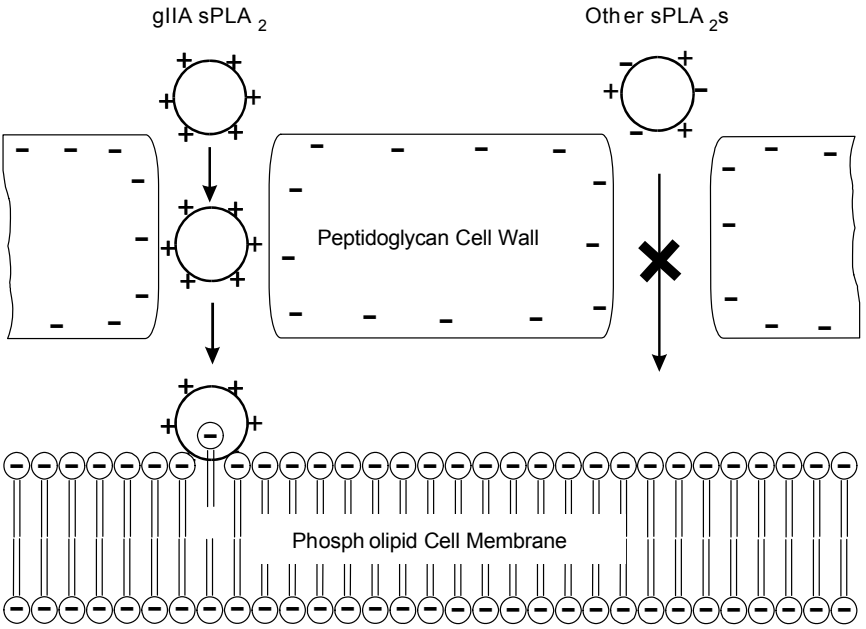

Fig. 8. A model of bacterial cell wall penetration and phospholipid hydrolysis by human group IIA secreted PLA_2 (gIIA $sPLA_2$). The extreme and global nature of the positive charge of this secreted PLA_2 has been proposed to allow the enzyme to move through the negatively charged cell wall mediated by the continual making and breaking of electrostatic bonds. For simplicity bacterial phospholipids are shown as being negatively charged, although the bacterial membrane will contain some zwitterionic phospholipids, normally phosphatidylethanolamine.

including roles that do not require catalytic activity. A non-catalytic role for the enzyme has recently been proposed involving uptake into cells in association with debris particles that contain anionic phospholipids (C.N. Birts, 2008).

2.3.1.3. Group IIB–F secreted PLA_2s. The group IIB includes the gaboon viper enzyme that is missing one of the highly conserved disulfides. Groups IIC–F are recently discovered mammalian enzymes of which the IIC is present only as a pseudo-gene in humans. These human genes, along with the IIA and V, map to the same chromosome locus; however, the function of these enzymes remains to be elucidated [20]. The interfacial kinetic and binding properties of these enzymes have been compared (A.G. Singer, 2002). A strong correlation is seen between the ability of individual enzymes to hydrolyze phosphatidylcholine vesicles and to hydrolyze the plasma membrane of mammalian cells in culture. The group IID enzyme, like the IIA and V enzymes, also binds to heparan sulfate proteoglycans. The group IIF enzyme has a unique proline-rich C-terminal extension, while hydrophobic residues near the C-terminus facilitate cell penetration, a feature that does not require catalytic activity (G.T. Wijewickrama, 2006).

2.3.1.4. Group III secreted PLA_2. The bee venom enzyme is placed in group III and is the primary allergen in bee venom. The enzyme is significantly larger than other $sPLA_2$s

and has less structural homology. The availability of a crystal structure has allowed extensive mutagenesis including subsequent spin-labeling used to define the nature of protein docking on the membrane surface (Y. Lin, 1998) (see Section 1.3.2). A group III human enzyme has recently been identified that possesses long N- and C-terminal extensions, which can be proteolytically removed. A potential role in stimulating tumor cell growth and angiogenesis has been suggested (M. Murakami, 2005).

2.3.1.5. Group V secreted PLA_2. This enzyme is the most studied human $sPLA_2$ except for the group IIA enzyme. The group V enzyme has a tryptophan on the interfacial binding surface, Trp-31, which is partly responsible for its ability to hydrolyze phospholipids in cell membranes. Exogenously added group V enzyme can catalyze phosphatidylcholine hydrolysis in the cell surface with subsequent activation of group IV $cPLA_2$ and enhanced leukotriene biosynthesis. Removal of Trp-31 by mutagenesis greatly reduces the effectiveness of added enzyme (S.K. Han, 1999). Like the IIA enzyme, the group V enzyme binds to heparan sulfate proteoglycans on the cell surface and is internalized. The ability of the enzyme to hydrolyze phospholipids in the plasma membrane with release of both lysophospholipid and fatty acid, and to bind to heparan sulfate proteoglycans could result in different effects on cell function depending on cell type. The production of group V PLA_2 knockout mice demonstrated that this $sPLA_2$ is involved in both eicosanoid generation and as a component of the phagocytic machinery of the cell [22]. The transgenic expression of this enzyme in mice produced a lethal phenotype characterized by respiratory failure in the early neonate that was attributed to aberrant hydrolysis of lung surfactant phospholipids (M. Ohtsuki, 2006). In fact, expression of this enzyme is highly elevated in human lungs with severe inflammation.

2.3.1.6. Group X secreted PLA_2. This enzyme is effective in the hydrolysis of phosphatidylcholine vesicles and the plasma membrane when added exogenously to adherent mammalian cells. Hydrolysis is accompanied by enhanced prostaglandin E2 production. The group X $sPLA_2$ does not bind to heparin so heparan sulfate proteoglycan-associated internalization seen with the group IIA and V enzymes cannot occur with this enzyme (S. Bezzine, 2000). The crystal structure of the human enzyme (Y.H. Pan, 2002) supports the binding to, and hydrolysis of, zwitterionic membrane surfaces of mammalian cells and lipoproteins. Transgenic expression of this enzyme in mice revealed that the enzyme exists as an inactive zymogen in most tissues, and is proteolytically activated during inflammation (M. Ohtsuki, 2006).

2.3.1.7. Group IX, group XI, group XII, and other secreted PLA_2s. The group IX and XI PLA_2s are represented by proteins from snail venom (conodipine-M) and green rice shoots, respectively. The mammalian group XII proteins include the XIIA enzyme that expresses minimal catalytic activity and a XIIB protein that lacks catalytic activity as a result of replacement of the active-site histidine with a leucine (M. Rouault, 2003). The roles of these enzymes may involve binding to an as yet unidentified receptor. Another protein containing a $sPLA_2$ motif is the viral capsid protein VP1 (Z. Zadori, 2001) classified as group XIII that is required for virus infectivity possibly involving hydrolysis of the endosomal membrane (G.A. Farr, 2005).

2.3.2. Phospholipases A_2 that involve a catalytic serine residue

2.3.2.1. Group IV cytosolic PLA_2. The group IV $cPLA_2$ has taken center stage as the phospholipase that is primarily involved in the regulation of prostaglandin and leukotriene biosynthesis (Chapter 13) as part of the inflammatory response [23]. Even though this enzyme translocates to a membranous fraction, it is recovered from the cytosolic fraction of the cell and hence is termed cytosolic. This enzyme is distinct from the mammalian $sPLA_2$s in its size (85 vs. 14 kDa), stimulation by Ca^{2+} (at micromolar vs. millimolar concentration), specificity at the *sn*-2 position of the substrate (arachidonate vs. no specificity), and catalytic mechanism. This enzyme shows minimal head-group specificity and phosphatidylcholine is the normal substrate. The discovery of the $cPLA_2$ has provided new insights into the cell signaling events that initiate the 'arachidonate cascade' described in Chapter 12.

The sequence of the $cPLA_2$ was deduced from the cDNA isolated from a number of species and is now referred to as $cPLA_2\alpha$ as the result of the discovery of further isoenzymes (β, γ, δ, ε, and ζ). The human α enzyme, mapped to chromosome 1, is highly conserved amongst mammalian species but the sequence can differ up to 20–30% between mammals and non-mammalian vertebrates. Distinct motifs of the enzyme have been identified including a N-terminal C2 domain and a larger C-terminal catalytic domain (Fig. 9). The complete crystal structure of human $cPLA_2\alpha$ (A. Dessen, 1999) provides a logical basis for discussion of the function and regulation of this important enzyme [24]. The properties and function of the β-isoenzyme have still to be resolved [23].

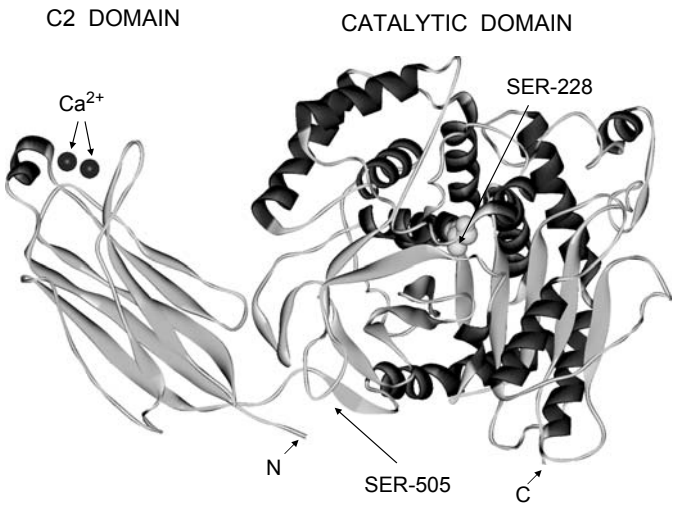

Fig. 9. Crystal structure of group IV cytosolic PLA_2 showing the C2 and catalytic domain. The two calcium ions bound to the C2 domain and the active-site Ser-228 are highlighted. The phospholipid interface would be parallel to the top surface of the molecule as shown. A flexible region between residues 499 and 538 is not seen in the crystal structure but contains Ser-505, the approximate position of which is indicated by the arrow. The N-and C-termini of the protein are indicated. Adapted from A. Dessen (1999) and Ref. [24]. (See color plate section, plate no. 9.)

The γ-isoenzyme lacks the C2 domain, is prenylated at the C-terminus, and lacks the regulatory phosphorylation sites of $cPLA_2\alpha$. The enzyme demonstrates high lysophospholipase activity and lacks the acyl chain selectivity of the α-isoenzyme suggesting a role in phospholipid remodeling [23]. The δ, ε, and ζ-isoezymes have been identified more recently (H. Chiba, 2004; T. Ohto, 2005) and are discussed [23]. The group IV isoenzymes (α–ζ) have also been designated A–F [15].

The catalytic domain: The $cPLA_2\alpha$ shows lysophospholipase and transacylase activities that are consistent with the formation of an acyl-serine intermediate characteristic of lipases and Ser-228 has been identified as the involved residue. In addition, Asp-549 has been demonstrated to be the second member of the predicted catalytic triad. However, histidine has not been identified as the third member and none of the19 histidine residues in the protein has been shown to play any catalytic role. At present, a dyad mechanism must be invoked while Arg-200 is in a position to stabilize the oxyanion intermediate. Interestingly, 1-palmitoyl lysophosphatidylcholine is degraded at a rate comparable to that of 2-arachidonoyl phosphatidylcholine, which raises the possibility that the enzyme serves multiple functions in the cell. The other isoenzymes of $cPLA_2$ also have these three conserved active-site residues (R, S, and D).

The C2 domain: The group IV $cPLA_2$ has a Ca^{2+}-dependent phospholipid-binding domain (CaLB or C2 domain) common to many proteins that translocate to membranes from the cytosol in the presence of Ca^{2+}. The activity of the $cPLA_2$ increases several fold as the Ca^{2+} concentration is increased to concentrations found in activated cells (300 nM). Since Ca^{2+} is not involved in the catalytic event, Ca^{2+} promotion of enzyme–membrane interaction probably accounts for calcium's stimulatory effect. The molecular mechanism by which Ca^{2+} binding promotes membrane interactions has been the subject of intensive investigations. The C2 domain consists of an anti-parallel β-sandwich composed of two four-stranded sheets (Fig. 9), while the structure is capped by three Ca^{2+}-binding loops known as the calcium-binding regions. It would appear that the binding of Ca^{2+} allows penetration of two of the loops into the membrane providing a stable membrane–protein interaction (O. Perisic, 1999; L. Bittova, 1999). The precise membrane-binding characteristics of a C2 domain depends on the protein under investigation (E.A. Nalefski, 2001; R.V. Stahelin, 2003) with the C2 domain of $cPLA_2$ preferring a phosphatidylcholine-rich interface. C2 domains that have been labeled with electron paramagnetic resonance probes have been used to analyze membrane docking on the phospholipid surface [10].

Immunofluorescence studies revealed that $cPLA_2$ translocates to specific intracellular membranes of the cell upon Ca^{2+} activation with the nuclear envelope being a preferred membrane. This location also contains the cyclooxygenase and 5-lipooxygenase (Chapter 12) and presumably allows facile transfer of the released arachidonic acid to these enzymes in the prostaglandin and leukotriene pathways. However, the exact site of translocation is contentious and appears to be specific to cell type and cell conditions, and appears to involve an accessory or receptor protein to achieve targeting specificity. The C2 domain shows the same membrane targeting specificity as the full-length enzyme (J.H. Evans, 2001). Co-localization of $cPLA_2$ with cyclooxygenase-2 (but not cyclooxygenase-1) as seen in endothelial cells (S. Grewal, 2005) is an attractive functional outcome.

Phosphorylation of cytosolic $PLA_2\alpha$: There are multiple sites for phosphorylation of the $cPLA_2$ and a number of protein kinases appear to use the enzyme as substrate.

A critical site is Ser-505, the site phosphorylated by mitogen-activated protein kinase. Phosphorylation at this site increases the activity of the enzyme both in vitro and in vivo. However, other phosphorylation sites may also be involved; in particular, Ser-727 may also have an important role in some cell systems [23]. The Ser-505 is located in a flexible loop that connects the C2 and catalytic domains (Fig. 9). It is possible that phosphorylation of this residue produces the optimum orientation of the two domains with respect to the membrane interface (S. Das, 2003). Overall, it is probable that the role of phosphorylation in translocation, membrane binding, and enzyme activation depends on the cell type and the nature of the stimulus [23].

Gene knockouts: The very large number of enzymes that have now been discovered with PLA_2 activity increases the difficulty of being able to unambiguously assign the role of a regulator of the inflammatory response involving arachidonic acid to one specific enzyme. Gene knockout studies in mice involving $cPLA_2\alpha$ have been particularly successful in both defining the primary importance of $cPLA_2$ in inflammation and identifying specific physiological roles [25,26]. Thus, $cPLA_2$ knockout mice have revealed important roles in fertility, generation of eicosanoids from inflammatory cells, brain injuries, and allergenic responses. Other forms of PLA_2 cannot replace these functions and hence the enzyme becomes a prime pharmacological target [26].

2.3.2.2. Group VI phospholipase A_2. This group of PLA_2s consists of the intracellular $iPLA_2$s [15] and at this time it is the group VIA (VIβ) enzyme for which most information is available [27–29]. A second $iPLA_2$ has also been described (D.J. Mancuso, 2000) and has been categorized as group VIB (or $iPLA_2\gamma$). More recently, four novel PLA_2s have been identified (groups VIC–F or δ–η) (M. van Tienhoven, 2002; C.M. Jenkins, 2004).

The group VIA (VIβ) enzyme is 85–88 kDa and consists of multiple splice variants that contain seven or eight ankyrin repeats (VIA-1 and VIA-2, respectively). The enzyme has broad phospholipid head group and acyl chain specificity and does not require Ca^{2+} for catalytic activity. It may be regulated by ATP and, importantly, is inhibited by a bromoenol lactone suicide substrate. The enzyme exhibits lysophospholipase and transacylase activity indicative of a catalytic serine (Ser-465 in VIA-1 and Ser-519 in VIA-2) and an acyl-serine intermediate. Some of the splice variants lack the C-terminal catalytic domain and may serve as inhibitory proteins for the group VIA PLA_2 (P.K.A. Larsson, 1998; A.D. Manguikian, 2004). Other mechanisms of regulation include interactions with calmodulin, phosphorylation, ankyrin interactions, proteolytic processing, and substrate availability [29]. Numerous cellular roles have been proposed for the VIA enzyme including a housekeeping role in phospholipid remodeling. Further functions involve more specific aspects of cell regulation including endothelial cell S phase progression that involves arachidonic acid release (S.P. Herbert, 2006) [29].

A role for this $iPLA_2$ in the control of insulin secretion from pancreatic islet β-cells [28] involves the release of arachidonic acid and a rise in cytosolic Ca^{2+}, a conclusion supported by β-cell transfection and knockdown studies, and supported by β-cell analysis in $iPLA_2\beta$ null mice (S.Z. Bao, 2004, 2006). The proposed role of this enzyme in a number of other aspects of cell signaling involving arachidonic release and eicosanoid formation have involved the use of bromoenol lactone, a compound that also inhibits

other enzymes including the VIB enzyme (D.J. Mancuso, 2000) and phosphatidic acid phosphohydrolase-1 (L. Fuentes, 2003).

A role of this $iPLA_2$ in cell apoptosis might involve caspase-3 proteolysis of the phospholipase to produce a more active enzyme (G. Atsumi, 2000). This $iPLA_2$ plays a central role in generating lysophosphatidylcholine, a compound used as an attractant signal during the clearance of apoptotic cells by macrophages (K. Lauber, 2004). Exposure of $iPLA_2$-derived lysophosphatidylcholine on the surface of apoptotic T cells is believed to facilitate the binding of IgM and complement factors resulting in clearance by macrophages (S.J. Kim, 2002).

The VIA enzyme is sensitive to intracellular calcium stores and is inhibited by binding to calmodulin. Release of calmodulin upon depletion of calcium stores leads to activation of the enzyme (M.J. Wolf, 1997). The binding site for calmodulin on the enzyme has been identified as primarily residing within the 15-kDa C-terminus of the protein (C.M. Jenkins, 2001). It is proposed that the activation of VIA during depletion of intracellular calcium stores results in lysophosphatidylcholine production, which activates store-operated Ca^{2+} entry (T. Smani, 2003, 2004).

The group VIB (VIγ) enzyme is under detailed investigation and initial results indicate that the protein is farnesylated (C.M. Jenkins, 2003) and has unusual properties distinct from the group VIA enzyme (M. Murakami, 2005) including hydrolysis of the *sn*-1 fatty acid of phosphatidylcholine leading to the formation of 2-arachidonoyl lysophosphatidylcholine (W. Yan, 2005), a potential precursor for a variety of lipid mediators.

2.3.2.3. Group VII and VIII PLA_2s. The group VII and VIII enzymes are better known as platelet-activating factor acetylhydrolases [30]. These enzymes catalyze hydrolysis of the *sn*-2 ester bond of platelet-activating factor (Chapter 9) and related pro-inflammatory phospholipids, thereby attenuating their bioactivity. The group VII enzyme exists as both secreted (plasma) and intracellular forms, and the plasma form has attracted most attention. The plasma form (VIIA), which is also known as lipoprotein-associated PLA_2, not only hydrolyzes platelet-activating factor but also hydrolyzes a range of oxidatively damaged phosphatidylcholines in cell membranes and lipoproteins that originally contained arachidonic acid at the *sn*-2 position. Such oxidative damage can be significant in reperfusion injury, cigarette smoking, and inflammation, and as such their formation is essentially uncontrolled. Therefore, the sole mechanism for regulating the biological impact of these compounds lies in their degradation by the acetylhydrolases.

The clinical importance of the group VII enzyme is highlighted by the lack of PAF-AH activity in a significant proportion of the Japanese population, and in the majority of cases this is due to a mutation, V279F. This mutation is associated with asthma, stroke, myocardial infarction, brain hemorrhage, and non-familial cardiomyopathy [30]. Despite the apparent importance of the enzyme in degrading platelet-activating factor, it is a possible therapeutic target because of its potential to hydrolyze oxidized phospholipids in low-density lipoproteins, the source of pro-inflammatory lipid mediators, lysoPC, and oxidized fatty acid derivatives.

Intracellular forms of the enzyme (VIIB) and the group VIII enzyme are also PAF-AHs but some are more selective and do not hydrolyze oxidized phospholipids [15,30].

2.3.2.4. Group XV PLA_2. This enzyme that is known as lysosomal PLA_2 is highly expressed in alveolar macrophages and has a classical lipase catalytic motif. The enzyme may play a role in the degradation of lung surfactant. Knockout mice for the group XV enzyme have a phenotype characteristic of a phospholipidosis with phospholipid accumulation in alveolar and peritoneal macrophages and the spleen (M. Hiraoka, 2005) [15].

2.4. Phospholipase C

2.4.1. Bacterial phospholipases C

PLCs have been known to be associated with bacteria since the classic demonstration by Macfarlane and Knight (1941) that α-toxin in *Clostridium perfringens* was a PLC (reviewed in Ref. [2]). The most extensively studied PLCs are those from *Bacillus cereus* and provided the first crystal structure for a PLC (E. Hough, 1989). Another PLC that is specific for phosphatidylinositol is also secreted in large amounts by *B. cereus* and has provided a crystal structure (D.W. Heinz, 1995).

2.4.2. Mammalian phospholipases C

These phospholipases are primarily involved in signal transduction. The structure and mechanism of action of phosphatidylinositol-specific PLCs from both mammalian and bacterial sources are reviewed in Refs. [31,32]. The structure of mammalian phosphatidylinositol-PLCδ is shown in Fig. 10 and highlights the domain structure. This protein was crystallized in the absence of its PH domain that is at the N-terminus. A 'tether and fix' model of membrane association is suggested (L.-O. Essen, 1996) whereby initial

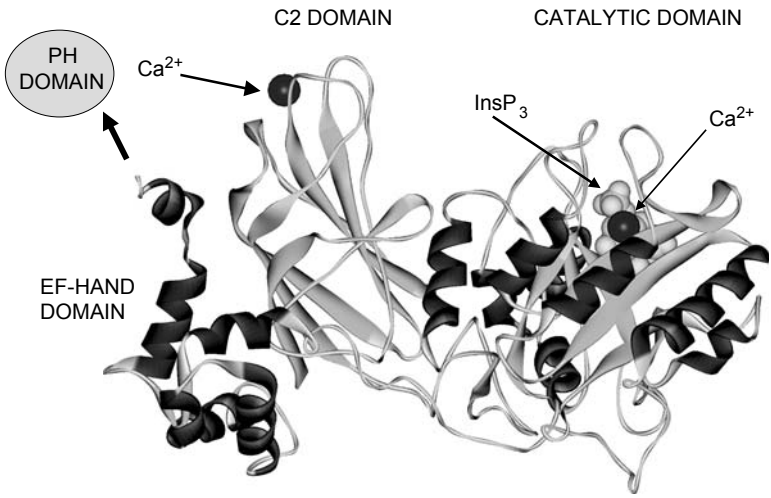

Fig. 10. Crystal structure of phosphatidylinositol-PLCδ showing the C2 and catalytic domains. The position of the PH domain that would be attached to the EF-hand domain is indicated. The membrane surface would be parallel to the top surface of the molecule as shown. Calcium ions are shown bound to the active site and to the C2 domain. The position of inositol trisphosphate in space-filling format at the active site is indicated. Adapted from L.-O. Essen (1996) and Ref. [32]. (See color plate section, plate no. 10.)

specific interaction is via the PH domain that targets membrane phosphoinositides followed by the binding of the C2 and catalytic domains. A two-step catalytic mechanism is proposed for both mammalian and bacterial enzymes [32] involving a cyclic phosphodiester intermediate, which in the case of the bacterial enzyme can be the major product of the reaction.

The major pathway of sphingomyelin degradation involves a special PLC, a sphingomyelinase. The enzyme is secreted by *B. cereus* while several mammalian sphingomyelinases play a prominent role in signal transduction in mammalian systems. The description of sphingolipids and their metabolism is covered in Chapter 13.

2.5. Phospholipase D

Classically, plants and bacteria have been the major sources for the purification of PLDs. The function of PLD in plants is not known although it may be involved in cell turnover and energy utilization during different cycles in plant life. Bacterial PLDs in some cases are toxins that can lead to severe cellular damage either alone or in combination with other proteins secreted from bacteria. These bacterial enzymes may also serve to help provide nutrients for the cell such as inorganic phosphate, as do the bacterial PLCs [2].

All PLDs characterized thus far act by a phosphatidate exchange reaction that involves a covalent phosphatidyl–enzyme as an intermediate [33]. For this reason, PLD can catalyze a 'base-exchange' reaction in which alcohols can substitute for water as the

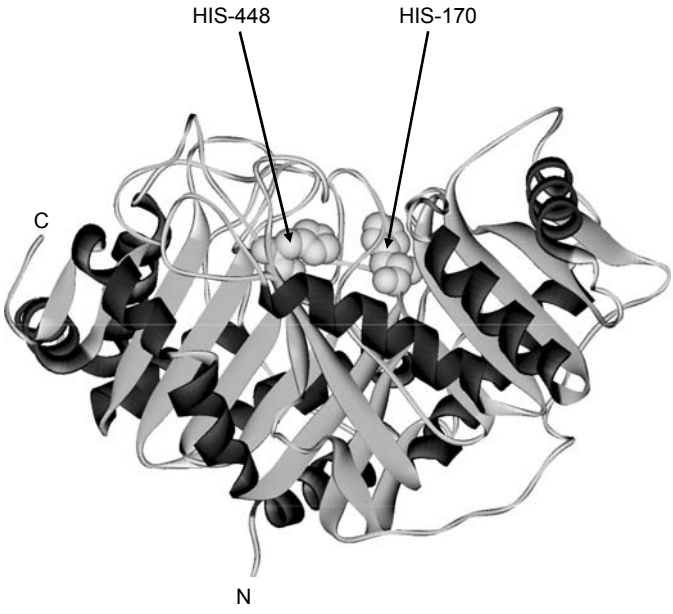

Fig. 11. Crystal structure of the PLD from *Streptomyces* sp. showing two active-site histidines in space-filling format together with the N- and C-termini of the protein. Adapted from I. Leiros (2000). (See color plate section, plate no. 11.)

phosphatidate acceptor. In fact, alcohols are better than water as phosphatidate acceptors; about 1% of an alcohol (e.g., ethanol) in water yields phosphatidylethanol almost exclusively and this property is the basis of some PLD assays.

The first crystal structure of a PLD (from *Streptomyces* sp.) has been published (I. Leiros, 2000) and is shown in Fig. 11. The positions of two active-site histidines, His-170 and His-448, are indicated and it is proposed that His-170 is the more likely residue to act as the nucleophile and thus be covalently modified during catalysis. This proposal has been confirmed more recently (I. Leiros, 2004) where crystal structures of reaction intermediates were obtained including identification of the catalytic water molecule.

Mammalian PLDs are now the subject of intense interest as they appear to be intimately involved in signal transduction (M. McDermott, 2004; G.M. Jenkins, 2005). Two mammalian PLDs have been identified and these contain several domains often associated with signal transduction proteins including a PH domain and a separate phosphatidylinositol-bis-phosphate-binding domain. PLC and PLD that act on glycosylphosphatidylinositol-anchored proteins on the cell surface are of growing importance and are discussed in Chapter 2.

3. Future directions

Since the last edition of 'Biochemistry of Lipids, Lipoproteins and Membranes' there has been much consolidation of our knowledge of phospholipases. New enzymes have been identified while mouse gene knockout studies are helping to clarify the role of individual phospholipases in cell function. The role of targeting of particular phospholipases to specific membrane locations remains a crucial area of investigation with the individual interfacial binding properties of individual enzymes playing a key role in this process. The participation of specific docking or receptor proteins in this targeting phenomenon remains a controversial area for many phospholipases. Our knowledge of the molecular interactions at membrane interfaces is still limited and will require advances in biophysical techniques including X-ray crystallography and NMR to elucidate such details. The discovery of an increasing number of non-mammalian phospholipases might help to identify unique pathways and processes in other organisms as well as providing potential drug targets for the future.

Abbreviations

Cmc	critical micellar concentration
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
PLs	phospholipases

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CHAPTER 12

The eicosanoids: cyclooxygenase, lipoxygenase, and epoxygenase pathways

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Contents

1. Introduction

1.1. Background, terminology, structures, and nomenclature

The term 'eicosanoids' is used to denote a group of oxygenated, 20-carbon fatty acids (Fig. 1) [1]. The major precursor of these compounds is arachidonic acid (AA; all *cis* 5,8,11,14-eicosatetraenoic acid), and the pathways leading to the eicosanoids are known collectively as the 'arachidonate cascade.' There are three major pathways within the cascade, including the cyclooxygenase (COX), lipoxygenase (LO), and epoxygenase pathways. In each case, these pathways are named after the enzyme(s) that catalyzes the first committed step. The prostanoids, which include the prostaglandins (PGs) and thromboxanes (Txs), are formed via the COX pathway. The first part of this discussion will focus on the prostanoids. Later in this chapter, we will describe the lipoxygenase and epoxygenase pathways.

The major eicosanoids are products of ω-6 essential fatty acids, and the essentiality of this group of fatty acids relates primarily to their functions as eicosanoids. Both the prostanoids and the leukotrienes (LTs) were discovered as vasoactive substances active on reproductive and pulmonary smooth muscle, respectively. The structures of the more stable prostanoids were determined in the late 1950s and early 1960s by workers at the Karolinksa Institute in Sweden and Unilever Laboratories in the Netherlands. The LTs were known initially as slow-reacting substances of anaphylaxis. Their structures were determined in the early 1980s. The details of the pathways and the enzymes and receptors for these pathways were characterized over the last 35 years. Because eicosanoid overproduction is associated with a number of pathologies, potent enzyme inhibitors and receptor antagonists have been developed that are widely used therapeutically.

The structures and biosynthetic interrelationships of the most important prostanoids are shown in Fig. 2 [1]. PG is the abbreviation for prostaglandin and Tx for thromboxane. Naturally occurring prostaglandins contain a cyclopentane ring, a *trans* double bond between C-13 and C-14, and an hydroxyl group at C-15. The letters following the abbreviation PG indicate the nature and location of the oxygen-containing substituents present in the cyclopentane ring. Letters are also used to label Tx derivatives (e.g., TxA and TxB). The numerical subscripts indicate the number of carbon–carbon double bonds in the side chains emanating from the cyclopentane ring (e.g., PGE_1 vs. PGE_2). In general, prostanoids with the '2' subscript are derived from AA; the '1' series prostanoids are formed from 8,11,14-eicosatrienoic acid (dihomo-γ-linolenic acid), and the '3' series compounds are derived from 5,8,11,14,17-eicosapentaenoic acid (EPA). Greek subscripts are used to denote the orientation of ring hydroxyl groups (e.g., $PGF_{2\alpha}$).

Prostanoids formed by the action of COXs have their aliphatic side chains emanating from C-8 and C-12 of the cyclopentane ring in the orientations shown in Fig. 2. Prostanoids formed via the COX pathway have their side chains *cis* to the cyclopentane ring.

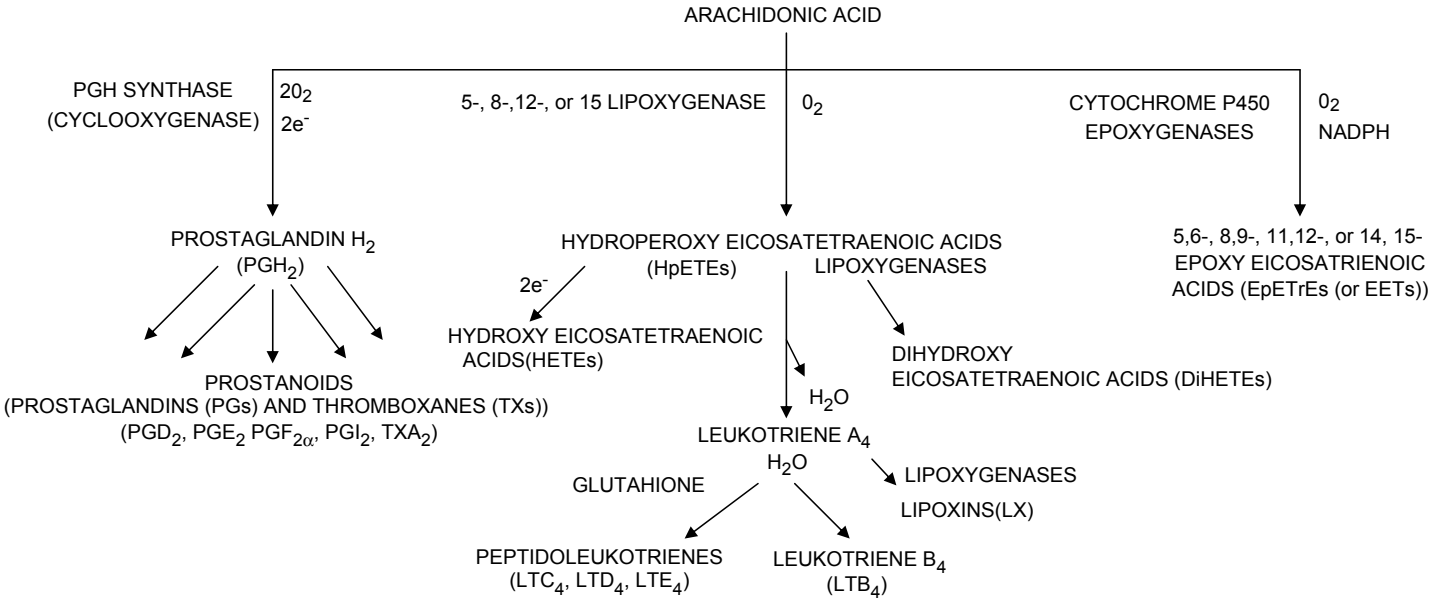

Fig. 1. Cyclooxygenase, lipoxygenase, and epoxygenase pathways leading to the formation of eicosanoids from arachidonic acid.

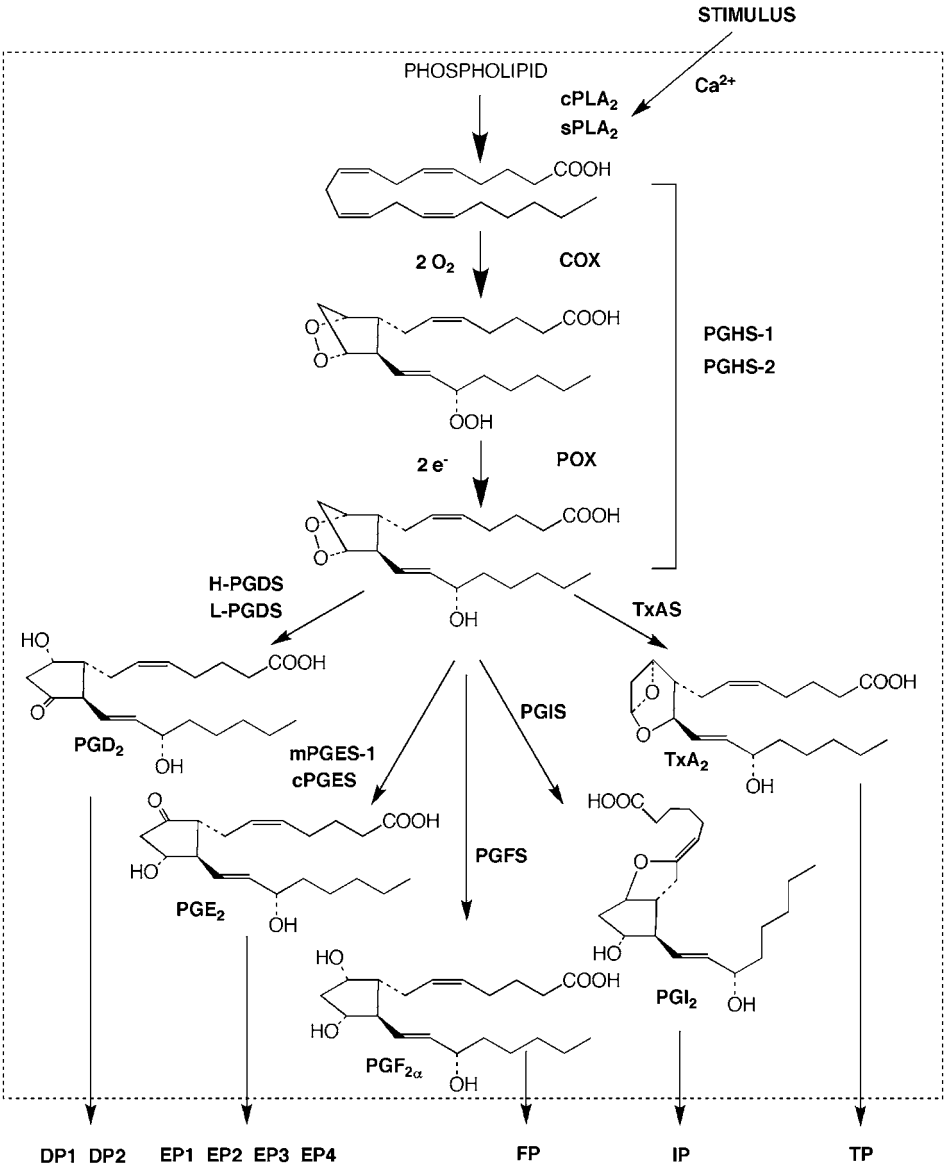

Fig. 2. Structures and biosynthetic relationships among the most common prostanoids. $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, nonpancreatic, secretory phospholipase A_2; PG, prostaglandin; PGHS, prostaglandin endoperoxide H synthase; COX, cyclooxygenase; POX, peroxidase; H-PGDS, hematopoietic PGD synthase; L-PGDS, lipocalin-type PGD synthase; cPGES, cytosolic PGE synthase; mPGES-1, microsomal PGE synthase-1; PGFS, PGF synthase; PGIS, PGI (prostacyclin) synthase; TXAS, TxA synthase. DP1, DP2, EP1, EP2, EP3, EP4, FP, IP, and TP are designations for the G-protein-linked PG receptors.

Another group of prostanoids known as isoprostanes are formed from AA and other fatty acids by nonenzymatic autooxidation. The side chains of isoprostanes are primarily in the *trans* orientation. Somewhat surprisingly, isoprostanes and their metabolites are found in greater quantities in urine than metabolites of prostanoids formed enzymatically via the COX. Particularly in pathological conditions that support autooxidation (e.g., CCl_4 toxicity), isoprostanes are produced in abundance, and they may be general markers for oxidant stress [2].

1.2. Prostanoid chemistry

PGs are soluble in lipid solvents below pH 3.0 and are typically extracted from acidified aqueous solutions with ether, chloroform/methanol, or ethyl acetate. PGE, PGF, and PGD derivatives are relatively stable in aqueous solution at pH 4–9 for short times; above pH 10, both PGE and PGD are subject to dehydration. PGI_2, which is also known as prostacyclin, contains a vinyl ether group that is very sensitive to acid-catalyzed hydrolysis; PGI_2 is unstable below pH 8.0. The stable hydrolysis product of PGI_2 is 6-keto-$PGF_{1\alpha}$. PGI_2 formation is usually monitored by measuring 6-keto-$PGF_{1\alpha}$ formation. TxA_2, which contains an oxane–oxetane grouping in place of the cyclopentane ring, is hydrolyzed rapidly ($t_{1/2}$ = 30 s at 37°C in neutral aqueous solution) to TxB_2; TxA_2 formation is assayed by quantifying TxB_2. PG derivatives are commonly quantified with immunoassays or by mass spectrometry using deuterium-labeled internal standards.

2. Prostanoid biosynthesis

Prostanoids are not stored by cells, but rather are synthesized and released rapidly (5–60 s) in response to extracellular hormonal stimuli. The pathway for stimulus-induced prostanoid formation as it might occur in a model cell is illustrated in Fig. 2 [1]. Prostanoid formation occurs in three stages: (i) mobilization of free AA [or 2-arachidonoylglycerol (2-AG); see below] from membrane phospholipids by cytosolic phospholipase A_2 ($cPLA_2$) and nonpancreatic, secretory (s) PLA_2; (ii) conversion of AA (or 2-AG) to the PG endoperoxide PGH_2 (or 2-PGH_2-glycerol) by a PG endoperoxide H synthase (PGHS, also known as cyclooxygenase (COX)); and (iii) cell-specific conversion of PGH_2 (or 2-PGH_2-glycerol) to one of the major prostanoids by one of a series of different synthases.

2.1. Mobilization of AA

PG synthesis is initiated by the interaction of various stimuli (e.g., bradykinin, angiotensin II, thrombin, PDGF, IL-1) with their cognate cell surface receptors (Fig. 2), which leads to the activation of one or more cellular lipases. Although in principle, there are a variety of lipases and phospholipases that could participate in this AA-mobilizing phase, the high molecular weight, Ca^{2+}-dependent, $cPLA_2$ and some of the $sPLA_2$s appear to be the relevant lipases (Chapter 11). The current consensus regarding the roles of PLA_2s in prostanoid synthesis is that typically $cPLA_2$ is involved directly in mobilizing AA for the constitutive PGHS-1 whereas $cPLA_2$ is involved in induction of $sPLA_2$ which, in turn, is involved in mobilizing AA for the inducible PGHS-2 (Y. Satake, 2004) [3].

2.2. Cytosolic and secreted phospholipases A_2

The predominant $cPLA_2$ isoform, $cPLA_{2\alpha}$, is found in the cytosol of resting cells but hormone-induced mobilization of intracellular Ca^{2+} leads to the translocation of $cPLA_2$ to the endoplasmic reticulum (ER) and nuclear envelope. There, $cPLA_{2\alpha}$ cleaves AA from the *sn2* position of phospholipids on the cytosolic surface of the membranes. The AA then traverses the membrane where it acts as a substrate for PGHSs which are located on the lumenal surfaces of the ER and the associated inner and outer membranes of the nuclear envelope [1]. The activity of $cPLA_2$ is also augmented by phosphorylation by a variety of kinases (M.A. Gijon, 2000). The translocation of $cPLA_2$ involves the binding of Ca^{2+} to an N-terminal CalB domain and then the binding of the Ca^{2+}/CalB domain to intracellular membranes [6]; Ca^{2+} is involved in translocation of the enzyme but not in the catalytic mechanism of $cPLA_2$ (A. Dessen, 1999).

There are ten different nonpancreatic $sPLA_2$s (Chapter 11). Two of these enzymes, Group IIA $sPLA_2$ and V $sPLA_2$, have been shown to be involved in releasing AA for prostanoid synthesis [3]. Stimulus-dependent AA mobilization by these $sPLA_2$s appears to occur independent of the ability of these enzymes to be released from cells. Expression of at least Group IIA $sPLA_2$ requires the presence of $cPLA_2$.

The crystal structure of $sPLA_2$ IIA from human synovial fluid has been determined. PLA_2s require high concentrations of Ca^{2+} (~1 mM) for maximal activity. Ca^{2+} is involved in phospholipid substrate binding and catalysis by $sPLA_2$s. Unlike cytosolic PLA_2, $sPLA_2$ shows no specificity toward either the phospholipid head group or the acyl group at the *sn*2 position. The levels of $sPLA_2$s are regulated transcriptionally in response to cell activation.

$cPLA_2$ is directly involved in the immediate AA release that occurs when a cell is stimulated with a circulating hormone or protease. For example, thrombin acting through its cell surface receptor activates $cPLA_2$ in platelet cells to cause AA release that results in TxA_2 formation. This entire process occurs in seconds. $cPLA_2$, on the other hand, plays a prominent role in 'late-phase' PG formation which occurs 2–3 h after cells have been exposed to a mediator of inflammation (e.g., endotoxin or interleukin-1β) or a growth factor such as platelet-derived growth factor and PGHS-2 has been induced.

2.3. Mobilization of 2-arachidonoylglycerol (2-AG)

2-AG may be an alternative substrate for PGHSs. Originally, it was found that PGHS-2, but not PGHS-1, converts 2-AG to 2-PGH_2-glycerol, and this intermediate is converted to the 2-prostanyl-glycerol derivatives but not 2-thromboxane-glycerol. It is not clear under what conditions or to what degree alternative substrates such as 2-AG are used in vivo to form products like the 2-prostanyl-glycerol derivatives or whether PGHS-1 might also be involved [4]. It is possible that these products have a unique set of biological roles that distinguish them from the classical PGs derived from AA itself. Although the pathway leading to the formation of 2-AG itself has not been defined in the context of prostanoid metabolism, 2-AG could be formed from phosphatidylcholine through the sequential actions of phospholipase C and acylglycerol lipase.

2.4. Prostaglandin endoperoxide H_2 (PGH_2) formation

Once AA is released, it can be acted upon by PGHS [5,6]. There are two PGHS isozymes — PGHS-1 and PGHS-2. PGHS-1 appears to use fatty acids such as AA exclusively as substrates. In contrast, PGHS-2 utilizes both fatty acids and 2-AG about equally well [5]. The PGHSs exhibit two different but complementary enzymatic activities (Fig. 2): (i) a COX (*bis*-oxygenase) which catalyzes the formation of PGG_2 (or 2-PGG_2-glycerol) from AA (or 2-AG) and two molecules of O_2 and (ii) a peroxidase which facilitates the two-electron reduction of the 15-hydroperoxyl group of PGG_2 (or 2-PGG_2-glycerol) to PGH_2 (or 2-PGH_2-glycerol) (Fig. 2). The oxygenase and peroxidase activities occur at distinct but interactive sites within the protein.

The initial step in the COX reaction is the stereospecific removal of the 13-pro-*S* hydrogen from AA. As depicted in Fig. 3, an AA molecule becomes oriented in the COX

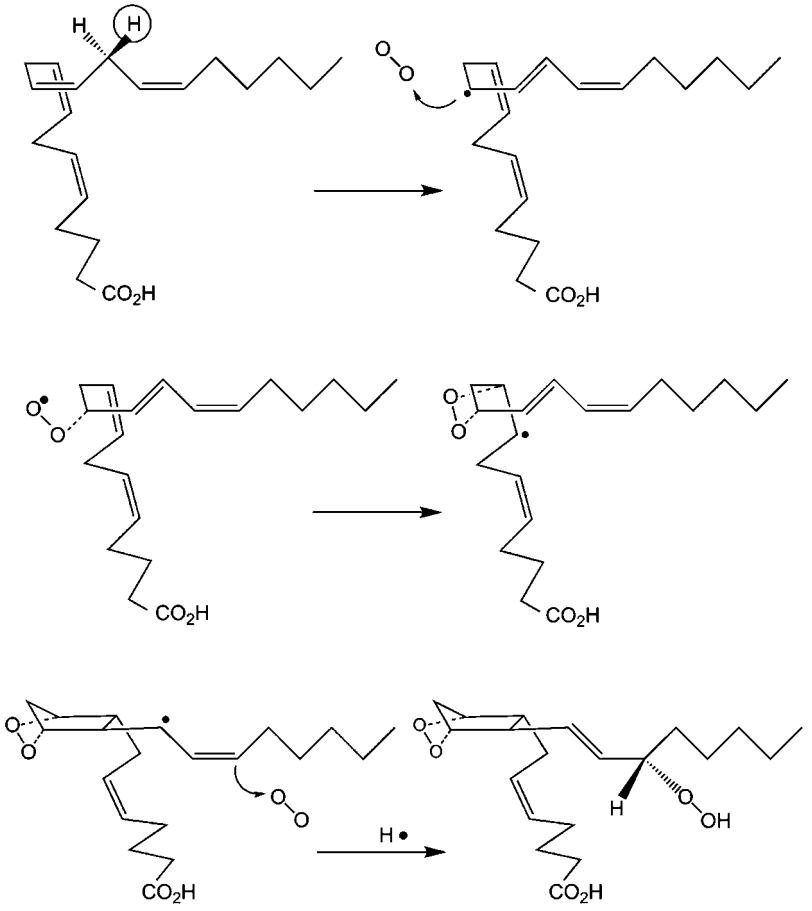

Fig. 3. Mechanism for the cyclooxygenase reaction showing the conversion of arachidonic acid and two molecules of oxygen to PGG_2.

active site with a kink in the carbon chain at C-9 [6]. Abstraction of the 13-pro-*S* hydrogen and subsequent isomerization leads to a carbon-centered radical at C-11 and attack of molecular oxygen at C-11 from the side opposite to that of hydrogen abstraction. The resulting 11-hydroperoxyl radical adds to the double bond at C-9, leading to intramolecular rearrangement and formation of another carbon-centered radical at C-15. This radical then reacts with another molecule of oxygen. The 15-hydroperoxyl group of PGG_2 can undergo a two-electron reduction to an alcohol yielding PGH_2 in a reaction catalyzed by the peroxidase activity of PGHSs.

2.5. PGHS active site

Depicted in Fig. 4 is a model of the COX and peroxidase active sites of ovine PGHS-1 [1]. The COX is an unusual activity that exhibits a requirement for hydroperoxide and undergoes a suicide inactivation [1,6]. The reason for the hydroperoxide-activating requirement is that in order for the COX to function, a hydroperoxide must oxidize the heme prosthetic group located at the peroxidase active site to an oxo-ferryl heme radical cation. This oxidized heme intermediate abstracts an electron from Tyr385. Finally, the resulting Tyr385 tyrosyl radical abstracts the 13-pro-*S* hydrogen from AA, initiating the COX reaction. Once the COX reaction begins, newly formed PGG_2 can serve as the source of the activating hydroperoxide; prior to PGG_2 formation, ambient cellular hydroperoxides apparently serve to initiate heme oxidation and COX catalysis. Ser530, the site of acetylation of PGHS-1 by aspirin (Section 2.7), is shown within the COX active site in Fig. 4. Also shown is Arg120. The guanidino group of this residue serves as the counterion for the carboxylate group of AA.

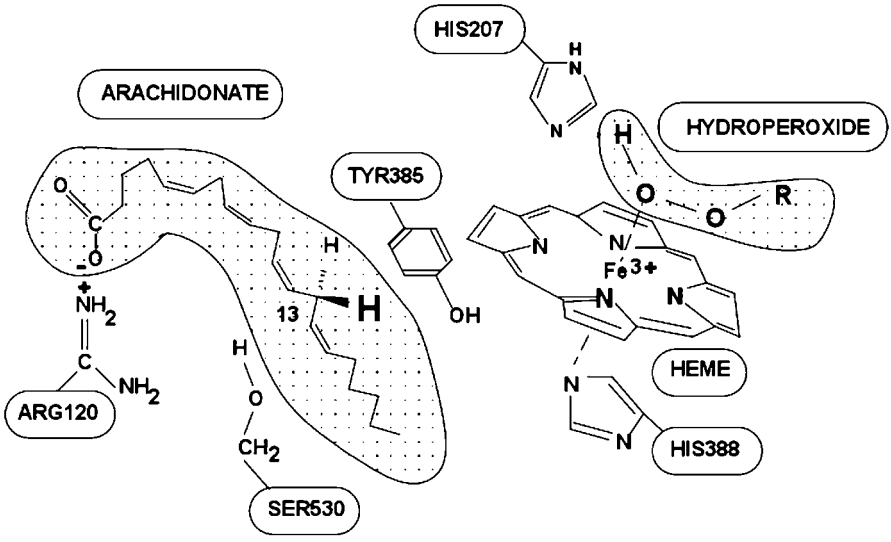

Fig. 4. Model of the cyclooxygenase and peroxidase active sites of the ovine PGH synthase-1 with arachidonic acid bound to the cyclooxygenase site and alkyl hydroperoxide (ROOH) bound to the heme group at the peroxidase site.

2.6. Physico-chemical properties of PGHSs

PGHS-1 was first purified from ovine vesicular gland, and most biochemical studies have been performed using this protein [1,5,6]. PGHS-1 is associated with the lumenal surfaces of the ER and the inner and outer membranes of the nuclear envelope. Detergent-solubilized ovine PGHS-1 is a dimer with a subunit molecular mass of 72 kDa. The dimer exhibits half of sites activity — only one COX site catalyzes an oxygenation at any given time. Thus, the enzyme must become a conformational heterodimer during COX catalysis. The biological advantage of this property is not known. The protein is *N*-glycosylated and is a hemoprotein containing one protoporphyrin IX per monomer. The sequences of cDNA clones for PGHS-1 from many mammals indicate that initially the protein has a signal peptide of 24–26 amino acids that is cleaved to yield a mature protein of 574 amino acids.

PGHS-2 was discovered in 1991 as an immediate early gene product in phorbol ester-activated murine 3T3 cells (D.A. Kujubu, 1991). PGHS-1 and PGHS-2 from the same species have amino acid sequences that are 60% identical [1,6]. The major sequence differences are in the signal peptides and the membrane-binding domains (residues 70–120 of PGHS-1); in addition, PGHS-2 contains a unique 18 amino acid insert near its carboxyl terminus. This 18 amino acid cassette and its associated *N*-glycosylation site are involved in ER-associated degradation of PGHS-2 [7].

The crystal structures of PGHS-1 and PGHS-2 have been determined. As noted earlier, two subunits of the enzymes form homodimers. Each monomer contains three sequential folding domains — an N-terminal epidermal growth factor-like domain of about 50 amino acids, an adjoining region containing about 70 amino acids that serves as the membrane-binding domain, and a C-terminal globular catalytic domain (Fig. 5).

PGHS-1 and PGHS-2 are integral membrane proteins. However, their interactions with membranes do not involve typical transmembrane helices. Instead, analysis of the crystal structures and membrane domain labeling studies have established that PGHSs interact *monotopically* with only one surface of the membrane bilayer [1]. The interaction involves four short amphipathic α-helices present in the membrane-binding domain noted above. The side chains of hydrophobic residues located on one surface of these helices interdigitate into and anchor PGHSs to the lumenal surface of the ER and the inner and outer membranes of the nuclear envelope (Fig. 5).

2.7. PGHSs and nonsteroidal anti-inflammatory drugs

PG synthesis can be inhibited by both nonsteroidal anti-inflammatory drugs (NSAIDs) and anti-inflammatory steroids. Both PGHS isozymes are pharmacological targets of common NSAIDs (e.g., aspirin, ibuprofen, naproxen). However, only PG synthesis mediated by PGHS-2 is inhibited by anti-inflammatory steroids, which block the synthesis of PGHS-2, at least in part, at the level of transcription [1].

PGHS-2 is also inhibited by ‘COX-2 inhibitors’ including rofecoxib and celecoxib. These drugs belong to a special class of NSAIDs specific for this isoform and are often referred to as COX-2 drugs. The selectivity of these latter drugs depends on subtle structural differences between the COX active sites of PGHS-1 and PGHS-2 [1]. These same

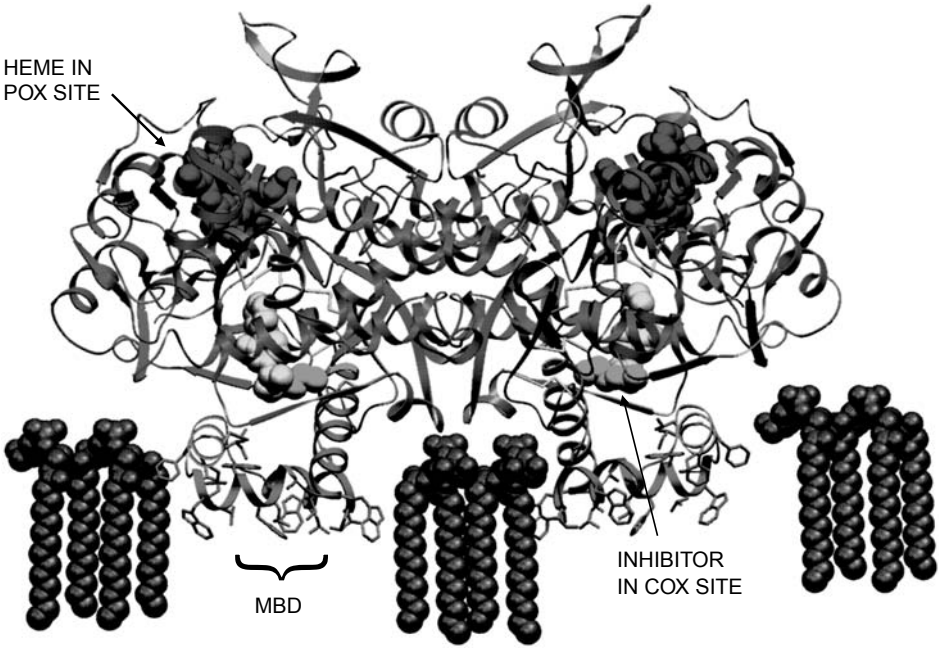

Fig. 5. Ribbon diagram of the structure of ovine PGH synthase-1 homodimer interdigitated via its membrane-binding domain (MBD) into the lumenal surface of the endoplasmic reticulum. (See color plate section, plate no. 12.)

differences account, at least in part, for the ability of PGHS-2 but not PGHS-1 to bind and oxygenate 2-arachidonoylglycerol. Rofecoxib, sold as Vioxx, was withdrawn from the U.S. market because of adverse cardiovascular effects due, at least in part, to an increase in the ratio of the prothrombotic compound TxA_2 formed via platelet PGHS-1 and the anti-thrombotic compound PGI_2 formed via vascular PGHS-2 [8].

The best-known NSAID is aspirin, acetylsalicylic acid. Aspirin binds to the COX active site, and, once bound, can acetylate Ser530 (Fig. 5) [1]. Acetylation of this active site serine causes irreversible COX inactivation. Curiously, the hydroxyl group of Ser530 is not essential for catalysis, but acetylated Ser530 protrudes into the COX site and interferes with the binding of AA.

Acetylation of PGHSs by aspirin has important pharmacological consequences. Besides the analgesic, anti-pyretic, and anti-inflammatory actions of aspirin, low-dose aspirin treatment — one 'baby' aspirin daily or one regular aspirin every three days — is a useful anti-platelet cardiovascular therapy (M.L. Capone, 2004). This low-dosage regimen leads to selective inhibition of platelet TxA_2 formation (and platelet aggregation) without appreciably affecting the synthesis of other prostanoids in other cells. Circulating blood platelets lack nuclei and are unable to synthesize new protein at appreciable rates. Exposure of the PGHS-1 of platelets to circulating aspirin causes irreversible inactivation of the platelet enzyme. Of course, PGHS-1 (and PGHS-2) inactivation also occurs in other cell types, but cell types other than platelets can resynthesize PGHSs relatively quickly. For new PGHS-1 activity to appear in platelets, new platelets must be formed. Because the

replacement time for platelets is about 12 days, it takes time for the circulating platelet pool to regain its original complement of active PGHS-1.

There are many NSAIDs other than aspirin [1]. In fact, this is one of the largest niches in the pharmaceutical market, currently accounting for about ten billion dollars in annual sales. Like aspirin, other NSAIDs act by inhibiting the COX activity of PGHS [1,5,8]. However, unlike aspirin, most of these drugs cause reversible enzyme inhibition simply by competing with AA for binding. A well-known example of a reversible NSAID is ibuprofen. All currently available NSAIDs inhibit both PGHS-1 and PGHS-2. However, inhibition of PGHS-2 appears to be primarily responsible for both the anti-inflammatory and analgesic actions of NSAIDs (L.J. Marnett, 1999). Dual inhibition of PGHS-1 and PGHS-2 with common NSAIDs causes unwanted ulcerogenic side effects [8]. Indeed, even COX-2 inhibitors, which were developed to circumvent this problem, interfere with wound healing [8].

Recently attention has been focused on COX-2 inhibitors as prophylactic agents in the prevention of colon cancer [9,10]. About 85% of tumors of the colon express elevated levels of PGHS-2 and classical NSAIDs and COX-2 inhibitors reduce mortality due to colon cancer.

2.8. Regulation of PGHS-1 and PGHS-2 gene expression

PGHS-1 and PGHS-2 are encoded by separate genes [7]. Apart from the first two exons, the intron/exon arrangements are similar. However, the PGHS-2 gene (~8 kb) is considerably smaller than the PGHS-1 gene (~22 kb). The PGHS-1 gene is on human chromosome 9, while the PGHS-2 gene is located on human chromosome 1.

Expression of the PGHS-1 and PGHS-2 genes is regulated in quite different ways [7]. PGHS-1 is expressed more or less constitutively in almost all tissues. Cells use PGHS-1 to produce PGs needed to regulate 'housekeeping activities' typically involving immediate responses to circulating hormones (Fig. 2). PGHS-1 is expressed during cell development (e.g., during megakaryocyte maturation). The PGHS-1 gene has a TATA-less promoter, a feature common to housekeeping genes. Induction of PGHS-1 gene expression is regulated by SP1 elements in the PGHS-1 promoter and by downstream intronic elements [7]. PGHS-1 protein is quite stable in cells [7].

PGHS-2 is absent from cells unless induced in response to cytokines, tumor promoters, or growth factors. PGHS-2 produces prostanoids which function during early stages of cell differentiation or replication; in many cases the newly formed prostanoids elicit cAMP formation which, in turn, promotes expression of specific genes [10,11]. There is some indirect evidence suggesting that at least some of the products formed via PGHS-2 operate at the level of the nucleus through peroxisomal proliferator-activated receptors to modulate transcription of specific genes (P.A. Scherle, 2000) (Chapter 10).

Much of what is known about PGHS-2 comes from studies on cultured fibroblasts, endothelial cells, and macrophages [7]. Typically, PGHS-2 is induced rapidly (1–3 h) and dramatically (20- to 80-fold). Platelet-derived growth factor, phorbol ester, and interleukin-1β induce PGHS-2 expression in fibroblasts and endothelial cells. Bacterial lipopolysaccharide, interleukin-1β, and tumor necrosis factor α stimulate PGHS-2 in monocytes and macrophages. While only a limited number of tissues and cell types have

been examined, it is likely that PGHS-2 can be induced in almost any cell or tissue with the appropriate stimuli. Importantly, as noted earlier, PGHS-2 expression, but not PGHS-1 expression, can be completely inhibited by anti-inflammatory glucocorticoids such as dexamethasone (R.C. Harris, 2001)) [7].

Transcriptional activation of the PGHS-2 gene is one important mechanism for increasing PGHS-2 protein expression. The PGHS-2 promoter contains a TATA box and at least six functional regulatory elements including an overlapping E-box and cAMP response element (CRE) close to the TATA box, an AP1 site, a CAAT enhancer binding protein (C/EBP) sequence, a nuclear factor κB (NF-κB) binding site, and a downstream CRE [7]. PGHS-2 gene transcription can be controlled by multiple signaling pathways including the cAMP pathway, the protein kinase C pathway (phorbol esters), viral transformation (*src*), and other pleiotropic pathways such as those activated by growth factors, endotoxin, and inflammatory cytokines. These latter agents (e.g., platelet-derived growth factor, lipopolysaccharide, interleukin-1β, tumor necrosis factor α) likely share convergent pathways involving the proximal CRE, the NF-κB site, and the C/EBP elements, two transcription factors common to inflammatory responses, and one or more of the established mitogen-activated protein kinase cascades (ERK1/2, JNK/SAPK, and p38/RK/Mpk2). In macrophages, bacterial endotoxin stimulates PGHS-2 expression through cooperative activation involving all of the known response elements in the PGHS-2 promoter [7].

2.9. Formation of biologically active prostanoids from PGH_2

Although all the major prostanoids are depicted in Fig. 2 as being formed by a single cell, prostanoid synthesis is somewhat cell specific [1]. For example, platelets form mainly TxA_2, endothelial cells form PGI_2 as their major prostanoid, and PGE_2 is the major prostanoid produced by renal collecting tubule cells. The syntheses of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TxA_2 from PGH_2 are catalyzed by PGD synthase (PGDS) [12] (J.M. Park, 2007), PGE synthase (PGES) [13], PGF_α synthase, PGI synthase (PGIS) (M. Wada, 2004), and TxA synthase (TXAS) (L.-H. Wang, 2001), respectively. Formation of $PGF_{2\alpha}$ involves a two-electron reduction of PGH_2, and a $PGF_\alpha S$ utilizing NADPH can catalyze this reaction. All other prostanoids are formed from PGH_2 via isomerization reactions involving no net change in oxidation state.

Glutathione-dependent and -independent PGDSs have been isolated. The glutathione-dependent, hematopoietic PGDS (H-PGDS) also exhibits glutathione-S-transferase activity. A glutathione-independent, lipocalin PGDS (L-PGDS) has been purified from brain and may be involved in producing PGD_2 that is involved in sleep regulation [14].

At least three different proteins have been shown to have PGES activity including a cytosolic PGE synthase (cPGES), an inducible, microsomal PGES (mPGES-1), and a non-inducible microsomal PGES (mPGES-2) [13]. cPGES appears to be more tightly coupled to PGHS-1. mPGES-1 has been reported to be more tightly coupled to PGHS-2 and interestingly, its expression like that of PGHS-2 is inhibited by anti-inflammatory steroids such as dexamethasone. All PGESs require reduced glutathione as a cofactor. Glutathione facilitates cleavage of the endoperoxide group and formation of the 9-keto group.

A NADPH-dependent PGF_{α} synthase activity has been partially purified from lung. Structurally, this enzyme is a member of the aldose reductase family of proteins. There is also a glutathione-dependent form of the enzyme [15]. The roles of these proteins in PGF synthesis in vivo remain to be established.

PGIS and TxAS are hemoproteins with molecular weights of 50–55,000. Both of these proteins are cytochrome P-450s. Both enzymes, like PGHSs, undergo suicide inactivation during catalysis. TxAS is found in abundance in platelets and lung. PGIS is localized to endothelial cells as well as both vascular and nonvascular smooth muscle. Both TxA and PGI synthases are believed to be on the cytosolic face of the ER. PGH_2 formed in the lumen of the ER via PGHSs diffuses across the membrane and is converted to a prostanoid end product on the cytosolic side of the membrane.

3. Prostanoid catabolism and mechanisms of action

3.1. Prostanoid catabolism

Once a prostanoid is formed on the cytosolic surface of the ER, it diffuses to the cell membrane and exits the cell probably via carrier-mediated transport (G. Reid, 2003). Prostanoids are local hormones that act very near their sites of synthesis. Unlike typical circulating hormones that are released from one major endocrine site, prostanoids are synthesized and released by virtually all organs. In addition, all prostanoids are inactivated rapidly in the circulation. The initial step of inactivation of PGE_2 is oxidation to a 15-keto compound in a reaction catalyzed by a family of 15-hydroxyprostaglandin dehydrogenases [16]. Further catabolism involves reduction of the double bond between C-13 and C-14, ω-oxidation, and β-oxidation. 15-Hydroxyprostaglandin dehydrogenases are reported to be tumor suppressors acting by reducing PGE_2 levels [17].

3.2. Physiological actions of prostanoids

Prostanoids act both in an autocrine fashion on the parent cell and in a paracrine fashion on neighboring cells [1,11]. Typically, the role of a prostanoid is to coordinate the responses of the parent cells and neighboring cells to the biosynthetic stimulus — a circulating hormone. The actions of prostanoids are mediated largely by G-protein-linked prostanoid receptors of the seven-transmembrane domain receptor superfamily [11].

One process that has been examined extensively is the platelet–vessel wall interaction involving PGI_2 and TxA_2 [8]. TxA_2 is synthesized by platelets when they bind to subendothelial collagen that is exposed by microinjury to the vascular endothelium. Newly synthesized TxA_2 promotes subsequent adherence and aggregation of circulating platelets to the subendothelium. In addition, TxA_2 produced by platelets causes constriction of vascular smooth muscle. The net effect is to coordinate the actions of platelets and the vasculature in response to deendothelialization of arterial vessels. Thus, prostanoids can be viewed as local hormones, which coordinate the effects of circulating hormones and other agents (e.g., collagen) that activate their synthesis.

3.3. Prostanoid receptors

The identification and characterization of prostanoid receptors has occurred during the past 10 years [11]. These results coupled with studies of PGHS and prostanoid receptor knockout mice have been critical to rationalize earlier results of studies on the physiological and pharmacological actions of prostanoids that had been somewhat confusing and difficult to interpret because PGs were found to cause such a wide variety of seemingly paradoxical effects.

The seminal step in understanding the structures of prostanoid receptors and their coupling to second messenger systems resulted from the cloning of receptors for each of the prostanoids [11]. Prostanoid receptor cloning began with the TxA/PGH receptor known as the TP receptor. cDNA encoding this receptor was cloned using oligonucleotide probes designed from protein sequence data obtained from the TP receptor purified from platelets. The results confirmed biochemical predictions that the TP receptor was a seven-membrane spanning domain receptor of the rhodopsin family. Subsequent cloning of other receptors was performed by homology screening using receptor cDNA fragments as cross-hybridization probes. All of these prostanoid receptors are of the G-rotein-linked receptor family.

It is now clear that there are nine PG distinct receptors each encoded by a different gene (Fig. 2 [11]. For example, in the case of PGE_2, four different prostaglandin E (EP) receptors have been identified and designated as EP1, EP2, EP3, and EP4 receptors. Based on studies with selective agonists for each of the EP receptors and their effects on second messenger production, it appears that EP1 is coupled to Ca^{2+} mobilization, EP2 and EP4 are coupled via G_s to the stimulation of adenylate cyclase, and EP3 receptors are coupled via G_i to the inhibition of adenylate cyclase.

In order to determine the physiological roles of various prostanoid receptors a number of knockout mice have been developed [18]. These studies indicate that prostacyclin receptors are involved in at least some types of pain responses, EP3 receptors are involved in the development of fever, EP2 and EP4 function in allergy and bone resorption, and EP1 receptors are involved in chemically induced colon cancer. The availability of cloned prostanoid receptors provides a rational and the appropriate technology to search for receptor agonists and antagonists that might provide some specificity beyond the currently available COX inhibitors, which prevent broadly the synthesis of all PGs.

The realization that the gene for PGHS-2 is an immediate early gene associated with cell replication and differentiation suggests that prostanoids synthesized via PGHS-2 may have nuclear effects. As noted above there have been several reports indicating that prostanoid derivatives can activate some isoforms of peroxisomal proliferator activated receptors (PPARs). There is evidence that PGI_2 can be involved in PPARδ-mediated responses such as decidualization and apoptosis (P.A. Scherle, 2000).

4. Leukotrienes and lipoxygenase products

4.1. Introduction and overview

LTs are produced by the action of 5-lipoxygenase (5-LO) which inserts a diatomic oxygen at carbon atom-5 of AA yielding 5(*S*)-hydroperoxy eicosatetraenoic acid (5-HpETE).

A subsequent dehydration reaction catalyzed by the same enzyme, 5-LO, results in formation of leukotriene A_4 (LTA_4), the chemically reactive precursor of biologically active LTs. As was the case with prostanoid biosynthesis, LT biosynthesis depends upon the availability of AA as a free carboxylic acid as the 5-LO substrate, which typically requires the action of $cPLA_2\alpha$ to release AA from membrane phospholipids. Also, LTs are not stored in cells, but synthesis and release from cells are rapid events following cellular activation. Interest in the LT family of AA metabolites arises from the potent biological activities of two products derived from LTA_4 — leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) (Fig. 6). LTB_4 is a very potent chemotactic and chemokinetic agent for the human polymorphonuclear leukocyte, while LTC_4 powerfully constricts specific smooth muscle such as bronchial smooth muscle, and mediates leakage of vascular fluid in the process of edema [19]. The name leukotriene was conceived to capture two unique attributes of these molecules. The first part of the name relates to those white blood cells derived from the bone marrow that have the capacity to synthesize this class of eicosanoid, for example the polymorphonuclear *leukocyte.* The last part of the name refers to the unique chemical structure, a conjugated *triene*, within these eicosanoids.

There are numerous other biochemical products of AA metabolism formed by lipoxygenase enzymes other than 5-LO. Other monooxygenases expressed in mammalian cells

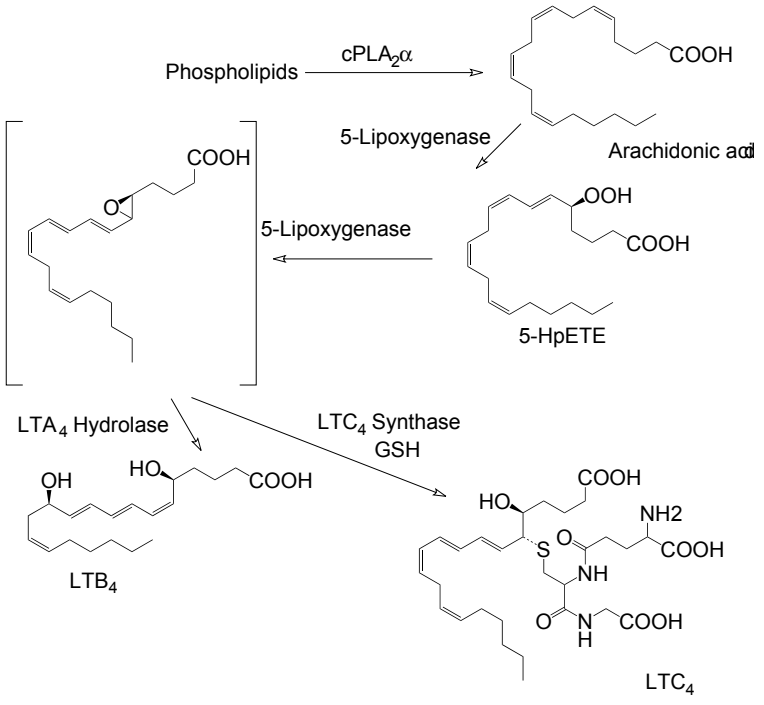

Fig. 6. Biochemical pathway of the metabolism of arachidonic acid into the biologically active leukotrienes. Arachidonic acid released from phospholipids by cytosolic (c) phospholipase $A_2\alpha$ is metabolized by 5-lipoxygenase to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and leukotriene A_4 (LTA_4) which is then enzymatically converted into leukotriene B_4 (LTB_4) or conjugated by glutathione to yield leukotriene C_4 (LTC_4).

include 12(*S*)-lipoxygenase (platelet), 12(*R*)-lipoxygenase (epidermis), 15-lipoxygenase-1, and 15-lipoxygenase-2. These enzymes are named in accordance with the carbon atom position of AA initially oxygenated even though other polyunsaturated fatty acids can be substrates. In addition, AA can be oxidized by specific isozymes of cytochrome P-450, leading to a family of epoxyeicosatrienoic acids (EETs). Methyl terminus (ω-oxidized) AA as well as lipoxygenase-like monohydroxy eicosatetraenoic acid (HETE) products are also formed by various P-450s. In general, much more is known about the biochemical role of PGs and LTs as mediators of biochemical events, but the other lipoxygenase products or cytochrome P-450 products are important metabolites; for example, the P-450 metabolites play an important role in kidney function. In accord with the body of information available, various pharmacological tools are available to inhibit 5-LO as well as specific LT receptors.

4.2. Leukotriene biosynthesis

The AA 5-lipoxygenase (5-LO, EC 1.13.11.34) is a non-heme iron metalloenzyme where iron is coordinated by three histidine residues, an asparagine, and the isoleucine carboxyl group in the active site. This 77,852 Da protein (human 5-LO) catalyzes the addition of molecular oxygen to the 1,4-*cis*-pentadienyl structural moiety closest to the carboxyl group of AA to yield a conjugated diene hydroperoxide, typical for all lipoxygenase reactions. The mechanism of 5-LO is thought to be similar to that of 15-lipoxygenase which has been studied in great detail [20] and begins with iron in the ferric state. In the reactive site, the pro-*S* hydrogen atom from carbon-7 of AA is removed as a proton and electron which enters the molecular orbitals of Fe(III) to form Fe(II). Molecular oxygen then adds to carbon-5 of the resulting radical to yield the hydroperoxy radical. The hydroperoxy radical abstracts an electron from Fe^{2+} to yield a hydroperoxide anion that can readily remove a proton from a water ligand of the catalytic iron to yield 5(*S*)-HpETE [5(*S*)-hydroperoxy-6,8,11,14-(*E*,*Z*,*Z*,*Z*)-eicosatetraenoic acid]. A second enzymatic activity of 5-LO (Fig. 7) catalyzes the stereospecific removal of the *pro-R* hydrogen atom at carbon-10 of the 5(*S*)-HpETE through a second cycle very similar in electron movement detail to the first 5-LO mechanism. In this case, the Fe^{3+} iron with a hydroxide anion ligand removes an electron from the carbon-10–hydrogen bond by the tunneling mechanism and the nascent proton is picked up by the hydroxide anion ligand. The radical site at carbon-10 then delocalizes over seven carbon atoms and the emerging high density of electron character at C-6 causes weakening of the peroxide (oxygen–oxygen bond) at C-5. The iron atom, now Fe^{2+}, can donate an electron to the forming hydroxyl radical to yield hydroxide anion, driving formation of the new carbon–oxygen bond of the epoxide. The hydroxide anion abstracts the proton from the water molecule coordinated with Fe^{3+} and thus regenerates Fe^{3+} with a hydroxyl anion as the sixth ligand. One unique feature of the 5-LO is that a fully active 5-LO with coordinated Fe^{3+} containing hydroxide anion as the sixth ligand is regenerated during one catalytic cycle by either the monooxygenase or the LTA_4 synthase mechanism. This is different from the COX mechanism where a second peroxidase mechanism is required to regenerate active enzyme.

The product of this reaction, 5(*S*),6(*S*)-oxido-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid, is LTA_4, a conjugated triene epoxide that is highly unstable. LTA_4 undergoes rapid

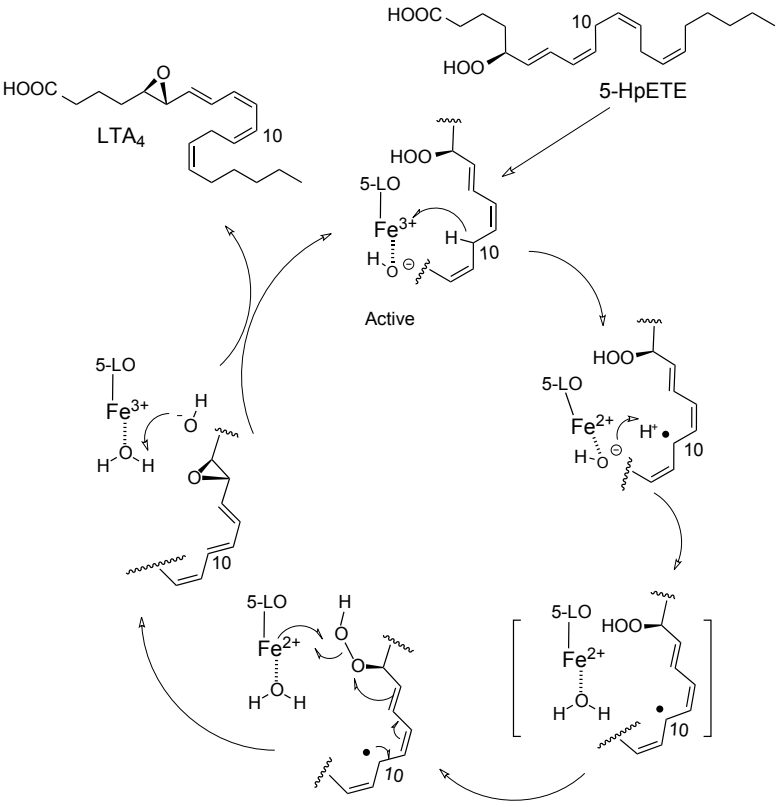

Fig. 7. Suggested mechanism for 5-lipoxygense converting 5-HpETE to LTA_4. The oxidation state of the iron ion and the nature of the sixth ligand to the coordination sphere of iron as either water or hydroxide ion are indicated as well as the transformation of 5-HpETE into the triene epoxide, LTA_4.

hydrolysis in water with a half-life of less than 3 s at pH 7.4 (room temperature) and also can react with proteins as well as DNA. Nonetheless, within cells, LTA_4 is stabilized by binding to proteins that remove water from the immediate environment of the epoxide structure. The nonenzymatic hydrolysis products of LTA_4 include several biologically inactive and enantiomeric 5,12- and 5,6-diHETEs. However, the hydrolysis of LTA_4 catalyzed by LTA_4 hydrolase [20] produces the biologically active LTB_4, 5(*S*),12(*R*)-dihydroxy-6,8,10,14-(*Z*,*E*,*E*,*Z*)-eicosatetraenoic acid (Fig. 6). A second pathway for LTA_4 metabolism is prominent in cells expressing the enzyme LTC_4 synthase [21] which catalyzes the addition of glutathione to carbon-6 of the triene epoxide yielding 5(*S*),6(*R*)-*S*-glutathionyl-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid (LTC_4) (Fig. 6). LTC_4 synthase is localized on the nuclear membrane and is a unique glutathione (S) transferase.

The formation of either LTC_4 or LTB_4 is controlled by the expression of either LTA_4 hydrolase or LTC_4 synthase by specific cell types. The human neutrophil, for example, expresses LTA_4 hydrolase and produces LTB_4 while the mast cell and eosinophil produce LTC_4, since they express LTC_4 synthase. Interestingly, cells have been found which do

not have 5-LO, but express either LTA_4 hydrolase (e.g., erythrocytes and lymphocytes) or LTC_4 synthase (e.g., platelets and endothelial cells). Studies have found that cells cooperate in the production of biologically active LTs through a process termed transcellular biosynthesis [22] where a cell such as the neutrophil or mast cell generates LTA_4 which is then released from the cell and is then taken up by either a platelet to make LTC_4 or red blood cell to make LTB_4. Despite the chemical reactivity of LTA_4, this process is known to be highly efficient and approximately 60 to 70% of the LTA_4 produced by the activated neutrophil can be released to another cell for transcellular biosynthesis of LTs (A. Sala, 1996).

4.3. Enzymes and proteins involved in leukotriene biosynthesis

4.3.1. 5-Lipoxygenase

The human 5-LO gene is unusually large and is present on chromosome 10. This gene covers more than 80 kb of DNA and has 14 exons that encode a 673 amino acid protein (without the initiator methionine residue) [23]. 5-Lipoxygenase has been purified from human, pig, rat, and guinea pig leukocytes, all with close to 90% homology. It is interesting to note that the purified or recombinant enzyme requires several cofactors for activity that include Ca^{2+}, ATP, fatty acid hydroperoxides, and phosphatidylcholine in addition to the AA and molecular oxygen substrates. Purified 5-LO was found to catalyze the initial oxidation of AA to yield 5-HpETE as well as the second enzymatic reaction to convert 5-HpETE into LTA_4. Recombinant human 5-LO has been expressed in osteosarcoma cells, cos-M6 cells, baculovirus-infected SF9 insect cells, yeast, and *Escherichia coli* [24].

Low concentrations of calcium ion (1–2 μM) are required for maximal activity of purified 5-LO, but the major role of calcium appears to be that of increasing lipophilicity of 5-LO through a C-2-like domain in order to translocate to the nuclear envelope. ATP has a stimulatory effect on 5-LO at 20 nM and lipid hydroperoxides are important to initiate the 5-LO catalytic cycle since they readily form Fe(III) from inactive 5-LO with Fe(II) by the pseudoperoxidase mechanism. Microsomal membranes as well as phosphatidylcholine vesicles can stimulate purified 5-LO activity since 5-LO performs the oxidation of AA at the interface between the membrane and cytosol in a manner similar to that of $cPLA_2\alpha$. Calcium ions increase the association of 5-LO with phosphatidylcholine vesicles that likely recapitulate events within the cell where 5-LO becomes associated with the nuclear membrane which is rich in phosphatidylcholine [25]. At the nuclear membrane AA is thought to be presented by a protein which has been termed 5-lipoxygenase activating protein (FLAP) [24]. In cells, such as the neutrophil and mast cells where 5-LO is found in the cytosol, 5-LO is catalytically active only when bound to a membrane, typically the nuclear membrane. In fact, in some cells 5-LO is found to be constitutively associated with the nuclear membrane, likely a result of a process of cellular activation while 5-LO is found in the alveolar macrophage within the nucleus itself [25].

4.3.2. 5-Lipoxygenase activating protein (FLAP)

FLAP was found during the course of development of the drug MK-886 by workers at Merck-Frosst in Montreal [24]. They found this drug bound to a novel protein that was essential for the production of LTs in stimulated intact cells and hence, the name ‘5-lipoxygenase

activating protein'. FLAP is a unique 161 amino acid-containing protein (18,157 Da). While the role of FLAP is not entirely clear, experiments using a ^{125}I-labeled photoaffinity analog of AA suggested that FLAP functions as a substrate transfer protein and in this manner stimulates 5-LO-catalyzed formation of LTs. LTC_4 synthase has 31% amino acid identity to FLAP with a highly conserved region possibly involved in lipid binding for both proteins [24]. FLAP likely exists as a homodimer or possibly a heterodimer with LTC_4 synthase.

4.3.3. LTA_4 hydrolase

LTA_4 hydrolase catalyzes the stereochemical addition of water to form the neutrophil chemotactic factor LTB_4. LTA_4 hydrolase (molecular weight 69,399) contains one zinc atom per enzyme molecule that is essential for the catalytic activity [19,26]. LTA_4 hydrolase is also a member of a family of zinc metalloproteases and exhibits protease activity. The finding of this activity led to the discovery of several drugs such as bestatin and captopril that are inhibitors of this enzyme. LTA_4 hydrolase is found in many cells including those which do not contain 5-LO and it is felt that these cells play an important role in transcellular biosynthesis of LTB_4 through cell–cell cooperation. LTA_4 is thought to be localized in the cytosol of the cell and is the only protein in the LT biosynthetic cascade that is not always membrane bound or translocated to the nuclear membrane following cellular activation. Therefore, in order to efficiently metabolize the chemically reactive LTA_4, either the LTA_4 hydrolase must come in close contact with the nuclear membrane during LTA_4 biosynthesis or a carrier protein must present LTA_4 to LTA_4 hydrolase in the cytosol. LTA_4 hydrolase is known to be efficiently suicide inactivated by LTA_4 when the electrophilic epoxide becomes covalently bound to the enzyme presumably within the active site (F.A. Fitzpatrick, 1990). A specific tyrosine residue covalently binds LTA_4 and this residue has been implicated as the potential proton donor in the suggested mechanism of LTA_4 hydration. The three-dimensional structure of LTA_4 hydrolase has now been determined by X-ray crystallography and a fairly detailed explanation of the enzymatic mechanism has now been proposed [26].

4.3.4. LTC_4 synthase

The conjugation of the tripeptide glutathione (γ-glutamyl-cysteinyl glycine) to the triene epoxide LTA_4 is carried out by LTC_4 synthase (EC 2.5.1.37). This enzyme is localized on the nuclear envelope of cells and has little homology to soluble glutathione (S) transferases. LTC_4 synthase does have some primary amino acid sequence homology to the recently described microsomal glutathione (S) transferases and FLAP [21]. Several microsomal glutathione (S) transferases have been found that catalyze formation of LTC_4 from LTA_4 and glutathione. LTC_4 synthase has a restricted distribution and is found predominantly in mast cells, macrophages, eosinophils, and monocytes. However, human platelets and endothelial cells have been found to contain LTC_4 synthase. Purified recombinant LTC_4 synthase will conjugate glutathione to both LTA_4 and LTA_4 methyl ester with a K_m of approximately 2–4 μM for the free acid and 7–10 μM for the methyl ester. The K_m for glutathione is approximately 2 mM. The drug MK-886 inhibits LTC_4 synthase with an IC_{50} of approximately 2–3 μM [24]. The gene for LTC_4 synthase is located on human chromosome 5, distal to that of cytokine, growth factor, and receptor genes (T.D. Bigby, 2000).

4.4. Regulation of leukotriene biosynthesis

The biosynthesis of LTs within cells is highly regulated and depends not only on the availability of AA and molecular oxygen, but also on the subcellular location of 5-LO and phosphorylation of critical serine residues. Resting neutrophils synthesize little, if any, LTs. However, following the elevation of intracellular calcium either through a physiological event such as phagocytosis or by pharmacological manipulation with the calcium ionophore A23187, the neutrophil produces a substantial amount of 5-LO products including LTs from either endogenous or exogenous AA. The need for an increase in intracellular calcium ion concentrations is a distinguishing feature of 5-LO that differentiates this enzyme from other lipoxygenases and COX-1 or -2.

A major determinant of 5-LO activity is the translocation of 5-LO to the nuclear membrane (Fig. 8). The site of localization of 5-LO is thought to be the inner nuclear membrane when 5-LO is within the nucleoplasm and on the outer nuclear membrane if 5-LO is located in the cytoplasm of the resting cell. The Ca^{2+}-dependent translocation event likely brings 5-LO to the same region where FLAP and $cPLA_2\alpha$ translocate. Regulation of LT biosynthesis is thus a process of assembly of the LT biosynthetic machine at a nuclear envelope site. It is this site where AA is released from nuclear membrane phospholipids, then converted to LTA_4, and ultimately conjugated with glutathione by nuclear membrane

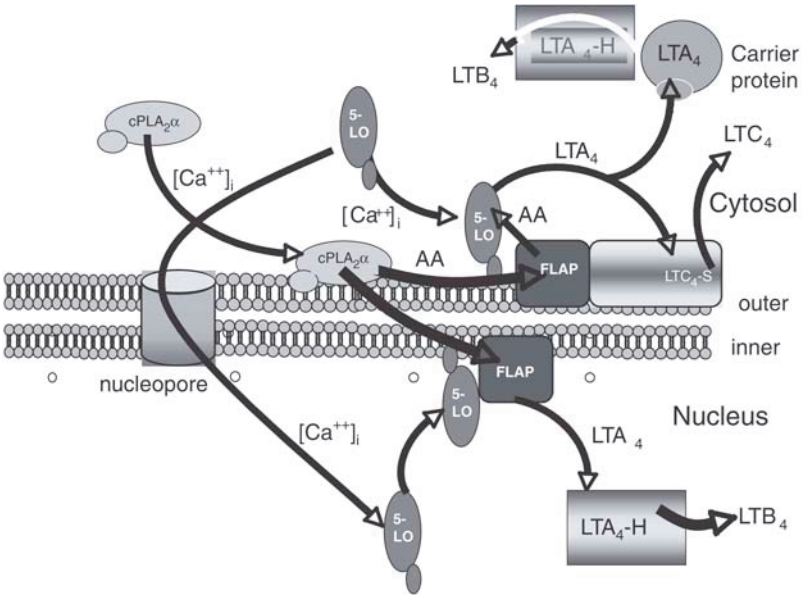

Fig. 8. Proposed model for the location of biosynthetic events occurring during leukotriene (LT) biosynthesis at the nuclear membrane of cells. Arachidonic acid is released from membrane glycerophospholipids by nuclear membrane-associated $cPLA_2\alpha$ and is then presented to 5-lipoxygenase via 5-lipoxygenase activating protein (FLAP). LTA_4 is either converted by nuclear membrane associated LTC_4 synthase (LTC_4-S) into LTC_4 or carried to LTA_4 hydrolase (LTA_4-H). These events can occur from the cytosolic side or the nucleoplasmic side of the dual lipid bilayer of the perinuclear region. (See color plate section, plate no. 13.)

LTC_4 synthase. The mechanism by which 5-LO is trafficked to the nuclear membrane is controlled by the C2-like domain of 5-LO that binds two Ca^{2+} ions which imparts high affinity for glycerophosphocholine lipids rich on the outer leaflet of either nuclear membrane [25]. 5-LO can be phosphorylated at S271 (MAPKAP kinase dependent) and S663 (ERK-1/2 dependent) to increase 5-LO activity and direct trafficking of 5-LO to the nucleoplasm. However, phosphorylation of S523, a protein kinase A-dependent process, inhibits 5-LO activity (see below).

The activity of 5-LO is also regulated by a suicide inactivation mechanism where LTA_4 rapidly inactivates 5-LO, likely through a covalent modification mechanism. Continued synthesis of LTs then requires synthesis of new 5-LO. The production of 5-LO is known to be regulated at the level of gene transcription as well as mRNA translation in addition to the translocation mechanism [24,25]. The 5-LO gene promoter contains several consensus-binding sites for known transcription factors including Sp1 and Egr-l. In a region located 212 to 88 base pairs upstream from the transcriptional start site of the human 5-LO gene, a highly rich G + C region is found that contains the consensus sequence for Spl and Egr-1 transcription factors. Furthermore, fairly common variations have been found in human populations in which deletions of one or two of the Spl binding motifs were observed [24]. This genetic polymorphism could have substantial effects on the induction of 5-LO transcription.

Various biochemical mechanisms can also alter LT biosynthesis within cells. Because of the complexity of 5-LO activation and the requirement of the Ca^{2+}-dependent translocation event, modification of the signal transduction pathways alters LT biosynthesis. For example, elevation of cellular levels of cyclic AMP inhibits LT biosynthesis even when synthesis is stimulated by the powerful calcium ionophore A23187. Adenosine and A2A receptor agonists inhibit production of LTs in human neutrophils, most likely through an enhanced production of cyclic AMP (P. Borgeat, 1999). The phosphorylation of S523 by cAMP activating protein kinase A inhibits 5-LO activity. The product of neutrophil LT biosynthesis, namely LTB_4, can also inhibit the synthesis of LTs when initiated during the course of phagocytosis. These effects can be observed at 1–3 nM LTB_4, are mediated through the LTB_4 receptor, and likely represent a feedback-like inhibition of LT biosynthesis through inactivation of 5-LO as well as cPLA2 (J. Fiedler, 1998). Pharmacological agents have been developed to inhibit LT production through direct action on 5-LO. The drug zileuton likely reduces activated 5-LO Fe(III) to the inactive 5-LO Fe(II) or prevents the oxidation of Fe(II) by lipid hydroperoxides, the pseudoperoxidase step, by serving as a competitive substrate [24]. Inhibitors of FLAP (Section 4.3.2) include MK-886 and the related agent BAY x1005. Because of its close similarity to the FLAP protein, LTC_4 synthase can be inhibited by MK-886 albeit at higher concentrations.

4.5. Metabolism of leukotrienes

The conversion of LTs into alternative structural entities is an important feature of inactivation of these potent biologically active eicosanoids. Metabolism of LTs is rapid and the exact pathway depends upon whether the substrate is LTB_4 or LTC_4. LTB_4 is rapidly metabolized through both oxidative and reductive pathways (Fig. 9) [27]. The most prominent pathway present in human neutrophils (CYP4F3) as well as hepatocytes

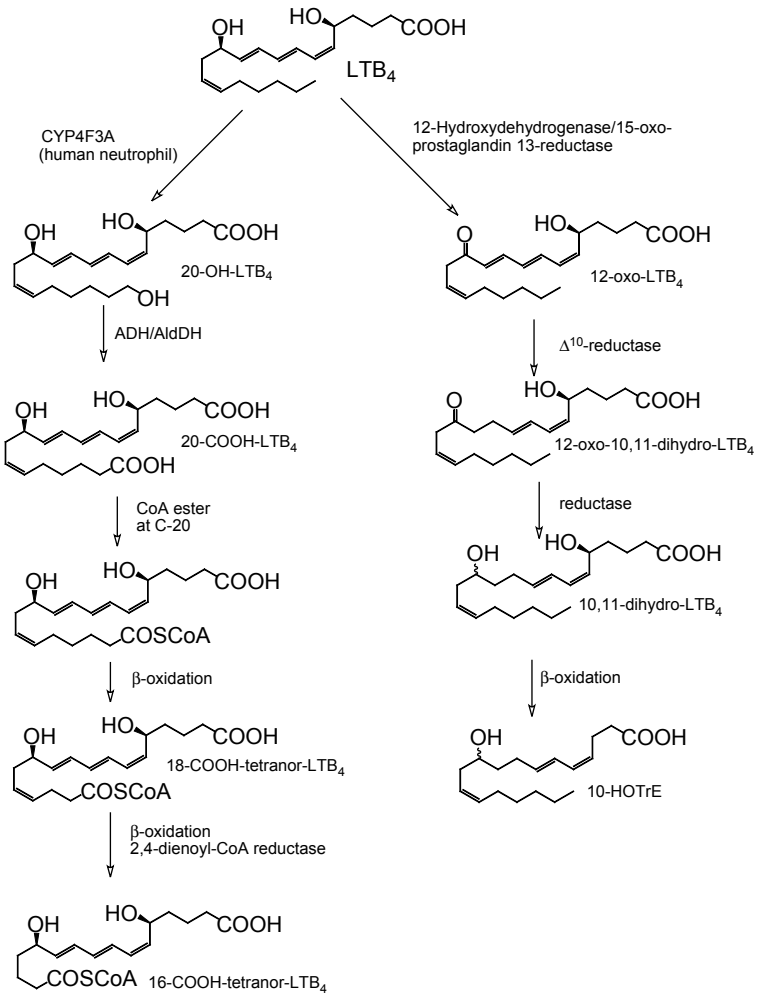

Fig. 9. Common metabolic transformations of leukotriene B_4 (LTB4) either by the cytochrome P-450 (CYP4F-family members) and ω-oxidation followed by β-oxidation, or by the 12-hydroxyeicosanoid dehydrogenase pathway which leads to reduction of the $\Delta^{10,11}$ double bond.

(CYP4F2) involves specific and unique cytochrome P-450s of the CYP4F family. cDNAs encoding 16 different proteins have now been cloned and expressed in several animal species [28] and each of these enzymes efficiently converts LTB_4 into 20-hydroxy-LTB_4. 20-Hydroxy-LTB_4 has some biological activity since it is a competitive agonist for the LTB_4 receptor. In the human neutrophil, 20-hydroxy-LTB_4 is further metabolized into biologically inactive 20-carboxy-LTB_4 by CYP4F3. In the hepatocyte and other cell types, 20-hydroxy-LTB_4 is further metabolized by alcohol dehydrogenase to form 20-oxo-LTB_4, then by fatty aldehyde dehydrogenase to form 20-carboxy-LTB_4 both of which reactions require NAD^+ (Fig. 9).

A unique reductive pathway is highly expressed in cells such as keratinocytes, endothelial cells, and kidney cells. This pathway involves an initial LTB_4 oxidation at the 12-hydroxyl group to form a 12-oxo moiety followed by reduction of the conjugated dienone and double bond $\Delta^{10,11}$ (Fig. 9). The products of the 12-hydroxy eicosanoid dehydrogenase pathway are devoid of biological activity and in certain cells represent the major pathway of inactivation. The 12-hydroxy dehydrogenase/15-oxo-prostaglandin-13-reductase, which is the committed enzyme of this pathway, has been crystallized and a three-dimensional structure reported (T. Shimizu, 2006).

A secondary metabolic pathway for LTB_4 in hepatocytes proceeds through fatty acid β-oxidation that requires initial ω-oxidation of the C-20 methyl terminus to 20-carboxy-LTB_4 followed by CoA ester formation [27]. β-Oxidation takes place both within the peroxisome as well as the mitochondria of the hepatocyte (Chapter 5). The importance of each oxidation processes in LTB_4 metabolism is highlighted by LTB_4 metabolism in human subjects with various genetic abnormalities. Deficiencies in peroxisomal metabolism (Zellweger disease) lead to a reduction in β-oxidation and in these individuals LTB_4 and 20-carboxy-LTB_4 are urinary excretion products (E. Mayatepek, 1999). Individuals with a deficiency in fatty aldehyde dehydrogenase, termed Sjögren-Larsson syndrome, excrete measurable levels of LTB_4 and 20-hydroxy-LTB_4 (M.A. Willemsen, 2001). None of these compounds can be measured in the urine of normal individuals even when exogenous LTB_4 is administered.

The metabolism of LTC_4 results in a major alteration of the biological activity of LTB_4 because the initial metabolites, LTD_4 and LTE_4, interact with different affinities with the receptors CysLT1 and CysLT2 (see below). The initial peptide cleavage reaction involves γ-glutamyl transpeptidase, an enzyme typically located on the outer cellular membrane of cells, to yield LTD_4 which has a very high affinity for the CysLT receptors (Fig. 10). Conversion of LTD_4 to LTE_4 likely involves a specific membrane-bound dipeptidase whose three-dimensional structure has recently been described. Sulfidopeptide LTs can also be metabolized specifically at the sulfur atom through oxidation reactions initiated by reactive oxygen species. More specific metabolic processing of the sulfidopeptide LTs include ω-oxidation by cytochrome P-450 followed by β-oxidation from the ω-terminus resulting in a series of chain-shortened products [27]. Formation of 20-carboxy-LTE_4 results in complete inactivation of the biological activities of this molecule due to lack of CysLT receptor recognition. Acetylation of the terminal amino group in LTE_4 generates *N*-acetyl-LTE_4, an abundant metabolite in rodent tissue. In humans, some LTE_4 is excreted in urine and has been used to reflect whole body production of sulfidopeptide LT in vivo.

4.6. Biological activities of leukotrienes

LTB_4 is thought to play an important role in the inflammatory process by way of its chemotactic and chemokinetic effects on the human polymorphonuclear leukocyte. LTB_4 induces the adherence of neutrophils to vascular endothelial cells and enhances the migration of neutrophils (diapedesis) into extravascular tissues. The biological activity of LTB_4 is mediated through two specific G-protein-coupled receptors termed BLT1 and BLT2 [29]. BLT1 is expressed almost exclusively in human polymorphonuclear leukocytes and to a much lesser extent on macrophages and in tissues such as thymus. The human BLT gene is located on

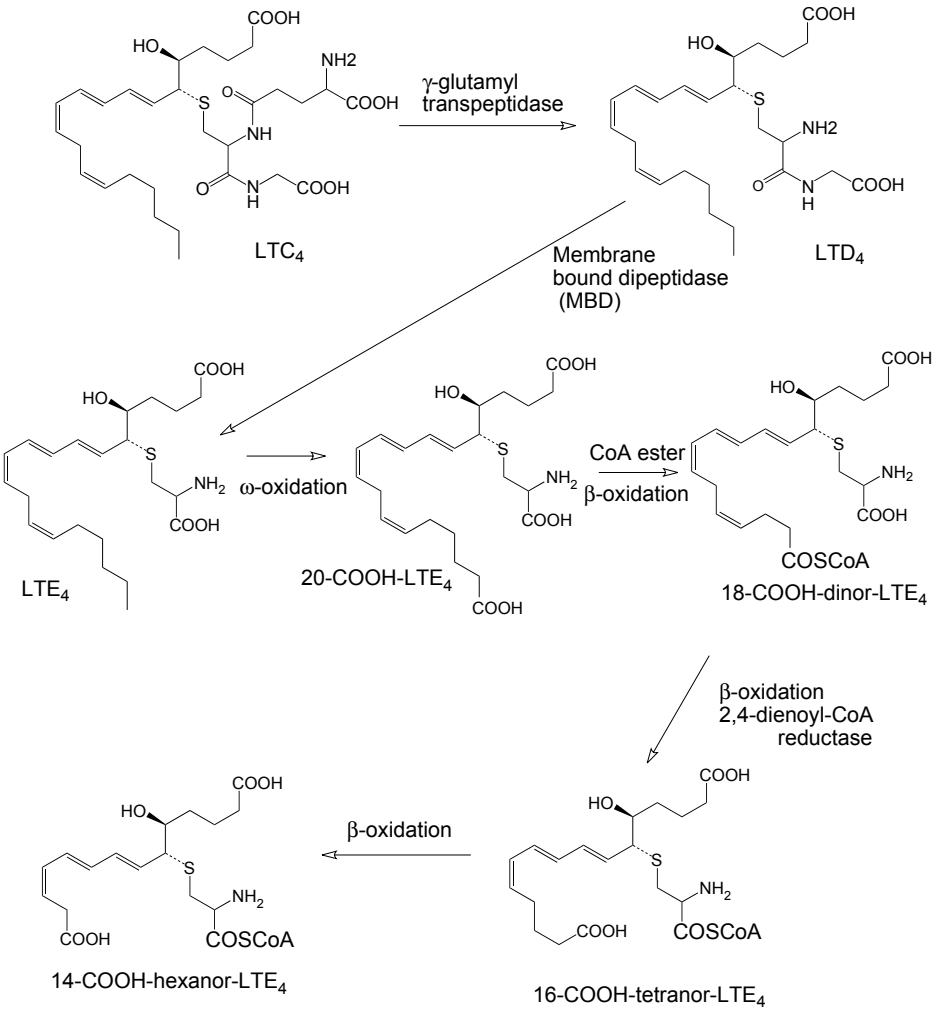

Fig. 10. Common metabolic transformations of leukotriene C_4 (LTC_4) to the biologically active sulfidopeptide leukotrienes, LTD_4 and LTE_4. Subsequent ω-oxidation of LTE_4 by cytochrome P-450 leads to the formation of 20-carboxy-LTE_4 which can undergo β-oxidation, after formation of the CoA ester, into a series of chain-shortened cysteinyl leukotriene metabolites.

chromosome 14. The chemotactic effect of LTB_4 is mediated through the BLT1 and BLT2 receptors. Several specific agents have been developed by pharmaceutical companies to inhibit the LTB_4 receptor; however, none has been fully developed to be used in humans.

LTC_4 and the peptide cleavage products LTD_4 and LTE_4 are mediators that cause bronchial smooth muscle contraction in asthma. These sulfidopeptide LTs also increase vascular leakage leading to edema. The discovery of LTs was a result of the search for the chemical structure of the biologically active principle called ‘slow reacting substance of anaphylaxis’ (R.C. Murphy, 1979). Two receptors for the cysteinyl LT have been

characterized and termed CysLT1 and CysLT2, and a third receptor that recognizes cysteinyl LTs has been recently described. The CysLT1 receptor is a G-protein-coupled receptor with seven transmembrane regions [30]. The CysLT1 receptor has limited distribution in tissues and is most abundant in smooth muscle of the lung and small intestine; LTD_4 and LTC_4 both activate this receptor. Several drugs are now available for inhibition of the CysLT1 receptor in human subjects. These are montelukast (IC_{50} 2–5 nM), pranlukast (IC_{50} 4–7 nM), and zafirlukast (IC_{50} 2–3 nM). The gene encoding the human CysLT1 receptor is located on the X chromosome [30].

4.7. Other lipoxygenase pathways

Numerous lipoxygenases occur within the plant and animal kingdoms and different lipoxygenases have been described in mammalian systems. These enzymes have several aspects in common. First, non-heme iron is an essential component of the catalytic activity of these enzymes and is held in place through histidine residues rather than by heme. Furthermore, these enzymes catalyze the insertion of molecular oxygen into polyunsaturated fatty acids, predominantly linoleic acid and AA, with the initial formation of lipid hydroperoxides. In general, the overall biological activities of the lipoxygenase products are not completely known and the significance of 12- and 15-lipoxygenase in humans remains undefined.

4.7.1. 12-Lipoxygenase

Two different enzymes termed 12-lipoxygenase (12-LO) catalyze the conversion of AA to 12-HpEET, which is subsequently reduced to 12-HETE (Fig. 11). The human platelet expresses one 12-LO [12(*S*)-lipoxygenase, EC 1.13.11.31] the cDNA of which has been cloned, sequenced, and found to encode a 662 amino acid protein with a molecular weight of 75,535. A second 12-LO is present in other mammalian systems including the mouse and rat and has been termed the 12(*R*)-lipoxygenase. The 12(*S*)-lipoxygenase is very similar in many respects to 15-lipoxygenase (15-LO) in terms of its substrate specificity and capability of forming both 12-HpETE and 15-HpETE from AA. The 12(*S*)-lipoxygenase has approximately 65% identity in primary structure to that of 15-LO from human reticulocytes. The 12(*R*)-lipoxygenase is less well studied but has also been described as the epidermal lipoxygenase from newly differentiated keratinocytes and the lipoxygenase that oxygenates AA at C-15 and C-8. Both 12/15-LO types are suicide inactivated. Several lines of evidence suggest that the 12-LO pathway of AA metabolism plays an important role in regulating cell survival and apoptosis (A.R. Brash, 1999).

4.7.2. 15-Lipoxygenase

The oxidation of AA at C-15 is catalyzed by 15-LOX1, a soluble 661 amino acid-containing protein with a molecular weight of 74,673. Many cells express this enzyme that also efficiently oxidizes linoleic acid to 13-hydroperoxyoctadecadienoic acid and, to a lesser extent, 9-hydroperoxyoctadecadienoic acid because of broad substrate specificity to both 12-HpETE and 15-HpETE [31]. One distinguishing feature of 15-LOX1 is that it can oxidize AA esterified to membrane phospholipids, thus forming esterified 15-HpETE. Expression of 15-LOX1 is enhanced by several interleukins, suggesting a role of this enzyme in events such as atherosclerosis.

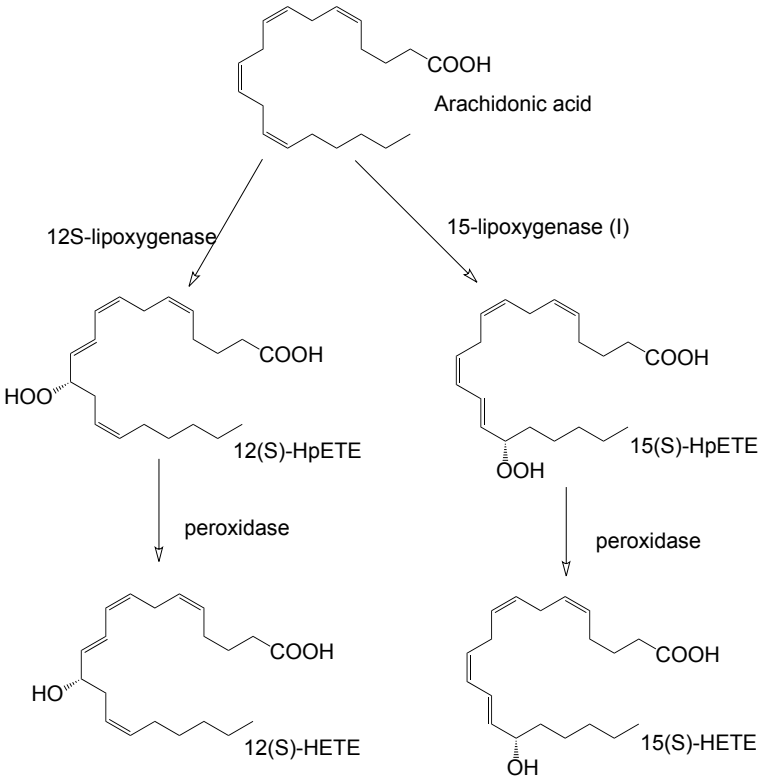

Fig. 11. Metabolism of arachidonic acid by 12- and 15-lipoxygenase pathways with corresponding stereospecific formation of hydroperoxyeicosatetraenoic acids (HpETE). Subsequent reduction of these hydroperoxides leads to the corresponding HETE at either carbon-12 or -15, which are thought to mediate biological activities of these enzymatic pathways.

The X-ray crystal structure of mammalian 15-LOX1 has revealed two domains, a catalytic domain and a β-barrel domain [32]. The β-barrel domain is similar to C2 domains found on other proteins and directs the binding of this enzyme to phospholipid membranes, the source of either AA or phospholipids for the oxidation process. The catalytic domain, which contains the histidine-coordinated Fe(III), holds the AA assisted by an ionic bond between R403 and the ionized carboxyl group of AA. The methyl terminus of AA is thus placed deep within a hydrophobic binding cleft in an arrangement that is likely similar to that of other lipoxygenases.

Mammalian 15-LOX1 is involved in the production of more complicated eicosanoids including the biologically active lipoxins [33]. Lipoxins are formed by the sequential reaction of 15-LO and 5-LO acting on precursor AA. For example, LTA_4 (the 5-LO product) can be converted to a lipoxin by the action of 15-LOX1.

There are a host of biological activities initiated by these 15-LOX1-dependent eicosanoids [31]. The unique activity of 15-LOX1 in oxidizing intact phospholipids has been featured in several hypotheses linking the oxidation of phospholipids in atherosclerotic

lesions to these lipoxygenases. The recent availability of strains of mice with a targeted deficiency in 5-, 12-, or 15-lipoxygenase (C. Funk, 2000) provides a powerful tool to ask specific questions concerning the role these lipoxygenases play in host defense reactions, cellular function, and disease processes.

5. Cytochrome P-450s and epoxygenase pathways

AA can be metabolized to a series of products characterized by the introduction of a single oxygen atom from molecular oxygen and formation of three different types of initial products catalyzed by various cytochrome P-450 mixed function oxidases (Fig. 12) [34,35]. The three classes of products include a series of HETEs formed by an allylic oxidation mechanism resulting in a family of conjugated dienes isomeric to the reduced products of a lipoxygenase reaction. P-450 metabolites formed by this mechanism have been characterized as 5-, 8-, 9-, 11-, 12-, and 15-HETE (Fig. 12), some of which are epimeric to the lipoxygenase-catalyzed products. A second class of reactions involves oxidation of the carbon atoms of AA with placement of a hydroxyl group either at the terminal carbon atom (ω) or at the penultimate carbon atom (ω-1), even proceeding through the ω-4 carbon atom position, with formation of a family of ω-oxidized monohydroxyeicosatetraenoic acids. Insertion of oxygen into the carbon–carbon bond results in the formation of a family of regio-isomeric *cis* epoxyeicosatetraenoic acids (EETs) from which the general pathway, the epoxygenase pathway, has been named. These regio-isomers include 14,15-, 11,12-, 8,9-, and 5,6-EETs which can be formed either as an *R,S,* or the *S,R* enantiomer (Fig. 12).

5.1. Epoxygenase P-450 isozymes

With the availability of recombinant P-450 isozymes, it has been possible to identify specific isozymes that metabolize AA. EET biosynthesis can be accomplished by CYPIA, CYP2B, CYP2C, CYP2D, CYP2G, CYP2J, CYP2N, and CYP4A subfamilies [32]. For each of these, unique EET regio-isomers are formed. For example CYP2C8 produces 14(*R*),15(*S*)-EET and 11(*R*),12(*S*)-EET with optical purities of 86 and 81%, respectively. However, it is likely that more than a single P-450 contributes to EET biosynthesis within a specific cell or tissue. Thus, the individual AA epoxygenase metabolite may depend upon expression of specific P-450 isoforms. It is thought that the majority of EET biosynthesis in the kidneys of humans and rats is the result of CYP2C expression in these tissues. However, the induction of specific P-450s can greatly alter the production of specific epoxygenase products.

5.2. Occurrence of EETs

Various EETs have been measured in tissues as well as physiological fluids such as urine (G. FitzGerald, 1990). Biologically active lipids, originally defined as an endothelium-derived hyperpolarizing factor and an inhibitor of Na^+/K^+ ATPase found in the thick ascending loop of Henley cells, were structurally characterized as 11(*R*),12(*S*)-EET and 20-HETE, both derived from cytochrome P-450-mediated metabolism of AA [35]. Interestingly, the EETs can readily form CoA esters and participate in reacylation of

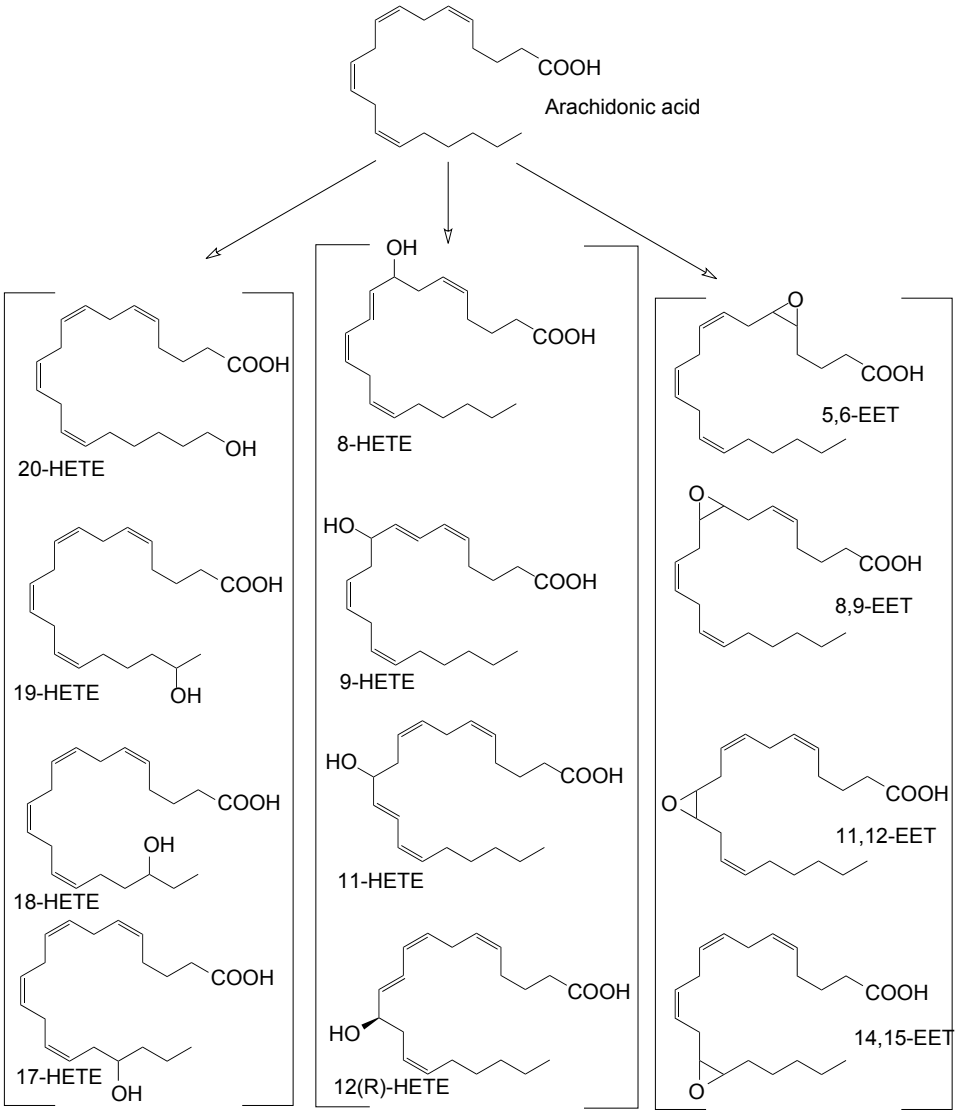

Fig. 12. Metabolism of arachidonic acid by cytochrome P-450 enzymes and the formation of three structurally distinct metabolite families. ω-Oxidation leads to a family of ω to ω-4 products including 20-HETE (ω-oxidation) and 19-HETE (ω-1 oxidation). The lipoxygenase-like mechanism of cytochrome P-450 metabolism leads to the formation of six different conjugated dienols, for which the structures of four are indicated. One unique biologically active lipoxygenase-like P-450 metabolite is 12(*R*)-HETE. The epoxygenase pathway leads to the formation of four regio-isomeric epoxyeicosatrienoic acid (EETs) all of which are biologically active.

lysophospholipids which results in the reincorporation of these oxidized metabolites of AA into phospholipid membranes, a biochemical feature not observed for PGs, Txs, or LTs. For example, human platelets contain 14,15-EET esterified within membrane phospholipids (Y. Zhu, 1995). The majority of EETs produced within cells are probably re-esterified to cellular glycerophospholipids.

5.3. Metabolism of EETs

A number of metabolic pathways operate on the primary epoxygenase metabolites of AA. Some of the more abundant pathways include CoA-dependent re-esterification as mentioned above as well as β-oxidation chain-shortening. A unique pathway involves epoxide hydrolase, a cytosolic enzyme that hydrates EETs to the corresponding vicinal dihydroxyeicosatrienoic acids [35]. A microsomal epoxide hydrolase can also metabolize EETs, but at a somewhat lower rate. The soluble epoxide hydrolase has substrate specificity, both in terms of the stereochemistry of the EET as well as its position in the AA chain. As expected, nonenzymatic hydration of these epoxides occurs especially under acidic conditions and can be accelerated during the isolation of these AA metabolites. Therefore, it is sometimes difficult to distinguish between nonenzymatic and enzymatic hydration of EETs. The 5,6-EET is a poor substrate for cytosolic as well as microsomal epoxide hydrolase; however, 5,6-EET is an efficient substrate for PGHS, leading to the formation of 5,6-epoxy-PGH_1. This reactive intermediate can subsequently be transformed into corresponding 5,6-epoxy-prostaglandins of the E, F, and I series or into an epoxy Tx analog. All of the EETs can also be substrates for lipoxygenases which introduce molecular oxygen at any 1,4-*cis*-pentadienyl position not interrupted by the epoxide ring. The EETs can also be conjugated with reduced glutathione catalyzed by glutathione (S) transferases. Studies of the metabolism of EETs by rat or mouse liver microsomal P-450 revealed the formation of a series of diepoxyeicosadienoic acids as well as monohydroxyepoxyeicosatrienoic acids. Interestingly, the diepoxides are further transformed into tetrahydrofurandiols via intermediate diol epoxides formed by soluble epoxide hydrolase. The characterization of specific products of EET and PGHS metabolism of AA have led to the observation of 5,6-epoxy-PGE_1 as a renal vasodilator with similar potency to that of PGE_2 and the metabolism of 8,9-EET by PGHS leading to 11-hydroxy-8. 9-epoxyeicosatrienoic acid which is a mitogen for rat glomerular mesangial cells.

5.4. Biological actions of EETs

Metabolites of AA derived from the epoxygenase P-450 pathway have been studied extensively in terms of their pharmacological properties. Potent effects have been observed in modulating various ion channels, membrane-bound transport proteins, mitogenesis, PPARα agonists, and activators of tyrosine kinase cascades [35]. EETs likely play an important role in mediating Na^+/K^+-ATPase activity and inhibiting the hydroosmotic effect of arginine vasopressin in the kidney. A picture has emerged for an important role of EETs in regulating renal vascular tone and fluid/electrolyte transport placing the EETs in the pathogenesis of hypertension (A.A. Spector, 2007).

6. Future directions

There is currently a reasonable understanding of the structures of PGHS-1 and -2, but many of the relationships between structure and function remain to be identified. For example, the peroxidase activities of PGHSs preferentially utilize alkyl hydroperoxides such as PGG_2 rather than hydrogen peroxide; the molecular basis for this specificity is not evident from simple observation of the structures. It will also be important to characterize further the membrane-binding domain of PGHSs in the context of the interaction of these domains with specific membrane lipids and whether this domain plays a role in governing substrate entry into the COX site.

Our understanding of the different biological roles of PGHS-I and PGHS-2 is only beginning to emerge. The functions of these two isozymes in apoptosis, carcinogenesis, angiogenesis, respiration, inflammation, pain, and reproduction need further exploration. Of key importance is understanding the reason for the existence of the two PGHS isozymes, and how coupling occurs between these enzymes, upstream lipases, and downstream synthases and receptors.

From a pharmacological perspective, it will be important to understand the molecular basis for the cardiovascular side effects of COX-2 inhibitors. The next few years should also see the development and testing of receptor antagonists that have the promise of being more specific than NSAIDs and COX-2 inhibitors. For example, it is thought that EP1 and EP4 play roles in colon cancer and that inhibition of one or both of these receptors may be useful in preventing colon carcinogenesis.

Considerable challenges remain in understanding the detailed biochemistry involved in the synthesis and release of biologically active LTs. Little is known about how these highly lipophilic molecules are released from cells; but even more curious is how the chemically reactive intermediate LTA_4, made on the perinuclear envelope, finds its way into a neighboring cell in the process of transcellular biosynthesis. The mechanism of 5-lipoxygenase is still poorly understood; however, a detailed X-ray structure of 5-lipoxygenase will likely reveal important facets relevant to translocation events of 5-lipoxygenase as well as the mechanism of suicide inactivation. Such information would be of great value in designing specific drugs as novel inhibitors of 5-lipoxygenase, a pharmacological approach highly successful for COX. Last but not least, little is known concerning a potential intracellular role for LTs. The currently known biological actions of LTs all involve cell membrane G-protein-linked receptors, yet an understanding of why biosynthesis of these lipophilic molecules occurs deep within the cell remains a mystery.

Abbreviations

2-AG	2-arachidonoylglycerol
AA	arachidonic acid
BLT (1 or 2)	leukotriene B_4 receptor (subclass 1 or 2)
COX	cyclooxygenase
cPGES	cytosolic PGE synthase
$cPLA_2$	cytosolic phospholipase A_2

CRE	cAMP response element
CYP4F(x)	cytochrome P-450 isozyme that carries out ω-oxidation of leukotriene B_4 (subclass x)
CysLT (1 or 2)	cysteinyl leukotriene receptor (subclass 1 or 2) for which leukotriene C_4, D_4, and E_4 are agonists
EET	epoxyeicosatetraenoic acid
EP	prostaglandin E receptor
ER	endoplasmic reticulum
FLAP	5-lipoxygenase activating protein
HETE	hydroxyeicosatetraenoic acid
HpETE	hydroperoxyeicosatetraenoic acid
LO	lipoxygenase
LT	leukotriene (followed by letter to designate structural type)
mPGES	microsomal PGE synthase
NSAID	nonsteroidal anti-inflammatory drug
PG	prostaglandin (followed by letter to designate structural type)
PGHS	prostaglandin endoperoxide H synthase
PLA_2	phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
Tx	thromboxane
TXAS	TxA synthase

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CHAPTER 13

Sphingolipids

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Contents

1. Introduction

Sphingolipids are found in essentially all animals, plants, and fungi, and in some prokaryotic organisms and viruses. As shown in Fig. 1A, they are composed of a sphingoid base ('sphingosine') [1] backbone to which a fatty acid may be attached through an amide bond and (or) a head group at the primary hydroxyl (Fig. 1B). The head groups range from a simple hydrogen (for sphingosine and ceramide) to more complex species, such as the phosphocholine moiety of sphingomyelin (SM) and the simple to complex glycans of glycosphingolipids, as illustrated in Fig. 1C.

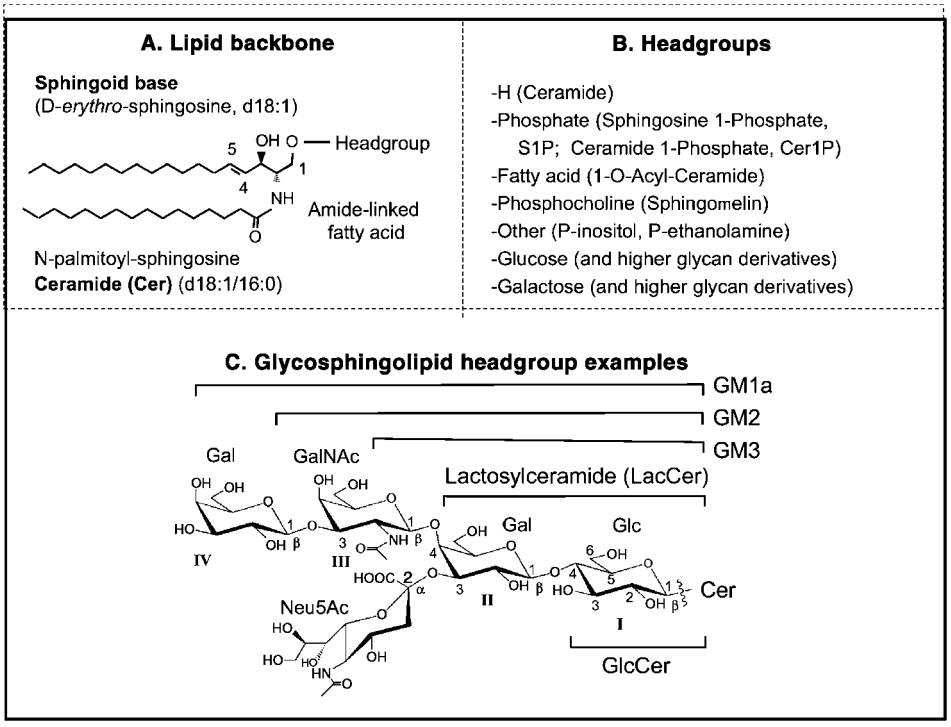

Fig. 1. Basic structures of sphingolipid backbones and head groups. (A) An example of a ceramide backbone, including commonly used names and abbreviations for the components; (B) common head groups attached directly to ceramide in mammalian sphingolipids; and (C) an example of a complex glycosphingolipid, ganglioside GM1a, and the names for the component parts. Abbreviations are as defined in text.

1.1. An overview of the functions of sphingolipids

Sphingolipids function as structural components of membranes, lipoproteins, skin, and other biomaterials, and as cell signaling modulators and mediators. As illustrated in Fig. 2, sphingolipids tend to associate with each other as well as with cholesterol and certain categories of proteins, such as those attached to the membrane via a glycosylphosphatidylinositol anchor (Chapter 2). The aggregates are generically referred to as 'rafts' unless they can be more specifically defined by co-localization with a marker for a distinct membrane structure, such as caveolin for caveolae (C.J. Cheng, 2006). Rafts are envisioned to be relatively small, distinct, and liquid-ordered subdomains of the plasma membrane that form and disperse rapidly due to the highly saturated alkyl chains and intermolecular hydrogen bonding of sphingolipids (alternatively, one can say that these components have such high phase-transition temperatures that they tend to phase separate from more mobile phosphoglycerolipids).

Rafts provide platforms to co-localize the components necessary for a cell function such as nutrient transport or cell signaling. The components of rafts are often studied by extracting biological samples with detergents such as Triton X-100 and analysis of the proteins and lipids that remain in the insoluble residue. In such cases, it is more accurate to describe them as 'detergent-resistant membranes' or 'Triton-insoluble membranes'

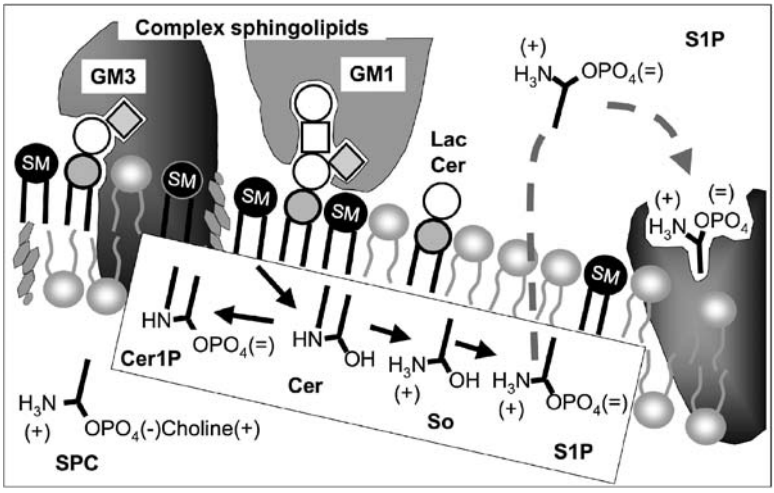

Fig. 2. Overview of sphingolipid structure and function. The schematic diagram represents a prototypical mammalian plasma membrane composed of sphingolipids (sphingomyelin, SM; lactosylceramide, LacCer; gangliosides GM1 and GM3; and the bioactive lipid backbones ceramide, Cer; ceramide 1-phosphate, Cer1P; sphingosine, So; sphingosine 1-phosphate, S1P; and sphingosylphosphocholine, SPC). Complex sphingolipids are mainly localized in 'rafts' and associated with proteins on the same membrane as well as the extracellular matrix and neighboring cells. The solid arrows represent a hypothetical signaling cascade in which complex sphingolipids are degraded to ceramide followed by formation of downstream signaling metabolites, including S1P, which is secreted from the cell (shown by the dashed arrow) and bound by an S1P receptor. The glycan symbols are the same as in Fig. 8.

(Chapter 1) because detergent extraction can cause redistribution of the molecular components and co-aggregation of molecules that are not normally associated with each other in vivo (M. Heffer-Lauc, 2005, 2007).

Glycosphingolipids additionally participate in cell adhesion and cell–cell communication by binding to proteins and carbohydrates from the extracellular matrix (E. Freire, 1994; A. Capela, 2006) and cells (R.L. Schnaar, 2004). This not only provides structure and organization, but also can modulate the activities of membrane receptors on neighboring cells or the same membrane (Fig. 2). For example, ganglioside GM3 inhibits activation of the epidermal growth factor receptor tyrosine kinase (S.J. Yoon, 2006). Some sphingolipids are used as binding sites by microorganisms, microbial toxins (e.g., the binding of GM1 by cholera toxin), and viruses (K. Hanada, 2006).

In addition to such functions for complex sphingolipids, the lipid backbones (ceramide, sphingosine 1-phosphate, and others) serve as intracellular and extracellular signals (Fig. 2). These signals can be produced by turnover of complex sphingolipids (both SM and glycosphingolipids) (R. Valaperta, 2006) and can be biosynthesized de novo, with the latter most often occurring in response to cell stress [2]. Some of the advantages of using the lipid backbones for signaling are that they also can effect biophysical changes in the membrane (reorganization of rafts, changes in membrane curvature, charge or permeability, etc.) and move from the site of synthesis to targets inside or outside of the cell since some of the metabolites (e.g., sphingosine and sphingosine 1-phosphate) have significant water solubility. Although they have been traditionally thought of as components of the plasma membrane and associated vesicles (endosomes, lysosomes, etc.), sphingolipids are now known to have regulatory functions in essentially all intracellular membranes.

These concepts have been discussed by G. van Meer in a recent overview of 'cellular lipidomics' [3] as well as in other reviews of signaling by complex [4] and simple [5] sphingolipids. Such sophisticated, multi-step, and multi-mediator mechanisms for cell regulation are difficult to characterize experimentally because perturbing the system to study a specific component usually causes changes elsewhere. One of the crippling limitations of the field has been the lack of methods to identify and quantify all of the molecules that are present in the cell — i.e., the 'sphingolipidome' — as a starting point for a systems biology analysis. Fortunately, '-omic' technologies are evolving rapidly for analysis of large number of sphingolipids [6–8] and the first computational pathway model for sphingolipids has been published for yeast [9], which have a relatively simple metabolic pathway.

1.2. A brief history of sphingolipids and disease

In *A Treatise on the Chemical Constitution of the Brain,* published in 1884 [1], J.L.W. Thudichum reported the isolation of novel compounds from brain (including those we now know as ceramides, SMs, cerebrosides, and sulfatides) and commented that upon hydrolysis they produced a compound that ' ... is of an alkaloidal nature, and to which, in commemoration of the many enigmas which it has presented to the inquirer, I have given the name of *Sphingosin*'. Herb Carter and colleagues, who established the definitive structure for sphingosine in 1947, proposed designation of ' ... those lipids derived from sphingosine as sphingolipids.' Over the last century, an astonishing number of

complex sphingolipids have been isolated and characterized [10,11] (for recent compliations, see online sources such as http://www.sphingomap.org, http://www.functionalglycomics.org/, and http://www.lipidbank.jp).

Thudichum, a practicing physician throughout most of his life (D.L. Drabkin, 1958), was searching for a better understanding of disease, but appreciated that ' ... to reach this goal of complete knowledge ... the medicinal chemist must ... not ... carry on research by a kind of fishing for supposed disease-poisons, of which, according to my view of the subject, the attempt of the boy to catch a whale in his mother's washing-tub is an appropriate parable' [1]. Thudichum's viewpoint was borne out when the first sphingolipid-associated disease, Gaucher disease, was shown to involve accumulation of glucosylceramide (GlcCer) (A. Aghion,1934; A. Rosenberg and E. Chargaff, 1958) due to a deficiency of glucocerebrosidase (by R. O. Brady in 1965, who was also instrumental in developing an enzyme replacement therapy). Many diseases are now known to be due to defects in sphingolipid turnover (see Section 4) [12] and progress is being made toward treating several by enzyme replacement, the use of inhibitors of sphingolipid synthesis, small-molecule chaperones that increase the activity of the residual enzyme, and — at some point in the future — gene therapy (M. Beck, 2007). The next sphingolipid–disease connection was the discovery that the binding target for cholera toxin is ganglioside GM1 (W.E. van Heyningen, 1971; J. Holmgren, 1973). A large number of microbial toxins (as well as viruses) are now known to recognize sphingolipids, including SM (K. Hanada, 2005).

The latter half of the 20th century also saw the characterization of the intermediates and enzymes of sphingolipid biosynthesis — starting with key work on the initial steps by R. Brady, W. Stoffel, and E. Snell — which enabled the first discoveries of diseases caused by disruption of sphingolipid biosynthesis by food-borne mycotoxins (E. Wang, 1991) and genetic defects (hereditary sensory neuropathy type I) (J.L. Dawkins, 2001; K. Bejaoui, 2001).

After decades of intense effort to elucidate the functions of complex sphingolipids, a surprising new paradigm surfaced in 1986: that the lipid backbones are highly bioactive compounds that participate in cell regulation (Y.A. Hannun, 1986). Over the subsequent two decades, signaling pathways have been found for every sphingolipid backbone shown in Fig. 2. These discoveries have prompted searches for compounds that modulate sphingolipid metabolism and signaling (M. Kester, 2003; T.E. Fox, 2006), and both synthetic and naturally occurring compounds (including sphingolipids themselves as components of the diet) [13] have shown promise in studies with animal models for atherosclerosis (E. Glaros, 2007), diabetes (W.L. Holland, 2007), HIV (T. Umehara, 2006), cancer (E.M. Schmelz, 2001), and other diseases. Early-stage human clinical trials have also begun (G.K. Schwartz, 2006).

Thudichum would surely be pleased to see that basic research on sphingolipids has, indeed, led to a better understanding of disease.

1.3. Structural variation and nomenclature for sphingolipids

The number of sphingolipid subspecies is not known, but there are hundreds of different combinations of sphingoid base backbones with amide-linked fatty acids, and over

400 different head-group variations (see http://www.sphingomap.org; the web site of the Consortium for Functional Glycomics, http://www.functionalglycomics.org; or the KEGG glycan resource tool, http://www.genome.jp/kegg/glycan/) [11]. Fortunately, an individual cell contains only a subset of these. The LIPID MAPS Consortium has recommended a systematic nomenclature [14] that draws heavily on IUPAC recommendations [15] but also displays the compounds in a manner that is consistent with other lipid classes.

1.3.1. Sphingoid bases

The 18-carbon sphingoid base shown in Fig. 1 has the chemical structure (2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol, but is typically referred to as sphingosine or (*E*)-sphing-4-enine. It is the major species found in most mammalian sphingolipids, followed by sphinganine (which lacks the 4,5-*trans*-double bond) and 4-hydroxysphinganine or (2*S*,3*S*,4*R*)-2-aminooctadecane-1,3,4-triol, which is sometimes called phytosphingosine (Fig. 3). A useful shorthand nomenclature is to give the number of hydroxyl groups: d for

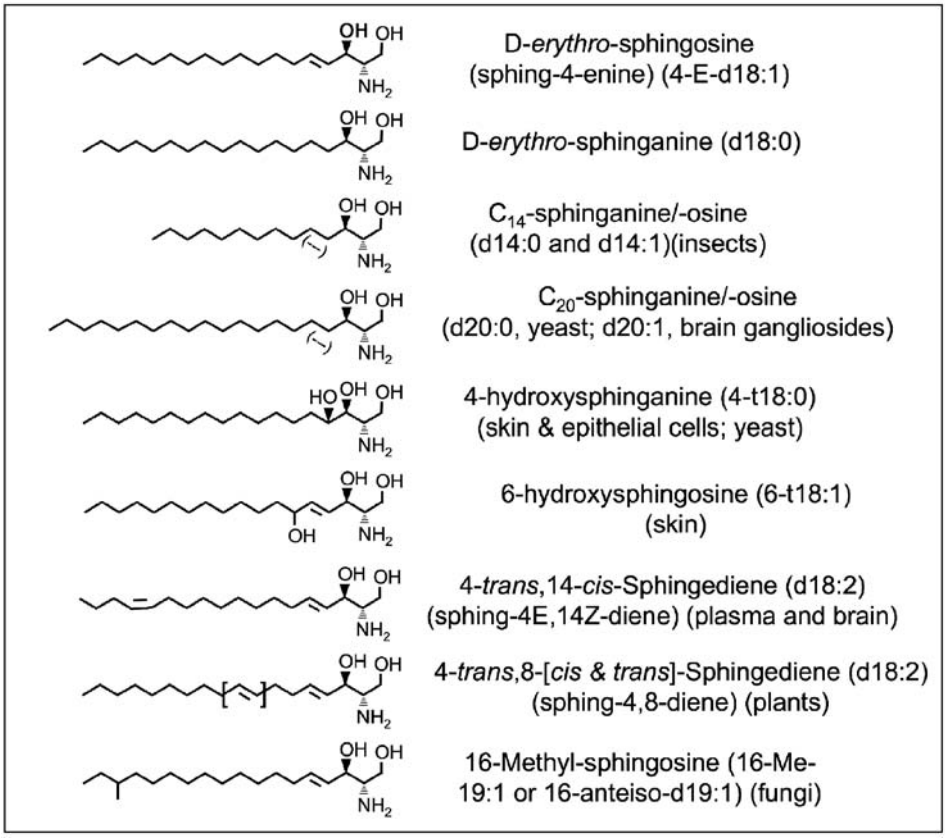

Fig. 3. Structures of major sphingoid bases of mammalian cells and examples of sphingoid bases found in other species.

the two (di-) hydroxyls of sphingosine and sphinganine, and t (tri-) for the additional hydroxyl in 4-hydroxysphinganine, followed by the chain length (typically 18 carbons) and number of double bonds (0, 1, or sometimes 2); therefore, sphingosine is abbreviated as d18:1 (other examples are given in Fig. 3). Also shown in this figure are the major structural variations in mammals: (a) C20-sphingosine (d20:1) in brain gangliosides; (b) an additional hydroxyl in skin; and (c) a second double bond, found in plasma and brain. In addition, there are trace amounts of other chain-length species (d17:1, d19:1).

Trace amounts of *N*- and *O*-methyl-sphingoid bases are sometimes found in mammalian sphingolipids and a sphingosine *N*-methyltransferase activity has been found in mouse brain (Y. Igarashi, 1989). We have also noted that *N*-methylation is induced by adding sphingoid bases to a variety of mammalian cell lines (Y. Liu, personal communication) or administered in vivo (P.R. Morales, 2007).

When considering sphingoid bases from all species [16], the structural variations include (Fig. 3): (i) alkyl chain lengths from 12 to 22 carbon atoms (e.g., C_{14}-sphingoid bases are prevalent in insects); (ii) hydroxyl groups at additional positions; (iii) double bonds at other sites along the alkyl chain (common in plants); and (iv) branching (methyl groups) at other positions (which is particularly prevalent in fungi) (K.A. Karlsson, 1970; S.B. Levery, 2005).

1.3.2. Ceramides

The majority of the sphingoid bases in cells are *N*-acylated with the long-chain and very-long-chain fatty acids (Fig. 4) to produce 'ceramides', as they are often referred

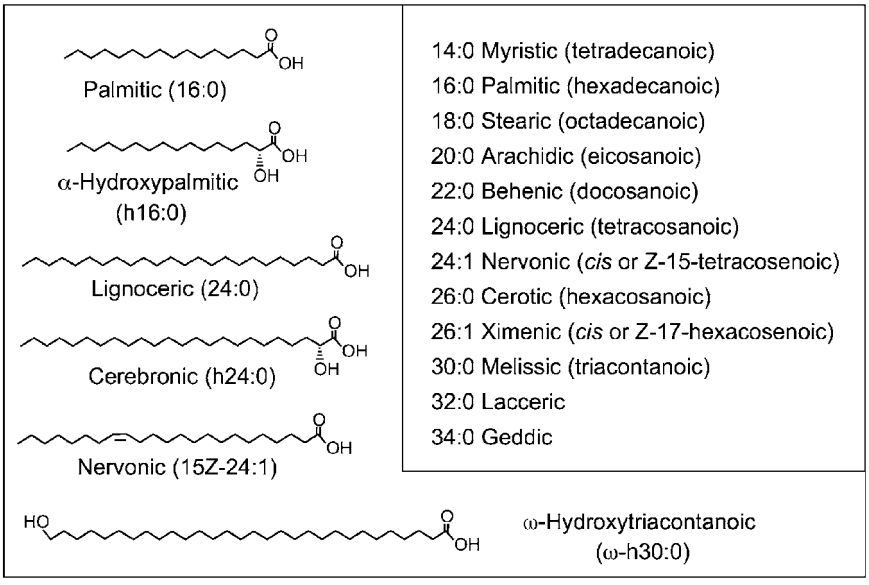

Fig. 4. Structures and names of representative fatty acids, including α- and ω-hydroxy fatty acids, in mammalian sphingolipids.

to generically. The fatty acids of Cer vary in chain length (typically 14–36 carbon atoms), degree of unsaturation (but are mostly saturated), and presence or absence of a hydroxyl group on the α- or ω-carbon atom.

The current convention is to use 'ceramide' to denote *N*-acylsphingosines, dihydroceramides for *N*-acylsphinganines, and 4-hydroxyceramides or phytoceramides for *N*-acyl-4-hydroxysphinganines. The fatty acyl chain length is usually presented as a prefix, such as C_{16}-Cer for *N*-palmitoylsphingosine. An even clearer nomenclature provides information about both the sphingoid base and the fatty acid; for example, d18:1/16:0 for *N*-palmitoylsphingosine.

On a practical level, the sphingolipid field is caught somewhat awkwardly between old and new nomenclatures due to differences in the information that one gets from traditional analytical methods such as radiolabeling followed by thin-layer chromatography versus more structure-specific methods such as mass spectrometry (MS).

1.3.3. Phosphosphingolipids

The simplest phosphosphingolipids are the sphingoid base 1-phosphates and the Cer 1-phosphates. The most prevalent phosphosphingolipid in most mammalian tissues is SM (Cer phosphocholine), which is also present in small amounts in cells as the 'lyso-' form, sphingosylphosphocholine (lyso-SM). Cer phosphoethanolamines are found in substantial amounts in some organisms, such as chickens (in liver) and the fruit fly, *Drosophila melanogaster*. Fungi, plants, and other organisms have additional species such as Cer phosphoinositols.

1.3.4. Glycosphingolipids

Glycosphingolipids have a carbohydrate (glucose or galactose for mammalian sphingolipids) attached to the 1-hydroxyl of Cer via a glycosidic bond in the β configuration. More complex sphingolipids have additional carbohydrates (or other modifications of GlcCer or GalCer, such as sulfation). For mammals, the major carbohydrates are glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), and fucose (Fuc). Acidic glycosphingolipids contain ionized functional groups such as phosphate, sulfate (sulfatides), or charged sugar residues such as sialic acid or, less frequently, glucuronic acid (GlcA).

'Sialic acid' is a family of compounds that are differentially expressed across species (A. Varki, 2002). Sialic acids may contain *N*-acetyl or *N*-glycolyl (i.e., hydroxyacetyl) groups at C_5, and are distinguished by names such as *N*-acetylneuraminic acid (Neu5Ac) (structure shown in Fig. 1C) and *N*-glycolylneuraminic acid (Neu5Gc). Human gangliosides have Neu5Ac, although Neu5Gc is expressed in human fetuses and tumors. Mice have both Neu5Ac and Neu5Gc, and studies of a mouse Neu5Gc knockout model have found that loss of the ability to produce this form of sialic acid has functional consequences (M. Hedlund, 2007). Two common modifications of sialic acid are 9-*O*-acetylation (A. Varki, 2006) and formation of an intramolecular lactone (e.g., GD1b-lactone) (S. Sonnino, 2006), which are cell-type specific and developmentally regulated.

Despite the availability of so many building blocks, nature has elected to limit the number of possible compounds by (i) adding only Glc or Gal directly to Cer; (ii) converting

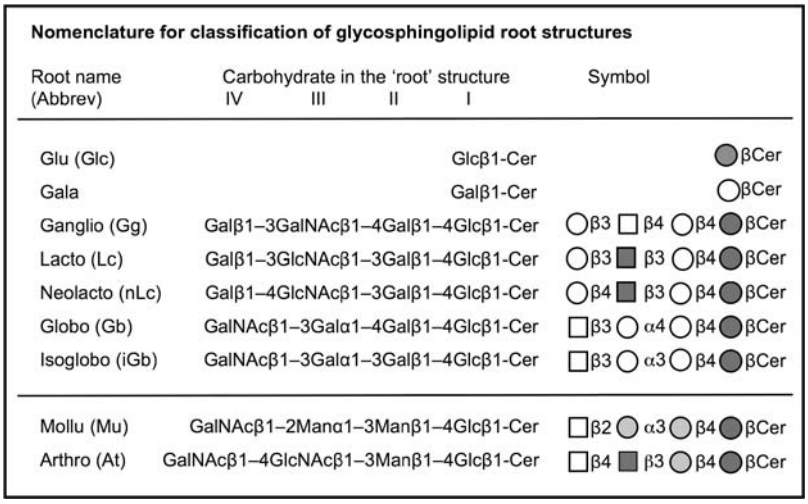

Fig. 5. Nomenclature and symbolic representations of the major subcategories of glycosphingolipids, from the simple (GlcCer and GalCer) to the root structures. All except Mollu and Arthro are present in mammalian cells. The glycan symbols are as in Fig. 8.

GalCer into a relatively small number of downstream metabolites, with the main products being sulfatides, which have a sulfate at the 3-position of the galactose; (iii) forming only a few immediate products from GlcCer (mainly lactosylceramide, LacCer) (Figs. 1C and 5); and (iv) producing a relatively limited number of tri- and tetra-hexosides from LacCer, which serve as so-called 'root structures' (Fig. 5) for formation of more complex sphingolipids by further chain extension and branching. This reduction in the number of potential metabolites is fortunate for investigators because it has been calculated that almost 20 million different tetrameric glycans could potentially be produced from nine common monosaccharides (Y. Nagai, 1997). Nonetheless, some of the glycans are very large — such as the tri- and tetra-sialosylpoly-*N*-acetyllactosaminyl gangliosides of human placenta that have >20 residues (S.B. Levery, 1999), and the polyglycosylceramides recognized by *Helicobacter pylori*, which have extended and highly branched *N*-acetyllactosamine core substituted by fucose and sialic acid (H. Karlsson, 2000).

The glycosphingolipid nomenclature has been systematized to allow subspecies to be described unambiguously [14,15]. Using these guidelines, one would name ganglioside GM1a: Neu5Acα2-3(Galβ13GalNAcβ1-4)Galβ1-4Glcβ1Cer (d18:1/16:0), although the colloquial names assigned by the 'Svennerholm' system [14,15] are still in common usage, in which isomers are designated by additional letters, for example:

Galβ1–3GalNacβ1–4Galβ1–4Glcβ1–1′Cer GM1a
|
Neu5Acα2–3

Neu5Acα2-3Galβ1–3GalNacβ1–4Galβ1–4Glcβ1–1′Cer GMlb

These compounds can also be named using the root structure (Fig. 5) and Roman numerals to show where the sialic acid (or other carbohydrate branchpoint) occurs along the chain, and Arabic superscripts to designate the hydroxyl to which it is linked:

II^3-α-*N*-acetylneuraminosyl-gangliotetraosylCer (II^3-α-Neu5NacGg$_4$Cer) for GM1a
IV^3-α-*N*-acetylneuraminosyl-gangliotetraosylCer (IV^3-α-Neu5NacGg$_4$Cer) for GM1b

A number of sphingolipids are referred to by their historic names as antigens and blood group structures, such as Forssman antigen (IV^3-α-GalNAc-Gb$_4$Cer), a globopentosylceramide that is found in many mammals (but probably not in humans), and the Lewis blood group antigens, which correspond to a family of α1-3-fucosylated structures (e.g. Lewis x, sialyl Lewis x).

1.3.5. Sulfatoglycosphingolipids (sulfatides)
Cerebrosulfatide (3′-sulfo-Galβ1-1′Cer, or GalCer-I^3-sulfate) is the major sulfoglycolipid of brain, kidney, the gastrointestinal tract and endometrium, and mammalian male germ cells. Two glucuronic acid (GlcA) sulfatoglycolipids (3-*O*-SO$_3$-GlcAβ1-3Galβ1-4GlcNAcβ1-3Galββ1-4Glcββ1-1′Cer and 3-*O*-SO$_3$-GlcAβ1-3(Galβ1-4GlcNAcβ1-3)$_2$ Galβ1-4Glcβ1-1′Cer) occur in the peripheral nervous system. Sulfatides are thought to be involved in neuronal cell differentiation, myelin formation and maintenance, and leukocyte adhesion to selectins.

1.3.6. Lysosphingolipids
Lysosphingolipids lack the amide-linked fatty acid of the Cer backbone, which makes them highly water soluble. A lysoglycosphingolipid with one hexose (e.g., glucosyl- or, most often, galactosylsphingosine) is colloquially referred to as a 'psychosine'. Elevation of psychosine in some sphingolipid storage diseases may play a role in the pathology (K. Suzuki, 1998)]. Other novel lysosphingolipids with alkyl groups on the sugar have been termed plasmalopsychosines (K.K. Sadozai, 1993; T. Hikita, 2001).

1.3.7. Sphingolipids covalently linked to proteins
Structural proteins of the cornified cell envelope of the skin permeability barrier are covalently attached to ω-hydroxyceramides and ω-hydroxyglucosylceramides through the ω-hydroxyl groups (T. Doering, 1999; M.E. Stewart, 2001; H. Farwanah, 2007), and for some organisms, such as yeast, Cers replace diacylglycerols in the covalently attached phosphatidylinositol glycan linkage that is used to attach the proteins to membranes (A. Conzelmann, 1995).

2. Biochemical properties and functions

Sphingolipids have very diverse properties because some are extremely water insoluble (Cers), whereas others (sphingosine 1-phosphate, most gangliosides, and polyhexosylceramides) are so water soluble that all or some remains in the aqueous phase when

standard lipid extracts are prepared using organic solvents. The properties of sphingolipids have been reviewed recently by F. Goni and A. Alonso (2006) [17] for sphingoid bases and Cers, and by B. Maggio (2006) for Cers and more complex species [18]). Methods for analyzing sphingolipids, sphingolipid-metabolizing enzymes, and other aspects of 'sphingolipidology' have been compiled in two volumes of *Methods in Enzymology* [19].

2.1. Sphingoid bases

A distinctive feature of sphingoid bases is that they can bear a net positive charge at neutral pH, which is rare among naturally occurring lipids. Nonetheless, the pK_a of the amino group is low for a simple amine and is influenced by its environment — i.e., between 7 and 8 in detergent micelles (A.H. Merrill, 1989) and 9 in phospholipid membranes (F. Lopez-Garcia, 1993). Thus, sphingoid bases behave as cationic amphiphiles, yet a portion can be uncharged at physiological pH, and hence flip across biological membranes , which may help explain why sphingoid bases are used for cell signaling (and are easily delivered to cells experimentally). Sphingosine has been noted to induce channels in membranes, and at high concentrations it is lysosomotrophic, presumably because it can be trapped by the by acidic pH of lysosomes.

Sphingosine 1-phosphate (S1P) is also water soluble (consistent with its function in signaling), but often difficult to handle as the zwitterion. Useful tips for handling sphingolipids are available on the Web site of Avanti Polar Lipids (http://www.avantilipids.com/SyntheticSphingosine-1-phosphate.asp). The high levels of S1P in blood (ca. 1 μM) are associated with high-density lipoproteins (HDL) (B. Zhang, 2005).

2.2. Ceramides

Cers are essentially insoluble in aqueous medium and are therefore difficult to administer to cells unless one uses potentially disruptive detergents, liposomes, or organic solvent mixtures (C. Luberto, 2000). This problem has often been bypassed using short-chain analogs, although their membrane properties are quite different [17]; however, short-chain Cers are metabolized to long-chain Cers after cellular uptake (B. Ogretmen, 2002), which may explain how they can often recapitulate biological effects of the natural species. Fluorescent Cer analogs, such as *N*-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoylceramide (NBD-Cer) and boron dipyrromethene difluoride (BODIPY), have proved very useful in studies of sphingolipid transport and metabolism (A. Dagan, 2000; R.E. Pagano, 2000) (Chapter 17).

The water insolubility of Cers, combined with strong intermolecular interactions, account for their participation in the water barrier of skin, where Cers are about one-third of the total lipid. In cell membranes, Cers tend to associate with rafts and caveolae and can affect membrane curvature. These may be important contributors to bilayer organization during cell signaling, for example, when SM is hydrolyzed to Cer in response to agonist activation of SMase [18]. Cer is also involved in cell signaling (as discussed in Section 5), and under certain conditions, Cers can form channels and induce 'leakiness' in membranes, such as mitochondria, which may contribute to the induction of apoptosis (L.R. Montes, 2002; L.J. Siskind, 2006).

2.3. Phosphosphingolipids

SM is the major phosphosphingolipid in mammalian tissues and lipoproteins, and has been studied extensively with respect to its role in the formation of sterol-enriched ordered membrane domains and cell signaling (J. Slotte, 2006). Like Cers (and most other complex sphingolipids), SMs typically have high phase-transition temperatures (>37°C), which is a factor in their tendency to associate with rafts.

The other significant phosphosphingolipid of mammals is Cer 1-phosphate, which has the potential to affect bilayer curvature, mediate processes such as phagocytosis (V. Hinkovska-Galcheva, 2005), and participate in cross-talk with other lipid signaling pathways, including the regulation of phospholipase A2 (C.E. Chalfant, 2005). Cer phosphoethanolamines, which are significant species in avian liver, insects, and other organisms, may also be present in mammals in small amounts (M.N. Nikolova-Karakashian, 2000). Cers derivatized with phosphoinositol and other glycans are found on other species, such as *Saccharomyces cerevisiae* and *Sporothrix schenckii* (R.C. Dickson, 2006).

2.4. Glycosphingolipids

Perhaps the most frequently utilized chemical property of glycosphingolipids is the greater stability of the glycosidic bonds between the carbohydrates and the amide-linked fatty acids versus the ester-linked fatty acids of glycerolipids, which allows the latter to be removed by basic hydrolysis and isolation of the (usually much lower abundance) glycosphingolipids by extraction and various types of chromatography [19]. It is additionally helpful that the more water-soluble glycosphingolipids tend to partition into the aqueous phase of organic solvent extracts of cells. Both of these properties have greatly facilitated studies of these species [19].

2.4.1. Biophysical properties

With respect to the biophysical properties of glycosphingolipids, it has been appreciated for some time (B. Maggio, 1986) that both the Cer backbones and the oligosaccharide chain have a marked influence on their thermotropic behavior, intermolecular packing, and surface electrical potential. As expected, considering the large head-group areas compared to the lipid backbones, the transition temperature and enthalpy of glycosphingolipids decrease proportionally to the complexity of the polar head group and the intermolecular spacing. In mixtures with phospholipids, increasing proportions of the phospholipids perturb the thermodynamic properties of the glycosphingolipids, and large changes in the molecular free energy, eccentricity, asymmetry ratio, and phase state of glycosphingolipid-containing structures can be triggered by small changes of the molecular parameters, lipid composition, and lateral surface pressure. These early findings match well the current concept regarding the functions of glycosphingolipids as molecules that can confer localized, unique properties in membrane microdomains as well as serve as information-rich ligands for specific head group–protein (and glycan–glycan) interactions. The functional consequences of these biophysical properties are for glycosphingolipids to [18]: (1) have ‘intrinsic codes’, meaning structural features that allow local molecular interactions that result in structural self-organization; (2) influence surface topography by projection of

molecular shape and miscibility of glycosphingolipids into formation of coexisting membrane domains; and (3) have effects beyond just the membrane interface as modulators of structural topology, bilayer recombination, and surface biocatalysis.

2.4.2. Receptor interaction

Complementary to these biophysical features, the complex carbohydrate head groups afford a high degree of specificity in binding to proteins, and sometimes the lipid backbones also participate. The categories of proteins that are known to have protein–glycosphingolipid interactions that are of functional significance (i.e., that alter the function of the protein) include the epidermal growth factor receptor with ganglioside GM3 (which appears to be modulated by GM3 interaction with *N*-linked GlcNAc termini of the receptor) (S.J. Yoon, 2006), fibroblast growth factor receptor and GM3 (M.S. Toledo, 2005), myelin-associated glycoprotein and gangliosides GD1a and GT1b (A.A. Vyas, 2002), platelet-derived growth factor receptor beta with ganglioside GM1 (J.L. Oblinger, 2003), and regulation of cell proliferation and survival by interactions between gangliosides and extracellular matrix proteins such as fibronectin and lamin, which appears to be altered by NEU3, a plasma membrane-associated sialidase that is up-regulated in colon tumors and suppresses apoptosis (K. Kato, 2006).

2.4.3. Localization

Glycosphingolipids are found in all cells of vertebrates. They are generally thought of as components of the plasma membrane, and in particular, subregions of the membrane such as 'rafts' that are often visualized by using antibodies against GM1 (M. Heffer-Lauc, 2005, 2007). On a more 'anatomical' level, GM1 and GM3 gangliosides have also been used to highlight distinct lipid microdomains within the apical plasma membrane domain of epithelial cells (P. Janich and D. Corbeil, 2007). However, the existence of some glycosphingolipids in intracellular membranes, and the functional significance of that localization, has been established for the GM1 of the nuclear envelope (R.W. Ledeen, 2007) and GD3 in mitochondria (C. Garcia-Ruiz, 2002). It seems likely that many other glycolipids will be found inside the cell (and probably with functional consequences) considering the dynamics of intracellular trafficking of membranes. In addition, a common property of gangliosides is that a substantial amount is shed from the plasma membrane of cells to the external environment, and may play a role in immunosurveillance (G. Lauc, 2006).

3. Biosynthesis of sphingolipids

Many of the features of pathways for sphingolipid metabolism are reasonably well characterized and genes have been identified for many of the initial biosynthetic steps [20].

3.1. Sphingoid bases and ceramide

3.1.1. Serine palmitoyltransferase

De novo sphingolipid biosynthesis begins with the condensation of palmitoyl-CoA and L-serine (Fig. 6) to form 3-ketosphinganine catalyzed by serine palmitoyltransferase (SPT).

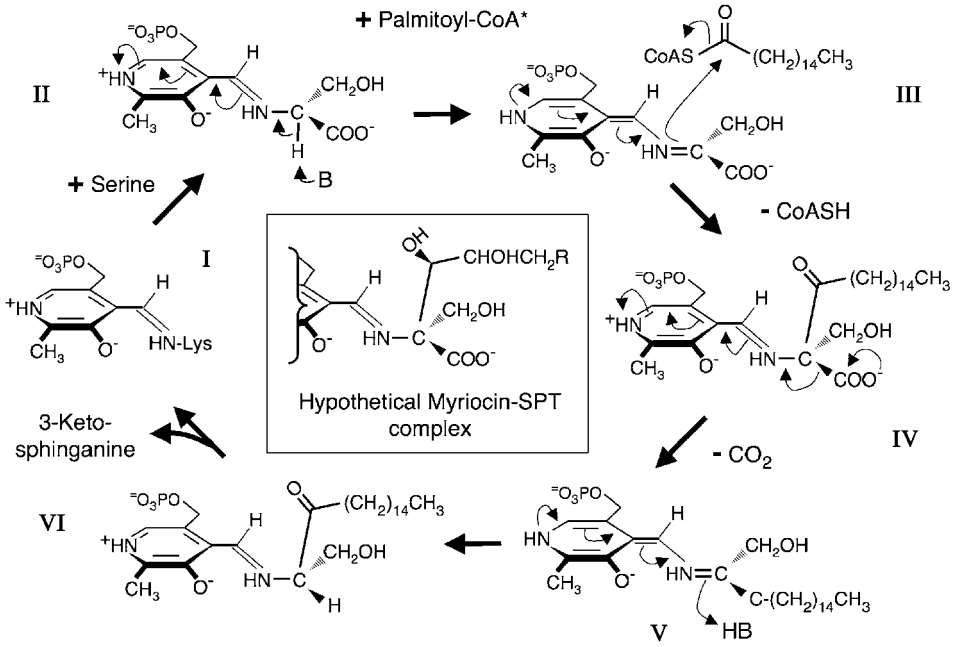

Fig. 6. A probable reaction mechanism for serine palmitoyltransferase (modified from K. Krisnangkura, 1976) and structure of the adduct that presumably accounts for the high-affinity inhibition by myriocin (inset). The asterisk on palmitoyl-CoA conveys that other chain-length sphingoid bases are produced utilizing the appropriate fatty acyl-CoA (e.g. dodecyl-CoA, $C_{12:0}$, to produce the d14:1 sphingoid base backbone of most insects).

The reaction proceeds with overall retention of configuration of C_2 of serine via the proposed mechanism (K. Krisnangkura, 1976) shown in Fig. 6.

As would be predicted from this mechanism involving pyridoxal 5′-phosphate, SPT undergoes 'suicide' inhibition by β-halo-L-alanines and L-cycloserine. However, more potent and selective inhibitors have been isolated from microorganisms, and myriocin (Y. Miyake, 1995) is not only mechanistically interesting (illustrated in Fig. 6 as the likely adduct that it forms with SPT) but is also commercially available and highly effective in suppressing de novo sphingolipid biosynthesis by cells in culture and in vivo (E.N. Glaros, 2007).

SPT is highly specific in the utilization of L-serine as its amino acid substrate. However, D-serine is a competitive inhibitor with an IC_{50} of ~0.3 mM, which is similar to the K_m for L-serine (K. Hanada, 2000). SPT is also highly selective for the co-substrate fatty acyl-CoA with the mammalian enzyme (R D. Williams, 1984; K. Hanada, 2000), utilizing palmitoyl-CoA ($C_{16:0}$) > pentadecanoyl- and heptadecanoyl-CoAs ($C_{15:0}$ and $C_{17:0}$) >> stearoyl-CoA ($C_{18:0}$) and essentially no unsaturated species except palmitelaidic (16:1 with a *trans*-double bond). This selectivity, combined with the abundance of palmitoyl-CoA (and scarcity of $C_{15:0}$ and $C_{17:0}$ fatty acids in most species except ruminants), accounts for the extremely high 18-carbon-chain-length specificity of most sphingoid bases in mammalian sphingolipids. Substrate supply also appears to be one of the factors that

affects the rate of de novo sphingolipid biosynthesis in mammalian cells (A.H. Merrill, 1988) and yeast (L.A. Cowart, 2007).

For mammals and yeast, at least two gene products (termed SPTLC1 and SPTLC2, or sometimes SPT1 and SPT2) are necessary for activity (R.C. Dickson, 2000) and appear to be physically associated (K. Gable, 2000; K. Hanada, 2000). Interestingly, another SPTLC2-like gene and gene product is expressed (SPTLC3) (T. Hornemann, 2006) ; therefore, one aspect of this pathway that warrants exploration is why there are multiple forms of this enzyme.

In the human genome, *SPTLC1* comprises 15 exons spanning ~85 kbp in the chromosome 9q21-q22 region, and *SPTLC2* comprises 12 exons spanning ~110 kbp in the chromosome 14q24.3-q31 region. The predicted MW of human SPT1 and SPT2 are 53 kDa and 63 kDa, respectively, and polypeptides of this approximate size are seen on Western blots of human and mouse cells using SPT1 and SPT2 antibodies (B. Weiss, 1997; K. Hanada, 2000). Higher MW bands are often seen on the Western blots, and these and other findings have been interpreted as evidence that SPT has an oligomeric structure (T. Hornemann, 2007). The SPT1 and SPT2 subunits have ~20% identity and have a single highly hydrophobic domain in the N-terminal region, which has been interpreted to represent a transmembrane domain (B. Weiss, 1997; K. Hanada, 1997). Indirect immunocytochemical analysis with epitope-tagged SPT1 indicated that the N- and C-termini are oriented toward the lumenal and cytosolic sides of the ER, respectively (S. Yasuda, 2003), which would place the majority of the active site region on the cytosolic side, as earlier suggested by the protease-sensitivity of SPT activity (E.C. Mandon, 1992). There appears to be another region of the polypeptide that is capable of spanning the membrane (G. Han, 2004).

There is evidence that SPT is associated with other proteins. In yeast, Dunn and co-workers have found a 10-kDa peptide, the product of the *Tsc3* gene, which associates with the SPT1/SPT2 complex (K. Gable, 2000) and is required for optimum SPT activity. No mammalian homolog of *Tsc3* has been found so far (K. Hanada, 2000); however, there may be an association with a class of endoplasmic reticulum (ER) proteins (termed Serinc1 to 5) (M. Inuzuka, 2005) that increase the synthesis of both phosphatidylserine and sphingolipids. Overexpression of Serinc1 in COS cells doubles SPT activity; therefore, Serinc may interact with SPT to facilitate serine utilization.

Using proteomic technologies (tandem affinity purification and MS) to discover protein–protein interactions, a substantial number of proteins have been identified as potential SPT2 (LCB2)-associated proteins in *Saccharomyces cerevisiae* (A.C. Gavin, 2002). These proteins are involved in various biological processes such as vesicle transport and nuclear import and export. A genome-wide yeast two-hybrid analysis in *Drosophila* (L. Giot, 2003) has suggested that SPT2 may interact with 13 proteins, including a proton transporter, organic cation transporter, hsc-70, and ribosomal proteins.

A protein that would be a logical interaction partner for SPT is 3-ketosphinganine reductase, which catalyzes the next step of the sphingolipid biosynthetic pathway. The gene for this reductase has been identified in yeast (T. Beeler, 1998) and in mammals (FVT1) (A. Kihara, 2004), and the sequence has coil–coil motifs that might interact with a similar motifs on SPT.

Mutations in SPTLC1 are associated with hereditary sensory and autonomic neuropathy type I (J.L. Dawkins, 2001; K. Bejaoui, 2001). The mutations occur at sites that

might not only interfere with the active site but also with protein–protein interactions. The crystal structure of SPT from *Sphingomonas paucimobilis* at 1.3-Å resolution (D.J. Campopiano, 2007) has located the pyridoxal 5′-phosphate cofactor bound covalently to Lys265 and the active site at the bottom of a deep cleft composed of residues from both subunits. Comparison of *in silico* models of the human SPT1/SPT2 heterodimer with this structure places the mutations in the active site and in locations that might affect its structure.

Although a large number of factors have been found to affect SPT mRNA, protein, or activity [21], most of the effects have been relatively small (several fold) (reviewed in K. Hanada, 2003, and J. Wei in Ref. [20]). Factors that appear to suppress sphingoid base synthesis are lipoproteins or addition of free sphingoid bases to cells in culture, and a phosphorylated but poorly degraded sphingoid base analog, *cis*-4-methylsphingosine (G. van Echten-Deckert, 1997). Up-regulation of sphingoid base synthesis has been seen mainly by endotoxin and cytokines, UV irradiation, retinoic acid, corticosteroids, phorbol esters, and resveratrol. Post-transcriptional regulation appears to be involved (D.K. Perry, 2000), especially in the rapid effects of heat shock (G.M. Jenkins, 2001). Interest in SPT is growing with the finding that inhibition of de novo sphingolipid biosynthesis with myriocin has several desirable health outcomes, including inhibition of atherosclerosis (T.S. Park, 2004; E.N. Glaros, 2007) and suppression of HIV replication (T. Umehara, 2006).

3.1.2. Synthesis of the N-acyl derivatives of sphingoid bases

As shown in Fig. 7, sphinganine is acylated to dihydroCers by a family of Cer synthases (CerSs, and sometimes referred to as Lass). The genes required for CerS activity were initially found in yeast (I. Guillas, 2001; B. Vallee, 2005)], then used to find the first gene encoding a mammalian CerS, CerS1 (K. Venkataraman, 2002), which was highly selective for stearoyl-CoA and formation of C_{18}-(dihydro)Cer. Most of the other CerSs are also selective, but to varying degrees (C. Riebeling, 2003; Y. Mizutani, 2005) (Fig. 7). The most definitive proof that the fatty acyl-CoA selectivity resides in CerS has been for CerS5/Lass5, which has been purified and retains selectivity for palmitoyl-CoA (S. Lahiri, 2005). Interestingly, all of the described CerS genes except CerS1 [22] contain a HOX domain, a transcription factor involved in developmental regulation.

Biosynthesis of the appropriate Cer subspecies is apparently very important because head and neck tumors have lower proportions of C_{18}-Cer than does neighboring normal tissue, and transfection of the CerS1/Lass1 gene into head and neck tumor cells in culture suppressed cell growth (S. Koybasi, 2004).

Fungi produce a number of inhibitors of CerS. The most studied are the fumonisins, which are produced by *Fusarium verticillioides*, a common contaminant of corn. Fumonisins were first discovered in a search for the causal factor(s) for human esophageal cancer in the Transkei region of South Africa and two diseases encountered in agricultural animals that eat contaminated food (W.F.O. Marasas, 2001). They were subsequently found to have many pathological effects: liver and kidney toxicity, renal cancer, immunosuppression (and in some cases immunostimulation), and birth defects, with the latter being seen not only in studies with experimental models (W.F.O. Marasas, 2004) but also in epidemiological studies of outbreaks of neural tube defects in the United States (S.A. Missmer, 2006).

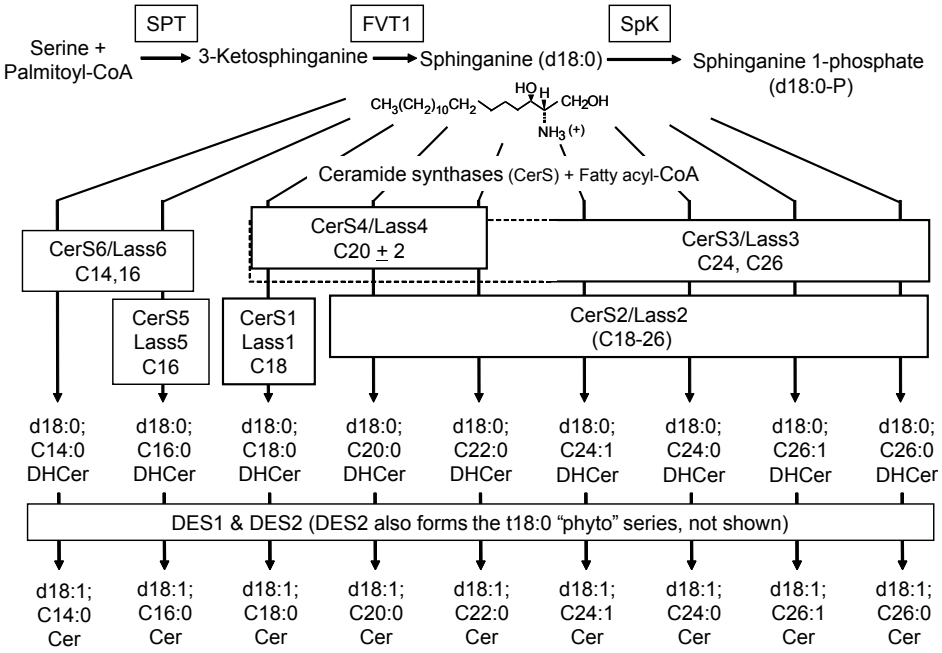

Fig. 7. A biosynthetic pathway for dihydroceramide (DHCer) and ceramide (Cer) backbones of sphingolipids. Shown at top is the de novo biosynthesis of sphinganine (d18:0) by serine palmitoyltransferase (SPT) and 3-ketosphinganine reductase (FVT1), followed by either acylation of sphinganine by CerS/Lass family of ceramide synthases (shown with their fatty acyl-CoA preferences) or phosphorylated by sphingosine kinase (SpK). *N*-acylsphinganines can be incorporated into more complex sphingolipids (Fig. 8) or oxidized to ceramides by dihydroceramide desaturase DES1 and DES2; DES2 is also capable of hydroxylating the 4-position to form 4-hydroxydihydroceramides, t18:0.

Inhibition of CerS by fumonisins (E. Wang, 1991) not only blocks complex sphingolipid formation but also causes accumulation of sphinganine and sphinganine 1-phosphate (M.C. Sullards, 2001). Elevation in both of these highly bioactive species may explain the paradox of how fumonisins can be cytotoxic for some cell types (via the cytotoxicity of sphingoid bases) but mitogenic for others (due to elevation of sphinganine 1-phosphate, which is mitogenic and an inhibitor of apoptosis). In contrast, the induction of neural tube defects appears to be due to the reduction of complex sphingolipid biosynthesis, resulting in a loss of folate transport since the folate-binding protein is a glycosylphosphatidylinositol-anchored protein that requires cholesterol and sphingolipids to function normally (V.L. Stevens, 1997).

Fumonisins have been used widely in studies of the roles of de novo synthesized Cer in cell regulation. Such studies must be interpreted with care because the elevation of sphinganine and sphinganine 1-phosphate may affect additional signaling pathways. In addition, *N*-acetylsphinganine (C_2-dihydroceramide) is also found in fumonisin-treated cells and in vivo (A.H. Merrill, 2001), and recent studies have shown that C_2-Cers can be phosphorylated (H. Van Overloop, 2007). Therefore, the number of potentially bioactive metabolites in fumonisin-treated cells is quite large.

Cer can also be made by reversal of ceramidase; however, this reaction appears to account for relatively little Cer synthesis under normal physiological conditions (S. El Bawab, 2001).

3.1.3. Desaturation and hydroxylation of dihydroceramides to form ceramides and 4-hydroxyceramides (phytoceramides)

The last step of Cer synthesis is the insertion of the 4,5-*trans*-double bond into the sphingoid base backbone, which occurs at the level of dihydroCer (Fig. 7) (D.E. Ong, 1973; J. Rother, 1992). After the genes encoding this family of desaturases were characterized in plants (P. Sperling, 2000), E. Heinz's lab used the sequences to clone and express in yeast the Δ^4-desaturases (dihydroCer desaturases) from humans, mice, *Drosophila*, and *Candida albicans*, as well as a murine bifunctional Δ^4-desaturase/C_4-hydroxylase that appeared to be responsible for the biosynthesis of the 4-hydroxyceramides (phytoceramides) (P. Ternes, 2002). The two mammalian genes are referred to as DES1 and DES2, and when expressed in mammalian cells, DES1 appears to have only desaturase activity whereas DES2 can perform both desaturation and 4-hydroxylation (F. Omae, 2004). In yeast, however, insertion of the 4-hydroxyl group occurs at the level of sphinganine (M.M. Grilley, 2000) and plants (P. Sperling, 2000).

DES would be predicted to play a very important role in cell regulation because dihydroceramides do not appear to be capable of activating many of the signaling targets of ceramides (A. Bielawaka, 1993). Nonetheless, dihydroceramides may have other signaling functions because they have been suggested to mediate at least some of the tumor suppression by γ-tocopherol (Q. Jiang, 2004) and fenretinide [5] (J.M. Kraveka, 2007), apparently via induction of autophagy [5] and inhibition of cell cycle progression (J.M. Kraveka, 2007). New DES inhibitors will be very helpful in further studies [23].

3.2. Biosynthesis of more complex sphingolipids

In mammals, Cer is at the branchpoint for biosynthesis of four major compounds: the two phosphosphingolipids, sphingomyelin (SM) and Cer 1-phosphate (Cer-P), and two glycosphingolipids, galactosylceramide (GalCer) and GlcCer, which are converted into hundreds of complex glycosphingolipids (http://www.sphingomap.com).

The initial head-group additions for Cer are shown in Fig. 8. Other pathways are shown in Refs. [20,24].

3.2.1. Sphingomyelin and ceramide phosphorylethanolamine

SM is synthesized by transfer of phosphorylcholine from phosphatidylcholine to Cer by SM synthase (SMS). This reaction liberates diacylglycerol, thus links glycerolipid and sphingolipid signaling pathways, although it is not known how frequently cells capitalize on this relationship for signaling purposes. There are two SMSs: SMS1 is localized to the Golgi, and SMS2 is localized to the plasma membrane (K. Huitema, 2004). The sequences of SMS1 and SMS2 suggest that they are integral membrane proteins with multiple membrane-spanning core domains (F.G. Tafesse, 2006). Characterization of cloned SMS1 and SMS2 has confirmed that they function as bi-directional lipid choline

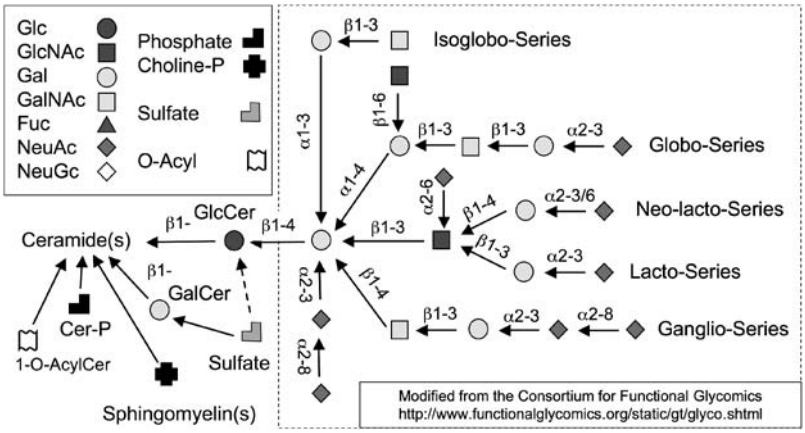

Fig. 8. Head-group additions to ceramide in a schematic representation. Starting with a given molecular subspecies of ceramide (Fig. 7), head groups can be added to make sphingomyelin by addition of a phosphocholine moiety (by transesterification from phosphatidylcholine), phosphate (from ATP), glucose (from UDP–Glc), galactose (from UDP–Gal), or a fatty acid (by transacylation from phosphatidylcholine or phosphatidylethanolamine). Subsequent metabolites are formed by sequential carbohydrate addition (from UDP–sugars except for GDP-fucose, GDP-mannose and CMP–sialic acid) or addition of sulfate.

phosphotransferases capable of converting phosphatidylcholine and Cer into SM and diacylglycerol and vice versa.

Knowledge about the regulation of SM biosynthesis is growing rapidly, with one of the interesting factors being the delivery of Cer to the enzyme. For the Golgi SMS1, this involves a Cer transport protein (CERT) that K. Hanada and co-workers have discovered (Chapter 17) (reviewed in Ref. [25]). CERT transfers Cers having C_{14}–C_{20} chain lengths (but not longer alkyl chains) as well as C_{16}-dihydroCer and phytoCer (K. Kumagai, 2005) from the ER to the *trans*-Golgi. CERT has a phosphoinositide-binding pleckstrin homology (PH) domain for Golgi-targeting and a lipid-transfer START domain. When CERT is phosphorylated, there is an auto-inhibitory interaction between the PH and START domains that inactivates both the phosphoinositide-binding and Cer transfer. Loss of SM and cholesterol from cells causes dephosphorylation of CERT, thereby activating Cer transport and restoring SM biosynthesis (K. Kumagai, 2007).

SM biosynthesis is modified by many additional factors, including 25-hydroxycholesterol, pioglitazone, expression levels of ABC proteins, development, and aging (M. El Alwani, 2005).

When SM metabolism is altered, it affects many aspects of cell behavior, such as the sensitivity of cells to proapoptotic agents (A.H. Van der Luit, 2006) and photosensitized photodamage (D. Separovic, 2007). In addition, SMS overexpression in mice causes significant decreases in HDL-SM and HDL-cholesterol and increases in non-HDL-SM and non-HDL cholesterol, which are signs of a higher atherogenic potential (J. Dong, 2006).

Much less is known about regulation of Cer phosphorylethanolamine synthesis. There is in vitro evidence, however, that Cer phosphorylethanolamine can be synthesized

from phosphatidylethanolamine and Cer in a reaction analogous to that catalyzed by SMS, and, once formed, can be methylated to SM (M. Malgat, 1986; M.N. Nikolova-Karakashian, 2000). The phosphosphingolipids of other organisms (such as the inositolphosphoceramides of yeast) have been studied more extensively. They are formed by transesterification (from phosphatidylinositol) (R.C. Dickson, 1999; A.S. Fischl, 2000) by transferases that share a conserved structural motif across yeast and pathogenic fungi, and which resembles somewhat a motif in lipid phosphatases (S.A. Heidler, 2000). Turnover of these inositolphosphoceramides involves a phosphohydrolase that is homologous to mammalian SMases (L.A. Cowart, 2006).

3.2.2. Glycosphingolipids

Pathways for the biosynthesis of the hundreds of glycosphingolipids (Fig. 8, Refs. [20,24], and www.sphingomap.org) appear complex, but are achieved using surprisingly few glycosyltransferases that commit precursors and intermediates to predictable products based on the specificities of the enzymes. The enzymes transfer a specific sugar from the appropriate sugar nucleotide (e.g., UDP–Glc, UDP–Gal, and CMP–sialic acid) to Cer or to the non-reducing end of the growing carbohydrate chain attached to Cer. The glycosyltransferases often recognize mainly the carbohydrate portion of the acceptor glycosphingolipid; however, there are instances where the backbone is a factor — such as the preferential incorporation of Cers with α-hydroxy fatty acids into GalCer (and sulfatides), whereas those with non-hydroxy-fatty acids are used to make GlcCer (I. van Genderen, 1995).

3.2.2.1. Biosynthesis of GlcCer and GalCer. GalCer and GlcCer are synthesized by UDP–Glc:glucosylceramide synthase (CGlcT) and UDP–Gal:galctosylceramide synthase (CGalT), respectively, the genes for which have been identified in numerous organisms (reviewed in T. Tencomnao, 2001). Relatively little is known at a biochemical level about these enzymes; however, CGlcT has a strongly hydrophobic Golgi anchor segment near the N-terminus (S. Ichikawa, 1996) and a catalytic C-terminal located in the cytosol (D.L. Marks, 1999). A growing number of factors have been found to regulate expression of CGlcT, including depletion of the amounts of GlcCer in the cell (A. Abe, 1996; I. Meivar-Levy, 1999), elevations in Cer (H. Komori, 2000), endotoxin and acute phase response mediators (R.A. Memon, 2001), anoxia and reoxygenation of endothelial cells (H. Zhao, 2003), and doxorubicin (Y. Uchida, 2004). GlcCer and protein-bound GlcCer play major roles in establishing the permeability barrier of skin, and CGlcT is up-regulated during epidermal barrier development (K. Hanley, 1997; R. Watanabe, 1998; T. Doering, 1999).

The synthesis of GlcCer can be inhibited by structural analogs of Cer (A. Abe, 2001). Use of these inhibitors has revealed how decreases in cellular levels of neutral glycosphingolipids and gangliosides (and elevation of Cer) causes cell cycle arrest (C.S.S. Rani, 1995), and how GlcCer synthesis appears to be a major determinant of survival of tumor cells (Y.Y. Liu, 2001; A. Senchenkov, 2001; V. Gouaze, 2005). Both inhibitors and gene knockout have been used to explore the consequences of severe GlcCer deficiency, and although elimination of detectable glycolipids is not lethal for some cell lines (S. Ichikawa, 1994), CGlcT knockout is embryonically lethal in mice (T. Yamashita, 1999).

Whereas GlcCer is synthesized on the cytosolic side of the Golgi apparatus (H. Coste, 1986; A.H. Futerman, 1991; D. Jeckel, 1992), GalCer is synthesized in the lumen of

the ER (H. Sprong, 1998, 2003). CGalT contains an ER retrieval signal (KKVK) at the C-terminus. The N-terminus, which is thought to contain the active site, faces the lumen of the ER (H. Sprong, 1998). UDP–Gal is transported to the lumen of the ER by UDP–Gal transporter 2 (UGT2), a splice variant of UGT1 (the transporter for UDP–Gal into the Golgi) that contains an ER-locating dilysine motif (KVKAS) (R. Kabuss, 2005). CGalT is highly expressed in oligodendrocytes and Schwann cells, with a pattern that matches that of myelination (A. Bosie, 1996), and the disruption of the mouse gene encoding CGalT leads to unstable myelin and progressive paralysis in mouse models (S.N. Fewou, 2005).

3.2.2.2. Biosynthesis of more complex glycosphingolipids. After GlcCer is made on the cytosolic aspect of the ER and/or early Golgi membranes (A.H. Futerman, 1991; D. Jeckel, 1992), the GlcCer must flip to the inside of the Golgi because the more complex glycosphingolipids are made in the lumen of the Golgi apparatus (H. Lannert, 1994). Studies with rat liver ER and Golgi membranes have found that transbilayer movement of spin-labeled GlcCer is rapid, saturable, and inhibitable by protease treatment, which suggests that the membranes contain a GlcCer flippase (X. Buton, 2002). The multiple-drug resistance pump (MDR1) has been found to be one of the transporters for GlcCer (M.F. De Rosa, 2004), but it appears to be involved only in the biosynthesis of neutral glycosphingolipids but not gangliosides, even though they theoretically share some of the same precursors (Fig. 8). These observations imply that there may be subcompartmentation within the Golgi for even early steps of glycosphingolipid biosynthesis, perhaps created by cooperation of enzymes that are responsible for forming a particular series of glycolipids.

Thus, the nature of the subsequent glycosylation reactions depends on the particular glycosyltransferases that are expressed in a given cell type (and their localization), the availability of the substrates, the presence of enzymes that may be competing for the same intermediates (and the kinetic properties of these enzymes), and the rates at which the precursors and products are trafficked through the Golgi. Since not all of the genes encoding the glycosyltransferases that are responsible for glycosphingolipid biosynthesis have been assigned, to keep abreast of this rapidly evolving subject, one can visit the Web sites for:

1. the Consortium for Functional Glycomics (http://www.functionalglycomics.org/),
2. the Complex Carbohydrate Research Center at the University of Georgia (http://www.ccrc.uga.edu/~moremen/glycomics/),
3. KEGG (http://www.genome.jp/kegg/glycan/GT.html),
4. LIPID MAPS (www.lipidmaps.org), and
5. other groups that are building bioinformatics tool to search these databases, for example: http://www.genedb.org/genedb/pathway_comparison_TriTryp/).

Thus, as a first level approach, one can attempt to predict which glycans will be present from gene expression profiles (and vice versa). As an example of the application of this strategy, the murine glycosyltransferases responsible for the expression of globo-series glycolipids have been analyzed with respect to the expression of Gb3 synthase and Gb4 synthase mRNAs versus the distribution of the products (Y. Fujii, 2005). Additional bioinformatics tools are being developed to predict glycan compositions based on knowledge about glycosyltransferase gene expression profiles (S. Kawano, 2005).

The partner for such computational approaches is to determine an in-depth glycan profile. An integrated mass spectrometric strategy has been developed to characterize the glycosphingolipids from cells based on release of the glycans using Cer glycanase followed by identification of the glycans using MS (S. Parry, 2007). This is a powerful approach, qualified mainly by its loss of information about the nature of the lipid backbones for these species, as well as the likelihood that some glycolipids may not be cleaved by Cer glycanase.

3.2.2.3. Combinatorial ganglioside biosynthesis. Another principle of glycosphingolipid biosynthesis is illustrated by the pathways for formation of the major gangliosides of most tissues. As illustrated in Fig. 9, gangliosides are synthesized by the stepwise transfer of neutral sugars and sialic acids by membrane-bound glycosyltransferases that are located in the regions of the Golgi apparatus that generally correspond to the order in which the sugars are added. For example, the sialyltransferase (SAT I in Fig. 9) catalyzing the synthesis of GM3 is in the *cis* Golgi, whereas the enzymes involved in later steps of ganglioside synthesis are localized in the more distal *trans* Golgi network.

There has been considerable progress in identification of the genes responsible for the key reactions in Fig. 9 [24], allowing these relationships to be tested by transfecting

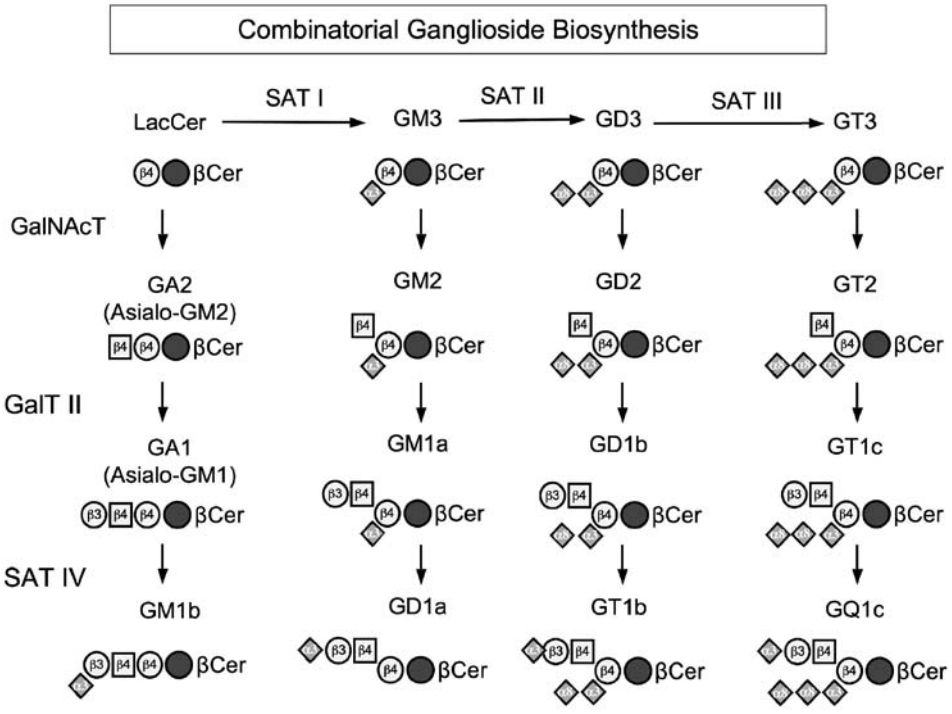

Fig. 9. Combinatorial nature of glycosphingolipid biosynthesis (modified from T. Kolter and K. Sandhoff, 2002). Glycosphingolipids are shown using symbols from Fig. 8. SAT refers to sialyltransferase; and GalNAcT and GalT to glycosyltransferases for UDP–GalNAc and UDP–Gal, respectively.

cells with the cDNAs encoding enzymes of this pathway and determining the types of glycosphingolipids that are elevated. For example, transfection of GalNAc-transferase cDNA into Chinese hamster ovary cells, which normally make mainly GM3, produced cells that now synthesize mainly GD2, whereas transfection of cells that are defective in sialylation yields GA2 (M.S. Lutz, 1994). The findings to date support the view that ganglioside synthesis can be viewed as a series of 'combinatorial' reactions (Fig. 9) that are catalyzed by a conservative number of key enzymes such that the ultimate ganglioside composition is determined by the relative activities of these enzymes, the availability of their substrates [26], and perhaps their sub-Golgi localization (A.S. Uliana, 2006).

Regulation of ganglioside biosynthesis involves both transcriptional and post-transcriptional factors, as has been elegantly reviewed for the nervous system [27]. Transcriptional control of key glycosyltransferases appears to account for many of the developmentally regulated tissue-selective variations in ganglioside amounts and types in mammalian organs, including large changes upon oncogenic transformation (R.W. Ledeen, 1998; S. Hakomori, 1998). The biosynthesis of gangliosides is also controlled through post-translational modification of glycosyltransferases (R.K. Yu, 2001) and factors such as the density of cells in culture (i.e., contact inhibition) (Z. Vukelic, 2001).

3.2.2.4. Sulfated glycosphingolipids. Sulfatide (3′-sulfo-GalCer) synthesis is catalyzed by GalCer sulfatotransferase, which utilizes the activated sulfate donor 3′-phosphoadenosine-5′-phosphosulfate. The cDNA encoding the sulfotransferase has been cloned (K. Honke, 1997) and knockout mice have been generated to analyze the biological roles of sulfoglycolipids and pathophysiology of their deficiency, which included neurological disorders due to myelin dysfunction and amelioration of monocyte infiltration in the kidney after ureteral obstruction, which provides evidence that sulfatide is an endogenous ligand of L-selectin (K. Honke, 2004). Investigation of the consequences of accumulation of sulfoglycolipids has also been conducted in arylsulfatase A-deficient mice (M. Molander-Melin, 2004). Regulation of sulfatide biosynthesis appears to reside in the activity of this sulfatotransferase.

4. Sphingolipid catabolism

Complex sphingolipids are lost from cells by multiple processes: (1) membrane internalization, recycling, and degradation; (2) turnover of membrane sphingolipids to release bioactive products that participate in cell signaling; and (3) secretion and shedding of sphingolipids from cells.

In general, sphingolipids are internalized with endocytic vesicles, sorted in early endosomes, and recycled back to the plasma membrane (often with remodeling of the sphingolipid) [28] or transported to lysosomes where they are degraded by specific acid hydrolases [12]. Given that lysosomal membranes are rich in sphingolipids, it has been unclear why they, too, do not undergo hydrolysis if the endocytosed membranes and lysosomal membranes are simply fused. This dilemma was solved when it was shown (W. Furst, 1992; W. Mobius, 1999) that endocytosed sphingolipids (and presumably other components) become invaginated into intraendosomal vesicles that are delivered into

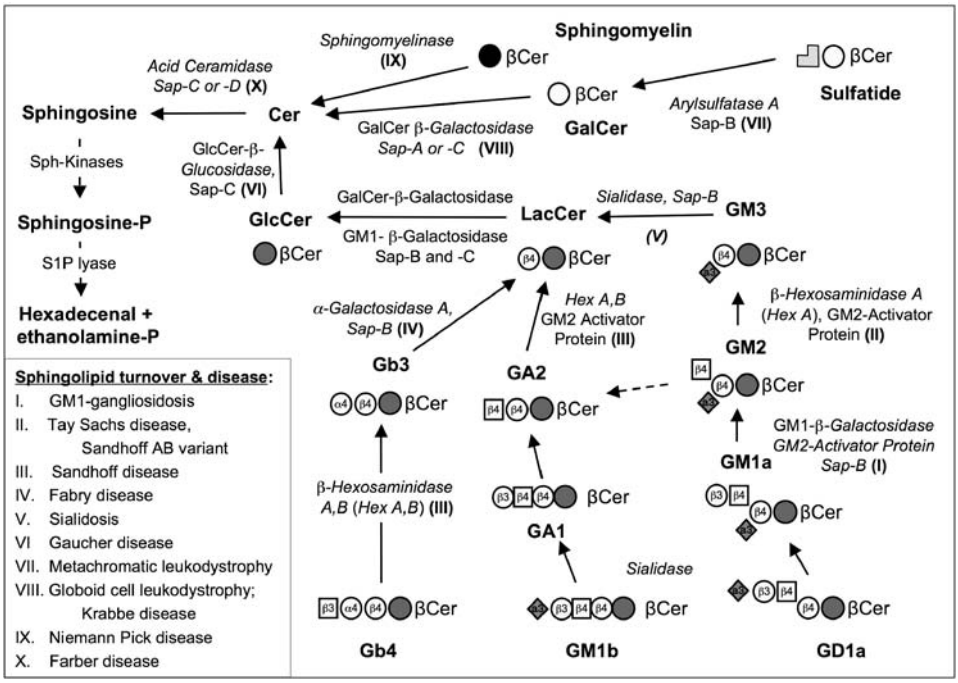

Fig. 10. Catabolism of complex sphingolipids and diseases associated with deficiencies in the enzymes (modified from T. Kolter and K. Sandhoff, 2006). Abbreviations are as in the text.

the lumen of the lysosome. Thus, hydrolytic enzymes contact the sphingolipids to be digested in the lumen rather than as part of the lysosomal membrane, which is additionally protected by an elaborate glycocalyx that lines the inner leaflet.

The major pathways for sphingolipid catabolism are summarized in Fig. 10. It is noteworthy that most of the steps have been associated with genetic diseases, except for the hydrolysis of LacCer, which is due to the presence of two enzymes — each of which can cleave that glycosphingolipid. Also noteworthy is that all degradative pathways converge on Cer. Many cells, nonetheless, contain at least small amounts of sphingosylphosphocholine and galactosylsphingosine (psychosine), for which receptors have been found, but they are probably produced in such small amounts that these sphingolipids do not contribute much to overall sphingolipid homeostasis.

4.1. Sphingomyelin

The lysosomal hydrolysis of SM to Cer and phosphocholine is catalyzed by acid SMase, a water-soluble, lysosomal glycoprotein that interacts with a sphingolipid-activator protein (SAP) and anionic lipids such as bis(monoacylglycerol)phosphate (BMP) to hydrolyze interfacial SM (T. Linke, 2001). Acid SMase possesses an N-terminal SAP-homology domain that appears to stabilize the correctly folded form of acid SMase as well as to facilitate the interfacial interaction with substrate in vivo (M. Kolzer, 2004). Acidic SMase is also secreted by many types of cells (I. Tabas, 1999; I. Takahashi, 2005).

Defects in the acid SMase gene (SMPD1) result in Niemann–Pick disease types A and B. Individuals with either form display accumulation of SM in reticuloendothelial cells scattered throughout the spleen, bone marrow, lymph nodes, liver, and lungs, but type A is the acute, lethal neuronopathic form. The severity of the disease has sometimes been related to the nature of the genetic defect (T. Takahashi, 1992) and the level of residual lysosomal SMase activity (D. Graber, 1994). However, the entire spectrum of phenotypic variation — ranging from subclinical retinal involvement to severe ataxia, cognitive deficits, and psychiatric disorders — has been found in even a cohort of patients who were homozygous for the same ancestral mutation W391G (V. Mihaylova, 2007). SM also accumulates in Niemann–Pick disease type C; it is not due to genetic defects in SMase per se but to defects in either the NPC-1 or NPC-2 genes, which encode proteins involved in cholesterol trafficking (M.C. Patterson, 2003) (Chapter 17).

Acid SMase-deficient mice have been generated (K. Horinouchi, 1995) and have been useful both as models for Niemann–Pick disease and to study the roles of acid SMase in cell signaling (P. Santana, 1996; D.J. Sillience, 2001). Cer derived from aberrantly high acid SMase activity also appears to play a role in disease because activation of acid SMase and generation of Cer has been found to play a key role in the liver cirrhosis and anemia in Wilson disease, which is caused by accumulation of Cu(2+) in cells (P.A. Lang, 2007).

Cells also have neutral SMases [29] that are found in multiple cellular compartments, including the plasma membrane and the nuclear membrane. At least one form of neutral SMase resides in sphingolipid-enriched microdomains and is inhibited by the caveolin-scaffolding domain (R.J. Veldman, 2001). Three neutral SMases have been cloned (nSMase1, nSMase2, and Smpd3) and at least one of their functions is to participate in cell signaling [29]. A human intestinal alkaline SMase gene has also been cloned and shown to encode a novel ecto-enzyme involved in the digestion of dietary SM (R.D. Duan, 2003).

All of the above SMases are ‘C’ type SMases that produce Cer and choline phosphate; an SMase D (which produces Cer 1-phosphate and choline) is found in the venom of brown recluse spider, *Corynebacterium pseudotuberculosis* (which commonly infects sheep), *Vibrio damsela* (an aquatic bacterium that causes wound infections in humans), and the human pathogen *Arcanobacterium haemolyticum*. The venom SMase D produces much of the tissue damage caused by these organisms (A.P Truett, 1993) by sustained activation of inflammation (S.H. Farsky, 2005). Interestingly, SMase also acts as a lysophosphatidylcholine phosphodiesterase to produce lysophosphatidic acid, another inflammatory mediator (L.A. van Meeteren, 2004).

4.2. Glycosphingolipids

Glycosphingolipids are catabolized by the stepwise hydrolysis of the terminal monosaccharides through the concerted action of a series of specific exoglycosidases (Fig. 10) [12]. There is often a requirement for SAPs, SAP-A, -B, -C, or -D, and GM2-activator protein, which is required for GM2 hydrolysis but can also accommodate other glycosphingolipids. Acidic lipids, and in particular BMP, are additionally involved in vivo as stimulators of glycosidase activity and are components of the lysosomal perimeter membrane.

A number of inherited diseases are caused by mutations in the structural genes encoding these enzymes and activator proteins that result in reduced enzymatic activity, loss of the appropriate targeting signals for transport to lysosomes, or alteration of the domains that interact with other subunits of the enzyme and/or activator proteins (Fig. 10). The GM2 gangliosidoses often serve as a prototype for how sphingolipidoses can result from several types of genetic defects since these diseases arise from mutations in the α or β subunits of hexosaminidases A, B, or S, or of the GM2-activator protein (R.A. Gravel, 1995) [12]. Hex A is an αβ heterodimer that degrades negatively charged and uncharged substrates, whereas Hex B is the ββ homodimer that cleaves mainly *N*-acetylgalactosamine residues from uncharged substrates such as GA2, globotetraosylceramide, and oligosaccharides (Fig. 10). Therefore, mutations in the structural gene for the α subunit result in partial or complete loss of Hex A activity (Tay–Sachs disease), and mutations in the β subunit affect both Hex A and Hex B (Sandhoff disease). Hex S is a homodimer of αα subunits, but is of secondary importance for GM2 hydrolysis and participates in sulfatide hydrolysis (S.T. Hepbildikler, 2002). The phenotypes of these two gangliosidoses are similar, but they are easily distinguished by measuring Hex A and Hex B activities and by the accumulation of Gb_4Cer in Sandhoff disease but not in Tay–Sachs disease (GM2 accumulates in both disorders). The AB variant has normal Hex A, B, and S activity when measured in vitro, but there is a defective (or absent) GM2-activator protein.

Mouse models are available for Tay–Sachs disease (α-chain deficiency), Sandhoff disease (β-chain deficiency), and GM2-activator deficiency (T. Kolter, 1998). The mice have phenotypes that are only slightly different from human GM2 gangliosidoses. However, the severity and course of the disease differs, and this has been attributed to species differences in the sialidase activity of the mouse versus human. The mouse sialidase accepts GM2 as a substrate (shown by the dashed arrow in Fig. 10) (K. Sango, 1995) and converts it to GA2, which cannot be further degraded since the responsible enzyme is also deficient.

The reasons that these biochemical defects trigger the neurodegeneration seen in the GM2 gangliosidoses are not fully understood. However, an inflammatory response involving macrophage/microglial cells (and mediators that they produce, such as macrophage-inflammatory protein 1α) is important because elimination of macrophage-inflammatory protein 1α expression resulted in a substantial decrease in macrophage/microglial cell infiltration and pathology, and reduced neuronal apoptosis. Furthermore, Sandhoff disease mice showed improved neurological status and a longer lifespan (Y.P. Wu, 2004). Mouse models have also been useful for studies of the effectiveness of gene therapy using primary microglial cells (D. Tsuji, 2005) and hippocampal neurons from embryonic Sandhoff mice (A. Arfi, 2006) and in vivo by injection of a non-replicating Herpes simplex vector encoding the Hex A alpha-subunit (S. Martino, 2005) and stereotaxic intracranial inoculation of adeno-associated viral vectors with the complementing β-hexosaminidase A (M.B. Cachon-Gonzalez, 2006).

4.3. Ceramide

The major pathway for catabolism of the Cer backbone is shown in Figs. 10 and 11, which illustrate the complexity of Cer metabolism.

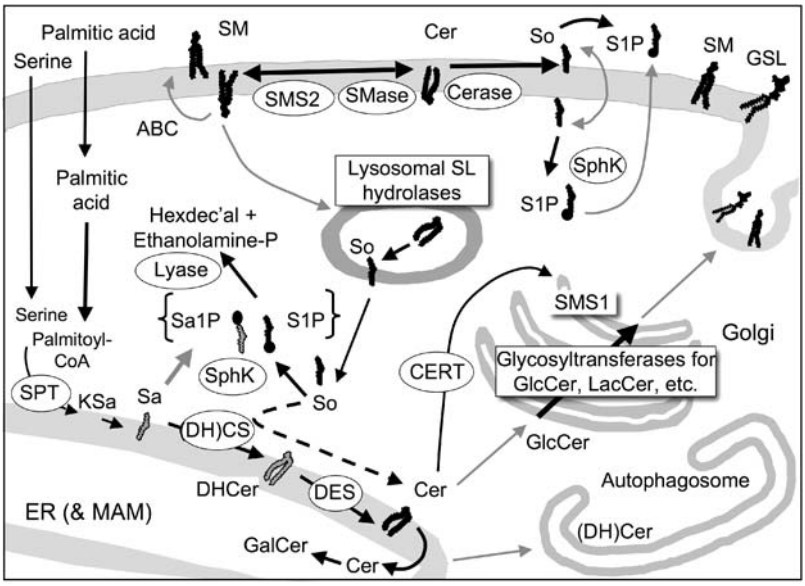

Fig. 11. Cellular sphingolipid dynamics. The diagram shows the fate of sphingolipids made de novo in the endoplasmic reticulum (ER) and mitochondria-associated membranes (MAM) from serine and palmitoyl-CoA by serine palmitoyltransferase (SPT) to sphinganine (Sa), and formation of dihydroceramide (DHCer) by ceramide synthases (CS) and desaturation of DHCer to Cer by DHCer desaturase (DES). Cer is trafficked by vesicles to the *cis*-Golgi or by CERT to the *trans*-Golgi, where head groups are added for glycosphingolipids (GSL) and sphingomyelin (by SM synthase, SMS1). The exception is GalCer, which is made in the lumen of the ER. After GlcCer synthesis, the glycosphingolipids are made in the lumen of the Golgi and subsequently transported to the plasma membrane. SM is also synthesized in the plasma membrane by SM synthase (SMS2) and is turned over via sphingomyelinases (SMase) to Cer and the downstream metabolites sphingosine (So) and sphingosine 1-phosphate (S1P). The turnover of sphingolipids occurs in several intracellular locations (including autophagosomes), but for simplicity, the production in the plasma membrane and vesicles such as lysosomes are shown to illustrate how the products can be recycled, degraded (via S1P lyase, which also can turn over sphinganine 1-phosphate, Sa1P), or in the case of S1P, secreted from the cell (S1P is also synthesized extracellularly). Transbilayer movement of sphingolipids involves transporters such as ATP-binding cassette (ABC) family pumps.

In lysosomes, Cers are hydrolyzed to free sphingoid bases and long-chain fatty acids by a ceramidase that has an acidic pH optimum. The lysosomal ceramidase is a water-soluble glycoprotein that hydrolyzes membrane-bound Cer in an interfacial reaction that requires stimulation by SAP-C or SAP-D and anionic phospholipids such as BMP (A. Klein, 1994; T. Linke, 2001). The human acidic ceramidase is a heterodimer of 40-kDa and 13-kDa subunits synthesized as a single precursor polypeptide and is targeted to the lysosome via the mannose 6-phosphate receptor (K. Ferlinz, 2001).

Additional ceramidases with neutral and alkaline pH optima have been found in various cell compartments (M. Tani, 2000, 2005) including mitochondria (El Bawab, 2000) and appear to be involved in signaling. A neutral ceramidase is expressed in human intestine and released into the intestinal lumen (L. Ohlsson, 2007), and mice lacking this

ceramidase are deficient in the intestinal degradation of Cer (M. Kono, 2006) and are also more susceptible to colon cancer (R.L. Proia, personal communication).

4.4. Sphingosine

The first step in the turnover of sphingosine is phosphorylation to S1P by ATP-dependent sphingosine kinases (SphK) that fall in two gene families (SphK1 and SphK2) [30]. Two forms of SphK1 are expressed in mice (A. Kihara, 2006) and although they differ in only a few N-terminal amino acids, they have different enzyme activities and stabilities. SPHK1a is present mostly as monomers whereas SPHK1b is lipid-modified, which reduces activity and favors formation of homo-oligomers.

SphK2 is structurally similar to SphK1 (S. Spiegel, 2007), with five evolutionarily conserved domains. The two kinases differ mainly in the central region and N-terminus, and have a putative BH3 domain. SphK2 has been localized to the nucleus and ER, and differs in kinetic properties from SphK1.

Little is known about the regulation of SphK2, but SphK1 is activated by stimulation of cells by factors such as phorbol ester or VEGF, and this appears to be via activation of ERK2, phosphorylation of SphK1 on serine 225, and translocation to the plasma membrane. Calcium has also been suggested to induce translocation of SphK1 to the plasma membrane via an association of Ca^{2+}/calmodulin with SphK1 (C.M. Sutherland, 2006). SphK1 and SphK2 have opposing roles in the regulation of Cer biosynthesis. Overexpression of SphK2 increases Cer synthesis whereas SphK1 decreases it, and conversely, down-regulation of SphK2 reduces sphingosine incorporation into Cer whereas down-regulation of SphK1 increases Cer synthesis (M. Mayceka, 2005).

A wide range of sphingoid bases are phosphorylated by sphingosine kinases, including analogs such as the clinical immunosuppressant FTY720, which is phosphorylated by SphK2. This is thought to be required for FTY720 to serve as an agonist for S1P receptors; however, unphosphorylated FTY720 can also target the CB1 cannabinoid receptor (S.W. Paugh, 2006). Much effort is being expended in the development of sphingosine kinase inhibitors (J.W. Kim, 2005; K.J. French, 2006) because S1P is involved in cancer cell growth and migration, as well as other important biological functions. In addition, an antibody against S1P has been reported to be effective in cancer suppression (R.A. Sabbadini, 2006).

If S1P is not secreted or dephosphorylated by specific S1P phosphatases, as well as by more general lipid phosphatases, it is cleaved irreversibly to ethanolamine phosphate and *trans*-2-hexadecenal by S1P lyase (J. Zhou, 1998; P.P. Van Veldhoven, 2000). As shown first in the 1970s by W. Stoffel and coworkers, the phosphoethanolamine can be utilized for the synthesis of phosphatidylethanolamine (Chapter 8), and *trans*-2-hexadecenal can be reduced to the alcohol and incorporated into alkyl ether lipids. Under certain conditions, degradation of sphingoid bases can account for as much as one-third of the ethanolamine in phosphatidylethanolamine (E.R. Smith, 1995). It is interesting that both the first enzyme of sphingoid base metabolism (SPT) and the last enzyme, the lyase, are pyridoxal 5′-phosphate-dependent.

S1P lyase expression is significantly down-regulated in human colon cancer tissues (as are S1P phosphatases), which should increase the stability of S1P that might contribute to carcinogenesis (B. Oskouian, 2006).

5. Overlaps between backbone sphingolipid signaling and sphingolipid metabolism

As is evident from Fig. 11, the biosynthesis and turnover of sphingolipids is a complex process that involves not only the production of many highly bioactive compounds (sphingoid bases, Cers, etc.) but also the correct balancing of de novo biosynthesis versus recycling of existing compounds, the trafficking of compounds to the correct intracellular and extracellular destinations, and coordination of cellular sphingolipid homeostasis with sphingolipids that the cells take up from extracellular lipoproteins and other sources.

No study has integrated all these facets of sphingolipid metabolism, although G. Tettamanti has given an excellent discussion of the major issues for the salvage pathways for glycosphingolipid metabolism [28]. Some expansion of our thinking about sphingolipid trafficking and turnover will be needed to accommodate the finding that autophagy not only provides another pathway for turnover of cellular constituents but also that autophagy can be regulated by Cer and S1P (R. Ghidoni; 1999; G. Lavieu, 2006) and by dihydroCer [5]. It is therefore not surprising that not only the bioactive 'signaling' metabolites that are produced by complex sphingolipid turnover, but also the intermediates of de novo synthesis, can profoundly affect cell behavior, and even cause disease. Indeed, sphingolipid biosynthesis can be described as both 'necessary and dangerous' [21]. There are now a large number of natural agonists, drugs, toxins and toxicants, and even intermediates of common metabolic pathways (such as palmitoyl-CoA) that can alter cell behavior at least in part by affecting sphingolipid biosynthesis.

Fig. 12 summarizes the cellular targets that currently appear to be regulated by backbone sphingolipids and these should be borne in mind because whenever the level of one sphingolipid is modulated, it is likely that others also change, especially when sphingolipids are added to cells exogenously in amounts typically higher than the cell usually produces.

6. Sphingolipidomics

To appreciate fully how many mediators contribute to a given cell behavior, one needs to know the types and amounts of numerous species. Hence, a 'sphingolipidomic' analysis is likely to uncover changes in metabolites and regulatory interactions that were not necessarily the focus of a particular study. Although methods are not yet available to measure all of the sphingolipidome, substantial progress has been made toward this goal. Sphingolipids can be analyzed by a variety of methods (for an overview, volumes 311 and 312 of *Methods in Enzymology* [20], which cover many aspects of sphingolipid analysis by traditional means such as thin-layer chromatography and immunochemistry). However, the most powerful approach today is tandem MS using various ionization methods (mainly electrospray ionization and matrix-assisted laser desorption) and tandem mass analyzers that are best matched to the project needs, such as triple quadrupole or tandem quadrupole–linear ion trap for MS/MS and MS^3 [6,7], respectively, or quadrupole time-of-flight (Z. Vukelic, 2006) or Fourier transform MS (V.B. Ivleva, 2004; Z. Vukelic, 2005) for higher mass accuracy. The methods currently available are able to analyze useful

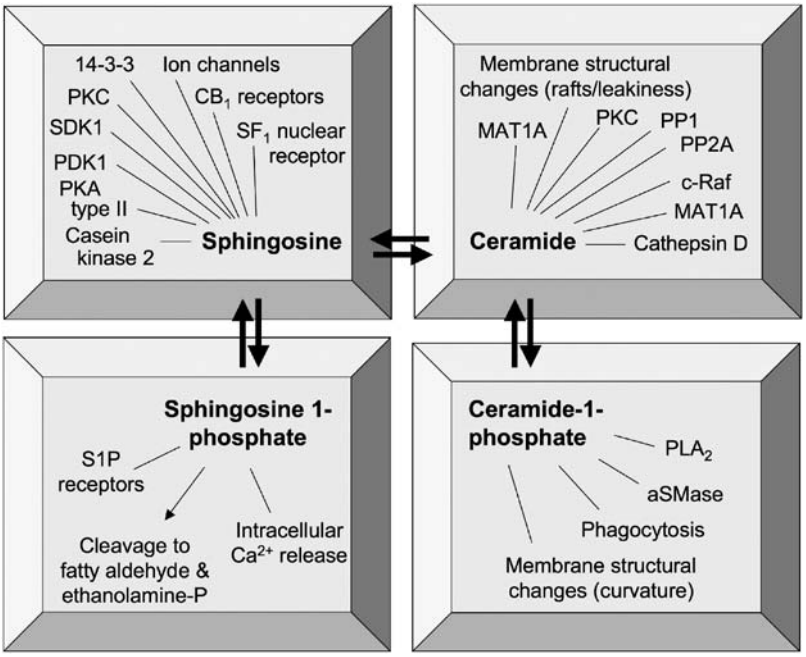

Fig. 12. Some signaling targets and pathways affected by sphingolipid backbones that are metabolically interrelated. PKA, protein kinase A; PDK1, 3-phosphoinositide-dependent kinase 1; SDK1, sphingosine-dependent kinase 1; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; aSMase, acid sphingomyelinase; PLA_2, phospholipase A_2; S1P, sphingosine-1-phosphase; MAT1A, methionine adenosyltransferase (liver specific); SF1, steroidogenic factor 1. The biophysical properties of ceramides and ceramide 1-phosphates may play important roles in membrane structure, including tendencies to form rafts, membrane curvature, and leakiness.

subsets of the sphingolipidome such as: all of the biosynthetic intermediates to, and including, the branchpoint where Cer is incorporated into phosphosphingolipids (SM, Cer phosphoethanolamine, or Cer phosphate), glycoshingolipids (GlcCer and GalCer), or turned over to the backbone sphingoid base (A.H. Merrill, 2005); all of the backbone subspecies that are involved in cell signaling (A.H. Merrill, 2005; A. Bielawski, 2006); sulfatides (in Alzheimer's disease) (H. Cheng, 2003); and lipids that are enriched in viral membranes (B. Brugger, 2006) or detergent-resistant membranes (M. Valsecchi, 2007), lipoproteins (U. Sommer, 2006), tissues such as hippocampus (M. Vukelic, 2006), and in sphingolipid storage diseases such as Fabry's disease (D. Touboul, 2005; T. Fujiwaki, 2006). The glycan survey method using Cer glycanase [8] can be used to complement these methods even though it does not give information about the lipid backbone.

6.1. Use of mass spectrometry for sphingolipidomics

MS is not a 'one size fits all' technology. Therefore, it is useful to understand some of its principles, limitations, and promising new applications. The first challenge in MS analysis is to determine conditions where the compounds of interest are converted to

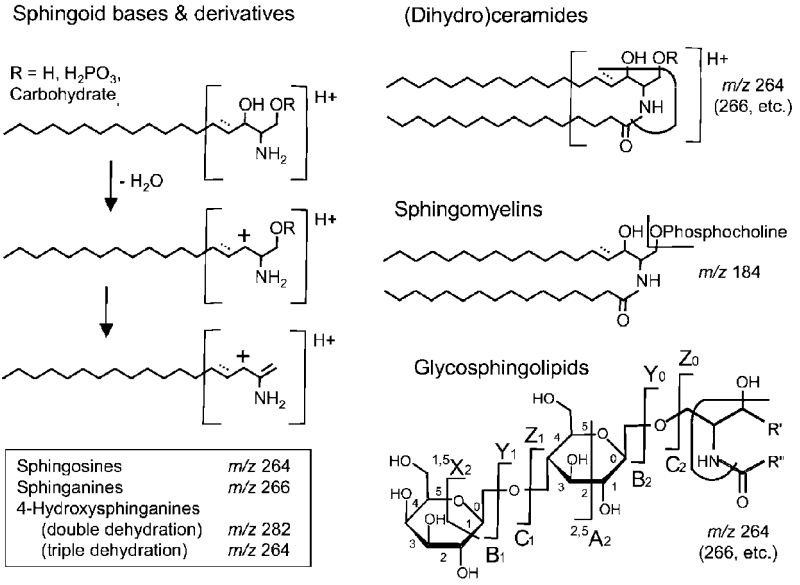

Fig. 13. Major fragmentation sites of sphingolipids that are useful for analysis by electrospray tandem mass spectrometry [6].

ions that are useful as unique and sensitive identifiers. Fortunately, sphingolipids are relatively easily analyzed because, as shown in Fig. 13 and reviewed in Ref. [6], using electrospray ionization: (i) long-chain bases and many complex sphingolipids readily form $(M + H)^+$ ions (i.e., positive ions from the original molecule plus a proton) in the positive ion mode; (ii) sphingoid base 1-phosphates, sulfatides, SMs, and gangliosides form strong $(M - H)^-$, $(M - 15)^-$ or $(M - nH)^{n-}$ ions, respectively, in negative ion mode; and (iii) the fragmentation profiles for most sphingolipids provide information about the head groups and backbone sphingoid bases and fatty acids, provided that one has the capacity for tandem MS (MS/MS and MS^n) analysis.

A common analytical complication for sphingolipids is for more than one compound to give the same *m/z*, for example , the isomers GlcCer versus GalCer or gangliosides GM1a versus GM1b (if they have the same backbones). These may be distinguishable by their fragmentation patterns using tandem MS, but it is often desirable for them to be resolved chromatographically for unambiguous quantitation in complex mixtures. Fortunately, various types of chromatography (liquid and thin-layer) have been used alone and in combination with MS for analysis of sphingolipids for decades [20].

For MS analysis to be quantitative, investigators must have access to a sufficiently complete series of internal standards that can be added to samples so their recoveries can be used to account for differences in extraction efficiency, ionization, and fragmentation. This is still a major limitation to a complete, quantitative sphingolipidomic analysis; however, a number of individual internal standards and internal standard 'cocktails' have been identified by the Lipid Maps Consortium (http://www.lipidmaps.org) and are commercially available from Avanti Polar Lipids (Alabaster, AL). A related challenge is to

obtain information about metabolic flux of sphingolipids, since an MS measurement will reveal the amount of a compound, but not its origin. The latter is usually determined by adding a stable isotope precursor that is incorporated into the compounds of interest, then analysis of the different isotopomers by MS (K.Y. Tserng, 2004) [6].

6.2. Use of mass spectrometry for tissue imaging

What is missing from most lipidomics analysis is the ability to locate which cells contain the analytes that are being measured in an extract from a tissue or cell culture. Fortunately, MS methods have also been developed for analysis of sphingolipids in situ using samples such as thin slices of tissue, cultured cells, or model membranes. In one approach, tissue slices are placed on a metal plate, imbedded as uniformly and non-disruptively as possible with a compound that can be used for matrix-assisted laser desorption mass spectrometry (i.e., a compound that will absorb laser light, volatilize, and ionize nearby compounds of interest in the sample), then a laser beam is moved incrementally across the sample to generate discrete mass spectra over the selected *m/z* range (S. Roy, 2006; A.S. Woods, 2006; S.N. Jackson, 2007). The specific *m/z* of interest can be selected and plotted in *x*, *y*-space to yield a molecular image of the biological sample that can be cross-referenced with more traditional histological markers. The resolution of this technique is ~50–100 μm; therefore, each data set is usually for a small number of cells. Other types of sample ionization have also proven useful for imaging MS, such as secondary ion MS (P. Sjovall, 2004; S. Roy, 2006, K. Borner, 2006) and desorption electrospray ionization (J.M. Wiseman, 2006 ; M. Kraft, 2006).

When applied to brain tissue, imaging MS showed that the cerebellar cortex contains small amounts of SM but larger amounts of sulfatides and gangliosides GM1, GD1, and GT1, whereas the cerebellar peduncle contains sulfatides and primarily GM1 with smaller quantities of GD1 (A.S. Woods, 2006), and that chain-length variants of GalCer are differentially localized in white matter, with C_{18}-subspecies being associated with cholesterol-rich regions whereas C_{24}-subspecies are found primarily in areas that are also enriched in Na^+ and K^+ (K. Borner, 2006). While imaging MS has limitations with respect to its ability to resolve isomeric and isobaric compounds and to provide quantitative information, the ability to know the cellular — and eventually subcellular — location of the analytes of interest will be a major complement to the ability to analyze the molecular subspecies profile and amounts using the other sphingolipidomic MS methods.

7. Perspectives and future directions

Since the 2002 edition of this book, 'sphingolipidology' has entered the era of 'sphingolipidomics' with the availability of tools for moderate-to-high-throughput analysis of many of the genes, enzymes, and metabolites of the sphingolipidome. A new challenge is to integrate this information into comprehensive 'systems' models using computational tools that are capable of handling large amounts of information, visualization tools that express the findings in forms that can be more easily interpreted, and models for pathway analysis, as have been recently applied to studies of yeast (F. Alvarez-Vasquez, 2005).

These skills are typically found in disciplines that heretofore have not been engaged much by sphingolipid researchers. Hence, one might say that sphingolipid research has matured from the stage where it stands on mainly two legs, chemistry and biology, to where it needs a third, bioengineering — which is perhaps one interpretation of the riddle of the Sphinx.

Acknowledgments

This chapter draws heavily on information from previous editions of this book, thus I gratefully acknowledge the contributions of my co-authors for those versions: Charles C. Sweeley and Konrad Sandhoff. I also thank Akemi Suzuki for helpful suggestions.

Abbreviations

Cer	ceramide
CGalT	galctosylceramide synthase
CGlcT	glucosylceramide synthase
DES	dihydroceramide desaturase
DHCer	dihydroceramide
ER	endoplasmic reticulum
ESI-MS/MS	electrospray tandem mass spectrometry
Fuc	fucose
G, with subscript for the subclass	ganglioside
Gal	galactose
GalNAc	N-acetylgalactosamine
Gb, with subscript for the number of carbohydrates	globoside
Glc	glucose
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
GM2-AP	G_{M2}-activator protein
Hex A or B	hexosaminidase A or B
Lac	lactose
Man	mannose
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
Pal-CoA	palmitoyl-CoA
S1P	sphingosine 1-phosphate
Sa1P	sphinganine 1-phosphate
SAP	sphingolipid-activator protein
Ser	serine
SM	sphingomyelin

Smase	sphingomyelinase
SPT	serine palmitoyltransferase
UDP–sugar	uridine dinucleotide phosphate sugar

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CHAPTER 14

Cholesterol biosynthesis

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Contents

1. Introduction

Cholesterol's structure, biosynthetic pathway, and metabolic regulation have tested the ingenuity of chemists, biochemists, and cell biologists for over 100 years. The 20th century began with the pioneering work of Heinrich Wieland, who deduced the structure of cholesterol and bile acids, for which Wieland was awarded the Nobel Prize in Chemistry in 1926. How is such a complex molecule acquired by the cell? Investigation into cholesterol acquisition required the development of isotopic tracer methods in Rudi Schoenheimer's laboratory in the 1930s. Schoenheimer found not only that mice synthesize cholesterol, but also that cholesterol synthesis is regulated by the amount of cholesterol in the diet. Konrad Bloch and David Rittenberg then showed that the ring structure and side chain of cholesterol are derived from acetate, and they identified intermediates in the pathway. Subsequent work by Konrad Bloch, John Cornforth, and George Popjak succeeded in establishing the biosynthetic origin of all 27 carbons of cholesterol. For their elegant work, Bloch was awarded the Nobel Prize in Chemistry in 1964 and Cornforth received the Nobel Prize in Chemistry in 1975.

By the 1980s, the cholesterol biosynthetic pathway was understood to be a complex pathway of over 40 cytosolic and membrane-bound enzymes, at least two of which are subject to feedback regulation by the end product, cholesterol, and oxygenated forms of cholesterol (called oxysterols). Genes encoding the key enzymes were cloned, which revealed the transcriptional and post-translational control of these enzymes. Michael Brown and Joseph Goldstein were awarded the Nobel Prize in Physiology or Medicine in 1985 for their comprehensive work on feedback regulation of cholesterol metabolism. Subsequently, the mechanisms of regulation were elucidated on a molecular level, although it was still not clear how cholesterol elicits all of the regulation. Today we know that the Schoenheimer result is due to feedback regulation of over a dozen enzymes in the cholesterol biosynthetic pathway. Factors that regulate cholesterol synthesis have been found to also control the synthetic pathways for fatty acids, triacylglycerols, and NADPH. Furthermore, the evidence is rapidly building that cholesterol's precursors and metabolites might serve as biologically active signaling molecules.

Fig. 1 is an overview of the metabolic and transport pathways that control cholesterol levels in mammalian cells (reviewed in Ref. [1]). Cholesterol is synthesized from acetyl-CoA via the isoprenoid pathway. At least 14 enzymes in the biosynthetic pathway are regulated by cellular cholesterol levels [2], of which the four key enzymes are shown.

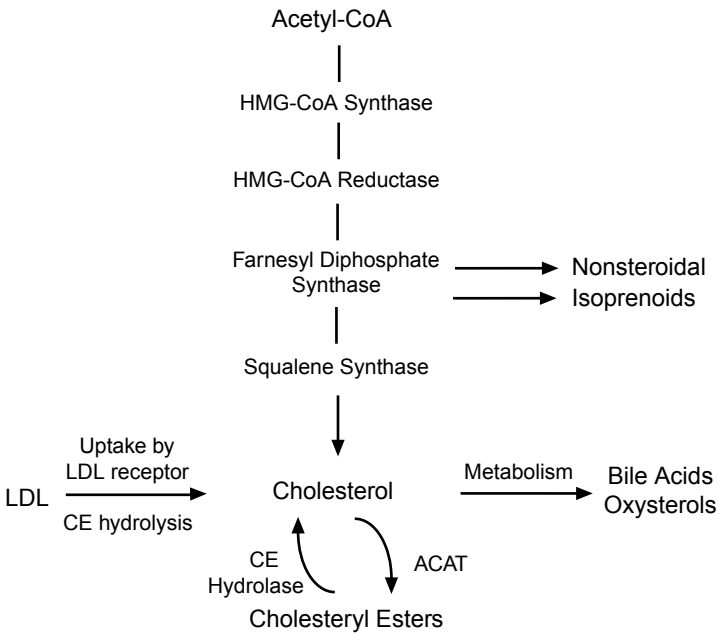

Fig. 1. Overview of the metabolic and transport pathways that control cholesterol levels in mammalian cells. Cholesterol is synthesized from acetyl-CoA and the four key enzymes that regulate cholesterol synthesis are indicated. Cells also obtain cholesterol by uptake and hydrolysis of LDL's cholesteryl esters (CE). End products derived from cholesterol or intermediates in the pathway include bile acids, oxysterols, cholesteryl esters, and non-steroidal isoprenoids. ACAT, acyl-CoA:cholesterol acyltransferase.

Essential non-steroidal isoprenoids, such as dolichol, prenylated proteins, heme A, and isopentenyl adenosine-containing tRNAs, are also synthesized by this pathway. In extrahepatic tissues, most cellular cholesterol is derived from de novo synthesis [3], whereas hepatocytes obtain most of their cholesterol via the receptor-mediated uptake of plasma lipoproteins, such as low-density lipoprotein (LDL). LDL is bound and internalized by the LDL receptor and delivered to lysosomes via the endocytic pathway, where hydrolysis of the core cholesteryl esters (CE) occurs (Chapter 20). The cholesterol that is released is transported throughout the cell. Normal mammalian cells tightly regulate cholesterol synthesis and LDL uptake to maintain cellular cholesterol levels within narrow limits and supply sufficient isoprenoids to satisfy metabolic requirements of the cell. Regulation of cholesterol biosynthetic enzymes takes place at the level of gene transcription, mRNA stability, translation, enzyme phosphorylation, and enzyme degradation. Cellular cholesterol levels are also modulated by a cycle of cholesterol esterification mediated by acyl-CoA:cholesterol acyltransferase (ACAT) and hydrolysis of the CE, by cholesterol metabolism to bile acids and oxysterols, and by cholesterol efflux.

2. The cholesterol biosynthetic pathway

Fig. 2 takes a closer look at the cholesterol biosynthetic pathway. Sterols are synthesized from the two-carbon building block, acetyl-CoA. The soluble enzyme acetoacetyl-CoA thiolase interconverts acetyl-CoA and acetoacetyl-CoA, which are then condensed by 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase to form HMG-CoA. There are two genes for HMG-CoA synthase (F.G. Hegardt, 1999). One encodes a mitochondrial enzyme that is involved in ketogenesis, which predominates in the liver. In extrahepatic tissues, the most abundant form of HMG-CoA synthase is a soluble enzyme of 53 kDa, encoded by another gene that is highly regulated by supply of cholesterol (G. Gil, 1986). Like acetoacetyl-CoA thiolase, HMG-CoA synthase has classically been described as a cytosolic enzyme because it is found in the 100,000 $\times$ g supernatant of homogenized cells and tissues. However, both enzymes contain peroxisomal targeting sequences [4] and may reside in multiple cellular compartments.

HMG-CoA reductase catalyzes the reduction of HMG-CoA to mevalonate, utilizing two molecules of NADPH. HMG-CoA reductase is a 97-kDa glycoprotein of the endoplasmic reticulum (ER) (L. Liscum, 1985) and peroxisomes [4]. Analysis of the ER enzyme's domain structure revealed an N-terminal membrane domain with eight transmembrane spans (E.H. Olender, 1992), a short linker, and a C-terminal catalytic domain facing the cytosol. Transmembrane spans 2–5 share a high degree of sequence similarity with several other key proteins in cholesterol metabolism; this region is termed the sterol-sensing domain (Section 3.5). Elucidation of the crystal structure of the HMG-CoA reductase catalytic domain indicated that the active protein is a tetramer (E.S. Istvan, 2000), which is consistent with biochemical analysis. The monomers appear to be arranged in two dimers, with the active sites at the monomer–monomer interface. The dimer–dimer interface is predominantly hydrophobic.

HMG-CoA reductase is the rate-determining enzyme of the cholesterol biosynthetic pathway and, like HMG-CoA synthase, is highly regulated by the supply of cholesterol.

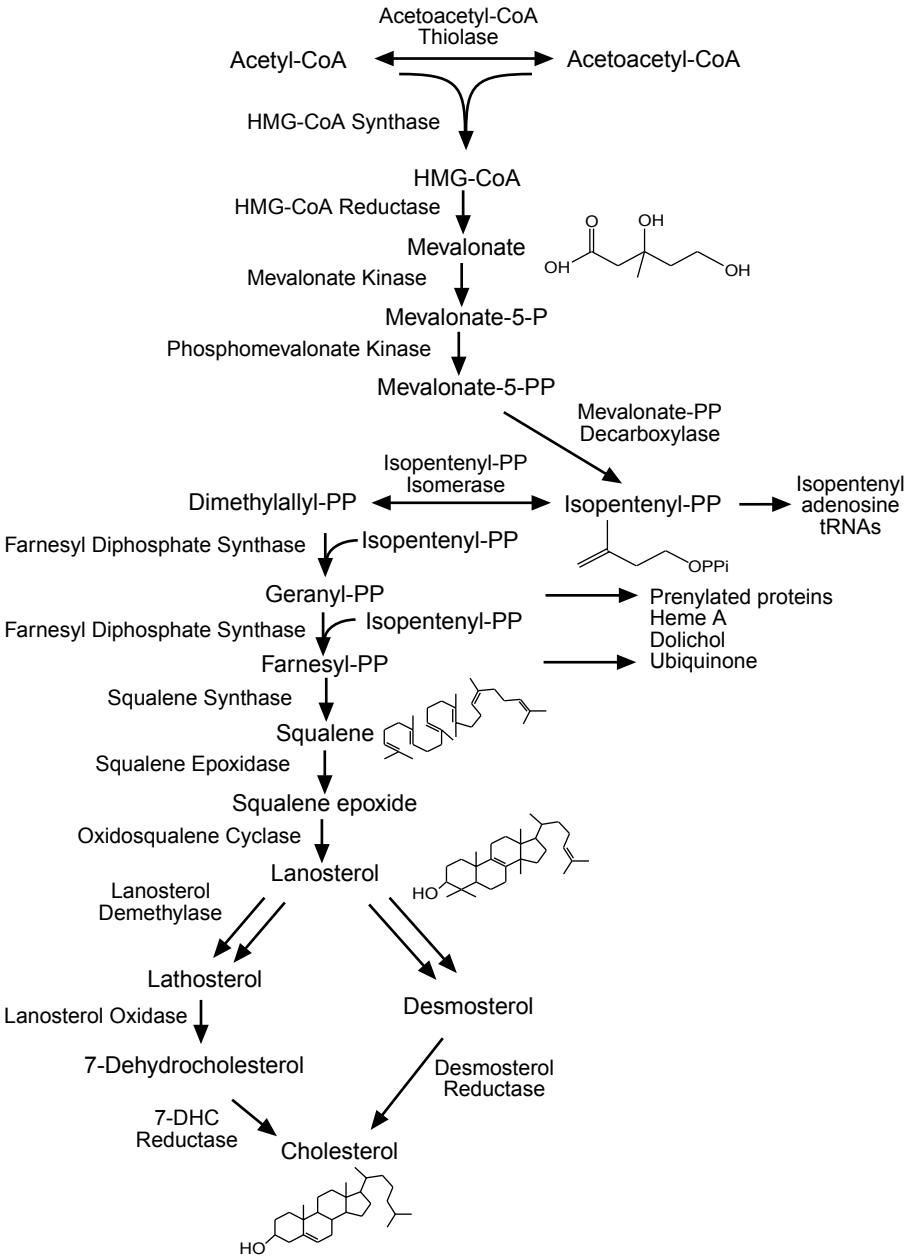

Fig. 2. The cholesterol biosynthetic pathway. Some of the major intermediates and end products are indicated. Double arrows indicate that multiple enzymatic steps are involved. HMG, 3-hydroxy-3-methylglutaryl; PP, diphosphate; DHC, dehydrocholesterol.

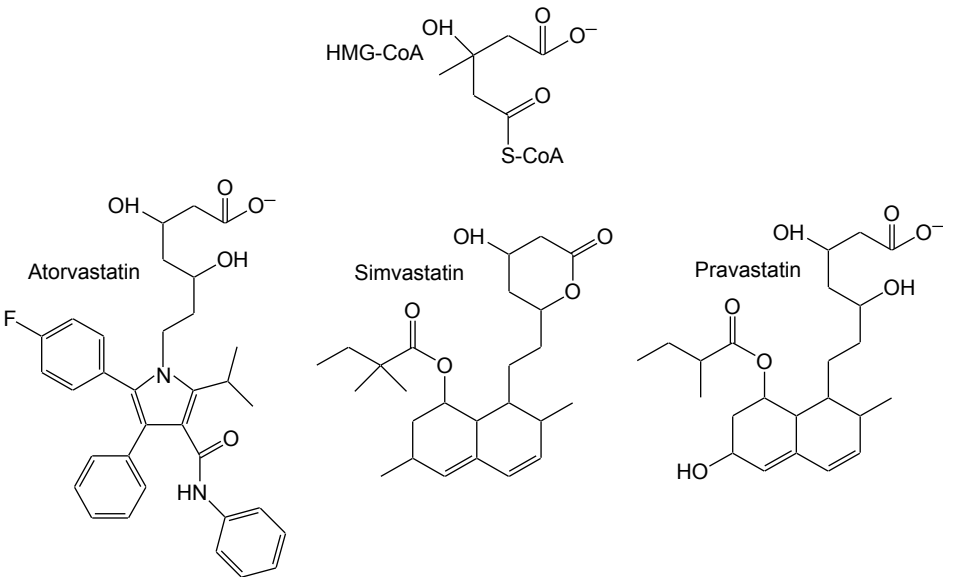

Fig. 3. Chemical structures of HMG-CoA and several statin inhibitors of HMG-CoA reductase. Atorvastatin (Lipitor), simvastatin (Zocor), and pravastatin (Pravachol) are widely prescribed cholesterol-lowering drugs.

Thus, the enzyme has received intense scrutiny as a therapeutic target for treatment of hypercholesterolemia. The enzyme is inhibited by a class of pharmacological agents, called statins, which have an HMG-like moiety and a bulky hydrophobic group [5] (Fig. 3). Statins occupy the HMG-binding portion of the active site, preventing HMG-CoA from binding (E.S. Istvan, 2001, 2002). Also, the bulky hydrophobic group causes disordering of several catalytic residues. Thus, statins are potent, reversible competitive inhibitors of HMG-CoA reductase with K_i values in the nanomolar range. Elevated plasma cholesterol levels are a primary risk factor for coronary artery disease, and statin inhibition of HMG-CoA reductase effectively reduces plasma cholesterol levels and decreases overall mortality. Statins also exert cholesterol-independent, or 'pleiotropic', effects that improve endothelial function, attenuate smooth muscle cell proliferation, and reduce inflammation (J.K. Liao, 2005). The complete inhibition of HMG-CoA reductase by statins will kill cells even if exogenous cholesterol is supplied because complete inhibition deprives cells of all mevalonate-derived products, including essential non-steroidal isoprenoids. To survive, cells must produce a small amount of mevalonate that, when limiting, is used preferentially by higher affinity pathways for non-steroidal isoprenoid production (S. Mosley, 1983).

Mevalonate is metabolized to farnesyl-diphosphate (farnesyl-PP) by a series of enzymes localized in peroxisomes (Fig. 2). First, mevalonate kinase phosphorylates the 5-hydroxy group of mevalonic acid. The enzyme is a homodimer of 40 kDa that is subject to feedback inhibition by several isoprenoid intermediates (S.M. Houten, 2000). Mutations in the mevalonate kinase gene lead to the human genetic disease mevalonic aciduria

(Section 2.2). The product of mevalonate kinase, mevalonate-5-P, is then phosphorylated to form mevalonic acid-5-PP, which is decarboxylated and dehydrated by mevalonate-PP decarboxylase to form isopentenyl-PP. Isopentenyl-PP is in equilibrium with its isomer, dimethylallyl-PP. Farnesyl-PP synthase catalyzes the head-to-tail condensations of two molecules of isopentenyl-PP with dimethylallyl-PP to form geranyl-PP and then farnesyl-PP. The enzyme is part of a large family of prenyltransferases that synthesize the backbones for all isoprenoids, including cholesterol, steroids, prenylated proteins, heme A, dolichol, ubiquinone, carotenoids, retinoids, chlorophyll, and natural rubber (K.C. Wang, 2000). Farnesyl-PP synthase can be inhibited by nitrogen-containing bisphosphonates, which results in reduced prenylation of small GTP-binding proteins (R.G. Russell, 2006). This inhibition affects the activity of osteoclasts, which are bone cells that resorb bone tissue, and lead to osteoclast apoptosis; therefore, bisphosphonates are a major class of drugs used for increasing bone density in bone diseases.

Squalene synthase is a 47-kDa protein of the ER and catalyzes the first committed step in cholesterol synthesis. The enzyme condenses two molecules of farnesyl-PP and subsequently reduces the presqualene-PP intermediate to form squalene. A large N-terminal catalytic domain faces the cytosol, anchored to the membrane by a C-terminal domain. This orientation may allow the enzyme to receive the hydrophilic substrates from the cytosol and release the hydrophobic product into the ER membrane for further metabolism [6]. Squalene synthase is highly regulated by the cholesterol content of the cell. Thus, this enzyme plays an important role in directing the flow of farnesyl-PP into the sterol or non-sterol branches of the pathway (M.S. Brown, 1980) [6]. Squalene synthase is inhibited by zaragozic acid, also called squalestatin (A. Baxter, 1992). Squalestatin has elicited interest as a potential cholesterol-lowering drug because it inhibits cholesterol synthesis without affecting the production of non-steroidal isoprenoids; however, it has been found that squalestatin fails to mimic the beneficial anti-inflammatory effect of statins, indicating that production of inflammatory cytokines and chemokines is regulated by non-steroidal isoprenoids (L. Diomede, 2001).

Squalene is converted into the first sterol, lanosterol, by the action of squalene epoxidase and oxidosqualene:lanosterol cyclase. The catalytic mechanism for the cyclase's four cyclization reactions was revealed when the crystal structure of the human enzyme was obtained (R. Thoma, 2004). Oxidosqualene:lanosterol cyclase is considered an attractive target for developing inhibitors of the cholesterol biosynthetic pathway because its inhibition leads to the production of 24,25-epoxycholesterol (M.W. Huff, 2005). This oxysterol is a potent ligand activator of the liver X receptor (LXR) and leads to expression of several genes that promote cellular cholesterol efflux, such as ABCA1, ABCG5, and ABCG8 (Section 4.1). Thus, inhibitors of oxidosqualene:lanosterol cyclase could be therapeutically advantageous because they would reduce cholesterol synthesis and promote cholesterol efflux (M.W. Huff, 2005).

Lanosterol is converted to cholesterol by a series of oxidations, reductions, and demethylations. The required enzyme reactions have been defined and metabolic intermediates identified; however, the precise sequence of reactions between lanosterol and cholesterol remains to be established [7] (Fig. 4). There is evidence for two alternative pathways that differ in the point at which the $\Delta 24$ double bond is reduced (Section 2.3). Both 7-dehydrocholesterol (DHC) and desmosterol have been postulated to be the

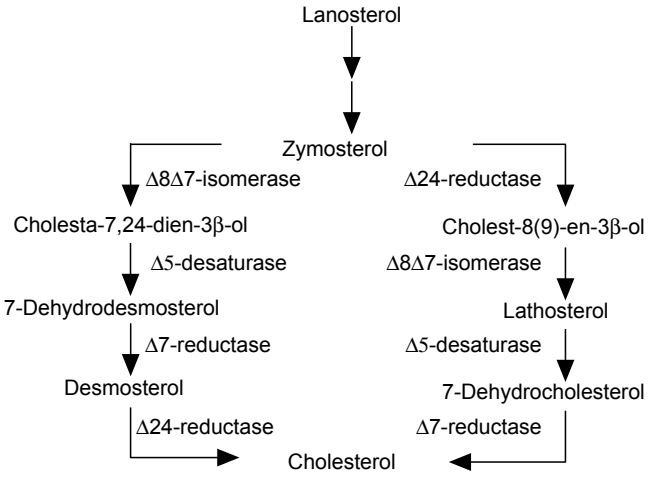

Fig. 4. Final steps in the cholesterol biosynthetic pathway. Alternate steps have been proposed for the conversion of zymosterol to cholesterol, which differ in the point at which the Δ24-reductase reaction occurs. Adapted from Waterham and Wanders [7] and Kelley and Hennekam [11].

immediate precursor of cholesterol. One of the key enzymes in the latter part of the pathway is 7-DHC Δ7-reductase, a 55-kDa integral membrane protein. Mutations in the gene for 7-DHC Δ7-reductase cause the human genetic disease Smith–Lemli–Opitz syndrome (SLOS) (Section 2.3).

2.1. Enzyme compartmentalization

Where does cholesterol synthesis take place? All of the enzymes that convert acetyl-CoA to farnesyl-PP have classically been thought of as cytosolic enzymes, with the exception of HMG-CoA reductase, which is typically depicted as an ER enzyme with the catalytic site facing the cytosol. Enzymes that convert farnesyl-PP to cholesterol are classically described as microsomal. However, there is evidence that many of the enzymes in this pathway are also, or exclusively, peroxisomal [4].

Evidence in favor of peroxisomal involvement in cholesterol biosynthesis is the following. The molecular cloning of cDNAs encoding many of these enzymes revealed peroxisomal targeting sequences (W.J. Kovacs, 2003). The availability of specific antibodies allowed immunocytochemical localization to peroxisomes [4] (W.J. Kovacs, 2006). Fibroblasts from individuals with peroxisome biogenesis disorders showed reduced enzymatic activities of cholesterol biosynthetic enzymes, reduced rates of cholesterol synthesis, and lower cholesterol content [4]. Together these data suggest that peroxisomes may play a role in all steps in the cholesterol biosynthetic pathway, except the conversion of farnesyl-PP to squalene. The latter reaction is catalyzed by squalene synthase, which is found solely in the ER.

HMG-CoA reductase is the one anomaly in that immunocytochemistry and immunoblotting have localized HMG-CoA reductase to both the ER and the peroxisomes;

however, no peroxisomal targeting motif has been found in the HMG-CoA reductase protein sequence. Furthermore, the peroxisomal HMG-CoA reductase has an apparent molecular weight of 90 kDa whereas the ER enzyme has 97 kDa (W.H. Engfelt, 1997). The peroxisomal enzyme exhibits other distinct properties: it is resistant to statin inhibition, the enzyme's activity is not regulated by phosphorylation, and the protein's turnover is not regulated by mevalonate. Altogether, this evidence suggests that the ER and peroxisome enzymes are functionally and structurally distinct (N. Aboushadi, 2000).

Not all investigators are convinced that peroxisomes are involved in cholesterol biosynthesis. In contrast to Olivier and Krisans [4], Hogenboom and colleagues have found no deficiency in cholesterol biosynthesis in fibroblasts from patients with a peroxisomal biogenesis disorder (S. Hogenboom, 2003). In addition, using a variety of biochemical and microscopic techniques, they found that mevalonate kinase, phosphomevalonate kinase, and mevalonate pyrophosphate decarboxylase are cytosolic, not peroxisomal enzymes (S. Hogenboom, 2004).

It is not clear why cholesterol synthesis might be compartmentalized such that intermediates cycle between peroxisomes and the cytosol. One possibility is to permit the shunting of acetyl-CoA derived from peroxisomal β-oxidation of long-chain fatty acids preferentially into the cholesterol biosynthetic pathway rather than allowing it to be released into the cytosol for incorporation into cellular fatty acids (W.J. Kovacs, 2006).

2.2. Mevalonic aciduria

Cholesterol synthesis is essential for normal development and maintenance of tissues that cannot obtain cholesterol from plasma lipoproteins, such as brain [3]. Furthermore, the biosynthetic pathway supplies non-steroidal isoprenoids that are required by all cells. Thus, it is not surprising that metabolic defects in the cholesterol biosynthetic pathway have devastating physiological consequences [8,9].

The first recognized human metabolic defect in the biosynthesis of cholesterol and isoprenoids was mevalonic aciduria [10]. Mevalonic aciduria is an autosomal recessive disorder that is quite rare, with only 30 known patients (D. Haas, 2006). In normal individuals, a small amount of mevalonic acid diffuses from cells into the plasma at levels proportional to the rate of cellular cholesterol formation. Patients with the severe, classical form of mevalonic aciduria excrete 10,000–200,000 times the normal amount of mevalonic acid because they have severely reduced amounts of mevalonate kinase activity. Their clinical features include failure to thrive, anemia, gastroenteropathy, hepatosplenomegaly, psychomotor retardation, hypotonia, ataxia, cataracts, and dysmorphic features [10]. Surprisingly, patients with severe deficiencies in mevalonate kinase show normal plasma cholesterol levels and cultured mevalonic aciduria fibroblasts have a rate of cholesterol synthesis that is half that of normal cells. Close examination of cholesterogenic enzymes in mevalonic aciduria fibroblasts has revealed a 6-fold increase in HMG-CoA reductase activity, which is postulated to compensate for the low mevalonate kinase activity. Thus, mevalonate is overproduced.

Individuals with low, but measurable, mevalonate kinase activity have a milder form of the disease called hyperimmunoglobulinemia D syndrome. Approximately 180 of these patients have been reported worldwide; they suffer from recurrent inflammatory

spells, lymphadenopathy, arthralgia, gastrointestinal problems, and skin rashes (D. Haas, 2006). Both the severe and the milder forms of the disease result from missense mutations that likely affect the stability and/or folding of the mevalonate kinase protein (S.H. Mandey, 2006).

2.3. Smith–Lemli–Opitz syndrome

A second metabolic defect in cholesterol synthesis leads to Smith–Lemli–Opitz syndrome (SLOS) (B.U. Fitzkey, 1999) [11]. SLOS is one of the most common autosomal recessive disorders, with estimates of incidence ranging from 1 in 10,000 to 1 in 60,000 of live births [12]. Individuals with SLOS have markedly elevated levels of plasma 7-DHC and low plasma cholesterol levels. 7-DHC Δ7-reductase activity is deficient in SLOS patients' samples, and cloning of the 7-DHC Δ7-reductase gene led to identification of over 100 missense and many null mutations in SLOS patients (P.E. Jira, 2003).

Severely reduced cholesterol synthesis is predicted to have severe consequences for development of the fetus because cholesterol is obtained only from the maternal circulation during the first trimester [11]. In addition, the brain is predicted to be severely affected because plasma lipoproteins cannot cross the blood–brain barrier and most, if not all, cholesterol needed for brain growth and function is synthesized locally (S.D. Turley, 1998) [3,13]. Indeed, severely affected SLOS infants who died soon after birth were found to have functionally null 7-DHC Δ7-reductase alleles [13], whereas less severely affected individuals likely have some residual 7-DHC Δ7-reductase catalytic activity. Simvastatin has been found to increase serum and cerebral spinal fluid cholesterol levels in SLOS patients that have partial 7-DHC Δ7-reductase activity, possibly due to transcriptional upregulation of the allele with residual activity (P.E. Jira, 2000).

Patients with SLOS have mental retardation and microcephaly, which is consistent with the idea that cholesterol synthesis is required for normal brain development. Clinical features also include failure to thrive, and characteristic craniofacial, skeletal, and genital anomalies. Is the clinical phenotype due to a lack of cholesterol or due to the cellular accumulation of 7-DHC? Two studies provided evidence in favor of the former. First, the clinical phenotype of a SLOS rat model was corrected by a cholesterol-rich diet (W. Gaoua, 2000). Second, a multicenter clinical trial showed that SLOS children fed a diet supplemented with cholesterol for 6–15 months had improved growth and neurodevelopment (i.e., language and cognitive skills) (M. Irons, 1997; E.R. Elias, 1997). However, not all studies agreed. A 6-year longitudinal study showed no improvement in cognitive, motor, or adaptive skills in response to cholesterol supplementation (D.M. Sikona, 2004). Furthermore, mice with uncontrolled cholesterol synthesis due to knockout of Insig-1 and Insig-2 regulatory proteins (Section 3.1) exhibited a buildup of precholesterol sterol intermediates, which led to facial clefting that resembles the SLOS phenotype (L.J. Engelking, 2006). The latter two studies are consistent with the cellular accumulation of 7-DHC causing the SLOS phenotype.

What are the final steps in the cholesterol biosynthetic pathway? SLOS may provide an answer to that question. As noted above, there is evidence for two alternative pathways for conversion of lanosterol to cholesterol, which differ in the point at which the Δ24 double bond is reduced [11]. In both pathways, lanosterol is demethylated to form zymosterol

Table 1
Inborn errors of sterol biosynthesis

Syndrome	Metabolic defect
Mevalonic aciduria	Mevalonate kinase
Smith–Lemli–Opitz	Sterol Δ7-reductase
Desmosterolosis	Sterol Δ24-reductase
Rhizomelic chondrodysplasia punctata (CDP)	Pex7 peroxisomal enzyme import
CDP X-linked dominant (CDPX2)	Sterol Δ8,Δ7-isomerase
Congenital hemidysplasia with ichthyosis and limb defects (CHILD syndrome)	Sterol Δ8,Δ7-isomerase, sterol C-4 demethylase
Greenberg skeletal dysplasia	Sterol Δ14-reductase
Lathosterolosis	Sterol Δ5-desaturase

These syndromes and their corresponding metabolic defects are reviewed in Kelley and Herman [9].

(Fig. 4). Then, zymosterol can be metabolized sequentially by a Δ24-reductase, Δ8,Δ7-isomerase, and Δ5-desaturase to form 7-DHC, which is reduced at the Δ7 position to form cholesterol. Alternatively, zymosterol can be metabolized by the Δ8,Δ7-isomerase and Δ5-desaturase to form 7-dehydrodesmosterol. 7-Dehydrodesmosterol is metabolized by the Δ7-reductase to form desmosterol and then by the Δ24-reductase to form cholesterol. The fact that the SLOS deficiency in Δ7-reductase leads to a buildup of 7-DHC rather than 7-dehydrodesmosterol is interpreted to mean that the former pathway is the principal one. However, the latter pathway must also be used because desmosterol is an abundant cholesterol precursor in certain tissues. It has been suggested that the final steps in the cholesterol biosynthetic pathway are tissue specific. Perhaps, in SLOS cells, any 7-dehydrodesmosterol that accumulates is metabolized by the available Δ24-reductase to form 7-DHC.

2.4. Other enzyme deficiencies

Other inborn errors of sterol biosynthesis are summarized in Table 1 [9]. Rhizomelic chondrodysplasia punctata, like Zellweger syndrome, exhibits defective sterol synthesis due to the lack of key peroxisomal enzymes of cholesterol biosynthesis. Conradi–Hunermann syndrome (known as CDPX2) and most cases of congenital hemidysplasia with ichthyosis and limb defects (known as CHILD syndrome) are due to mutations in the sterol Δ8,Δ7-isomerase gene, which is located on the X chromosome. Mutations in a single gene may lead to different syndromes with similar, but distinct, pathologies due to the mosaicism of X-chromosome inactivation. A few cases of CHILD syndrome may be due to mutations in the sterol C-4 demethylase gene, also located on the X chromosome.

3. Regulation of cholesterol synthesis

Isoprenoid synthesis is regulated by sterol and non-sterol components of the biosynthetic pathway, oxysterols, and also by physiological factors. The cholesterol content of the

cell controls several enzymes in the biosynthetic pathway, but historically the focus has been on the rate-limiting enzyme HMG-CoA reductase. Different regulators have different mechanisms of action. For example, sterols have been shown to regulate HMG-CoA reductase at the level of transcription, whereas non-sterols regulate HMG-CoA reductase mRNA translation. Both sterols and non-sterols are needed for regulation of HMG-CoA reductase protein degradation. Physiological factors that influence cholesterol synthesis include diurnal rhythm, insulin and glucagon, thyroid hormone, glucocorticoids, estrogen, and bile acids. These factors regulate HMG-CoA reductase by transcriptional, translational, and post-translational mechanisms.

3.1. Transcriptional regulation

HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway and combined regulation of HMG-CoA reductase synthesis and turnover can alter steady-state levels of the enzyme by 200-fold. HMG-CoA reductase is regulated in parallel with many other enzymes in the cholesterol biosynthetic pathway, as well as the LDL receptor. This coordinate regulation occurs because each gene has a similar sequence (cis-acting element) within the promoter that recognizes a common trans-acting transcription factor. Availability of the transcription factor that binds to the promoter sequence is influenced by the cellular cholesterol content.

Fig. 5 illustrates the current model of cholesterol-mediated transcriptional regulation [14]. The 5′-flanking regions of cholesterol-regulated genes have one to three copies of a 10-bp non-palindromic nucleotide sequence termed the sterol regulatory element (SRE). SREs are conditional positive elements that are required for gene transcription in cholesterol-depleted cells. The SRE sequence found in the LDL receptor gene is 5′-ATCACCCCAC-3′. SREs were readily identified in the HMG-CoA synthase, HMG-CoA reductase, farnesyl-PP synthase, and squalene synthase genes; however, there is not a strict SRE consensus sequence and identifying functional SREs has been difficult (P.A. Edwards, 2000).

The transcription factor that binds the SRE is termed the SRE-binding protein (SREBP) [2]. The first SREBP to be identified was the protein that bound to the LDL receptor promoter. Cloning of SREBP cDNAs revealed that there are two SREBP genes that produce three distinct proteins. SREBP-1a and -1c are derived from one gene that contains two promoters and differ in the length of the N-terminal transactivation domain, whereas SREBP-2 is derived from a second gene and is 45% identical to SREBP-1a.

SREBPs induce transcription of the genes encoding every known enzymatic step of cholesterol biosynthesis (Y. Sakakura, 2001). They also control the synthesis of fatty acids (via fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase), triacylglycerols, and phospholipids (via glycerol-3-phosphate acyltransferase) [2]. This regulation was illustrated using transgenic mice over-expressing the nuclear forms of SREBP-1a, -1c, and -2 in liver. Over-expression of each isoform resulted in activation of a full spectrum of cholesterol and fatty acid biosynthetic enzymes; however, absolute levels of induction of each enzyme and the subsequent liver phenotype varied according to the SREBP isoform expressed. From these data, it was concluded that SREBP-1a is a strong activator of both cholesterol and fatty acid synthesis and is likely to be important in

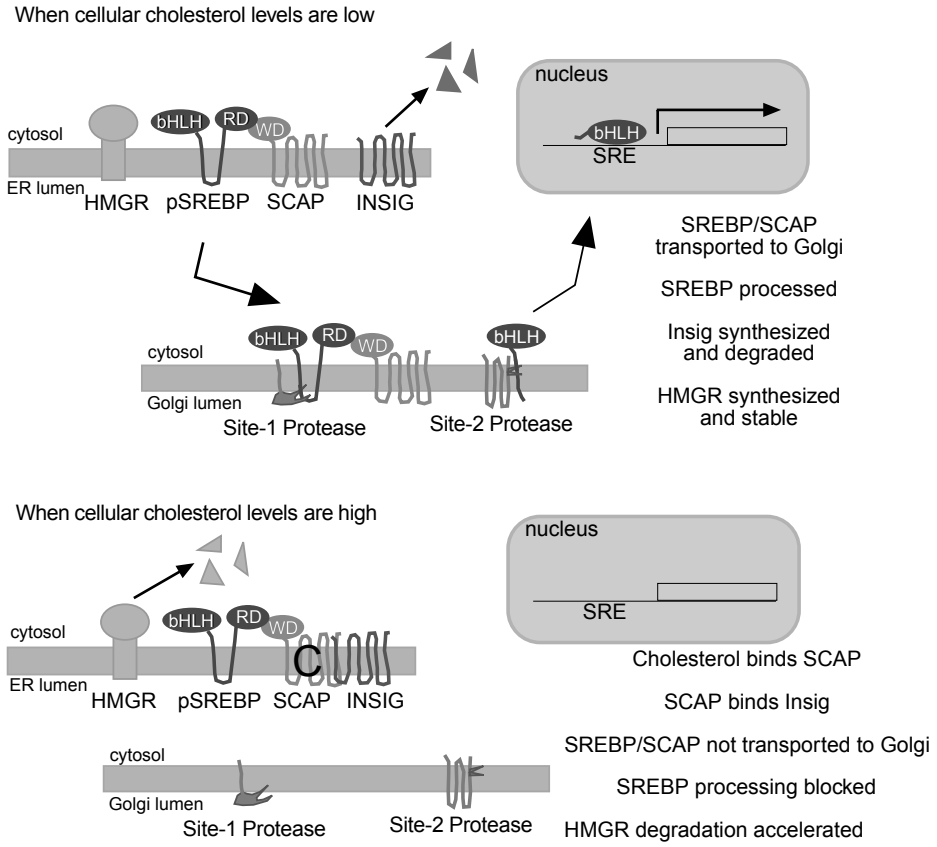

Fig. 5. Current model of cholesterol regulation of SREBP proteolysis. The sterol regulatory element-binding protein (SREBP) precursor is inserted into the endoplasmic reticulum (ER) membrane. The SREBP regulatory domain (RD) interacts with the SREBP cleavage-activating protein (SCAP), likely through SCAP's WD repeats. When cholesterol levels are low, SCAP escorts SREBP to the Golgi where the transcription factor is released by site-1 protease cleavage of a lumenal loop followed by site-2 protease cleavage within a transmembrane span. The mature SREBP translocates into the nucleus and activates gene transcription. HMG-CoA reductase (HMGR) is synthesized and stable in the ER membrane, whereas Insig is synthesized and quickly degraded. In cholesterol-fed cells, cholesterol binds SCAP, which induces SCAP to bind Insig. SCAP and Insig form a stable ER complex, anchoring the SREBP precursor in the ER. Thus, the SREBP precursor is not proteolyzed to release the basic helix-loop-helix (bHLH) transcription factor. Degradation of HMG-CoA reductase is accelerated. (See color plate section, plate no. 14.)

rapidly dividing cells that require lipid for membrane production. SREBP-1c is involved in regulation of fatty acid synthesis and glucose metabolism; it is the predominant form in liver and adipocytes. SREBP-2 preferentially activates cholesterol biosynthetic genes and the LDL receptor, and primarily responds when the liver's demand for cholesterol rises.

Newly synthesized SREBPs are translocated into the ER membrane as inactive 125-kDa membrane-bound precursors. They have three functional domains: an N-terminal domain containing a basic helix-loop-helix leucine zipper transcription factor, two

membrane spanning segments, and a C-terminal regulatory domain. The sequential two-step cleavage of the full-length precursor SREBP and release of the 68-kDa N-terminal transcription factor are influenced by the cellular cholesterol content.

Identification of the proteins required for regulated SREBP cleavage was accomplished using somatic cell genetic approaches. Mutant Chinese hamster ovary cells with abnormal regulation of cholesterol and fatty acid metabolism, which were selected over the past 20 years, have proved invaluable for achieving this goal [15,16]. Using expression cloning strategies, genes were isolated that restored SREBP-mediated transcription in each mutant. Elegant work by the Brown and Goldstein's laboratory led to identification of two proteases and an escort protein required for SREBP precursor cleavage.

When cellular cholesterol levels are low (Fig. 5), the C-terminal regulatory domain of the SREBP precursor interacts with the C-terminus of a chaperone, SREBP cleavage-activating protein (SCAP), through SCAP's four Trp–Asp (WD) repeats [17]. SCAP forms a complex with the CopII proteins Sec23 and Sec24, and is carried to the Golgi via CopII vesicles (L.-P. Sun, 2005). SCAP thereby escorts the SREBP precursor to the Golgi. In the Golgi, the lumenal loop of the SREBP precursor is clipped by a site-1 protease, which is a subtilisin-like serine protease; however, the two halves of the protein remain membrane anchored. Then an extremely hydrophobic zinc metalloprotease, site-2 protease, clips the N-terminal SREBP intermediate within the first membrane spanning segment, releasing the soluble transcription factor, mature SREBP. Upon translocation to the nucleus, the mature SREBP binds SRE sequences within the promoters of target genes and enhances their transcription.

Among the proteins synthesized under these conditions are the ER resident proteins, HMG-CoA reductase, and Insig-1. HMG-CoA reductase is a long-lived protein when cholesterol levels are low; however, Insig-1 is rapidly ubiquitinated and degraded (Y. Gong, 2006). A closely related isoform, Insig-2, is expressed at low, constitutive levels and is not regulated by SREBPs (D. Yabe, 2002).

When cellular cholesterol levels are raised to a threshold level, the cholesterol binds to SCAP, which causes SCAP to undergo a conformational shift [14,18]. SCAP no longer interacts with the CopII proteins (L.-P. Sun, 2005); instead, it binds to an Insig and forms a stable complex (T. Yang, 2002). Thus, SCAP and the SREBP precursor no longer travel to the Golgi and the SREBP precursor is not proteolyzed to produce the mature SREBP. As a result, transcription of target genes declines to basal levels.

Three lines of evidence indicate that SREBP–SCAP trafficking is controlled by cholesterol (A. Nohturfft, 1998, 1999, 2000). First is the finding that the SREBP precursor's N-linked carbohydrates are endoglycosidase H resistant (trimmed by Golgi mannosidases) when cellular cholesterol levels are low, and endoglycosidase H sensitive when cellular cholesterol levels are high. Thus, release of the mature SREBP transcription factor appears to coincide with transport to the Golgi. Second, the sterol-dependent movement of SCAP has been directly visualized in cultured cells transfected with a green fluorescent protein–SCAP fusion protein. Third, in vitro vesicle-budding assays have demonstrated that sterols suppress SREBP–SCAP complexes from entering into vesicles departing from the ER.

SCAP binds cholesterol via a five-transmembrane domain of approximately 180 amino acids, termed the sterol-sensing domain. This domain has 25% identity and 55%

similarity with a corresponding region in HMG-CoA reductase and is critical for SCAP's cholesterol-regulated SREBP escort function since certain amino acid substitutions in the sterol-sensing domain lead to constitutive activity (X. Hua, 1996; A. Nohturfft, 1996). SCAP proteins with missense mutations in the sterol-sensing domain fail to interact with Insig, and likely undergo no cholesterol-induced conformational shift.

SCAP was shown to bind specifically to cholesterol, but not to oxysterols (A. Radhakrishnan, 2004); yet oxysterols regulate the trafficking of SREBP to the Golgi like cholesterol does. This anomaly is explained by recent studies from the Brown and Goldstein's laboratory showing that oxysterols bind to Insig, which leads to Insig binding to SCAP (A. Radhakrishnan, 2007). Thus, both SCAP and Insig serve as sterols sensors for the SREBP pathway. SCAP's interaction with CopII proteins can be disrupted by cholesterol binding to SCAP or by oxysterol binding to Insig, which binds to SCAP. In either case, a shift is induced in SCAP conformation that prevents a sorting signal (Met–Glu–Leu–Ala–Asp–Leu) from interacting with the CopII protein Sec24.

3.2. mRNA translation

HMG-CoA reductase is also subject to translational control by a mevalonate-derived non-sterol regulator (D. Peffley, 1985; M. Nakanishi, 1988). This component of the regulatory mechanism can be observed only when cultured cells are acutely incubated with statins, which block mevalonate formation. Under those conditions, sterols have no effect on HMG-CoA reductase mRNA translation; however, mevalonate reduces the HMG-CoA mRNA translation by 80% with no change in mRNA levels. Translational control of hepatic HMG-CoA reductase by dietary cholesterol was shown in an animal model in which polysome-associated HMG-CoA reductase mRNA was analyzed in cholesterol-fed rats (C.M. Chambers, 1997). It was found that cholesterol feeding increased the portion of mRNA associated with translationally inactive monosomes and decreased the portion of mRNA associated with translationally active polysomes. The mechanism of HMG-CoA reductase translational control has not been elucidated.

3.3. Phosphorylation

Many key metabolic enzymes are modulated by phosphorylation–dephosphorylation and it has long been known that HMG-CoA reductase catalytic activity is inhibited by phosphorylation (Z.H. Beg, 1973). Rodent HMG-CoA reductase is phosphorylated on Ser 871 by an AMP-activated protein kinase that uses ATP as a phosphate donor (P.R. Clarke, 1990). However, examination of HMG-CoA reductase activity in rat liver showed that phosphorylation–dephosphorylation could not account for the long-term regulation that occurred with diurnal light cycling, fasting, or cholesterol-supplemented diet (M.S. Brown, 1979). Approximately 75–90% of HMG-CoA reductase enzyme was found to be phosphorylated (inactive) under all physiological conditions. This reservoir of inactive enzyme may allow cells to respond transiently to short-term cholesterol needs.

The AMP-activated kinase that phosphorylates and inactivates HMG-CoA reductase also phosphorylates and inactivates acetyl-CoA carboxylase. It has been suggested that,

when cellular ATP levels are depleted causing AMP levels to increase, the resultant activation of the kinase would inhibit cholesterol and fatty acid biosynthetic pathways, thus conserving energy (D.G. Hardie, 1992). Consistent with this hypothesis, cholesterol synthesis was reduced when ATP levels were depleted by incubation with 2-deoxy-D-glucose (R. Sato, 1993). However, cholesterol synthesis was not reduced when ATP levels declined in cells expressing a Ser 871 to Ala mutant form of HMG-CoA reductase, which is not phosphorylated (R. Sato, 1993). Therefore, HMG-CoA reductase phosphorylation appears to be important for preserving cellular energy stores rather than end product feedback regulation.

3.4. Proteolysis

Raising the cellular cholesterol content not only stops transcription of the genes encoding the cholesterol biosynthetic enzymes, but also leads to accelerated degradation of the rate-limiting enzyme, HMG-CoA reductase [19]. In cholesterol-depleted cells, HMG-CoA reductase is a stable protein that is degraded slowly ($T_{1/2}$ = 13 h) (J.R. Faust, 1982). If cholesterol repletion simply stopped transcription of the HMG-CoA reductase gene, it would lead to a slow decline in HMG-CoA reductase enzyme activity owing to stability of the protein; however, in presence of excess sterols or mevalonate, there is rapid ($T_{1/2}$ = 3.6 h) (J.R. Faust, 1982) and selective degradation of the enzyme, which results in more precise control of cellular sterol synthesis.

The HMG-CoA reductase membrane domain is necessary and sufficient for regulated degradation. Expression of the cytosolic catalytic domain results in a stable protein that is not subject to regulated degradation (G. Gil, 1985), whereas expression of the N-terminal membrane domain linked to a reporter protein results in regulated degradation of the reporter (D. Skalnik, 1988). Transmembrane span 2 was shown to be necessary for regulated degradation (H. Kumagai, 1995). Evidence that HMG-CoA reductase is polyubiquitinated prior to proteolysis has been provided for the yeast [19] and mammalian (T. Ravid, 2000) enzymes.

What are the signals for accelerated HMG-CoA reductase degradation (Fig. 6)? Cholesterol itself has little effect on HMG-CoA reductase turnover; instead, the sterol signal for HMG-CoA reductase degradation comes from the cholesterol biosynthetic precursor, lanosterol (B.-L. Song, 2004, 2005). DeBose-Boyd and colleagues have shown that lanosterol acts by inducing binding of HMG-CoA reductase to Insig via HMG-CoA reductase's sterol-sensing domain (N. Sever, 2003). The binding requires a tetrapeptide sequence YIYF in the second transmembrane span of reductase. Once bound to Insig, HMG-CoA reductase is ubiquitinated on lysine 248, which is adjacent to transmembrane span 2, and the enzyme is then proteolyzed. Grp78, a membrane-bound E3 ubiquitin ligase, also binds Insig and is required for the lanosterol-regulated ubiquitination and degradation of HMG-CoA reductase (B.L. Song, 2005). Analysis of numerous related sterols point to the 4,4-dimethyl moiety of lanosterol as being critical for its potent regulatory action (B.-L. Song, 2005). Lanosterol stimulation of HMG-CoA reductase turnover would have the beneficial effect of shutting down further lanosterol synthesis while permitting the continued metabolism of potentially cytotoxic lanosterol intermediates to cholesterol [14].

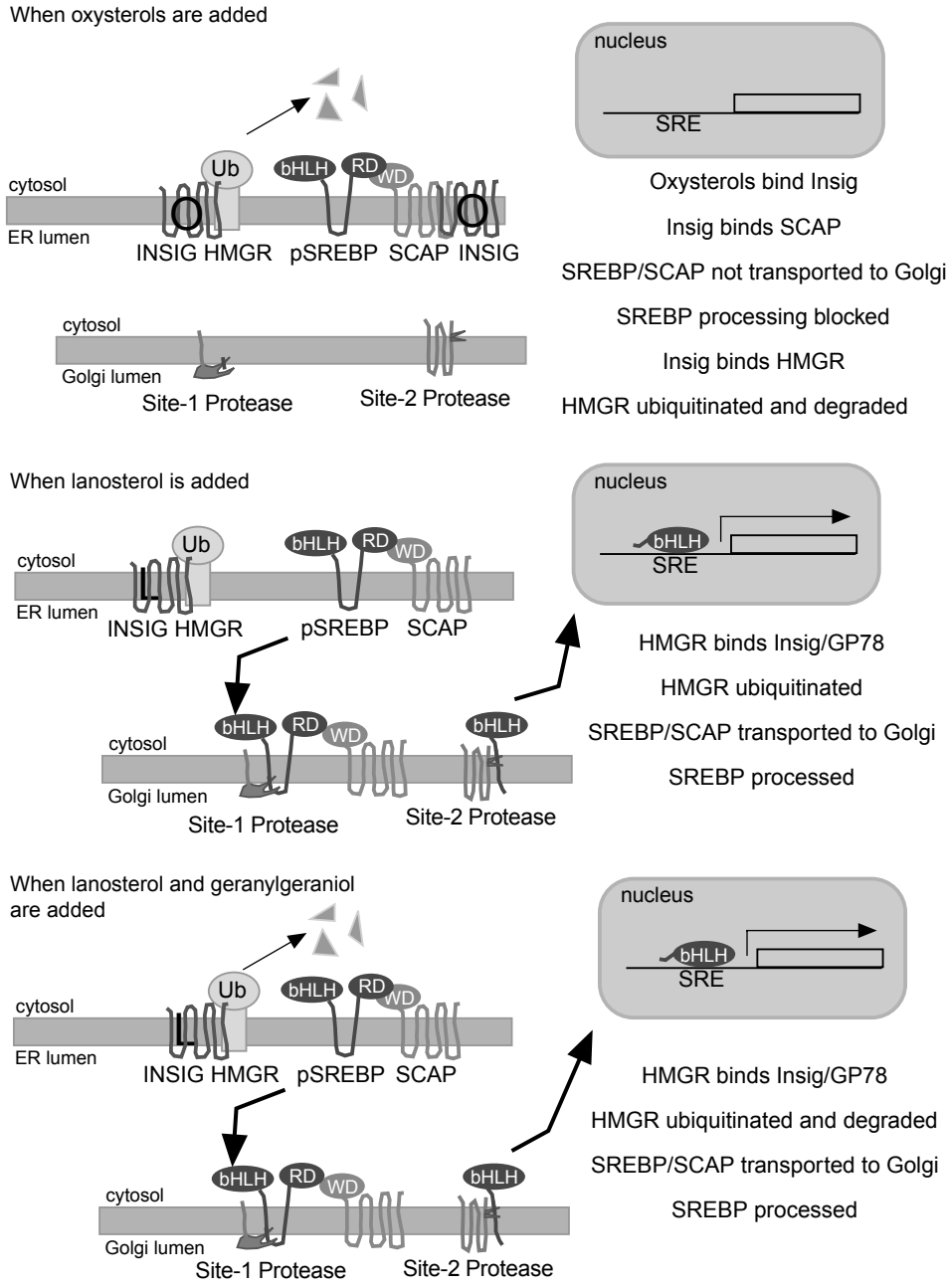

Fig. 6. Sterol regulation of SREBP processing. Oxysterols bind to Insig and induce Insig to anchor SCAP/SREBP in the ER membrane. Oxysterols also lead to HMG-CoA reductase (HMGR) ubiquitination and degradation. Lanosterol causes Insig-Gp78 complexes to bind to HMG-CoA reductase, leading to HMGR ubiquitination. SREBP is transported to the nucleus and processed. If geranygeraniol is also present, the ubiquitinated HMG-CoA reductase is extracted from the membrane and degraded. SREBP transport and processing continues. (See color plate section, plate no. 15.)

In mammalian cells, the addition of sterols leads to accelerated HMG-CoA reductase degradation only when the sterols are accompanied by a mevalonate-derived non-sterol signal (M. Nakanishi, 1988; J. Roitelman, 1992; T.E. Meigs, 1997). There is strong evidence that the non-sterol isoprenoid signal for HMG-CoA reductase degradation is geranylgeraniol or a geranylgeranylated protein (N. Sever, 2003). Possible candidate geranylgeranylated proteins involved in HMG-CoA reductase degradation include the Rab proteins, a superfamily of proteins that regulates every major step in membrane trafficking. It has long been known that the regulated turnover of HMG-CoA reductase requires ongoing protein synthesis (K. Chun, 1990; J. Roitelman, 1992; T. Ravid, 2000); it is likely that the ongoing synthesis serves to supply a geranylgeranylated protein.

The Hampton's laboratory first identified the proteins that are involved in HMG-CoA reductase degradation, the so-called HRD proteins, in a yeast genetic approach. HRD2p encodes a subunit of the 26S proteosome, whereas HRD1p and HRD3p encode components of the E3 ubiquitin ligase [19]. There are two HMG-CoA reductase isozymes in yeast. Hmg1p is an abundant, long-lived protein, whereas Hmg2p is a less abundant protein for which the half-life is regulated. In contrast to the mammalian enzyme, which requires both sterols and non-sterols for regulated turnover, the yeast Hmg2p isozyme can be suppressed by a non-sterol signal alone, although sterols enhance the non-sterol regulation. Regulation of yeast Hmg2p degradation absolutely requires two amino acids in the membrane domain: they are lysines at positions 6 and 357. Replacement with arginine leads to stabilization of the protein and no ubiquitination [19].

3.5. Sterol-sensing domain

The sterol-sensing domain of HMG-CoA reductase mediates the enzyme's binding to Insig, which leads to reductase ubiquitination and degradation. Sterols also induce the association of SCAP to Insig; however, this results in a stable complex that is retained in the ER but not degraded. HMG-CoA reductase and SCAP are not the only cellular proteins with sterol-sensing domains.

Sterol-sensing domains with a high degree of sequence similarity are found in several other proteins with obvious connections to cholesterol homeostasis [20]. One is the biosynthetic enzyme 7-DHC Δ7-reductase. The function of the sterol-sensing domain of 7-DHC Δ7-reductase is not clear; however, amino acid substitutions in that region cause the loss of 90% of catalytic activity (S.H. Bae, 1999). Another sterol-sensing domain-containing protein is NPC1, a 1278-amino acid glycoprotein found in late endosomes and lysosomes. NPC1 is hypothesized to play a role in trafficking of cholesterol, gangliosides, and other cargo from late endosomes to destinations throughout the cell [21]. Mutations in NPC1 lead to the predominant form of Niemann–Pick C disease, a human genetic disease characterized by progressive neurodegeneration. The biological function of NPC1 is still not clear, but structure–function analysis indicates that the sterol-sensing domain is important and binds to cholesterol (N. Ohgami, 2004). Mutations that cause amino acid substitutions within the sterol-sensing domain lead to a rapidly progressing, infantile form of the disease, whereas amino acid substitutions throughout the rest of the protein lead to juvenile or adult onset of disease [21].

NPC1L1 is a protein with 42% identity with NPC1 (J.P. Davies, 2000). Several lines of evidence indicate that NPC1L1 plays a role in the intestinal absorption of cholesterol and plant sterols (S.W. Altmann, 2004) [22]. NPC1L1 is found on the plasma membrane of enterocytes in the proximal jejunum. NPC1L1 homozygous knockout mice absorb significantly less cholesterol than control mice and are insensitive to the effects of ezetimibe, a cholesterol absorption inhibitor that lowers plasma LDL cholesterol levels.

Two other proteins with sterol-sensing domains, patched and dispatched, are involved in developmental patterning (A.P. McMahon, 2000). Patched is the receptor for the morphogen, sonic hedgehog, which is the only known protein with a covalently attached cholesterol moiety (Chapter 2). Dispatched is the plasma membrane protein required for secretion of cholesterol-modified hedgehog. Hedgehog binding to patched leads to a signal transduction cascade that activates transcription of specific genes. Mutations in patched cause basal cell nevus syndrome, which is characterized by developmental abnormalities and basal cell carcinomas (R.L. Johnson, 1996). A direct role for patched and dispatched in cholesterol metabolism has not been established; however, cholesterol biosynthesis has been shown to play a crucial role in sonic hedgehog-dependent pathways. Either cholesterol itself or specific oxysterols are required for sonic hedgehog signal transduction (R.B. Corcoran, 2006). Also, exposure of embryos to inhibitors of cholesterol biosynthesis, such as Triparanol, AY-9944, or BM 15.766, cause profound developmental defects that resemble those in sonic hedgehog mutant embryos (J.A. Porter, 1996). These developmental defects are likely due to accumulation of pre-cholesterol sterol intermediates (L.J. Engelking, 2006).

4. Metabolism of cholesterol

Cellular cholesterol levels are regulated, not only by feedback inhibition of cholesterol synthesis, but also by feed-forward regulation of cholesterol metabolism. Excess cholesterol is metabolized to oxysterols. In addition to blocking SCAP-facilitated proteolysis of SREBP and thereby downregulating endogenous cholesterol synthesis and LDL receptor levels, oxysterols also activate bile acid synthesis (Chapter 15) and cholesterol esterification, which further reduces the cellular content of unesterified cholesterol.

4.1. Oxysterols

Oxysterols are oxygenated derivatives of cholesterol that signal cholesterol excess [23]. They are potent suppressors of cholesterol synthesis (A.A. Kandutsch, 1973, 1974). Their effectiveness has been attributed to their ability to diffuse into and through cells to activate regulatory processes, thus bypassing the need for receptor-mediated entry. It was long assumed that cholesterol was the natural regulator of cholesterol synthesis and that oxysterols were contaminants found in commercial supplies of cholesterol or formed upon storage of stock cholesterol solutions. Now it is known that there are many naturally occurring oxysterols that have diverse actions on cellular lipid metabolism [24]. 25-Hydroxycholesterol is the most studied oxysterol; however, other oxysterols are as, or more, physiologically important.

Oxysterols can be formed enzymatically by the action of at least three distinct hydroxylases [25]. The mitochondrial sterol 27-hydroxylase participates in an alternative pathway of bile acid biosynthesis, hydroxylating cholesterol and several other intermediates in the bile acid synthetic pathway. 27-Hydroxycholesterol formed in peripheral tissues is a potent inhibitor of endogenous cholesterol synthesis. It is also thought to be secreted into the bloodstream and transported to the liver, where 27-hydroxycholesterol binds the LXR nuclear hormone receptor (Chapter 15). LXR forms a heterodimer with the retinoid X receptor and activates transcription of genes encoding bile acid biosynthetic enzymes. The physiological significance of sterol 27-hydroxylase is illustrated by the genetic disease cerebrotendinous xanthomatosis, which is caused by mutations in the sterol 27-hydroxylase gene [26]. The absence of this critical hydroxylase activity precludes the mobilization of excess cholesterol from peripheral tissues and leads to cholesterol deposition and xanthoma development.

24-Hydroxylase is an ER enzyme predominantly expressed in a specific subset of neurons in the brain. Bjorkhem and colleagues have provided strong evidence that 24-hydroxylase maintains cholesterol homeostasis in the brain, which cannot participate in high-density lipoprotein-mediated reverse cholesterol transport [27]. 24-Hydroxycholesterol is secreted from the brain across the blood–brain barrier into the circulation, is taken up by the liver, and metabolized into bile acids. Mice lacking the cholesterol 24-hydroxylase gene show 40% reduction of brain cholesterol synthesis, but no change in steady-state level of brain cholesterol (E.G. Lund, 2003). This indicates that synthesis of new cholesterol and formation of 24-hydroxycholesterol are closely coupled. Mice lacking cholesterol 24-hydroxylase also exhibit severe deficiencies in spatial, associative, and motor learning, and in hippocampal long-term potentiation (T.J. Kotti, 2006). The effects on long-term potentiation can be reversed by treatment with geranylgeraniol, but not cholesterol. These data indicate that cholesterol synthesis in the brain is required to ensure a constant supply of the isoprenoid geranylgeraniol, likely for post-translational modification of a protein critical for learning.

25-Hydroxycholesterol is a potent regulator of SREBP proteolytic processing when added exogenously to cells. It acts by binding to Insig and causes Insig to bind SCAP (A. Radhakrishnan, 2007) (Fig. 6). Oxysterol-bound Insig also binds to HMG-CoA reductase and accelerates the enzyme's ubiquitination and degradation (N. Sever, 2003). The enzyme that produces 25-hydroxycholesterol, 25-hydroxylase, resides in the same sub-cellular compartment as Insig [25]; thus, it is postulated that 25-hydroxycholesterol may serve as the physiologically relevant oxysterol regulator of cholesterol synthesis.

Oxysterols activate transcription of several genes that play key roles in maintaining bodily cholesterol homeostasis by binding to the LXR subfamily of nuclear hormone receptors [28]. The LXR subfamily contains two members, LXRα and LXRβ, which are activated by ligand binding, heterodimerize with the retinoid X receptor, and bind to response elements in the promoter of key genes in cholesterol and lipid metabolism. Activated genes include SREBP-1c and fatty acid synthase, which increase fatty acid synthesis; cholesteryl 7-α hydroxylase and apo E, which lead to cholesterol clearance; CE transfer protein and lipoprotein lipase, which affect triacylglycerol metabolism; and NPC1, ABCA1, ABCG1, ABCG5, and ABCG8, which increases cholesterol efflux. LXRs are implicated in multiple metabolic and inflammatory pathways that are important in

the pathogenesis of cardiovascular and metabolic diseases [28]. Their important role in cholesterol homeostasis is illustrated by the phenotype of LXRα-null mice, which show dramatic adrenomegaly, due to a build-up of cholesterol and CE. When fed a cholesterol-enriched chow, the LXRα-null mice show severe hepatomegaly and a pale liver, due to hepatic cholesterol build-up (D.J. Peet, 1998).

Another protein that binds oxysterols with high affinity was first reported by Kandutsch (1977) and called oxysterol-binding protein (OSBP). At the time the protein was purified and cDNA cloned (F.R. Taylor, 1989; P.A. Dawson, 1989), OSBP was expected to be a cytosolic protein that translocated into the nucleus and repressed transcription of cholesterogenic genes when oxysterols were present. Given our current knowledge of transcriptional control, we might expect OSBP to bind to the SREBP precursor, SCAP, or Insig in the ER or site-1 protease or site-2 protease in the Golgi to interfere with SREBP proteolytic processing. However, a direct role for OSBP in transcriptional control has not been demonstrated. In fact, knockdown of OSBP in HeLa cells has no effect on 25-hydroxycholesterol inhibition of cholesterol synthesis (T. Nishimura, 2005).

How might OSBP transduce signals? One reasonable hypothesis is that when cellular cholesterol levels are high, cholesterol hydroxylation occurs. The resultant oxysterol would then bind to OSBP, and would translocate to the Golgi and signal suppression of cholesterol synthesis. However, the opposite occurs. OSBP associates with Golgi membranes when cholesterol levels are depleted. Addition of cholesterol causes the detachment of OSBP from the Golgi and localization to the cytosol. The story is made more complex by the finding that the human OSBP family has at least 12 members, with sequence similarity in the C-terminal ligand-binding domain, whereas *Saccharomyces cerevisiae* has seven related proteins [23]. The yeast homologs, called Osh proteins, appear to have overlapping functions since deletion of any one Osh protein has no effect, but deletion of all seven is lethal (C.T. Beh, 2001). Analysis of combination deletion strains points to a role for Osh proteins in sterol homeostasis.

4.2. Cholesteryl ester synthesis

Excess cholesterol can also be metabolized to CE. ACAT is the ER enzyme that catalyzes the esterification of cellular sterols with fatty acids. In vivo, ACAT plays an important physiological role in intestinal absorption of dietary cholesterol, in intestinal and hepatic lipoprotein assembly, in transformation of macrophages into CE laden foam cells, and in control of the cellular free cholesterol pool that serves as substrate for bile acid and steroid hormone formation. ACAT is an allosteric enzyme, thought to be regulated by an ER cholesterol pool that is in equilibrium with the pool that regulates cholesterol biosynthesis. ACAT is activated more effectively by oxysterols than by cholesterol itself, likely due to differences in their solubility. As the fatty acyl donor, ACAT prefers endogenously synthesized, monounsaturated fatty acyl-CoA.

The cloning of the human ACAT gene and its orthologs, as well as the subsequent generation of ACAT-deficient mice, led to the realization that two ACAT isozymes contribute to the enzyme activity [20,29]. Human ACAT-1 was cloned using an expression cloning strategy. The gene encodes an integral membrane protein of 550 amino acids that is present in almost all cells and tissues examined. Orthologs were identified in other

mammalian species, as well as *Drosophila melanogaster* and *Candida elegans*. The first indication of multiple ACATs came from the cloning of two ACAT-related enzymes (ARE1 and ARE2) from *S. cerevisiae* (H. Yang, 1996). The inactivation of both yeast genes was required to eliminate sterol esterification. In addition, ACAT-1-deficient mice showed the expected depletion of CE in adrenals, ovaries, testes, and macrophages, but no changes in intestinal cholesterol absorption or hepatic cholesterol esterification (V.L. Meiner, 1996). This result indicated that a second ACAT must be present in those mouse tissues.

The cloning of ACAT-2 (R.A. Anderson, 1998; S. Cases, 1998; P. Oelkers, 1998) revealed a protein of similar size to ACAT-1, with a novel N-terminus but a C-terminus highly similar to ACAT-1. In adult humans, ACAT-2 is expressed in the apical region of intestinal enterocytes and in hepatocytes. Disruption of the ACAT-2 gene in mice led to dramatic reduction in cholesterol absorption and prevention of hypercholesterolemia (A.K.K. Buhman, 2000). The data suggest that, in humans, ACAT-1 plays a critical role in foam-cell formation and cholesterol homeostasis in extrahepatic tissues, whereas ACAT-2 has an important role in absorption of dietary cholesterol [29]. Interest in ACAT inhibitors as a therapeutic strategy has been revived by liver-specific knockdown of ACAT-2 in atherosclerotic-prone mice, which significantly reduced aortic atherosclerotic lesions (T.A. Bell, 2006).

5. Future directions

Fifty years ago, it was recognized that hepatic cholesterol synthesis was subject to feedback regulation by dietary cholesterol (R.G. Gould, 1950). Only in the last two decades have the mechanisms been elucidated for transcriptional and degradative regulation of the rate-limiting enzyme, HMG-CoA reductase. Both forms of regulation require that proteins sense the local cholesterol concentration. Rising cholesterol levels cause HMG-CoA reductase to be ubiquitinated and degraded by the proteosome. They cause SCAP to remain associated with Insig in the ER rather than translocating to the Golgi. A challenge that is just being met is to determine how HMG-CoA reductase and SCAP transduce the signal of increased cellular cholesterol content into action, i.e., protein degradation or movement to Golgi.

HMG-CoA reductase and SCAP are not the only cellular proteins equipped with a sterol-sensing domain. Does the sterol-sensing domain in 7-DHC Δ7-reductase confer cholesterol-mediated feedback regulation upon this last step in the biosynthetic pathway? What is the function of the sterol-sensing domains in NPC1, NPC1L1, patched, and dispatched? Is their sub-cellular location, or their binding to another protein, altered by cholesterol? Are they regulated by cholesterol itself or another biologically active sterol?

Abbreviations

ACAT	acyl-CoA:cholesterol acyltransferase
CE	cholesteryl ester

DHC	dehydrocholesterol
ER	endoplasmic reticulum
HMG	3-hydroxy-3-methylglutaryl
Insig	insulin-induced gene
LDL	low-density lipoprotein
LXR	liver X receptor
OSBP	oxysterol-binding protein
PP	diphosphate
SCAP	SREBP cleavage-activating protein
SLOS	Smith–Lemli–Opitz syndrome
SRE	sterol regulatory element
SREBP	SRE-binding protein

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CHAPTER 15

Metabolism and function of bile acids

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Contents

1. Introduction

Bile acids make up a group of sterol-derived compounds that act as detergents to facilitate the digestion and absorption of fats and fat-soluble molecules in the intestine, and to keep cholesterol from precipitating in bile. In mammalian species, the cholesterol side chain is trimmed to yield C24-sterol derivatives. In other vertebrate species, the hydroxylation of the side chain does not lead to its removal and the products of the biosynthetic pathway are referred to as bile alcohols. Invertebrate species do not synthesize sterol bile acids. Over the last few years, much information has been gained about the function of bile acids and the mechanisms that regulate their synthesis. The focus of this chapter is to provide a general overview of bile acid biochemistry and to review recent discoveries that have advanced our understanding of bile acid metabolism and function in mammals.

The concept of bile was developed around the late 1600s to mid-1700s. It was early in the 1800s when bile solutes were crudely isolated. Among the components identified were the amino acid taurine (identified in ox bile, hence its name), cholesterol, and a nitrogenous acid. The term ‘cholic acid’ was initially applied to the acidic component but this was changed to the generic term ‘bile acid’ shortly after. By the mid-1800s, taurine- and glycine-conjugated bile acids could be distinguished and it was also around this time that the idea that bile acids were responsible for solubilizing cholesterol in bile emerged. More than half a century ago, it became evident that bile acids are synthesized from

cholesterol [1]. Bile acids are the major solutes in bile. Typical mammalian bile consists of about 82% water, 12% bile acids, 4% phospholipids (mostly phosphatidylcholines), 1% unesterified cholesterol, 0.3% bilirubin, and the remainder as assorted solutes (including proteins).

2. Bile acid structure

The structure of bile acids holds the key to their ability to act as efficient detergents. In general, cholesterol is modified by epimerization of the pre-existing 3β-hydroxyl group, saturation and hydroxylation of the steroid nucleus and trimming of the side chain [2]. Fig. 1 shows the positions of the carbons in the steroid nucleus that are modified during bile acid biosynthesis. Under normal physiological conditions, one or two hydroxyl groups are added to the steroid nucleus. These modifications render the sterol less hydrophobic, enabling it to interact with an aqueous environment more efficiently. The hydroxyl groups of many bile acids are oriented towards one face of the steroid nucleus giving the molecule an amphipathic character. After trimming three carbons from the side chain, the 'free' bile acids are covalently linked to one of two amino acids (either taurine or glycine) to form 'conjugated' bile acids. Conjugated bile acids readily ionize, allowing these polar molecules to efficiently interact with both hydrophobic and hydrophilic substances (Fig. 2).

The number and specific orientation of the hydroxyl groups added to the steroid nucleus vary according to animal species. Table 1 lists the bile acids that are commonly found in the bile of different mammalian species. Some bile acids, such as cholic acid and chenodeoxycholic acid, are common to many mammalian species whereas others are unique

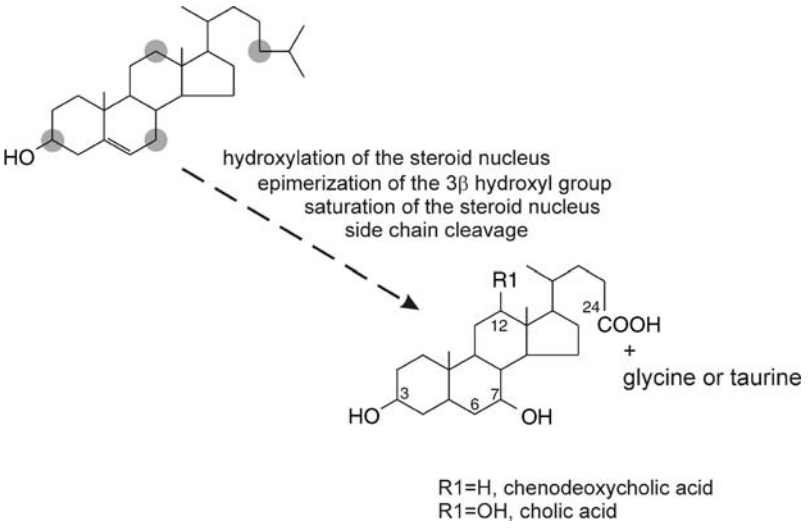

Fig. 1. Conversion of cholesterol into bile acids. The carbons in the cholesterol molecule that accept modification in the process of bile acid synthesis are circled.

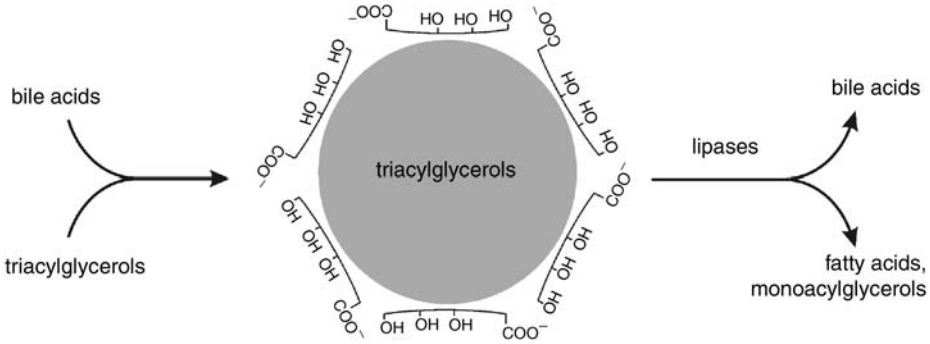

Fig. 2. Interaction of bile acids with triacylglycerols. Lipid-soluble nutrients may be present in the triacylglycerol droplet. Lipases hydrolyze the triacylglycerols to liberate fatty acids and monoacylglycerols.

Table 1

Abundant bile acids found in the bile of selected mammalian species

Bile acid	Location[a] and orientation of hydroxyl groups	Species
Chenodeoxycholic acid	3α, 7α	Bear, hamster, human, pig
Ursodeoxycholic acid	3α, 7β	Bear
Deoxycholic acid	3α, 12α	Cat, human, rabbit
Hyocholic acid	3α, 6α, 7α	Pig
β-Muricholic acid	3α, 6β, 7β	Mouse, rat
Cholic acid	3α, 7α, 12α	Bear, cat, hamster, human, mouse, pig, rabbit, rat

[a]See Fig. 1 for the location of the modified carbons within the steroid moiety.

to certain species. Ursodeoxycholic acid, which is abundant in bear bile, has been found to be therapeutically useful for treating primary biliary cirrhosis and dissolving gallstones. It is chemically and biologically distinct from its isomer chenodeoxycholic acid, which differs only in the orientation of the hydroxyl group attached to carbon 7 of the steroid nucleus. Among the mammalian species that are commonly studied in the laboratory, only the hamster shows a biliary bile acid composition that is comparable to that of humans.

Fig. 3 shows the synthesis of taurine from cysteine via oxidation and decarboxylation reactions. Taurine is very rare in plants but is abundant in animal tissue, particularly in the brain. The bile acids of carnivores are mostly conjugated to taurine whereas those of herbivores are conjugated to glycine. Both taurine- and glycine-conjugated bile acids are found in the bile of omnivores. The bile of cats contains taurine-conjugated bile acids exclusively. Cats appear to have a requirement for taurine because withdrawal of dietary taurine causes the degeneration of the retina leading ultimately to blindness. Taurine deficiency does not appear to have any significant consequences in other mammalian species. Conjugated bile acids are more acidic than unconjugated bile acids due to the carboxyl group contributed by the amino acid. Consequently, conjugated bile acids readily ionize and exist mainly as bile salts at physiological pH. The functional significance

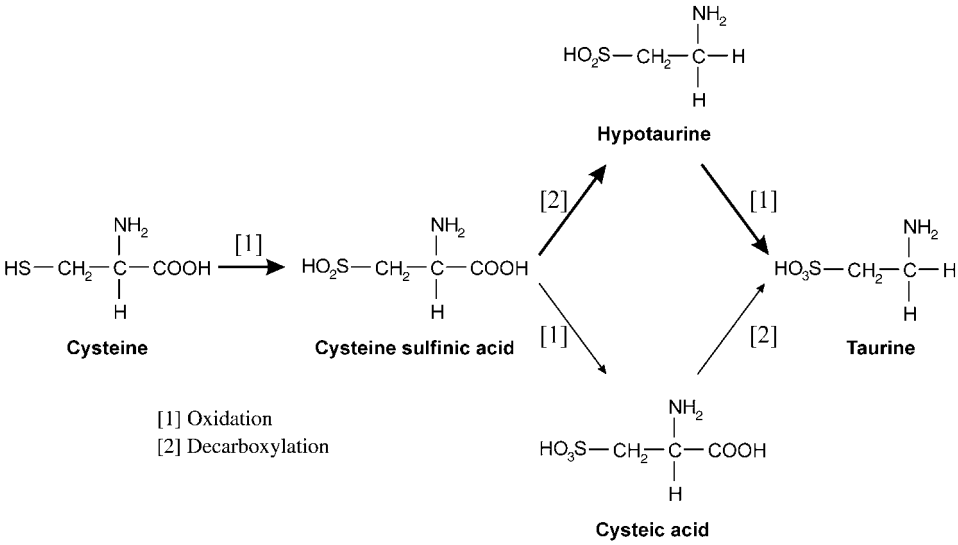

Fig. 3. Synthesis of taurine from cysteine. The major pathway for the formation of taurine is via hypotaurine.

of the choice of amino acid used for conjugation is not clear. Cultured rat hepatoma cells show differential sensitivity to taurine- and glycine-conjugated bile acids [3]. In these cells, glycine-conjugated bile acids are toxic and induce cell death by apoptosis whereas taurine-conjugated bile acids are well-tolerated and even promote cell survival.

3. Biosynthesis of bile acids

Classical studies elucidated the major steps in the bile acid biosynthetic pathway mainly by analyzing the metabolites formed from labeled cholesterol and oxysterols. Several distinct reactions occurring in various subcellular compartments (cytosol, endoplasmic reticulum, mitochondria, and peroxisomes) are necessary to transform cholesterol into bile acids [4]. Reactions involving modifications of the steroid nucleus occur in the endoplasmic reticulum and mitochondria. The removal of the cholesterol side chain involves peroxisomes. Many of the enzymes that catalyze these reactions have been purified, their cDNAs cloned and ectopically expressed in a variety of cultured cell lines. In addition, the impact of overactivity and deficiency of some of these enzymes in the formation of bile acids in vivo has been studied through the use of gene therapy, transgenic and targeted gene disruption techniques.

3.1. The classical and alternative bile acid biosynthetic pathways

The classical biosynthetic pathway operates entirely in the liver (Fig. 4). First, α-hydroxylation occurs on carbon 7 of the cholesterol steroid nucleus. This reaction is catalyzed by the

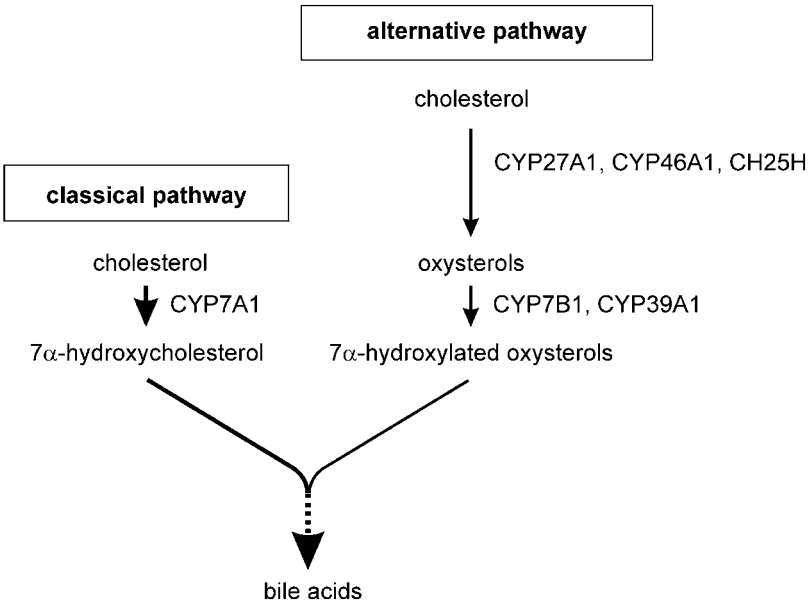

Fig. 4. The bile acid biosynthetic pathways. The classical pathway operates entirely in the liver and cholesterol 7α-hydroxylase (CYP7A1) initiates the pathway. In other tissues, the entry of cholesterol into the alternate pathways is facilitated by sterol 27-hydroxylase (CYP27A1), cholesterol 24-hydroxylase (CYP46A1), and cholesterol 25-hydroxylase (CH25H). The oxysterols generated by these enzymes are 7α-hydroxylated by oxysterol 7α-hydroxylases CYP7B1 and CYP39A1, and the products enter the latter steps of the classical pathway.

microsomal cytochrome P-450 monooxygenase referred to as cholesterol 7α-hydroxylase (CYP7A1) and is the rate-limiting step of the classical pathway. Several of the enzymes that participate in the transformation of cholesterol into bile acids belong to the cytochrome P-450 family. In general, this class of enzymes catalyzes the hydroxylation of various organic compounds using molecular oxygen as a cosubstrate. The heme-containing monooxygenases recognize specific molecules, or a group of related compounds, and work in concert with NADPH:cytochrome P-450 oxidoreductase which supplies electrons for the reactions. CYP7A1 shows selectivity towards cholesterol. Bile acid output from the liver is correlated with the CYP7A1 activity, and it is generally considered that the classical pathway is the source of the bulk of the bile acids made by the liver.

The existence of an alternate pathway for the synthesis of bile acids was suspected because it was possible for oxysterols to be converted into bile acids (N. Wachtel, 1968). It is now recognized that a variety of oxysterols produced by an assortment of cell types can be converted into bile acids. The production of these oxysterols is catalyzed by several sterol hydroxylases: sterol 27-hydroxylase (CYP27A1) (J.J. Cali, 1991), cholesterol 25-hydroxylase (CH25H) (E.G. Lund, 1998), and cholesterol 24-hydroxylase (CYP46A1) (E.G. Lund, 1999). Cholesterol 25-hydroxylase is not a cytochrome P-450 monooxygenase, unlike the two other enzymes. Almost all of the 24-hydroxycholesterol that ends up in the liver originates from the brain, and it has been suggested that the production of

this oxysterol is a major mechanism for eliminating excess cholesterol from this organ (I. Bjorkhem, 2001). CYP27A1 is also important in the latter stages of bile acid synthesis in the liver, as it is the major enzyme that catalyzes the hydroxylation of the side chain which facilitates the cleavage of the sterol side chain. The oxysterols generated outside the liver are 7α-hydroxylated, mainly by oxysterol hydroxylases distinct from CYP7A1. The CYP7B1 oxysterol 7α-hydroxylase prefers 25-hydroxycholesterol and 27-hydroxycholesterol, whereas CYP39A1 oxysterol 7α-hydroxylase is selective for 24-hydroxycholesterol (J. Li-Hawkins, 2000). Although CYP7A1 shows a high preference towards cholesterol, some oxysterols can also be accepted as substrates.

The latter steps required to complete the synthesis of bile acids occur only in the liver and are common to both the classical and alternative pathways. Consequently, the liver is the only organ in the body capable of producing bile acids. The isomerization of the 3β-hydroxyl group and the saturation of the steroid nucleus involve 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (K. Wikvall, 1981), 3-oxo-Δ^4-steroid 5β-reductase (O. Berseus, 1967), and 3α-hydroxysteroid dehydrogenase (A. Stolz, 1987). The activities of these enzymes are necessary for the formation of normal bile acids. The enzyme sterol 12α-hydroxylase (CYP8B1) catalyzes the addition of a hydroxyl group to carbon 12 of the steroid nucleus, and therefore controls the production of the cholic and chenodeoxycholic acids. Changes in the ratio of cholic to chenodeoxycholic acid affect the overall hydrophobicity of the bile acid pool.

The cDNA encoding an enzyme capable of catalyzing the 6α-hydroxylation of the steroid nucleus was cloned from pig liver (K. Lundell, 2001). This enzyme, named CYP4A21, is believed to be responsible for the formation of hyocholic acid, a bile acid typically found in porcine bile. β-muricholic acid is made from chenodeoxycholic acid but the detailed steps are not completely worked out. This bile acid is a major component of mouse and rat bile. The conversion of lithocholic acid (a 3α-monohydroxylated bile acid) into β-muricholic acid has also been observed, but the enzymes responsible for catalyzing the reactions are not known. Intestinal bacteria are thought to be responsible for modifying the steroid nucleus to form 7β-hydroxylated bile acids. However it has been noted that bear liver has the capacity to produce ursodeoxycholic acid, indicating the existence of hepatic enzymes that can catalyze the direct 7β-hydroxylation of the steroid nucleus or epimerization of the 7α-hydroxyl group (L.R. Hagey, 1993).

3.2. Mutations affecting key enzymes involved in bile acid biosynthesis

Bile acid synthesis represents a major pathway for cholesterol catabolism. In humans, bile acid excretion can account for the disposal of up to ~0.5 g of cholesterol per day. In animal studies, direct stimulation of bile acid synthesis by increasing the abundance of CYP7A1 enzyme in the liver through gene therapy, reduces the concentration of cholesterol in the plasma (D.K. Spady, 1995, 1998; L.B. Agellon, 1997). Consequently, it was reasonably expected that inhibiting bile acid synthesis by repression of CYP7A1 would impair cholesterol catabolism and lead to an increased concentration of plasma cholesterol. In mice, the complete loss of CYP7A1 function results in a high incidence of neonatal lethality due mainly to inefficient absorption of fats and fat-soluble vitamins [5]. CYP7A1-deficient mice that manage to survive beyond the weaning period synthesize bile acids via the

alternative pathway [6]. The original CYP7A1-deficient strain did not exhibit hypercholesterolemia, but in mice from a subline developed by selective breeding, plasma cholesterol concentration is higher than in wild-type mice (S.K. Erickson, 2003). In humans, CYP7A1 deficiency is rare, and individuals with this condition do display hypercholesterolemia and appear to be predisposed to premature atherosclerosis (C.R. Pullinger, 2002). It is not clear if the loss of CYP7A1 activity affects human neonatal survival.

Mutations in human CYP27A1 cause cerebrotendinous xanthomatosis (J.J. Cali, 1991). This disorder, which is characterized by neurological defects and premature atherosclerosis, may be the consequence of sterol accumulation in neural and other tissues. Cerebrotendinous xanthomatosis patients have reduced capacity for normal bile acid synthesis but produce large amounts of bile alcohols, consistent with the importance of CYP27A1 in the removal of the cholesterol side chain. Interestingly, deficiency of CYP27A1 in mice does not elaborate the full complement of defects observed in humans with cerebrotendinous xanthomatosis [7]. The basis for the difference is not completely understood. It has been suggested other cytochrome P-450 enzymes, CYP3A4 in particular, partially compensate for the missing functions supplied by CYP27A1 in the murine species (A. Honda, 2001). Indeed, CYP27A1-deficient mice are still capable of producing normal C24 bile acids but overall bile acid synthesis is markedly diminished. This finding confirms that CYP27A1 activity is quantitatively important in side chain cleavage, but that hydroxylation of another carbon in the side chain can permit some side chain cleavage. McArdle RH-7777 rat hepatoma cells are deficient in both CYP7A1 and CYP27A1, and no longer possess the capacity to synthesize bile acids. However, reinstatement of CYP7A1 activity enables these cells to synthesize C24 bile acids despite the absence of CYP27A1 activity. CYP27A1 deficiency causes hypertriglyceridemia and hepatomegaly in mice, indicating that CYP27A1 function affects other metabolic processes in this species (J.J. Repa, 2000).

Anomalous expression of the genes encoding the two other known hydroxylases that facilitate the entry of cholesterol into the bile acid biosynthetic pathway does not appear to have much effect on the systemic metabolism of cholesterol. The *Cyp46a1* gene, which encodes cholesterol 24-hydroxylase, is expressed in only a small but specific subset of neurons in the brain. It was recently found that mice lacking CYP46A1, although not showing obvious physical abnormalities, have severe learning deficiencies [8]. The *Ch25h* gene, which encodes cholesterol 25-hydroxylase, is expressed at a low level in a wide variety of tissues. A controversial observation is that altered CH25H expression in the brain may predispose to the development of Alzheimer's disease (M. Riemenschneider, 2004; A. Papassotiropoulos, 2005). It is of interest to note that defective formation of oxysterols via CYP27A1, CYP46A1, and CH25H is associated with some form of neurological dysfunction. These associations support the idea that the synthesis of bile acids from oxysterols is an important route for the catabolism of excess cholesterol.

The importance of the CYP7B1 oxysterol 7α-hydroxylase in bile acid synthesis has also been studied in mice. Mice lacking this microsomal enzyme are viable and do not exhibit obvious defects in cholesterol or bile acid metabolism [9]. The notable feature in CYP7B1-deficient mice is the accumulation of 25- and 27-hydroxycholesterol in plasma and cells, suggesting that CYP7B1 is important for the catabolism of these oxysterols into bile acids. In humans, mutations in CYP7B1 result in severe neonatal liver disease characterized by cholestasis (arrest of bile flow) and cirrhosis (damage and scarring of

liver tissue resulting from chronic impaired liver function) [10]. Mutations in 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase and 3-oxo-Δ^4-steroid 5β-reductase are also known to cause progressive intrahepatic cholestasis [11–13]. The effect of CYP39A1 oxysterol 7α-hydroxylase mutations in animals has not yet been described.

The importance of peroxisomes in the cleavage of the cholesterol side chain during bile acid synthesis is well illustrated in Zellweger syndrome. This genetic disorder is characterized by peroxisome deficiency and accumulation of large amounts of bile alcohols in the plasma of afflicted patients (R.J. Wanders, 1987). Mice carrying an induced mutation in the *Scp2* gene also accumulate bile alcohols similar to those seen in Zellweger patients [14]. The *Scp2* gene encodes two proteins: the cytosolic sterol carrier protein-2 and the peroxisomal sterol carrier protein-x. SCPx contains the entire SCP2 sequence plus an N-terminal domain that has a β-ketothiolase activity (U. Seedorf, 1994). The basis for the accumulation of bile alcohols in mice homozygous for a mutant *Scp2* gene is the deficiency in peroxisomal β-ketothiolase activity supplied by SCPx.

4. Transport of bile acids

4.1. Enterohepatic circulation

Bile acids circulate between the liver and intestines via bile and portal blood. The path traced by bile acids between these two organs is depicted in Fig. 5, and is referred to as

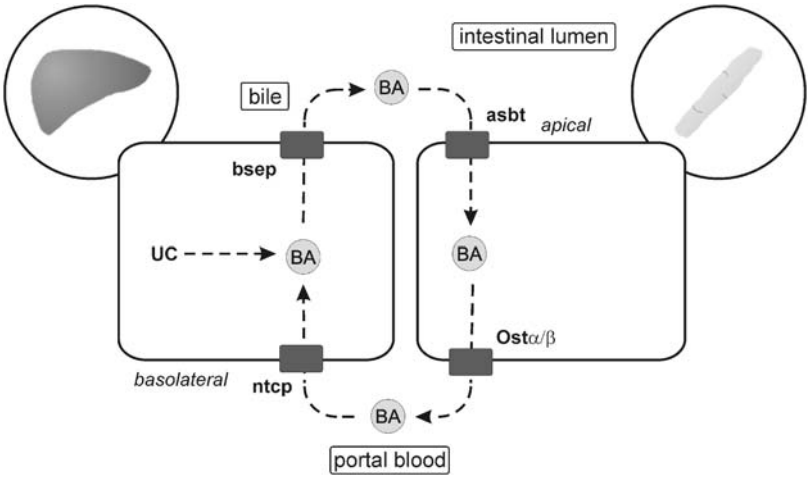

Fig. 5. Transport of bile acids in the enterohepatic circulation. The left and right sides of the figure depict a liver and intestinal cell, respectively. Bile acids (BA) are made from unesterified cholesterol (UC) in the liver. The movement of bile acids in the enterohepatic circulation is vectorial. The major transporters thought to be responsible for the entry and exit of bile acids in liver and intestinal cells are sodium/taurocholate cotransporting polypeptide (ntcp; SLC10A1), bile salt export pump (bsep; ABCB11), apical/sodium bile acid cotransporter (asbt; SLC10A2), and organic solute transporters α/β, (Ostα/β).

the enterohepatic circulation [15]. A number of transporters involved in the transport of bile acids have been described [16]. Hepatocytes recover bile acids from portal blood by an active process involving sodium/taurocholate cotransporting polypeptide (ntcp; SLC10A1). The recovered bile acids, along with newly synthesized bile acids, are secreted into bile via the bile salt export pump (bsep; ABCB11). This protein belongs to the ATP-binding cassette (ABC) family of transporters [17]. Mutations in human bsep are known to cause progressive familial intrahepatic cholestasis type 2 [18]. However, targeted disruption of the murine *Abcb11* gene does not recapitulate the human disease phenotype in mice [19]. This finding is another example illustrating a difference between human and murine bile acid metabolism.

The secreted bile acids are stored in the gallbladder prior to being released into the small intestine. An exception occurs in the rat (but not in the mouse), which lacks a gallbladder and thus continuously releases bile into the intestine. Gallbladder emptying is hormonally induced by cholecystokinin in response to feeding. Recently, it was found that FGF15/19 can promote gallbladder filling [20]. The primary bile acids (the products of bile acid biosynthesis in the liver) are metabolized by enteric bacteria to produce deconjugated (i.e., lacking taurine or glycine), and/or dehydroxylated derivatives referred to as secondary bile acids. The secondary bile acids may be further modified by sulfation and/or glucuronidation. The deconjugated bile acids ('free' bile acids) are absorbed along the entire axis of the intestines. The majority of the conjugated bile acids are recovered in the terminal ileum via an active process involving the apical/sodium bile acid transporter (asbt; SLC10A2) [21]. In humans, mutations in asbt cause primary bile acid malabsorption (P. Oelkers, 1997). A heteromeric transporter, composed of organic solute transporters α and β (Ostα/β), has been identified as the basolateral exporter of bile acids in enterocytes (P. Dawson, 2005).

As already mentioned, the major lipids that are present in bile are the bile acids, phospholipids (mainly phosphatidylcholines, PC), and unesterified cholesterol. The solubility of cholesterol in bile is dependent on the ratio of these molecules. The acyl chain composition of biliary PC (predominantly C16:0 at the *sn*-1 position and either C18:1 or C18:2 at the *sn*-2 position) are distinct from that normally found in bulk cell membranes (predominantly C18:0 at the *sn*-1 position and C20:4 at the *sn*-2 position). The secretion of PC into bile requires a canalicular membrane protein referred to as mdr2 (ABCB4), another transporter belonging to the ABC family of transporters. The concentration of PC in the bile of mice that are deficient in mdr2 is extremely low [22]. The secretion of bile acids into bile is not affected by mdr2 deficiency. However, cholesterol concentration in the bile of mdr2-deficient mice is diminished, indicating that the secretion of cholesterol into bile is dependent on biliary PC. In humans, mutations in MDR3 (the human equivalent of mdr2) cause a condition known as progressive familial intrahepatic cholestasis type 3 [18]. The secretion of cholesterol into bile is facilitated by a heterodimer of two 'half' ABC-type transporters called ABCG5 and ABCG8. The ABCG5/8 complex is localized to the apical membranes of hepatocytes as well as of enterocytes. Loss-of-function mutations in the genes encoding either ABCG5 or ABCG8 decrease biliary cholesterol output but these mutations appear to have a greater impact on plant sterol metabolism, causing massive accumulation of plant sterols in blood and tissues (sitosterolemia) [18].

4.2. Intracellular transport of bile acids

The mechanism for the intracellular transport of bile acids is less understood than the uptake and secretion of bile acids by liver and intestinal cells. Although several intracellular proteins are capable of binding bile acids, it is not yet clear if these proteins are in fact involved in the transcellular transport of bile acids [23]. The candidate protein in intestinal cells is the ileal lipid binding protein (ilbp; FABP6, also referred to as IBABP). This protein, a member of the intracellular lipid binding protein family [24], is abundantly expressed in the distal portion of the small intestine where asbt is found. It has been suggested that ilbp and asbt interact to form a macromolecular bile acid transport system in intestinal cells. The protein providing the equivalent function in liver cells is not known. The liver-fatty acid binding protein does not bind bile acids efficiently. High level expression of the human bile acid binder in hepatoma cells capable of active bile acid uptake does not appear to influence bile acid transport [3].

5. Molecular regulation of key enzymes in the bile acid biosynthetic pathways

Bile acid synthesis is modulated by a variety of hormonal and nutrient factors. Alterations in bile acid metabolism have been documented in response to thyroid hormones, glucocorticoids, and insulin. It is also well known that cholesterol and bile acids have opposite effects on the activity of the bile acid biosynthetic pathway (Fig. 6). A major advance into the understanding of the mechanisms that regulate bile acid synthesis came with the cloning of the rat CYP7A1 cDNA (D.F. Jelinek, 1990), which permitted the expression of the *CYP7A1* gene to be monitored at the molecular level. Many of the details relating to the molecular mechanisms involved in regulating bile acid synthesis have been elucidated using both cultured cells and genetically modified mouse strains.

Feeding rats with a cholesterol-enriched diet induces bile acid synthesis. This increase is attributable to the rise in CYP7A1 activity, which catalyzes the rate-limiting step of the classical pathway (Fig. 6). Interruption of the return of bile acids to the liver, by

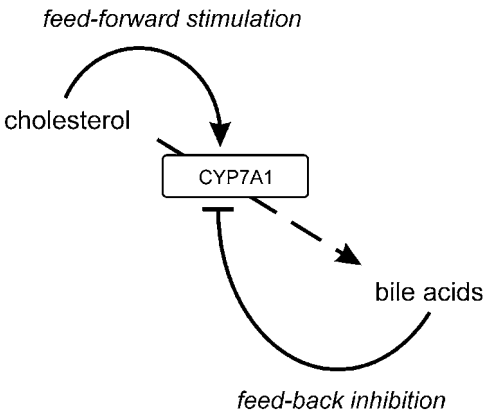

Fig. 6. Regulation of bile acid synthesis.

diverting bile or by feeding a bile acid-binding resin, also stimulates the synthesis of bile acids. In contrast, re-introduction of bile acids into bile-diverted rats reverses the stimulatory effect, indicating that bile acid synthesis is subject to end-product inhibition. It was later established that the enzyme activity of CYP7A1 closely parallels CYP7A1 mRNA abundance, suggesting that *Cyp7a1* gene transcription is the major determinant of CYP7A1 activity.

In many of the early studies, crystalline cholesterol was added directly to the standard rodent chow and fed to the animals. Although this experimental condition was useful in illustrating the stimulation of the *CYP7A1* gene in response to dietary cholesterol, it does not normally occur in nature. The use of semi-purified diets has revealed that the composition of the fat in which cholesterol is presented has a marked influence on the ability of cholesterol to regulate *CYP7A1* gene expression (S.K. Cheema, 1997). It has also become apparent that the fat component of the diet is capable of stimulating murine *CYP7A1* gene expression, independent of exogenous cholesterol. Changes in the activities of enzymes involved in bile acid metabolism have also been documented in response to fasting and refeeding, as well as altered thyroid hormone, corticosteroid, and insulin status.

The regulation of the classical and alternative bile acid biosynthetic pathways has been studied mostly in mice and rats. The data indicate that the classical pathway is under stringent regulation, with much of the control exerted on the *CYP7A1* gene, whereas the alternative pathway appears to operate constitutively. There is also emerging evidence indicating that the synthesis of bile acids in humans is only moderately regulated, unlike in mice and rats.

5.1. Transcriptional control

Several transcription factor-binding sites have been mapped in the *CYP7A1* gene promoter, and most of these bind transcription factors that are members of the nuclear receptor superfamily (Table 2). Some of these receptors, notably the liver X receptor α (LXRα; NR1H3) and peroxisome proliferator-activated receptor α (PPARα; NR1C1), bind to their target elements as heterodimers with retinoid X receptor α (RXRα; NR2B1).

Table 2
Transcription factors shown to have functional interaction with the *CYP7A1*/*Cyp7a1* gene promoter

Transcription factor	Species
BTEB (Klf9)	Rat
C/EBPβ	Rat
COUP-TFII (NR2F2, ARP-1, EAR3)	Rat
DBP	Rat
HNF-1 (Tcf-1)	Human
HNF-3α (Foxa1)	Human, hamster, rat
HNF-4α (NR2A1, Tcf4)	Human, hamster, rat
LRH-1 (FTF, NR5A2)	Human, rat
LXRα (NR1H3)	Rat
PPARα (NR1C1)	Mouse
TRα (NR1A1) and TRβ (NR1A2)	Human

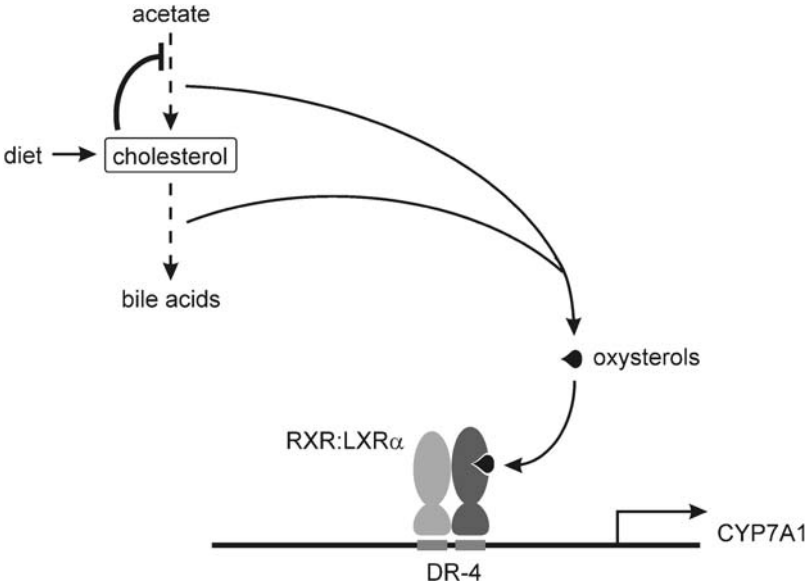

Fig. 7. Induction of *Cyp7a1* gene expression by oxysterol-activated LXRα:RXRα. Both the bile acid and cholesterol biosynthetic pathways generate oxysterols. The binding site of LXRα:RXRα in the *Cyp7a1* gene promoter is a DR-4 element (a direct repeat of the hexanucleotide hormone response element separated by 4 nt).

The stimulation of *CYP7A1* gene expression by cholesterol involves LXRα (Fig. 7), an oxysterol-activated transcription factor [25]. In cultured cells, induction of the rat *Cyp7a1* gene promoter by oxysterols is dependent on LXRα. In LXRα-deficient mice, the *Cyp7a1* gene is no longer induced by cholesterol feeding (D.J. Peet, 1998). The oxysterols that serve as potent ligands for LXRα are likely generated by the early steps in the alternative bile acid biosynthetic pathway (i.e., 25-hydroxycholesterol and 27-hydroxycholesterol), and by the cholesterol biosynthetic pathway (i.e., 24(*S*),25-epoxycholesterol). It is notable that 24(*S*),25-epoxycholesterol is also capable of repressing 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (T.A. Spencer, 1985).

Fatty acids and their metabolites can stimulate the murine *Cyp7a1* gene promoter via PPARα:RXRα in hepatoma cells [26]. Interestingly, LXRα:RXRα and PPARα:RXRα heterodimers bind to overlapping regions in the murine *CYP7a1* gene promoter. It is currently not known how these transcription factors interact with the *Cyp7a1* gene promoter when both are simultaneously activated, or whether this is relevant to the earlier finding that the type of fat in the diet influences the response of the murine *Cyp7a1* gene to dietary cholesterol. The corresponding region of the *human CYP7A1* gene promoter does not interact with either LXRα:RXRα or PPARα:RXRα. In transgenic mice, the human *CYP7A1* gene is not stimulated by cholesterol feeding [27]. A differential interaction of thyroid hormone receptors (TRα; NR1A1 and TRβ; NR1A2) with the *Cyp7a1* gene promoters of different species has also been documented [28]. The human *CYP7A1* gene

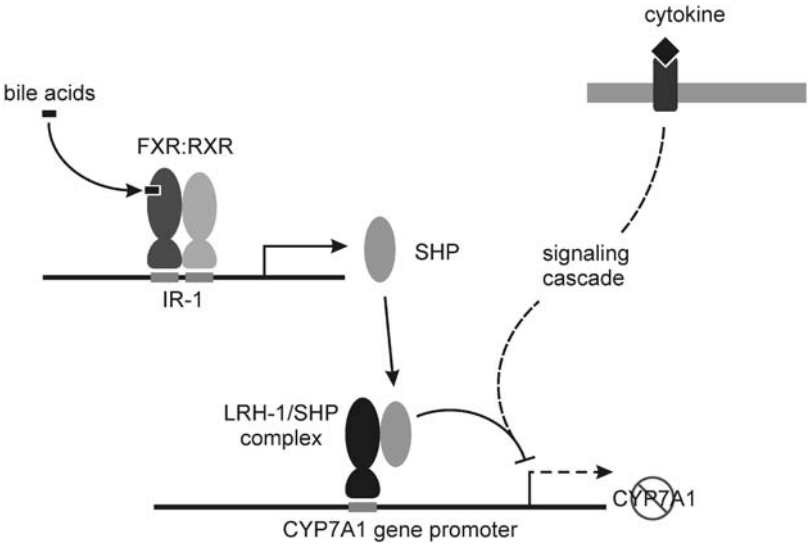

Fig. 8. Repression of *Cyp7a1* gene expression by bile acids. The liver receptor homolog-1 (LRH-1) binds to the *Cyp7a1* gene promoter to enable expression in the liver. A bile acid-activated FXR:RXR heterodimer binds to an IR-1 element (an inverted repeat of the hexanucleotide hormone response element separated by 1 nt) in the promoter of the gene that encodes the small heterodimer partner (SHP) and stimulates its expression. The binding of SHP to LRH-1 arrests the expression of the *Cyp7a1* gene. *Cyp7a1* gene expression can also be repressed via an alternate mechanism involving the binding of cytokines to membrane receptors and the activation of signaling cascades. The production of these extracellular messengers may be stimulated by bile acids (Section 5.1).

promoter binds to and is inhibited by the thyroid hormone receptor whereas the comparable region of the murine *Cyp7a1* gene promoter does not bind the thyroid hormone receptor. Murine *Cyp7a1* gene expression is stimulated by thyroid hormone, and mice lacking thyroid hormone receptor-β do not show this response to thyroid hormone treatment [29]. These differences may indicate that the promoters of the gene encoding CYP7A1 in different species are configured to respond to regulatory cues that are relevant to specific species.

An indirect mechanism for the inhibition of *Cyp7a1* gene expression by bile acids has been proposed (Fig. 8). The liver receptor homolog protein-1 (LRH-1; NR5A2) is a monomeric orphan nuclear receptor that binds to the *Cyp7a1* gene promoter to enable expression in the liver (M. Nitta, 1999). The binding of bile acids to the farnesoid X receptor (FXR; NR1H4), another transcription factor belonging to the nuclear receptor superfamily, has been documented (L.Z. Mi, 2003). Bile acids stimulate the expression of certain genes in an FXR-dependent fashion, supporting the notion that bile acids are the physiological ligands of FXR. FXR also stimulates the expression of the gene encoding the nuclear factor known as small heterodimer partner (SHP; NR0B2) (T.T. Lu, 2000; B. Goodwin, 2000). The interaction of SHP with LRH-1 renders the *Cyp7a1* gene promoter insensitive to stimulation by other transcription factors.

Studies on FXR-deficient mice indicate that bile acids can also regulate gene expression independently of FXR. However, SHP-deficient mice still show inhibition of *Cyp7a1*

and *Cyp8b1* gene expression in response to cholic acid feeding but not to a synthetic FXR agonist (T.A. Kerr, 2002). Other studies have demonstrated that the *Cyp7a1* gene can also be inhibited by bile acids by interfering with the recruitment of coactivators onto HNF-4α (NR2A1) on the *Cyp7a1* gene promoter (E. De Fabiani, 2003), or via a mechanism involving the JNK/c-jun pathway (J.H. Miyake, 2000; S. Gupta, 2001). It was recently discovered that fibroblast growth factor 15 (FGF15) (FGF19 in humans) also participates in the inhibition of *Cyp7a1* gene expression [30]. The expression of the murine gene encoding FGF15 in the small intestine is regulated by bile acids via FXR. FGF15 secreted into blood by enterocytes binds to the FGF receptor 4 on liver cells, causing the activation of a signaling cascade that ultimately leads to the inhibition of the *Cyp7a1* gene.

Bile acids inhibit the expression of the *Cyp8b1* gene (encodes the sterol 12α-hydroxylase) in parallel with the *Cyp7a1* gene. Suppression of *Cyp8b1* gene expression appears to be mediated through SHP, as LRH-1 is also required for *Cyp8b1* promoter activity (A. del Castillo-Olivares, 2000). The expression of the rat *Cyp8b1* gene is also inhibited by thyroid hormones, but it is not clear whether this effect involves the interaction of the thyroid hormone receptor with the rat *Cyp8b1* gene promoter (U. Andersson, 1999). Moreover thyroid hormones, unlike bile acids, exhibit opposite effects on the expression of *Cyp8b1* and *Cyp7a1* genes in rats.

5.2. Post-transcriptional control

The majority of the studies described in the literature dealing with the regulation of bile acid synthesis have focused on CYP7A1 and it is apparent that most of the control is exerted at the level of gene transcription. The CYP7A1 mRNA has a short half-life, attributable to the existence of multiple copies of the AUUUA motif in its 3′-untranslated region (L.B. Agellon, 1997). However, some bile acids can further accelerate the decay of chimeric mRNAs containing the 3′-untranslated region of the murine CYP7A1 mRNA in hepatoma cells, and this effect is independent of the AUUUA element [31]. The regulation of CYP7A1 enzyme activity by phosphorylation/dephosphorylation has been suggested, but the results from several studies are conflicting. Phosphorylated forms of CYP7A1 have been detected in HepG2 human hepatoblastoma cells (D. Stroup, 2005).

6. The expanding role of bile acids in metabolism

Since the initial discovery that bile acids are ligands of FXR, it has become apparent that these molecules also serve as ligands for two other nuclear receptors known as the pregnane X receptor (PXR; NR1I2) (J.L. Staudinger, 2001) and the vitamin D receptor (VDR; NR1I1) (M. Makishima, 2002). Both of these receptors prefer secondary over primary bile acids and induce the expression of genes involved in the latter steps of bile acid catabolism, suggesting that these receptors protect both the liver and intestine from the potential cytotoxic effects of hydrophobic secondary bile acids. Additionally, bile acids may play an important part in controlling bacterial growth in the small intestine (T. Inagaki, 2006).

A connection between bile acid, triacylglycerol, and carbohydrate metabolism has also emerged. The loss of FXR in mice results in increased hepatic and plasma triacylglycerol concentrations. Previously, a decrease in plasma triacylglycerol concentration was observed in human subjects that were fed chenodeoxycholic acid (B. Angelin, 1978). More recently, it has been shown that cholic acid feeding can prevent hypertriglyceridemia in mice (M. Watanabe, 2004). The proposed mechanism for this effect is the inhibition of genes involved in lipogenesis by attenuating sterol response element binding protein-1C [32] function via SHP. Mice lacking FXR also exhibit impaired glucose tolerance and reduced insulin sensitivity [33]. A study showing that glucose could alter FXR gene expression in rat hepatocytes suggested the possibility that bile acids and glucose metabolism may be linked (D. Duran-Sandoval, 2004). Indeed, bile acid feeding inhibits the expression of genes involved in glucose formation (e.g., genes encoding phosphoenolpyruvate kinase, glucose-6-P phosphatase, fructose-1,6-bisphosphate phosphatase), and mice lacking FXR are not able to decrease the expression of these genes in response to bile acids.

The previously described membrane proteins capable of binding bile acids are transporters responsible for their internalization or externalization. Recently, bile acids were shown to be a ligand for a G coupled cell surface receptor called TGR5 (Y. Kawamata, 2003). This receptor is found in macrophages (including Kupffer cells), sinusoidal endothelial cells, and brown adipocytes. Binding of bile acid to TGR5 causes the activation of a mitogen-activated protein kinase signaling cascade and the rise of intracellular cyclic AMP levels as demonstrated by in vitro studies. The full significance of this interaction on whole body energy metabolism became evident from a study using mice lacking cAMP-dependent thyroid hormone activating enzyme type 2 iodothyronine deiodinase (encoded by the *Dio2* gene) where it was found that the high fat diet with added cholic acid had no effect on the body weight gain in these mice whereas it was attenuated in wild-type mice [34]. The apparent basis for this difference is the inhibition of lipid deposition in brown adipose tissue. It is proposed that the increase in intracellular cyclic AMP levels resulting from the binding of bile acids to TGR5 in brown adipocytes is a key step required for the induction of the type 2 iodothyronine deiodinase enzyme.

7. Future directions

The pathways leading to the synthesis, transport, and disposal of bile acids have been uncovered. It is clear that these processes are under tight and coordinated control. The use of genetically modified mice has been instrumental in illustrating the importance of bile acids in regulating the activities of several metabolic pathways. Over the last few years, it has become apparent that bile acids possess a much wider repertoire of biological activities than originally thought, extending well beyond the classically known role of a detergent aiding in the solubilization of lipids. By serving as a ligand for several nuclear receptors, bile acids have the capacity to regulate the expression of genes involved not only in its own metabolism, but also key genes involved in fat and carbohydrate metabolism. Moreover, the ability of bile acids to modulate signaling cascades not only in liver but also in peripheral tissues firmly establishes their ability to modulate a variety of cellular

processes within the body. In the coming years, it will be exciting to learn how this collection of molecules that were once thought of as simple catabolic end-products of cholesterol orchestrate the acquisition and processing of energy-rich lipids, as well as the storage and use of energy extracted from these substances.

Abbreviations

ABC	ATP-binding cassette
CYP7A1	cholesterol 7α-hydroxylase
CYP7B1	oxysterol 7α-hydroxylase
CYP8B1	sterol 12α-hydroxylase
CYP27A1	sterol 27-hydroxylase
FXR	farnesoid X receptor
LXRα	liver X receptor α
PC	phosphatidylcholine
PPARα	peroxisome proliferator-activated receptor α
SHP	small heterodimer partner
RXR	retinoid X receptor

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CHAPTER 16

Lipid assembly into cell membranes

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Contents

1. Introduction

A fundamental problem of cell biology and biochemistry is the elucidation of the mechanisms by which the specific components of subcellular membranes are assembled into mature organelles. The major components of all cell membranes are lipids and proteins. The presence of discrete structural motifs contained in the primary sequence of proteins directs a large number of post-translational processes that enable their sorting among different membrane compartments [1]. The sorting process for proteins is essentially absolute such that plasma membrane proteins are never found in the mitochondria or vice versa. In contrast, lipid molecules do not contain discrete structural subdomains that exclusively direct their movement to specific membranes. The distribution of lipids among different organelles is heterogeneous, but (with a few exceptions) is not usually absolute. These observations indicate that specialized sorting and transport machinery must exist for lipid assembly into different membranes, but the mechanisms governing these processes are only now beginning to be understood at the molecular level.

2. The diversity of lipids

A multiplicity of individual lipids can contribute to membrane formation. The biological role of this lipid heterogeneity is not completely understood and the list of significant actions continues to grow. Some of the diversity contributes to membrane fluidity. Another role for lipid diversity is the storage of precursors that are metabolized to potent second messengers (e.g., diacylglycerol (DG), ceramide, sphingosine, sphingosine-1 phosphate, lyso-phosphatidic acid, inositol trisphosphate, and eicosanoids) (Chapter 12). In addition, many polyphosphoinositides function as membrane recognition and attachment sites for protein complexes involved in protein traffic and membrane fusion events [2]. Phosphatidylserine (PS) and polyphosphoinositides can also regulate attachment of cytoskeletal proteins to membranes. Minor lipids, such as lyso-bisphosphatidic, also play crucial roles in endosomal sorting processes. Segregated domains of cholesterol and sphingolipid form microdomains with unusual physical properties that contribute to protein sorting and are enriched in specific subsets of membrane proteins (Chapter 1).

In addition to the large numbers of chemically distinct lipid species that occur within a prokaryotic or a eukaryotic cell, there is another level of complexity — the asymmetric distribution of the lipids across the plane of the bilayer. Two striking examples of membrane lipid asymmetry were originally described in the red blood cell membrane (A. Verkleij, 1973), and the cytoplasmic membrane of *Bacillus megaterium* (J. Rothman, 1977). The data in Fig. 1

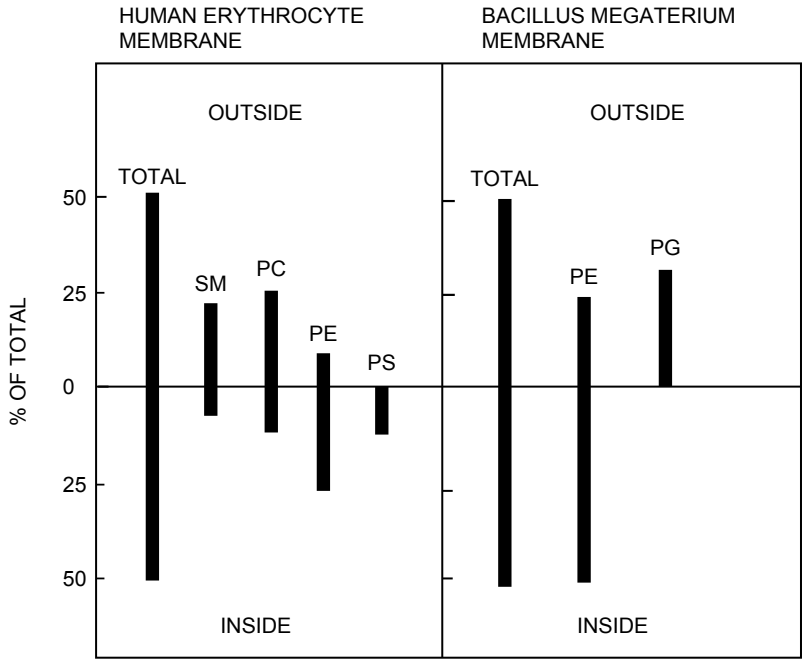


Fig. 1. The asymmetric distribution of lipids across the plane of the cell membrane of human erythrocytes and *Bacillus megaterium*.

demonstrate that in the red cell membrane, the outer leaflet of the lipid bilayer is composed primarily of sphingomyelin (SM) and phosphatidylcholine (PC), and the inner leaflet contains PS and phosphatidylethanolamine (PE), with lesser amounts of PC and SM. Relatively small amounts of phosphatidylinositol (PI) and its phosphorylated derivatives are also found in the erythrocyte membrane and these anionic lipids are distributed such that the majority are localized to the inner leaflet of the bilayer. These lipid distributions across the bilayer for red blood cells have also largely been confirmed for nucleated eukaryotic cells. In the prokaryote *B. megaterium*, the distribution of PE has been shown to be asymmetric, with 30% of this lipid present on the outer leaflet of the bilayer and 70% on the inner leaflet. PE comprises about 70%, and phosphatidylglycerol (PG) about 30%, of the total phospholipid. Thus, nearly all the PG is in the outer leaflet of the bilayer.

Yet another level of complexity is found in cells that possess multiple membrane systems. The gram-negative bacteria have both inner and outer membrane systems that differ in lipid composition (Chapters 1 and 3). In animal eukaryotes there are numerous membrane systems, the best characterized being endoplasmic reticulum (ER), Golgi, plasma, mitochondrial, endosomal, lysosomal, peroxisomal, and nuclear membranes. In higher plants, the eukaryotic organelle repertoire is expanded to include chloroplasts and other plastids, vacuoles, and glyoxysomes (Chapter 4). Several of these membrane systems have dramatically different lipid compositions, as shown in Table 1 (A. Colbeau, 1971; T.W. Keenan, 1970). These differences in lipid content raise a variety of interesting questions: how are the lipid compositions of different organelles established? How are these differences maintained? Are the different lipid compositions essential for organelle function?

3. Methods to study intra- and intermembrane lipid transport

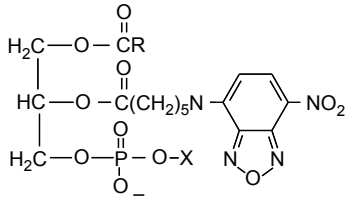
3.1. Fluorescent probes

Pagano and co-workers pioneered methods for the rapid insertion of fluorescent phospholipid analogs from liposomes into the plasma membranes of cultured cells [3,4]. Virtually all of these analogs exhibit slight water solubility and high hydrophobic partitioning coefficients that enable them to be efficiently and reversibly transferred to cell surface membranes at low temperature from liposomes or albumin complexes containing the fluorescent lipid. A commonly used fluorochrome is nitrobenzoxadiazole (NBD), which is conjugated either to short-chain fatty acids in the *sn*-2 position of glycerophospholipids or to the amine of sphingosine. Other analogs such as boron dipyrromethene difluoride (BODIPY) derivatized fatty acids have proved equally effective. The structures of some of these analogs are shown in Fig. 2. Subsequent to the insertion of these lipid analogs into cell membranes, the cells can be washed at low temperature to remove the donor liposomes or albumin complexes. In almost all cases, this procedure results in the pulse labeling of the outer leaflet of the plasma membrane with the fluorescent lipid analog. Upon warming the cells, the fluorescent lipid moves from the plasma membrane to the cell interior by a variety of pathways. The lipid analogs can also be removed from the outer leaflet of the plasma membrane at reduced temperature by washing cells with a solution that contains

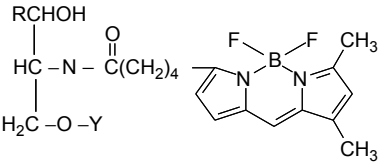
Table 1
Lipid compositions of subcellular organelles from rat liver

Phospholipid	Endoplasmic reticulum		Mitochondrial membranes		Lysosomal membrane	Nuclear membrane	Golgi membrane	Plasma membrane
	Rough	Smooth	Inner	Outer				
Lysophosphatidylcholine	2.9	2.9	0.6	–	2.9	–	5.9	1.8
Sphingomyelin	2.4	6.3	2.0	2.2	16.0	6.3	12.3	23.1
Phosphatidylcholine	59.6	54.4	40.5	49.4	41.9	52.1	45.3	43.1
Phosphatidylinositol	10.1	8.0	1.7	9.2	5.9	4.1	8.7	6.5
Phosphatidylserine	3.5	3.9	1	1	–	5.6	4.2	3.7
Phosphatidylethanolamine	20.0	22.0	38.8	34.9	20.5	25.1	17.0	20.5
Cardiolipin	1.2	2.4	17.0	4.2	–	–	–	–
Phospholipid/protein (μmol P/mg)	0.33	0.47	0.34	0.46	0.21	–	–	0.37
Cholesterol/phospholipid molar ratio	0.07	0.24	0.06	0.12	0.49	–	0.152	0.76

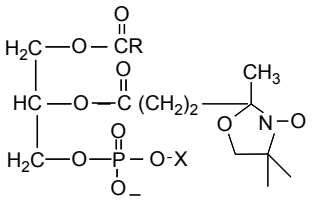
Values for individual lipids are percentage of total phospholipids phosphorus.



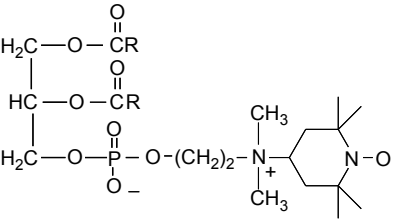
FLUORESCENT PHOSPHOLIPIDS
NBD-FLUOROCHROME



FLUORESCENT SPHINGOLIPIDS
BODIPY FLUOROCHROME



SPIN LABELED PHOSPHOLIPIDS
ACYL DERIVATIVE



SPIN LABELED PHOSPHOLIPIDS
HEAD GROUP DERIVATIVE

Fig. 2. General structural features of fluorescent and spin-labeled lipid analogs. The fluorescent lipids usually contain a short-chain fatty acid, amino-caproic acid, that is derivatized with 4-nitrobenzo-2-oxa-1,3-diazole (NBD), or valeric acid that is derivatized with a boron dipyrromethene difluoride (BODIPY) moiety. For fluorescent phospholipids, X can be hydrogen or the esterified forms of choline, ethanolamine, serine, or inositol. For fluorescent sphingolipids, Y can be hydrogen or the esterified forms of phosphocholine or mono- or oligosaccharides typical of glycosphingolipids. The spin-labeled lipids modified in the fatty acid portion contain a 4-doxylpentanoyl fatty acid in the *sn*-2 position. Those modified in the polar head group contain a tempocholine moiety in place of choline. The X substituent for the acyl spin-labeled lipids can be hydrogen or the esterified forms of choline, ethanolamine, or serine.

liposomes (e.g., composed of dioleoyl-PC) or albumin. When the lipid analogs are fluorescent their intracellular movement can be observed by fluorescence microscopy. In addition to their utility for examining the time-dependent changes in the fluorescence pattern within cells, these lipids can be extracted from cells and their chemical metabolism analyzed using thin-layer chromatography or liquid chromatography/mass spectrometry. The fluorescent sterols dehydroergosterol and NBD-cholesterol are also available for in vivo cellular studies. These sterols can be delivered to cells in reconstituted lipoproteins or from complexes with methyl-β-cyclodextrin (E. Kilsdonk, 1995).

3.2. Spin-labeled analogs

Paramagnetic analogs of phospholipids have also been used to investigate lipid transport phenomena in model membrane systems (R.D. Kornberg, 1971) and in biological membranes. Representative structures are shown in Fig. 2. Several of these spin-labeled lipid analogs that are modified in the fatty acid chain can be readily and reversibly transferred

from the bulk aqueous phase to biological membranes, in much the same way as the fluorescent lipid analogs. Since the amplitude of the electron spin resonance spectrum is proportional to the amount of spin-labeled lipid present, these analogs can be used to measure the depletion or retention of the lipids (M. Seigneuret, 1984). In a typical experiment, an intact red cell is incubated with trace amounts of spin-labeled phospholipid at reduced temperature. This treatment effectively pulse labels the outer leaflet of the plasma membrane. Upon warming, the spin-labeled lipids can either remain in the outer leaflet of the plasma membrane or be internalized by transport across the bilayer. If the cells are subsequently cooled and incubated in the presence of ascorbate, the electron spin resonance signal of lipid present in the outer leaflet (but not the inner leaflet) of the plasma membrane is quenched and the spectral difference can be used to determine both the rate and extent of transbilayer movement.

3.3. Asymmetric chemical modification of membranes

One method for ascertaining the distribution of lipids across the plane of the membrane bilayer is the use of membrane-impermeant reagents that react with the primary amines of PS and PE on only the external leaflet of the bilayer. Reagents such as trinitrobenzenesulfonate (TNBS), isethionylacetimidate, and fluorescamine are impermeant at reduced temperatures and the chemically modified lipids can be readily identified by thin-layer chromatography. When such reagents are used in conjunction with in vivo radiolabeling of the lipid, it is possible to discern the temporal and metabolic conditions required for the newly synthesized lipids to reach the compartment that is accessible to the chemical modifying reagents. A useful variation of this approach combines chemical reduction of NBD phospholipids with dithionite to eliminate fluorescence (J.C. McIntyre, 1991). When this latter technique is employed with fluorescence microscopy or spectrofluorometry, it can be extremely informative for resolving questions about transbilayer topology.

Specific pools of lipids on the external surface of cells can also be modified by the action of enzymes such as phospholipases, sphingomyelinases (A. Verkleij, 1973), and cholesterol oxidase (Y. Lange, 1985). These enzymes generate characteristic derivatives of the parental lipids that can be readily identified by thin-layer chromatography or mass spectrometry, providing another technique for identifying specific pools of lipid on the external surface of the cell membrane. Another means to sample lipids at membrane interfaces relies upon specific chemical desorption. The interaction between sterols and methyl-β-cyclodextrin provides a high-affinity interaction that can be used to selectively remove (or deliver) cholesterol and dehydroergosterol to membranes (E. Kilsdonk, 1995).

3.4. Lipid transfer proteins

In 1968, Wirtz identified a soluble intracellular protein derived from rat liver that was capable of binding PC and transferring it from one population of (donor) membranes to a second population of (acceptor) membranes (K. Wirtz, 1968). Since this initial observation, many of these proteins have been identified in virtually all mammalian tissues, in plants, and in yeast and other microorganisms [5]. The well-characterized phospholipid transfer proteins fall into three main categories: (i) those specific for PC; (ii) those with

high activity for PI and less, but significant, activity with PC, and in some cases SM (J. Westerman, 1995); and (iii) those with transfer activity with most phospholipids and cholesterol (this latter protein is referred to as the non-specific lipid transfer protein). In addition to the phospholipid transfer proteins, there are also intracellular proteins with high transfer activity for sphingolipids (T. Sasaki, 1990). The action of these proteins is usually a one-for-one exchange of lipid molecules between donor and acceptor membranes in vitro. Typically, the proteins equilibrate the lipid present in the outer leaflets of liposomes. The ability of these proteins to transfer lipids from accessible membrane compartments has made them useful tools for inserting lipids into, or removing them from, membranes and probing the transbilayer movement of phospholipids. The role of these proteins in membrane biogenesis will be discussed in Section 4.3.2.6.

3.5. Organelle-specific lipid metabolism

For a few lipids, distinct changes in structure also serve to define the arrival at certain organelles or their subcompartments. The enzyme PS decarboxylase is located at the inner mitochondrial membrane of mammalian cells (L.M.G. van Golde, 1974). The synthesis of PS, however, occurs primarily in the ER and related membranes (Chapter 8). Thus, the decarboxylation of PS can be used as an indicator of the transport of this lipid to the inner mitochondrial membrane [6]. Yeasts also contain a mitochondrial PS decarboxylase (Psd1p), and in addition, a second PS decarboxylase (Psd2p) is found in the Golgi. Mutations in the *PSD1* or *PSD2* genes of yeast make it possible to use the metabolism of PS to PE as an index of lipid transport to the locus of either the mitochondria or the Golgi. In yeast, the movement of PE (derived from either Psd1p or Psd2p) to the ER can also be followed by measuring its methylation to PC, since the PE-methyltransferases are present only in the ER. Site-specific metabolism also occurs for sphingolipids (Chapter 13). Ceramide, the hydrophobic precursor for all sphingolipids, is synthesized in the ER. The conversion of ceramide to SM occurs at the lumenal surface of the cis-medial Golgi (A.H. Futerman, 1990; D. Jeckel, 1990). Thus, SM synthesis from ceramide can be used to follow ceramide transport from ER to Golgi. The synthesis of glucosylceramide (GlcCer) also occurs at the Golgi but at the cytosolic side of the membrane. Subsequently, the GlcCer moves into the lumen of the organelle and is converted to lactosylceramide and more complex glycosphingolipids. As with phospholipids, each metabolic step that occurs in a separate organelle or with different topology from the precursor can serve as an indicator of lipid transport.

Important elements of sterol metabolism can also be used to elucidate where in the cell a particular precursor has moved [7]. The arrival of cholesteryl esters within lysosomes is revealed by cleavage of the fatty acid to yield free cholesterol. The subsequent transport of cholesterol to the ER can be monitored by the action of acyl-CoA:cholesterol acyltransferase (Chapter 14) that results in the formation of new molecular species of cholesteryl esters. In addition, sphingomyelinase treatment of the cell surface induces cholesterol movement from the plasma membrane to the ER where its arrival can likewise be monitored by the action of acyl-CoA:cholesterol acyltransferase. Import of cholesterol into mitochondria (usually restricted to steroidogenic cells) can be followed by side-chain cleavage reactions that produce pregnenolone [8]. Movement of pregnenolone out of mitochondria can be followed by oxidations at positions 3, 17, and 21, which occur in the ER.

4. Lipid transport processes

The movement of lipids within the cell can be divided into two different general classes of transport: intramembrane transport, which entails the transbilayer movement of the lipid molecule; and intermembrane transport, which is the movement of lipid molecules from one distinct membrane to another. In some cases, the transmembrane movement of a phospholipid is coupled to a process that also removes the lipid from the membrane in which it was resident, and these events have the character of the lipid being vectorially pumped across and out of the membrane. Extensive reviews of these processes have been published [6,7,9–12].

4.1. Intramembrane lipid translocation and model membranes

The observation that biological membranes can be asymmetric with respect to transbilayer disposition of lipid components (Fig. 1) initially raised basic questions about how such asymmetry was established and maintained. An important issue that needed to be resolved on theoretical grounds was whether lipids in model membranes could undergo spontaneous transbilayer movement. A simple consideration of the events that occur in the transbilayer movement of a zwitterionic molecule such as PC suggests that at least two energetically unfavorable events must occur. The first is desolvation of the molecule and the second is movement of the charged portion of the lipid through the hydrophobic portion of the bilayer.

Direct experiments to examine the transbilayer movement of phospholipids (R.D. Kornberg, 1971) made use of spin-labeled analogs of PC in which the choline moiety was replaced with the tempocholine probe, *N*,*N*-dimethyl-*N*-(1′-oxyl-2′,2′,6′,6′-tetramethyl-4′-piperidyl)-ethanolamine (Fig. 2). These workers found that only the electron spin resonance signal generated by molecules in the outer leaflet of unilamellar liposomes could be rapidly quenched by ascorbate. The electron spin resonance signal from lipid molecules initially residing at the inner leaflet of liposomes was accessible to ascorbate with a $t_{1/2}$ of $>$6.5 h, indicating slow transbilayer lipid movement (Fig. 3).

Additional evidence for slow transbilayer phospholipid movement in liposomes came from experiments using [^{3}H]PC-labeled liposomes and PC transfer protein. In the presence of excess unlabeled acceptor membranes, only the PC in the outer leaflet of the liposome membrane was rapidly transferred (J.E. Rothman, 1975). The [^{3}H]PC initially present in the inner leaflet of the membrane moved to the outer leaflet with a $t_{1/2}$ of 11–15 days (Fig. 3). Further evidence demonstrating slow transbilayer movement of phospholipids was obtained from unilamellar liposomes containing 90% PC and 10% PE. In these liposomes, the PE initially residing in the outer leaflet of the membrane was rapidly modified by isethionylacetimidate. The PE at the inner leaflet remained refractory to modification by TNBS (i.e., did not undergo transbilayer movement) with a $t_{1/2}$ of $>$80 days (Fig. 3).

In contrast to phospholipids, non-polar lipids such as DG behave differently. B.R. Ganong (1984) synthesized a structural analog of DG in which the *sn*-3 hydroxyl

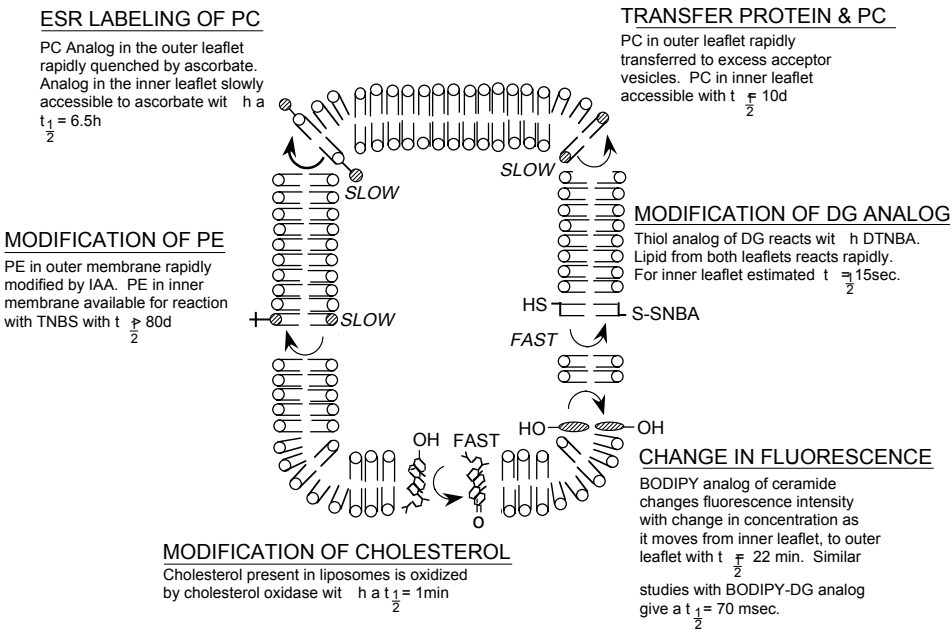


Fig. 3. Summary of key experiments examining transbilayer lipid movement in liposomes. Reaction of dithiobisnitrobenzoic acid (DTNBA) with R-SH gives R-S-SNBA. Each of the experiments was specifically designed to initially sample only the outer leaflet of the bilayer and then at subsequent periods detect the movement of lipid from the inner to the outer leaflet of the bilayer. IAA, isethionylacetimidate; TNBS, trinitrobenzenesulfonate; DG, diacylglycerol; DTNBA, dithiobisnitrobenzoic acid (Ellman's reagent).

group was replaced by an SH group that could be detected with dithiobisnitrobenzoic acid (Ellman's reagent). When liposomes containing the thiol analog of DG were reacted with Ellman's reagent, the $t_{1/2}$ for transmembrane movement was determined to be 15 s. Concentration-dependent changes in the fluorescent properties of BODIPY lipids have also been used to estimate transbilayer movement of DG and ceramide (J. Bai, 1997). By the fluorescence method, the $t_{1/2}$ for transbilayer movement of BODIPY-DG is 70 ms and that of BODIPY-ceramide is 22 min (Fig. 3). These results strongly suggest that the polar moiety of phospholipids is the portion of the molecule that greatly retards transbilayer movement of these molecules in model membranes. Cholesterol is another non-polar lipid whose transmembrane movement has been examined. Treatment of PC/cholesterol liposomes with cholesterol oxidase demonstrated that the entire cholesterol pool could be readily oxidized with a $t_{1/2}$ of 1 min at 37°C (J.M. Backer, 1981) (Fig. 3).

Thus, studies with model membranes provide clear evidence that the transbilayer movement of phospholipids is a very slow process in this system, whereas the process appears to be rapid for non-polar lipids. The results imply that if transbilayer movement of phospholipids does occur in biological membranes, it must be a facilitated process.

4.2. Intramembrane lipid translocation and biological membranes

4.2.1. Prokaryotes

The primary consideration in the genesis of any biological membrane is the location of the synthetic apparatus that manufactures the subunits of the membrane and its relationship to the final distribution of its products [13]. In *Escherichia coli*, substantial evidence indicates that the synthesis of phospholipids occurs at the inner (cytoplasmic) membrane by enzymes that have their active sites on the cytoplasmic surface of the inner membrane. Such an orientation allows free access of water-soluble substrates and reaction products to the cytosol.

In experiments performed with *B. megaterium*, Rothman (1977) used chemical modification with TNBS, under conditions where the probe did not enter the cell, to distinguish between PE molecules located on the outer and inner sides of the cell membrane. This technique was coupled with pulse-chase experiments with [^{32}P]inorganic phosphate and [^{3}H]glycerol and demonstrated that newly synthesized PE is initially found on the cytoplasmic surface of the cell membrane and is rapidly translocated to the outer leaflet of the membrane with a $t_{1/2}$ of 3 min at 37°C. Although the translocation is rapid, it does not occur coincident with synthesis, but rather, with a significant delay after the molecule is synthesized. In addition, the translocation can continue in the absence of PE synthesis. These findings indicate that lipid synthesis and translocation are two distinct events.

The energetic requirements for transmembrane movement of phospholipids have been investigated in bacterial membranes (K.E. Langley, 1979). Using *B. megaterium* and a TNBS probe, these studies demonstrated that the transbilayer movement of newly synthesized PE was unaffected by inhibitors of ATP synthesis and protein synthesis. Thus, the driving force for phospholipid translocation in *B. megaterium* is independent of metabolic energy, lipid synthesis, and protein assembly into cell membranes.

Additional work with closed vesicles derived from *B. megaterium* membranes demonstrates that NBD analogs of PE, PG, and PC can translocate across the membrane with a $t_{1/2}$ of 30 s at 37°C (S. Hraffnsdottir, 1997). Similar types of experiments conducted with closed vesicles isolated from *E. coli* inner membrane reveal that NBD phospholipids traverse the bilayer with a $t_{1/2}$ of 7 min at 37°C (R. Huijbregts, 1996). This latter process is insensitive to protease and *N*-ethylmaleimide treatments and does not require ATP. Collectively, the data indicate that transbilayer lipid movement is rapid and does not require metabolic energy in bacterial membranes that harbor the biosynthetic enzymes for phospholipids. The basic characteristics of lipid translocation in the intact cell appear to be retained in isolated membranes.

The mechanisms regulating the transbilayer movement of phospholipids have been probed by biochemical and genetic studies. Although some studies demonstrate that reconstitution of liposomes with bacterial membrane proteins can facilitate ATP-independent transbilayer phospholipid movement (J. Kubelt, 2002), no specific proteins or genes have been clearly defined. An alternative to specific transporters has been proposed by de Kruijff and colleagues, who emphasize a generic lipid transport role for the hydrophobic membrane spanning peptides of subsets of membrane proteins [13]. Both synthetic membrane spanning peptides and single transmembrane domains from membrane proteins, inserted into liposomes, facilitate transmembrane

phospholipid movement. Thus, the bacterial membranes that function in lipid biosynthesis appear to have a complement of proteins that non-specifically enable phospholipid mixing between membrane bilayers.

In gram-negative bacteria, lipopolysaccharide (LPS) is the major lipid constituent of the outer leaflet of the outer membrane (Chapters 1 and 3). The core structure of LPS consisting of lipid A–Kdo_2–Hep_3–Glc_3–Gal_2 (also known as Ra-LPS) is synthesized at the inner aspect of the inner membrane of the bacteria. Ra-LPS must be translocated across the inner membrane, modified by carbohydrate to create the O-antigen component, transported across the periplasmic space, and then assembled into the outer aspect of the outer membrane. The translocation of Ra-LPS across the inner membrane is catalyzed by the bacterial ATP-binding cassette (ABC) family transporter, MsbA (Fig. 4) [14]. *E. coli* strains defective in Ra-LPS transport show accumulation of extensively invaginated membranous structures within the inner membrane compartment. These strains accumulate Ra-LPS and PE in the inner membrane and also fail to transport PE to the outer membrane. The latter observation has led to the postulation that PE may also be a substrate for the MsbA transporter. However, several direct tests of MsbA as an obligate PE translocase have not provided definitive evidence for this role.

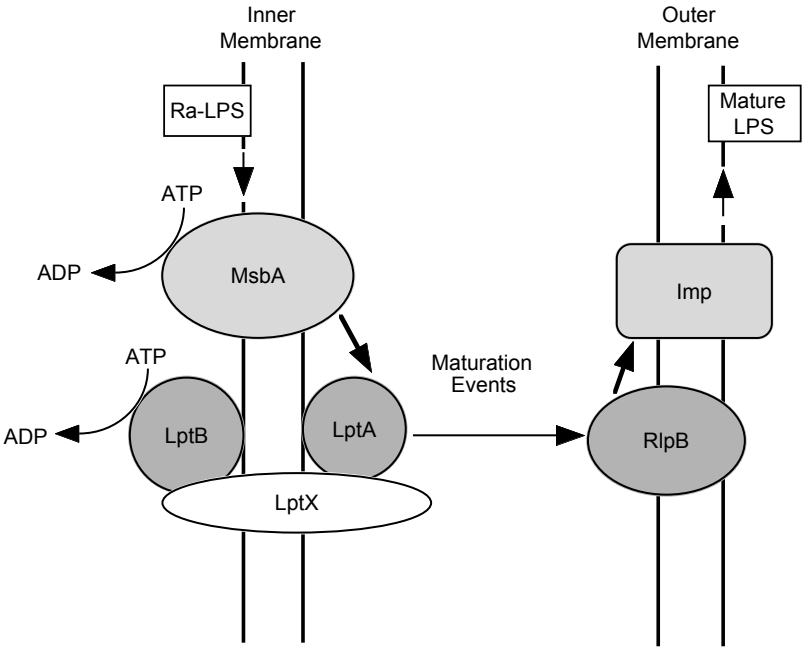


Fig. 4. Genetic and biochemical elements of transbilayer and intermembrane transport of Ra-LPS. Ra-LPS is synthesized on the cytoplasmic face of the inner membrane of gram-negative bacteria. MsbA transports the Ra-LPS to the outer aspect of the inner membrane in an ATP-dependent reaction. The Ra-LPS is transferred to LptA, a subunit of a multipartite ATPase. The LptX is proposed to physically couple ATP hydrolysis by LptB to the transport function of LptA. LptA transfers the LPS to RlpB, which subsequently transfers the molecule to Imp. The Imp completes the transfer of the LPS to the outer aspect of the outer membrane.

4.2.2. Eukaryotes

4.2.2.1. Transbilayer movement of lipid at the endoplasmic reticulum In eukaryotic systems a detailed pattern of synthetic asymmetry has emerged with respect to the topology of the enzymes of phospholipid synthesis in rat liver microsomal membranes. Protease mapping experiments (D.E. Vance, 1977; R. Bell, 1981) have indicated that the active sites of the phospholipid synthetic enzymes are located on the cytosolic face of the ER. Thus, in both prokaryotic and eukaryotic systems, it appears that the site of synthesis of the bulk of cellular phospholipid is the cytosolic side of the membrane. This asymmetric localization of synthetic enzymes strongly implicates transbilayer movement of phospholipids as a necessary and important event in membrane assembly that is required for the equal expansion of both leaflets of the bilayer [13].

The transbilayer movement of phospholipids in microsomal membranes has been measured using several different approaches. Phospholipid transfer proteins were used to probe the transbilayer movement of lipids in preparations of liver microsomes that were first radiolabeled with lipid precursors in vivo (D.B. Zilversmit, 1977). The results from these experiments provided evidence that PC, PE, PS, and PI from both membrane leaflets were exchanged between labeled microsomes and excess acceptor membranes with a maximal $t_{1/2}$ of ~45 min.

In a different approach, a water-soluble, short-chain (dibutyroyl) analog of PC was used to measure the rate of uptake and lumenal sequestration by isolated liver microsomes (W. Bishop, 1985). This PC analog was taken up in a time- and temperature-dependent manner. The kinetics of uptake were saturable with respect to substrate concentration and the transport activity was protease sensitive. The transporter was also shown to be stereospecific in its action and was unaffected by the addition of ATP. Virtually identical properties have also been described for a microsomal transporter that utilizes butyroyl-lyso-PC (Y. Kawashima, 1987).

Additional studies utilized spin-labeled analogs of PC, PE, PS, and SM, and the $t_{1/2}$ for the translocation of these lipid analogs from the cytosolic face to the lumenal face of the microsomes was calculated to be 20 min. The transport process did not require ATP and the translocation of each class of lipid showed identical sensitivity to inhibition by *N*-ethylmaleimide. Furthermore, different species of lipid showed transport kinetics that were consistent with mutual competition for a single transporter. These results indicate that the ER has a relatively non-specific, ATP-independent transporter that is capable of translocating multiple species of lipid across the bilayer. Several attempts have been made to reconstitute the protein components of the ER necessary for ATP-independent transbilayer lipid movement within liposomes (J. Backer, 1987; S. Gummadi, 2002). These approaches have met with only limited success and thus far no specific proteins or genes have been directly implicated in the process.

Thus, the data from both bacteria and animal cells demonstrate that transbilayer movement of phospholipid occurs on a timescale of minutes, in an ATP-independent fashion in membranes that contain the majority of the enzymes involved in their biosynthesis. These intramembrane transport properties observed in the major biosynthetic membranes, however, are not generally true for other membrane systems. This is especially true of the plasma membrane, Golgi, endosomal, and lysosomal membranes.

4.2.2.2. Transbilayer lipid movement at the eukaryotic plasma membrane and endomembranes The transbilayer movement of lipids at the cell surface and within the Golgi of eukaryotes is being understood with increasing molecular and biochemical detail. Three fundamental classes of transport are now recognized and consist of the aminophospholipid translocases (also called flippases), the scramblases, and the ABC pumps. A schematic summary of some of the properties of these proteins is shown in Figs. 5 and 6.

Aminophospholipid translocases. Numerous studies using either short-chain versions of PS, or spin-labeled, or fluorescent analogs of PS and PE established the general

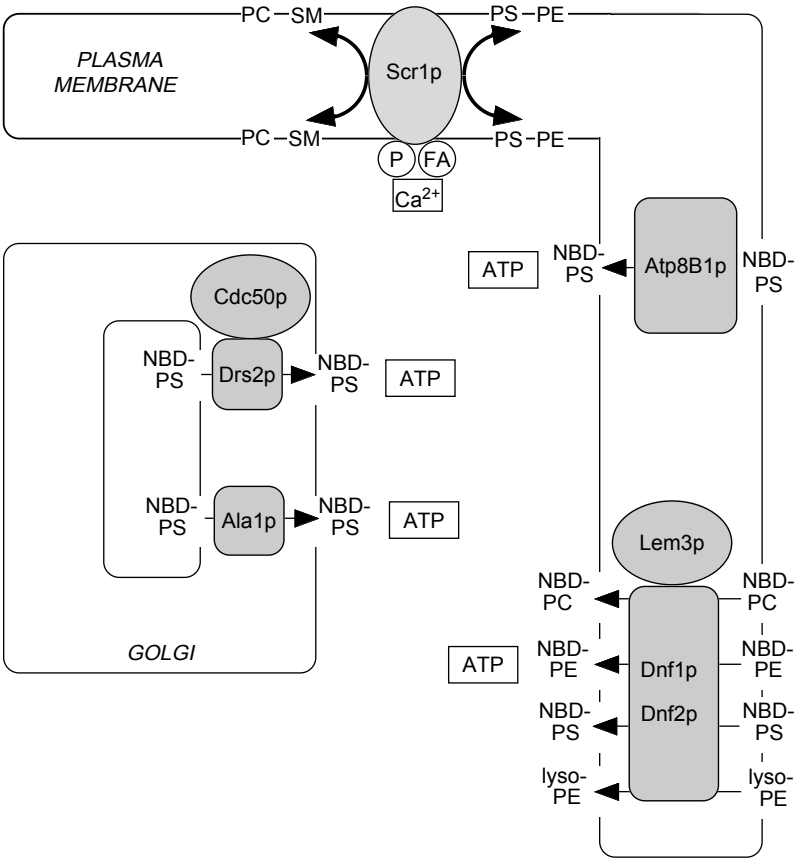


Fig. 5. Regulation of membrane asymmetry. The mammalian scramblase 1 (Scr1p) randomizes lipid distributions across the plasma membrane. The Scr1p activity is regulated by phosphorylation (P), acylation (FA), and Ca^{2+} levels. The P-type ATPases create membrane asymmetry by translocating aminophospholipids towards the cytosolic leaflets of plasma and Golgi membranes. Yeast Dnf1p and Dnf2p translocate all the diacyl aminoglycerophospholipids and lyso-PE. The correct localization of Dnf1p and Dnf2p requires the presence of Lem3p, which acts as a molecular chaperone. Atp8Blp is a mammalian protein that translocates NBD-PS. The yeast protein Drs2p and the plant protein Alalp translocate NBD-PS to the cytosolic surface of the Golgi. Localization of Drs2p to the Golgi requires Cdc50p function.

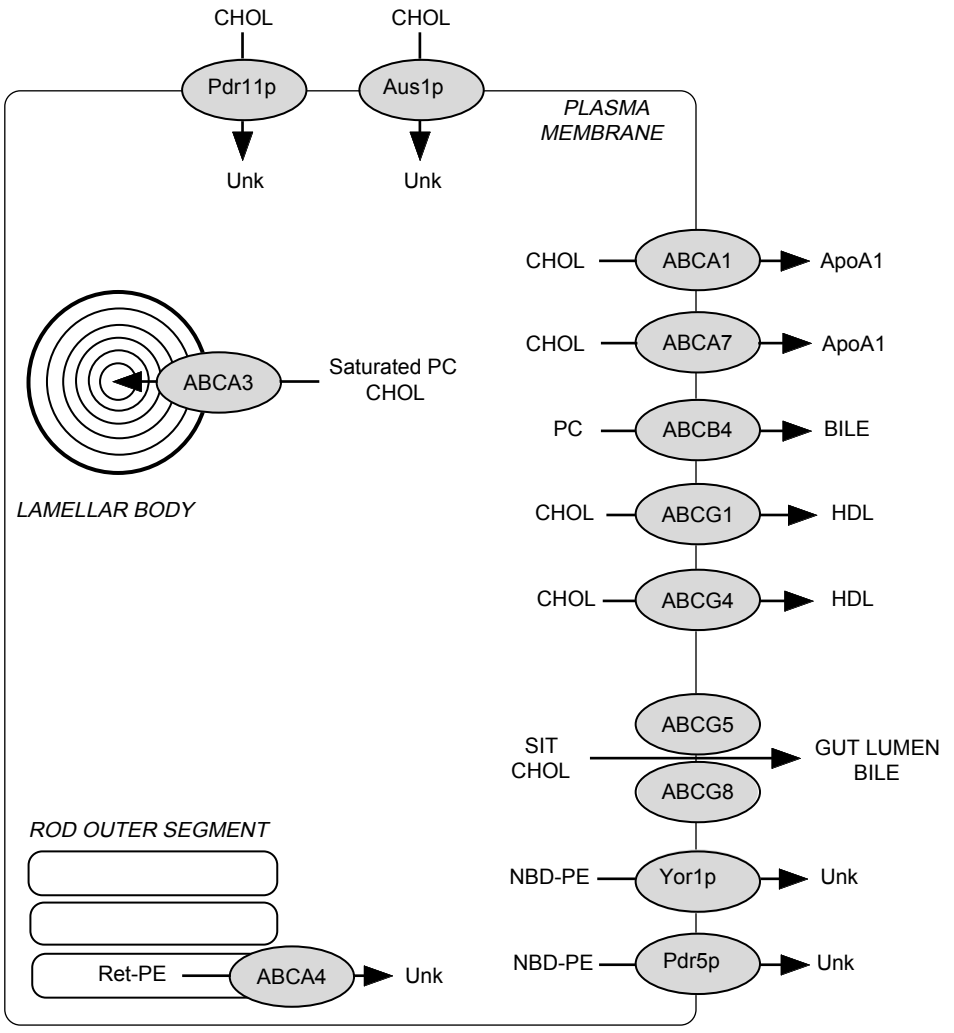


Fig. 6. A summary of important ABC transporters acting on sterols and phospholipids. In many cases, the ABC transporters are required for the movement of lipids across membranes and insert them into extramembranous acceptors. The known physiological acceptors are indicated, as determined by biochemical measurements. When not known, the acceptors are designated Unk. The transporters Pdr11p, Aus1p, Yor1p, and Pdr5p are found in yeast. ABCA3 is found primarily in alveolar type 2 cells of the lung in which lamellar bodies are pulmonary surfactant storage organelles. ABCA4 is found in rod and cone cells of the eye. CHOL, cholesterol; Ret-PE, retinylidene-PE; HDL, high-density lipoprotein; Apo A1, apolipoprotein A1; SIT, sitosterol.

properties of the plasma membrane translocases of erythrocytes and nucleated eukaryotic cells [15]. The major mammalian aminophospholipid translocases recognize PS and PE at the external surface of the plasma membrane and translocate them to the cytosolic side of the membrane. This flipping process requires ATP on the cytosolic side of the membrane. In erythrocytes, the affinity for PS is about 30-fold higher than for PE, and the equilibrium

distribution is 95% PS and 90% PE in the inner membrane. A general summary of several of the aminophospholipid translocases is given in Fig. 5. The cell surface aminophospholipid translocases are susceptible to protease digestion and inactivation with *N*-ethylmaleimide. Additional susceptibilities to inhibition by AlF_4 and Na_2VO_4 are typical of the P-type ATPase family of transporters. The P-type designation refers to the occurrence of a phospho-enzyme transport intermediate in the reaction cycle. The stoichiometry of ATP utilization per phospholipid translocation event is approximately one. All of the characteristics are consistent with the plasma membrane aminophospholipid translocases being ATPases that are activated by aminophospholipids (D. Daleke, 2000). One functional feature that distinguishes the P-type ATPases from the ABC transporters described later is that the action of the P-type ATPases implicated in lipid transport usually results in a transbilayer transport event and retention of the lipid substrate within the membrane, thus creating an asymmetry between the bilayer leaflets.

A chromaffin granule P-type ATPase, designated ATPase II, with properties similar to the plasma membrane protein, was purified to homogeneity and its cDNA cloned. The deduced protein sequence contains three P-type ATPase consensus sequences (X. Tang, 1996). The sequence information also identified this protein as a member of the P-4 ATPase family. The cDNA sequence of the ATPase II was used to identify the major yeast ortholog, which is the *DRS2* gene that encodes the Drs2p protein. The *drs2* acronym derives from a genetic screen that yielded mutations producing defects in ribosome synthesis. The initial studies with *DRS2* yielded conflicting results among different research groups with respect to defects in aminophospholipid transport. Despite these initial ambiguities, several laboratories identified homologs of *DRS2* in yeast, and critically tested their functions. One prominent yeast homolog of *DRS2* is the essential gene responsible for neomycin resistance, *NEO1*. The homologs related to the *DRS2* and *NEO1* genes were named *DNF1*, *DNF2*, and *DNF3* [10]. The *DNF* designation is an acronym for *DRS2/NEO1* Family. The picture that has now emerged from these studies is that the proteins, Dnf1p and Dnf2p, encoded by the *DNF1* and *DNF2* genes, are found primarily in the plasma membrane, but also appear to cycle through the endosomal compartment. In contrast, the Drs2p and Dnf3p, encoded by the *DRS2* and *DNF3* genes are localized to the Golgi. Examination of NBD-lipid transport at the plasma membrane reveals that Dnf1p and Dnf2p are translocases that act on NBD-PS, NBD-PE, and NBD-PC. In contrast, Drs2p and Dnf3p act as translocases that transport NBD-PS in the Golgi. In each case, the direction of ATP-dependent transport is to move the lipid substrates from the non-cytosolic side to the cytosolic side of the membrane bilayer.

The localization and activity of Dnf1p and Dnf2p is regulated by an additional protein designated Lem3p, which appears to act as a chaperone that targets the P-type ATPases to the plasma membrane. Two independent genetic screens identified Lem3p as a factor essential for the transbilayer transport of aminophospholipids (U. Kato, 2002; P. Hanson, 2003). The Lem3 acronym derives from an unrelated genetic analysis in which the gene was identified as a ligand effector modulator. The localization and activity of Drs2p (and probably Dnf3p) in the Golgi is also regulated by an accessory protein designated Cdc50p (K. Saito, 2004). The acronym Cdc derives from an unrelated genetic analysis in which the gene was identified as being involved in the cell division cycle. The Cdc50p (like Lem3p) appears to be necessary for proper localization of Drs2p to the Golgi. An additional protein

Ala1p (Aminophospholipid ATPase) has been identified as the homolog of Drs2p in plants (E. Gomes, 2000) and has been implicated in PS translocation in the Golgi.

A closely related human homolog of the *DRS2* gene is the human *ATP8B1* gene (also known as *FIC1* because of its involvement in familial intrahepatic cholestasis) [16]. The protein encoded by the human gene localizes to the bile canalicular membrane of the liver (Chapter 15). Mutations in the human gene are characterized by defective bile acid secretion from the liver. The lesions cause either progressive familial intrahepatic cholestasis or benign recurrent intrahepatic cholestasis. Heterologous expression of the *ATP8B1* gene in CHO-K1 cells results in enhanced NBD-PS transport at the plasma membrane. Although the gene-disease relationship is clear for *ATP8B1* and its characterized mutations, the connection between NBD-lipid transport and bile acid secretion by the liver is not known [15]. There is no evidence that Atp8B1p is a bile acid transporter and the data suggest that the function of this protein is somehow linked to the intracellular trafficking or activity of the bile acid transporter. The involvement of the Atp8B1p could also be through maintenance of correct lipid asymmetry in the bile canalicular membrane, which is necessary for the proper function of a bile acid transporter. Alternatively, plasma membrane lipid asymmetry may be required for correct endocytic recycling or recruitment of the bile acid transporter to the plasma membrane.

The utilization of NBD-lipid substrates has raised persistent questions about whether the transport of these analogs reflects the true transport of the corresponding native phospholipids. There is reasonable data in the literature to conclude that the analogs faithfully report the activity for genuine phospholipids. Further support for this conclusion comes from recent studies in yeast demonstrating that the natural lipid, lyso-PE, is a substrate for the Dnf1p, Dnf2p/Lem3p transporter (W. Riekhof, 2006). The flux rates measured with lyso-PE are very high and are capable of supporting all the needs of growing cells for the synthesis of both PE and PC, demonstrating that this transport process is coupled to biologically relevant aspects of membrane biogenesis. After its import into cells, lyso-PE is rapidly acylated to form PE, which is distributed to multiple organelles including the ER where the PE is methylated to form PC. These findings not only clearly define the action of Dnf1p, Dnf2p/Lem3p as a transporter of a natural lipid, but also raise the possibility that this and related transporters also work on other lyso-phospholipids.

Bidirectional transporters. The bidirectional transporters at the plasma membrane randomize the lipid distribution across the plane of the bilayer, and are commonly referred to as scramblases [17]. The action of scramblase is summarized in Fig. 5, and is similar to that of the previously described transbilayer transporter present in the ER. Scramblase protein was first functionally identified in erythrocytes but is also present in nucleated cells. The scramblase shows no lipid specificity and essentially collapses the asymmetry of lipids at the cell surface. Phospholipids, SM, and glycosphingolipids all serve as substrates. The randomizing function of the plasma membrane protein is activated by Ca^{2+} and does not require ATP.

The scramblase protein was purified to homogeneity and its cDNA cloned (Q. Zhou, 1997). A Ca^{2+}-binding domain is found in the cytosolic region adjacent to the transmembrane domain. Post-translational modifications to the scramblase that alter activity include acylation and phosphorylation. There is currently much interest in the regulation of scramblase function as it plays a critical role in the externalization of PS, a process

that is important for the recognition of apoptotic cells by phagocytes (V.A. Fadok, 1992). In addition to regulation by Ca^{2+} and oligomerization, the scramblase activity can be enhanced by phosphorylation directed by protein kinase C-delta (S. Frasch, 2000). The protein is also a substrate for the protein tyrosine kinase c-Abl (J. Sun, 2001). Four genes (*PLSCR1-4*) encoding different isoforms of the protein have now been identified (T. Wiedmer, 2000). In contrast to Plscr1p, Plscr2p is found in the nucleus and Plscr3p in the mitochondria. Plscr3p appears to play a role in promoting the exposure of cardiolipin on the outer membrane of the organelle, an event that is linked to apoptotic pathways (J. Liu, 2003). The molecular details of how the scramblases promote the transbilayer movement of lipids remain obscure and will require more extensive structure–function analysis.

ABC transporters. The ABC transporters are a large family of proteins involved in moving molecules across membranes in ATP-dependent reactions. A subset of this family transports molecules that include xenobiotics, bile acids, lipids, and other hydrophobic compounds [12,18,19]. The ABC family of proteins is structurally distinct from the P-type ATPases. A general functional characteristic of the ABC transporters that differentiates them from the P-type ATPases is that the ABC protein action usually results in extrusion of the transported molecule from the membrane, resulting in lipid transport to some hydrophobic acceptor, consisting of a protein or lipid complex, or a separate membrane. In 1993, Smit and co-workers described a transgenic mouse with null alleles for the ABC transporter, *MDR2*, that exhibited a profound defect in transporting PC into bile. These findings led Reutz (1994) to examine the activity of the *mdr2* protein as a PC translocase. The heterologous expression of the *MDR2* cDNA in yeast leads to incorporation of *mdr2* protein into yeast secretory vesicles and acquisition of the ability to translocate NBD-PC across the bilayer. The translocation process is time-, temperature-, and ATP-dependent. These findings indicate that the *mdr2* protein acts as a PC transporter. Unequivocal experiments in transgenic mice establish that the human MDR3 protein and mouse *mdr2* have identical function [20]. A unifying nomenclature has been established for members of the ABC family; the mouse *mdr2* and human MDR3 proteins are now designated as ABCB4.

Numerous other ABC transporters have now been implicated in lipid transport processes and the list is growing at an explosive rate [12,18,19]. A summary of the transporters and their functions appears in Fig. 6, and their association with specific diseases or phenotypes appears in Table 2. The ABCA1 protein promotes cholesterol export from cells to high-density lipoproteins and constitutes an important element of 'reverse cholesterol transport' (Chapter 19). Defects in ABCA1 function are responsible for Tangier disease (familial hypoalphalipoproteinemia). In vitro transport activities similar to those for ABCA1 are also found for ABCA7. The ABCA3 protein has been implicated in PC import into the intracellular storage organelle for pulmonary surfactant (lamellar body) present in lung alveolar type 2 cells. Defects in ABCA3 cause pulmonary surfactant deficiency in newborns. The ABCA4 transporter catalyzes the removal of a retinaldehyde adduct of PE, retinylidene-PE, from the disc membrane of the rod outer segment in the photoreceptor cells of the eye. Defects in ABCA4 reduce or eliminate this removal of retinylidene-PE and lead to the accumulation of precursors of lipofuscin within the disc membrane. The subsequent homeostatic turnover of the disc membranes upon

Table 2
Mammalian genetic diseases of cellular sterol and phospholipid transport

Gene	Transported lipids	Diseases/phenotypes
ABC transporters		
ABCA1	Cholesterol, PC	Familial hypoalphalipoproteinemia, Tangier disease
ABCA3	PC, cholesterol	Pulmonary surfactant insufficiency
ABCA4	*N*-Retinylidene-PE	Stargardt macular dystrophy, age-related macular degeneration?
ABCB4	PC, cholesterol	Hepatic cholestasis
ABCG1	PC, SM, cholesterol	Pulmonary lipidoses (mice)
ABCG5, ABCG8	Sitosterol, cholesterol	Sitosterolemia, hypercholesterolemia
P-type ATPases		
ATP8B1	NBD-PS, PS?, PE?	Progressive familial intrahepatic cholestasis, benign recurrent intrahepatic cholestasis
Other families		
NPC1	Cholesterol	Niemann–Pick type C disease
NPC2	Cholesterol	Niemann–Pick type C disease
NPC1L1	Cholesterol, plant sterols	Ezetimibe resistance
StAR	Cholesterol	Congenital adrenal hyperplasia

phagocytosis by the pigmented epithelium results in the deposition of lipofuscin in this cell layer and progressive development of Stargardt macular dystrophy (R. Allikmets, 1997; J. Weng, 1999). Progressive ABCA4 dysfunction has also been proposed as a causative element in the development of age-related macular dystrophy, the major cause of blindness in developed countries. The ABCG1 protein participates in transport of cholesterol, SM, and PC, and null alleles for the encoding gene in mice lead to abnormal lipid metabolism within the lung and pulmonary lipidoses. ABCG4 is closely related to ABCG1 and appears to act in an overlapping manner with it for transfer of cholesterol and phospholipid to partially lipidated apo A1 (Chapter 19). The heterodimeric ABCG5 and ABCG8 transporter is defective in individuals with sitosterolemia (M. Lee, 2001). Under normal conditions, intestinal absorption of the plant sterol, sitosterol, appears to be minimized by an efflux pumping mechanism that continually translocates the sterol back to the lumen of the gut. In individuals with ABCG5 or ABCG8 defects, this pumping mechanism is lost, and blood and tissue levels of this deleterious sterol increase dramatically. ABCG5 and ABCG8 also function to secrete cholesterol and plant sterols into bile from the liver.

ABC family lipid efflux transporters have also been identified in yeast, and gene products associated with yeast oligomycin resistance, Yor1p, and pleiotropic drug resistance, Pdr5p, have been shown to facilitate the removal of NBD-PE from cells (A. Decottignies, 1998). Two additional ABC transporters in yeast, Aus1p (the acronym derives from ABC protein involved in uptake of sterols) and Pdr11p, are upregulated in response to growth of cells on sterols, and are required for transporting extracellular sterols to the ER for the synthesis of steryl esters [21].

Although there is abundant genetic evidence implicating ABC transporters in sterol transport, the precise mechanism of action is unknown. The simplest model predicts that sterols are the substrates for transporters that translocate the lipid across and out of a given

membrane to an exogenous acceptor. Alternatively, an indirect model proposes that the transporters pump phospholipid out of the membrane and the sterol follows passively down a chemical potential gradient. Additional indirect models propose that ABC transporters may regulate membrane phospholipid packing and asymmetry in a manner that enables the sterol to interact with exogenous acceptor molecules, such as apolipoproteins that promote desorption of the lipid from the membrane. Detailed purification and reconstitution studies will be required to finally elucidate the mechanisms.

Plants also utilize ABC family members in complex lipid transport processes [22]. In plants, DG moieties must be transported to a collection of galactosyltransferases located within the chloroplast for the synthesis of galactolipids (Chapter 4). Although this system is complex and requires the interplay of multiple membrane compartments that include the ER, outer chloroplast envelope, and inner chloroplast envelope, the details of a few steps are now apparent (Fig. 7). One critical event is the transport of phosphatidic acid across the inner chloroplast membrane for the purpose of ultimately providing DG moieties for use in galactolipid synthesis. This transport of phosphatidic acid requires the action of the Tgd1p protein, encoded by the *TGD1* gene, present in the inner chloroplast membrane.

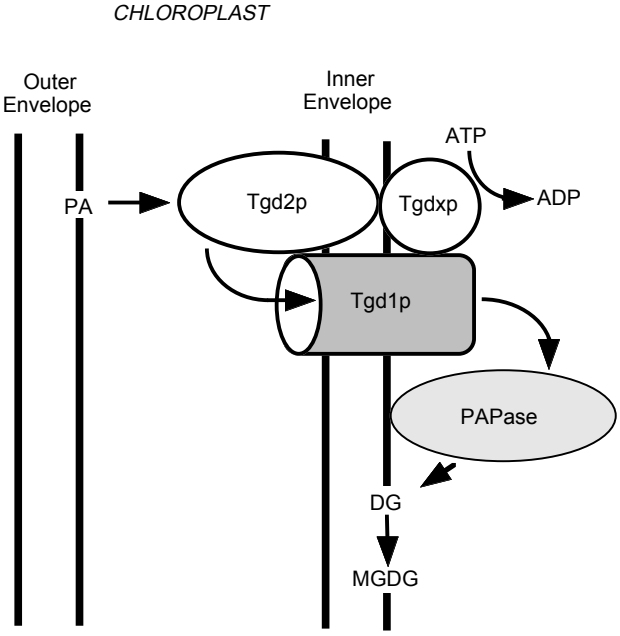


Fig. 7. Lipid transport between and across chloroplast membranes. The inner envelope of the chloroplast contains a multipartite ABC transporter for translocating phosphatidic acid (PA) into and across the membrane. The Tgd2p acts as a PA-binding protein that can transfer the lipid from the outer envelope to Tgd1p in the inner envelope. The Tgd1p translocates the lipid across the inner envelope and provides the substrate to phosphatidic acid phosphatase (PAPase) for the generation of diacylglycerol (DG) in the inner envelope. The diacyglycerol is used for monogalactosyl-diacylglycerol (MGDG) synthesis. The chemical driving force for lipid translocation comes from a proposed TgdXp subunit.

The acronym Tgd derives from the property of mutants in this gene that accumulate tri-galactosyl-diacylglycerol. The Tgd1p has the characteristics of a permease subunit of a multicomponent ABC transporter. A second protein, Tgd2p, has the characteristics of a substrate recognition subunit of a multicomponent ABC transporter, and exhibits phosphatidic acid-binding activity. A third protein component, the predicted nucleotide-binding subunit (TgdXp), is yet to be identified. However, the emerging picture is that a three-protein complex Tgd1p/Tg2p/TgdXp present in the inner envelope of the chloroplast constitutes an active ABC transporter that first recognizes phosphatidic acid in the juxtaposed outer envelope membrane, through a binding reaction with Tgd2p, and then transports the lipid across the inner envelope membrane by the action of Tgd1p. The driving force for these events is postulated to be coupled to the ATP-binding protein TgdXp. Subsequently, the transported phosphatidic acid is delivered to a lipid-phosphatase for generating the DG units required for galactolipid synthesis within the chloroplast. One should appreciate that this scheme couples transbilayer transport to the intermembrane transport of phosphatidic acid.

4.3. Intermembrane lipid transport

From a theoretical perspective a number of processes could contribute to the intermembrane transport of lipids. These include (i) monomer solubility and diffusion, (ii) soluble carriers such as lipid transfer proteins, (iii) carrier vesicles, (iv) membrane apposition and lipid transfer, and (v) membrane fusion processes. Lipids such as fatty acids, lysophosphatidic acid, and CDP-DG might have sufficient water solubility to allow for some monomeric transport, but most other lipids are likely to require one of the other potential mechanisms due to their extremely low solubility.

4.3.1. Transport in prokaryotes

The presence of multiple membrane systems in organisms, such as gram-negative bacteria, photosynthetic bacteria, and the eukaryotes, raises significant questions about the mechanisms of lipid transport for membrane biogenesis. In a 'simple' organism such as *E. coli*, there are two membrane systems: the inner or cytoplasmic membrane and the outer membrane (Chapters 1 and 3) [14]. The entire apparatus for synthesis of phospholipids and Ra-LPS is located at the cytoplasmic face of the inner membrane. Consequently, there must exist a mechanism for exporting phospholipids and Ra-LPS from the inner membrane to the outer membrane.

In some of the earliest studies of phospholipid transport between the inner and the outer membranes of *E. coli*, radiolabeling of PE revealed that the specific radioactivity of this lipid was 5-fold higher in the inner membrane than the outer membrane, immediately following a 30-s pulse with [^{3}H]glycerol (A.M. Donohue-Rolfe, 1980). During the chase period, the specific radioactivity of the outer membrane increased, while that of the inner membrane decreased. After several minutes, the specific activities of both membranes asymptotically approached the same value, which indicated radioequilibration between the membranes. The $t_{1/2}$ for the translocation of PE was determined to be 2.8 min. The transport in these studies was independent of protein synthesis, lipid synthesis, and ATP synthesis. It appeared, however, to be dependent upon the cell's proton motive force. Analysis of LPS

synthesis and transport revealed similar properties of precursor (Ra-LPS) synthesis in the inner membrane and transport to the outer membrane with a $t_{1/2}$ of 1.2 min. However, the LPS transport process is ATP-dependent and unlike phospholipid, radioactive LPS present in the outer membrane does not re-equilibrate with the inner membrane.

As described in Section 4.2.1, Ra-LPS utilizes MsbA for translocation across the inner membrane of gram-negative bacteria (Fig. 4). Recently, four genes have been implicated in the subsequent transport and assembly of Ra-LPS between the inner membrane and the outer membrane. Genetic experiments identify the LptA and LptB gene products as participants in the LPS transport process [23]. Both genes encode subunits of a multipartite ABC transporter with the LptA protein being periplasmic, and the LptB protein being cytosolic and harboring the ATP-binding domain. A transmembrane linker protein (LptX) functionally connecting LptA and LptB proteins has been proposed, but not yet demonstrated. Deletion of either LptA or LptB prevents maturation of the outer membrane and results in the accumulation of abnormal membrane structure within the periplasmic space. Such membrane structures would be consistent with LPS transport intermediates accumulating after release from the inner membrane, but prior to assembly into the outer membrane. A third protein implicated in the transport process is Imp, a beta barrel motif protein of the outer membrane thought to be essential for conveying the LPS to the outer aspect of the outer membrane. The fourth identified protein is Rlb, an essential protein that interacts with Imp [24]. The current model proposes that Ra-LPS is assembled at the inner aspect of the inner membrane and then translocated across the bilayer by the ATP-dependent action of MsbA. Subsequently, the Ra-LPS is transferred to LtpA, which facilitates the transit of Ra-LPS (or its more mature forms containing O-antigen) to the outer membrane for transfer to an Imp–RlpB complex (Fig. 4). This model is in its early stages and will require refinement and critical testing, but it presents an important conceptual picture of membrane–membrane interactions in which tethered protein components appear to play key roles in moving lipids between two compartments.

4.3.2. Transport in eukaryotes

Currently, there is a broad understanding of the elements of interorganelle transport for several different lipid classes. In many studies, the questions have been narrowly focused to the movement of one lipid class between a donor and an acceptor compartment that are temporally, metabolically, and geographically segregated within the cell. With increasing frequency, genes responsible for the direct transport of lipids between membranes, or the regulation of the processes, are being discovered. The discussion of these processes is organized by class of lipid and then by membrane systems investigated.

4.3.2.1. Phosphatidylcholine

Transport of newly synthesized PC from the ER to the plasma membrane. The principal site of PC synthesis is the ER and Golgi (Chapter 8). The transport of PC from its sites of synthesis to the plasma membrane has been examined using pulse-chase labeling with a [^{3}H]choline precursor for PC, and rapid plasma membrane isolation with cationic beads (M. Kaplan, 1985). These studies reveal that PC transport is an extremely rapid process occurring with a $t_{1/2} \approx 1$ min (Fig. 8). This transport is unaffected by metabolic poisons that deplete cellular ATP levels, disrupt vesicle transport, or alter cytoskeletal arrangement.

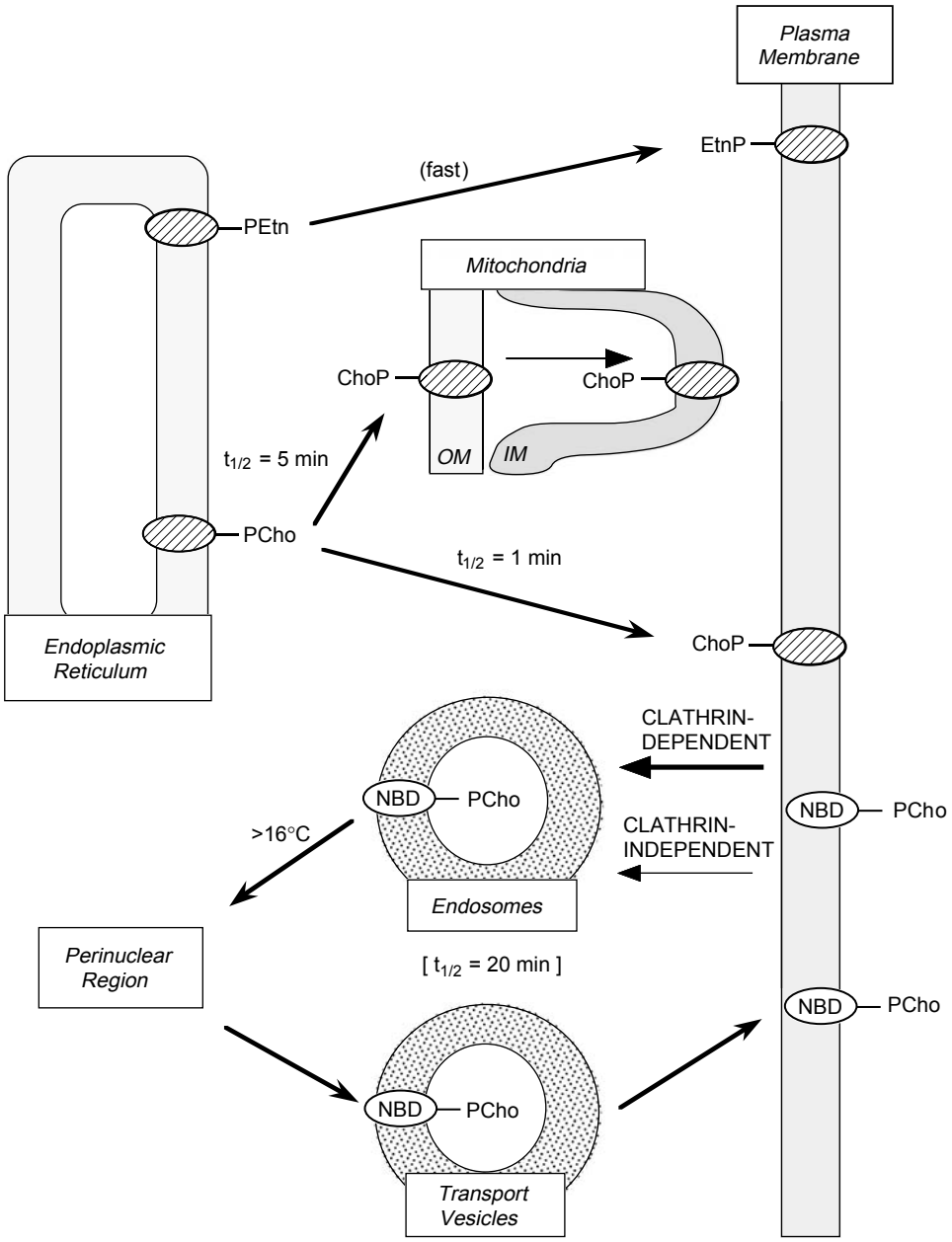


Fig. 8. Interorganelle transport of PC and PE from the ER and plasma membranes. The structure ⬭ represents the diacylglycerol portion of the phospholipid, and (NBD) represents the fluorescent diacylglycerol of phospholipids. PCho and PEtn are the abbreviations for phosphocholine and phosphoethanolamine, respectively. OM and IM are abbreviations for the outer and inner membranes of the mitochondria. The $t_{1/2}$ for PC transport from the perinuclear region of the cell to the plasma membrane is shown in brackets and estimated to be 20 min. The majority of NBD-PCho transport from the plasma membrane occurs by a clathrin-dependent mechanism.

The mechanism of this transport is presently unknown, but the results are consistent with a soluble carrier mechanism such as PC transfer protein, or transport at zones of apposition between membranes that facilitate rapid intermembrane transfer. Work by Pichler and colleagues has identified a subfraction of the ER that associates closely with the plasma membrane in yeast (H. Pichler, 2001). Future studies examining the effects of agents or mutations that disrupt these intracellular membrane associations will be critical for determining their role in lipid traffic.

Transport of newly synthesized PC from the ER to the mitochondria. Using conventional subcellular fractionation techniques, the transport of nascent PC to the mitochondria of baby hamster kidney cells was examined by pulse-chase experiments with a [^{3}H]choline precursor (M.P. Yaffe, 1983). These experiments show that the newly made PC pool equilibrates between the outer mitochondrial membrane and the ER in approximately 5 min (Fig. 8). Similar studies performed in yeast (G. Daum, 1986) also revealed that the PC pool rapidly equilibrates between the ER and the mitochondria. Addition of metabolic poisons did not eliminate the PC radioequilibration in yeast. Studies with isolated mitochondria demonstrate that PC loaded into the outer mitochondrial membrane can be transported to the inner membrane in an energy-independent manner (M. Lampl, 1994). Consistent with this finding is the observation that PC rapidly moves across the membrane of vesicles derived from mitochondrial outer membranes prepared from either mammalian cells or yeast (D. Dolis, 1996; M. Janssen, 1999). In addition to PC, monomethyl-PE and dimethyl-PE also rapidly equilibrate between the ER and mitochondria, and at least superficially appear to use the same machinery for transport and equilibration of these phospholipid pools between the membranes (A. de Kroon, 2003). In contrast to the methylated derivatives of PE, neither PE nor PS appears to undergo this rapid exchange between the membranes.

Transport of exogenous PC analogs from the cell surface to intracellular organelles. Clear evidence for the movement of PC from the plasma membrane to intracellular organelles has been obtained using the fluorescent lipid analog NBD-PC and cultured mammalian cells (R. Sleight, 1984). The fluorescent lipid can be pulse labeled into the outer leaflet of the plasma membrane at 2°C. Upon warming the cells, the fluorescent lipid is transported from the plasma membrane to the perinuclear region of the cell in the proximity of the Golgi apparatus and the centrioles, via an ATP-dependent process (Fig. 8). The lipid transport occurs by endocytosis and the process can be disrupted by reducing the temperature to 16°C, which causes the PC to accumulate in endosomal vesicles. The endocytosis of NBD-PC occurs via clathrin-independent (inhibited by nystatin) and clathrin-dependent (inhibited by chlorpromazine) pathways. The clathrin-dependent route predominates and accounts for more than 70% of the NBD-PC endocytosis (R. Singh, 2003). The kinetics for endocytosed NBD-PC transport from intracellular membranes back to the plasma membrane occurs with $t_{1/2} = 20$ min. During the transit cycle, the NBD-PC remains restricted to the non-cytosolic face of the respective membranes. The kinetics of this vesicle-based recycling of PC between the cell interior and the plasma membrane are markedly different from those for transport of newly synthesized PC to the cell surface. The disparity between the two transport processes suggests that there is restricted intermixing of nascent and recycling pools of PC.

Similar experiments in yeast reveal a different type of NBD-PC routing within the cell (Fig. 9). Treatment of cells with NBD-PC at 2°C yields intense labeling of both mitochondria and ER that is energy dependent. The NBD-PC is first translocated across the

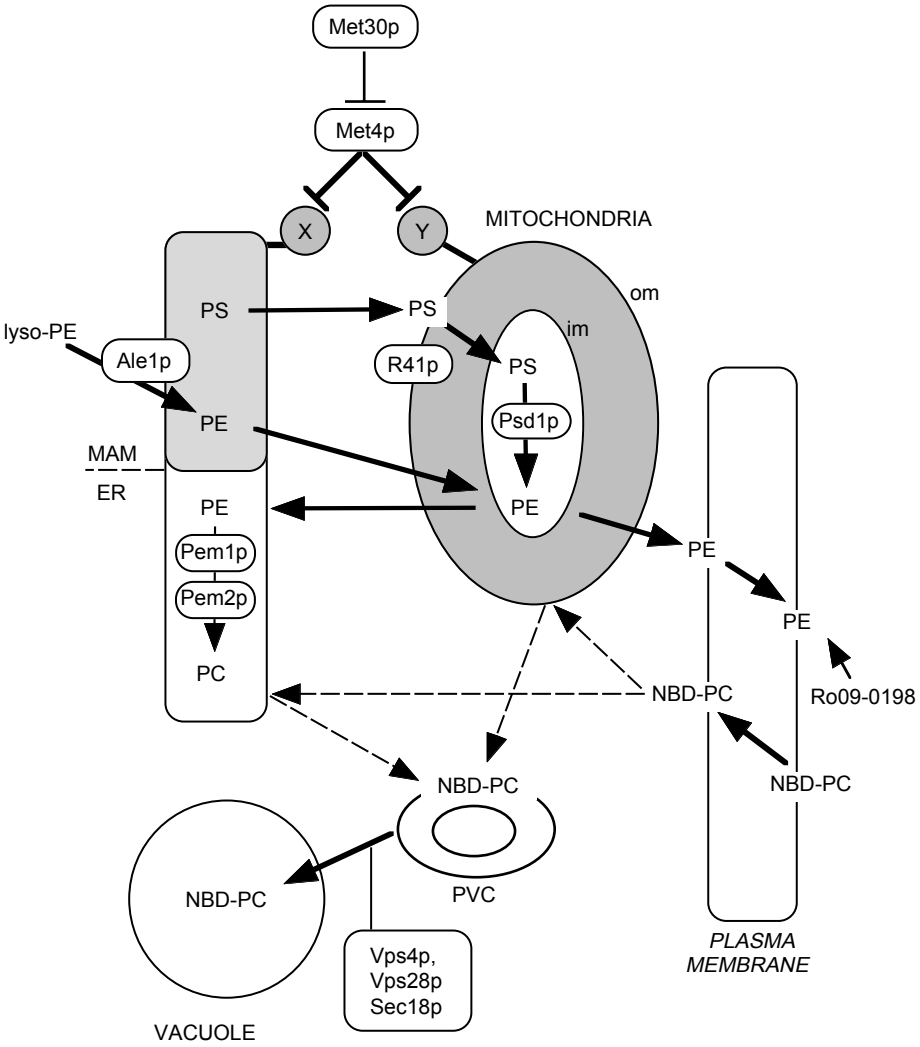


Fig. 9. Genetic elements of PS, PE, and PC transport involving ER, MAM, mitochondria, and the yeast vacuole. The figure is a composite of known transport events in mammalian and yeast systems. PS synthesized in the MAM is transported to the mitochondria under control of the ubiquitin ligase Met30p and the transcription factor Met4p. Active Met4p negatively regulates the interaction between mitochondria and MAM hypothesized to require proteins X and Y on the organelles. Import of PS into mitochondria is defective in the CHO R41 cell line and the putative R41p is postulated to regulate the lipid transport between the outer and inner membranes. PE generated in the mitochondria can be exported to the plasma membrane, or the ER. Methylation of PE in the ER by methyltransferases (Pem1p and Pem2p) produces PC. LysoPE can be taken up by the MAM and efficiently acylated by Alelp to form PE. This latter pool of PE can be rapidly assimilated into the mitochondria. PE in the plasma membrane can serve as a ligand for the cytotoxic drug Ro 09-0198, which can be used to screen for mutant strains defective in the synthesis and transport of the lipid. NBD-PC is taken up by yeast cells at 2°C by the action of Dnf1p, Dnf2p, and Lem3p (see Fig. 5), and non-specifically partitions to ER and mitochondria. Upon warming the cells, the NBD-PC concentrates in the prevacuolar compartment (PVC) and is then transported to the vacuole by processes requiring the proteins Vps4p, Vps28p, and Sec18p.

plasma membrane by the P-type ATPases (the ATP-dependent step) described earlier, followed by non-specific partitioning within the cell, primarily to the hydrophobic environments provided by ER and mitochondrial membranes (A. Grant, 2001). Subsequent warming of the cells promotes the transfer of the lipid to the prevacuolar compartment by mechanisms that remain undefined. From the prevacuolar compartment, the NBD-PC is transported to the vacuole. The data strongly suggest that the concentration of NBD-PC in the prevacuolar compartment is accompanied by the restriction of the lipid to the lumenal surface of the organelle. The transport to the vacuole requires the action of three gene products known to participate in the vesicular traffic of proteins to the vacuole, Vps4p, Vps28p, and Sec18p (the acronyms derive from vacuolar protein sorting mutants, and secretory mutants). Vps4p is an ATPase belonging to a large family of such molecules (AAA-ATPases) involved in membrane trafficking phenomena. The Vps28p is part of a multiprotein complex known as ESCRT1 that depends upon ubiquitination of membrane proteins for regulating intermembrane protein transport. The Sec18p encodes an ATPase that is the yeast ortholog of the mammalian *N*-ethylmaleimide-sensitive factor, which is required for multiple membrane protein sorting and vesicle trafficking events, involving docking interactions between membrane compartments. Thus, the sorting of NBD-PC to the vacuole in yeast is intimately connected to the vesicle-based protein sorting machinery for this organelle.

4.3.2.2. Phosphatidylethanolamine

Transport of newly synthesized PE to the plasma membrane. When an ethanolamine precursor is used, the primary site of PE synthesis is the ER (Chapter 8). The appearance of newly synthesized PE at the external leaflet of plasma membrane has been determined using chemical modification of the cell surface with TNBS at reduced temperature (R. Sleight, 1983). The results indicate that the initial rate of transport of PE is rapid and proceeds without a lag (Fig. 8). The transport process is insensitive to metabolic poisons that disrupt vesicle transport and cytoskeletal structure. The rapid transport kinetics occur at rates consistent with a soluble carrier-mediated process or transfer at zones of apposition between membranes. Analysis of the kinetics of the process is complicated since only PE at the outer leaflet of the plasma membrane is measured, and the basal scramblase activity or the 'leakage' of the ATP-dependent aminophospholipid transporter activity within the plasma membrane may be a step required for the lipid to arrive at this location. Despite these complications, the results clearly indicate that the initial rate of arrival of PE at the plasma membrane occurs on a timescale that clearly distinguishes it from well-characterized vesicle transport phenomena, and is independent of processes involved in protein transport to the cell surface.

PE derived from a PS precursor that is decarboxylated at the mitochondria is also transported to the plasma membrane (J.E. Vance, 1991) (Fig. 9). This mitochondrial PE is transported to the plasma membrane, with greater efficiency than PE synthesized from an ethanolamine precursor. The mechanism of this translocation remains to be elucidated but the process is unaffected by brefeldin A, a fungal metabolite that alters the structure and function of the Golgi apparatus.

Transport of newly synthesized PE to mitochondria. Early studies examining the movement of newly synthesized PE from the ER to the mitochondria of hepatocytes demonstrated that the process was markedly slower ($t_{1/2} \approx 2$ h) than that observed for PC (M.P. Yaffe, 1983). These experiments used classical rate sedimentation to isolate the organelles.

More detailed studies indicate that such mitochondrial fractions are likely to contain another resolvable compartment, the mitochondria-associated membrane (MAM) that appears to be a specialized domain of the ER (J.E. Vance, 1990). Evidence obtained using CHO-K1 cells (Y. Shiao, 1995) indicates that nascent PE (made via CDP–ethanolamine) is transported to the MAM but not to the inner mitochondrial membrane. It remains unclear whether some of this PE is transported to the outer mitochondrial membrane. The results are consistent with little import of PE derived from ethanolamine into mitochondria. Furthermore, yeast mutants lacking a functional allele for PS decarboxylase 1 (*psd1Δ* strains) are markedly deficient in mitochondrial PE (P.J. Trotter, 1995). The reduced PE in mitochondria cannot be fully restored by PE synthesized in the ER from an ethanolamine precursor, or that made in the Golgi or vacuole by PS decarboxylase 2 (R. Birner, 2001; M. Storey, 2001). These latter findings clearly demonstrate that there is compartmentation and restricted transport of different pools of PE within cells.

Recent studies identify another route for producing the mitochondrial PE pool that is dependent upon transport and metabolism of lyso-PE. In yeast, lyso-PE supplied externally to the cell is imported by the plasma membrane P-type ATPase components (Dnf1p, Dnf2p, and Lem3p) described earlier in this chapter (Fig. 5) (W. Riekhof, 2006). The imported lyso-PE is transported from the plasma membrane to the ER and MAM. In the ER and the MAM compartments, the lyso-PE is acylated to form PE by an acyltransferase named Ale1p (the acronym derives from acyltransferase for lyso-ethanolamine lipid) (W. Riekhof, 2007) (Fig. 9). Unlike PE produced in the ER and Golgi by the CDP–ethanolamine and PS decarboxylase 2 pathways, the MAM pool of PE derived from lyso-PE completely replenishes the missing mitochondrial pool of PE that results from a *psd1Δ* mutation. These findings identify important differences in the sorting of pools of PE in the ER and the MAM.

4.3.2.3. Phosphatidylserine

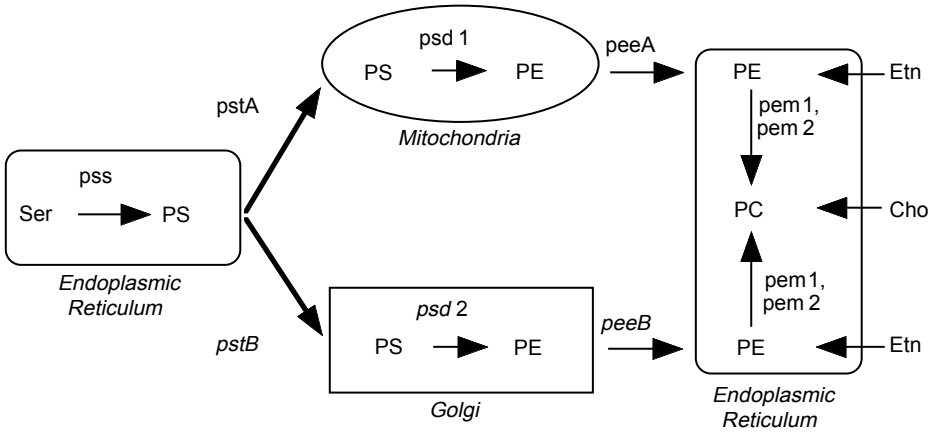
Transport of newly synthesized PS to the mitochondria. The location of PS decarboxylase at the inner mitochondrial membrane [6] provides a convenient method for determining the arrival of PS at this cellular location. The low steady-state level of PS at the mitochondrial inner membrane (Table 1) coupled with kinetic considerations indicates that PS is rapidly decarboxylated upon its arrival at the inner membrane. The general features of nascent PS transport are outlined in Fig. 9. The initial studies with intact mammalian cells that used PS decarboxylation as an indicator for lipid transport identified a clear ATP requirement for the transport process (D.R. Voelker, 1985). Subsequent reconstitution studies, with isolated organelles, established that mitochondria could interact with microsomes or liposomes and take up PS in an ATP-independent process. These findings indicated a requirement for ATP at a stage preceding the physical contact between the donor membrane and the outer mitochondrial membrane. Additional studies with isolated organelles provided evidence for a tight association between specialized elements of the ER and the mitochondria. These in vitro associations were also shown by electron microscopy to have in vivo counterparts (D. Ardail, 1993). Successful isolation of these specialized ER structures, now called the MAM, indicated that they are selectively enriched in a subset of lipid synthetic enzymes, especially PS synthase (J.E. Vance, 1990). In mammalian cells, pulse-chase experiments coupled with subcellular fractionation have now established that the PS destined for the mitochondria must transit through the MAM and some step of this process requires ATP (Y. Shiao, 1995). A MAM structure has also been identified and isolated from yeast cells (G. Daum, 1997).

The synthesis of PS and transport to the mitochondria have been successfully reconstituted using permeabilized cells [25]. The transport of PS to the mitochondria in permeabilized mammalian cells occurs in the absence of cytosol, displays an absolute requirement for ATP, and occurs with a $t_{1/2}$ of approximately 3 h at 37°C. This transport does not require ongoing synthesis of PS, and 45-fold dilution of the permeabilized cells does not alter the rate or extent of transport. These results are consistent with a membrane-bound transport intermediate that utilizes zones of close membrane apposition between the ER and the mitochondria. Although there is no absolute requirement for cytosol in the transport reaction, a soluble 9-kDa Ca^{2+}-binding protein, named S100B, that is highly conserved across mammalian species can enhance the transport several fold (O. Kuge, 2001). Permeabilized yeast have also been used to examine PS transport (G. Achleitner, 1995). Unlike mammalian cells, the transport of PS to yeast mitochondria does not require ATP.

Genetic approaches to identifying components involved in interorganelle aminophospholipid transport. Genetic tools constitute a powerful approach for identifying the components involved in lipid transport. The ability to isolate mutant strains defective in a given transport step, and then clone the genes by complementation of the mutation, can lead to clear molecular and mechanistic resolution of complex processes. The genetic aspects of PS and PE transport in eukaryotic systems are shown in Figs. 9 and 10. Understanding the genetic approach to examining aminophospholipid transport requires an appreciation that the synthesis and decarboxylation of PS and the methylation of PE are all geographically separate events within a cell. A basic hypothesis of the genetic approach is that specific genes, designated *PST* (for PS transport) and *PEE* (for PE export), either regulate or directly participate in the transfer process. Prominent methods for identifying mutant strains defective in these processes rely on the identification of drug-resistant mammalian cells (K. Emoto, 1999) or isolation of ethanolamine (Etn) auxotrophs (described in more detail in Ref. [6]) in suitable genetic backgrounds in yeast.

Studies with mammalian systems have identified a mutant line of CHO cells (R41) that is resistant to an antibiotic (Ro 09-0198) that recognizes plasma membrane PE and causes cytolysis (Fig. 9). The cells have normal enzyme activity for PS synthases and PS decarboxylase (Psd1p), but labeling with [^{14}C]serine reveals a defect in PE formation. Further analyses with isolated mitochondria demonstrate that the rate of transport of PS from the outer to the inner mitochondrial membrane occurs at approximately 40% of the rate in wild-type cells. By the scheme shown in Fig. 10, this cell line belongs to the *pstA* class of mutants with the putative protein, R41p, controlling PS transport between the outer and inner mitochondrial membranes (Fig. 9). Although the mutant line has a lesion in PS import into mitochondria, protein import into the inner membrane is unaffected. This cell line provides an important tool for cloning the complementing cDNA that encodes an element required for PS import into mitochondria.

In the yeast system, several genetic screens have been used to identify genes that participate in the transport of aminophospholipids. Genetic screening for Etn auxotrophs has yielded a number of useful mutant strains [6] defective in the PSTA and PSTB pathways (Fig. 10). The Etn auxotrophy works by providing cells with PE synthesized from the Kennedy pathways. A similar approach using choline auxotrophy can also be used to identify mutant strains defective in the PEEA and PEEB pathways. In this latter approach, PC can be synthesized from the Kennedy pathways. A mutant yeast strain designated



PATHWAY	SOURCE	GENE	PROTEIN
pstA	CHO-R41	-	-
	Yeast, *pstA* 1	*MET* 30	UBIQUITIN LIGASE
	Yeast, *met* 4	*MET* 4	TRANSCRIPTION FACTOR
pstB	Yeast, pstB1	*STT*4	PI-4 KINASE
	Yeast, pstB2	PSTB2	PI BINDING PROTEIN
	Yeast, psd2C2 Δ	PSD 2	PS DECARBOXYLASE

Fig. 10. Genetic analysis of aminoglycerophospholipid transport in eukaryotes. The transport of PS synthesized in the ER is regulated by *PSTA* and *PSTB* genes. The acronym stands for PS transport (either A or B pathways). Likewise, the transport of PE synthesized in the mitochondria or Golgi/vacuole is proposed to be regulated by *PEEA* and *PEEB* genes. The acronyms stand for PE export (either A or B pathways). Both known and proposed mutations along the metabolic and transport pathways appear in lower case italics. The table summarizes the mutants, genes, and proteins that have been identified in Chinese hamster ovary (CHO) cells and yeast. Other abbreviations: pss, PS synthase; psd, PS decarboxylase; pem, PE methyltransferase; Cho, choline; Etn, ethanolamine; Ser, serine.

pstA1 was identified and shown to be defective in PS transport between the MAM and mitochondria (M. Schumacher, 2002). Some of the general properties of the strain are (i) a requirement for Etn for growth, (ii) an inability to be rescued by choline, (iii) normal catalytic activity of PS synthase and mitochondrial PS decarboxylase 1, (iv) defective synthesis of mitochondrial PE, (v) reduced turnover of PS, (vi) reduced cellular and mitochondrial content of PE, and (vii) mitochondria of abnormal density. The mitochondria of *pstA1* cells sediment at higher density compared to those from wild-type strains when analyzed by centrifugation through sucrose gradients. The higher density of the mitochondria is a consequence of a reduced phospholipid:protein ratio of the organelle that results from reduced PS import. Reconstitution studies using different combinations of purified MAM and mitochondria from wild-type and mutant cells show that the *pstA1* strain is defective

in importing PS into the organelle in cell-free systems. Of particular interest is the finding that the transport defect is attributable to deficiencies in both the MAM as a donor compartment and the mitochondria as an acceptor compartment in the PS transport reaction. This finding indicates that the *pstA1* mutation occurs in a gene regulating the interactions between MAM and mitochondria (Figs. 9 and 10). The gene complementing the *pstA1* defect was cloned and identified as *MET30* (M. Schumacher 2002). The *MET30* gene encodes a substrate recognition subunit of a multiprotein ubiquitin ligase. The protein Met30p is required for the ubiquitination and inactivation of the transcription factor Met4p. Thus, inactivating mutations in Met30p (as in *pstA1* mutants) prevent downregulation of Met4p. The downstream effectors of Met4p that regulate PS transport are now being identified. The emerging model is that active Met4p acts as a negative regulator of PS transport to the mitochondria by increasing the expression of proteins/enzymes that inhibit the process. The negative regulation of PS transport by Met4p is turned off by Met30p. Collectively, these data suggest that proteins located on the MAM and mitochondria promote interactions between the organelles that are involved in docking and/or transport reactions. Active Met4p suppresses the interactions between the organelles and renders the MAM an incompetent donor and the mitochondria an incompetent acceptor. Conversely, active Met30p inactivates Met4p and enables interactions between the organelles that facilitate lipid transport.

Genetic analyses have also been used to identify genes involved in controlling PS transport between the ER and Golgi (Fig. 10). The screens have primarily focused on the PSTB pathway and have used mutagenesis and identification of Etn auxotrophs as a means to identify mutants defective in transport of PS to the locus of Psd2p in the Golgi [6]. The mutant strains identified, *pstB1*, *pstB2*, and *psd2-C2Δ*, all exhibit similar characteristics that include (i) a requirement of Etn for normal growth, (ii) normal PS synthase and PS decarboxylase 2 activity, (iii) defective synthesis of PE, (iv) decreased turnover of PS, and (v) decreased cellular content of PE. The growth defect of the *pstB1* strain is complemented by the gene *STT4*, which encodes a PI-4-kinase (Stt4p). The *pstB1* mutant strain is defective in PI-4-kinase activity that is corrected by the *STT4* gene (P. Trotter, 1998). These findings implicate polyphosphoinositides as potential regulatory molecules that control PS transport between the ER and the Golgi. Although most data identify Stt4p as a peripheral plasma membrane protein, a minor population of the protein has been identified in complexes containing the ER-associated tethering protein Scs2p (the acronym derives from suppressor of choline sensitivity). Scs2p belongs to a family of integral membrane proteins that can interact with proteins containing an FFAT motif (two phenylalanines within an acidic tract) [11,26]. These types of interactions are emerging as important recognition motifs involved in regulating lipid metabolism and transport.

The *pstB2* mutant is complemented by the gene *PSTB2*, which encodes a protein homologous to the phospholipid transfer protein, Sec14p (W. Wu, 2000). Sec14p binds PI and PC and transfers these lipids between membranes in vitro. The PstB2p protein also exhibits PI transfer activity, but does not transfer PC or PS in vitro. The PstB2p is amphitropic and is present in both soluble and membrane-bound forms. Reconstitution studies using different combinations of isolated organelles derived from wild-type and mutant cells demonstrate a requirement for PstB2p to reside on the acceptor membrane in

order for PS transport to occur. These findings suggest that PstB2p may exist in a complex with other proteins on the acceptor membrane that function in the docking and/or transport reactions between the donor and acceptor membranes.

The Psd2p resides on the Golgi and decarboxylates PS after its transport to the membrane. Psd2p is comprised of an active site-containing α subunit and a non-catalytic β subunit. The β subunit contains a C2 domain at the amino terminus. In general, C2 domains function as phospholipid and Ca^{2+}-binding domains in numerous proteins. Structural mutants of Psd2p lacking the C2 domain (Psd2p-C2Δ) are catalytically active and localize correctly within the cell. However, strains expressing the Psd2p-C2Δ at 10 times the normal level of enzyme activity behave as if they have a null allele of the gene in vivo (H. Kitamura, 2002). Reconstitution studies with isolated organelles and permeabilized cells reveal that deletion of the C2 domain of Psd2p disrupts PS transport between the ER and Golgi. Thus, the C2 domain of the enzyme participates in transporting PS to the proper location for catalysis. As described earlier, PstB2p is also required for PS transport between the ER and Golgi. Neither the C2 domain of Psd2p nor the PstB2p protein alone is sufficient for PS transport in vivo or in vitro. These findings suggest that a multiprotein complex consisting of Psd2p and PstB2p and perhaps several other proteins assembles on the acceptor membrane to create a docking and/or transport apparatus for moving PS between the membranes (Fig. 11).

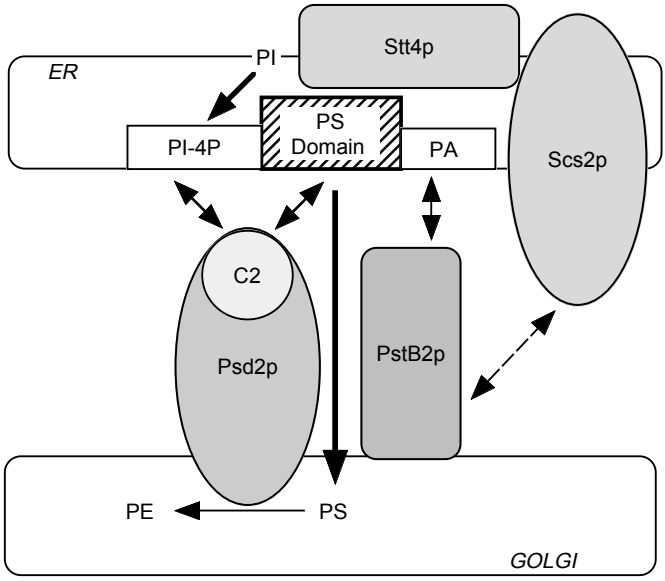


Fig. 11. Protein and lipid motifs regulating PS transport from the ER to the Golgi. PS transport between the ER and Golgi requires a PI-4 kinase (Stt4p), a lipid-binding protein (PstB2p), and the C2 domain of PS decarboxylase 2 (Psd2p). The Stt4p can be tethered to the ER by Scs2p. Lipid transport is most efficient from membranes enriched in PS, or PS plus phosphatidic acid (PA). Lipid recognition by Psd2p and PstB2p facilitates interactions between the membranes. Protein–protein interactions between the membranes involving PstB2p and Scs2p also occur, but require additional proteins, and are indicated by a dashed line.

Advances in developing reconstitution systems for investigating transport of PS to the location of Psd2p now make it possible to use liposomes of defined composition as the donor membrane compartment (W. Wu, 2004). In order for liposomes to function as donors they must possess several attributes. The transport reaction is most efficient from liposomes with a relatively low degree of curvature (>400 nm diameter) that are highly enriched in PS. Even modest surface dilution of PS, by 50% PC, can completely abrogate lipid transport between the membranes. However, if surface dilution occurs with phosphatidic acid instead of PC, high levels of PS transport between the membranes are maintained. In addition, surface dilution of PS with PI-4P does not produce the levels of inhibition found with PC. The results suggest that segregated domains of PS alone, or in the presence of phosphatidic acid and/or PI-4P, are the optimal physical arrangements in the donor membrane for the transport reaction. The findings with liposomes as donors raise the possibility that proteins in the biological donor membrane may segregate domains of specific lipids (such as PS) to facilitate their transport between the membranes (Fig. 11). The factors that induce such segregation by proteins in the donor membrane could depend upon external factors such as proteins in the acceptor membrane. Although such a model is speculative, it predicts a role for specific protein–protein and protein–lipid interactions between and within donor and acceptor membranes for recognition, docking, and facilitating interorganelle PS transport.

4.3.2.4. Sphingolipids

Ceramide transport from ER to Golgi. Ceramide is synthesized in the ER and the majority of this lipid is subsequently transported to the Golgi apparatus where it is metabolized to SM and glycosphingolipids such as GlcCer and lactosylceramide (Chapter 13). Measurement of the conversion of ceramide to either SM or glycosphingolipids can thus serve as an indicator of ceramide transport between organelles (Fig. 12). Hanada and coworkers have isolated mutant strains of Chinese hamster ovary cells that are resistant to a toxin, lysenin, that binds cell surface SM and causes cytolysis [27]. One class of mutants (LyA) is selectively defective in SM synthesis, but not glycosphingolipid synthesis, despite normal activity of SM synthase. These findings suggest that the routing of ceramide to SM and GlcCer synthases is different, and regulated by different gene products. Depletion of ATP in intact cells also yields arrest of ceramide (and BODIPY-ceramide) trafficking to SM synthase. When wild-type permeabilized cells are reconstituted with cytosol from the LyA cells, ceramide transport-dependent SM synthesis does not occur (T. Funakoshi, 2000). Thus, the LyA lesion resides in a soluble protein that participates in transport of nascent ceramide to the locus of SM synthase. In contrast to these findings, GlcCer synthesis failed to exhibit a clear requirement for cytosol for ceramide transport to the enzyme.

LyA cells are sensitive to sterol depletion with methyl-β-cyclodextrin and this phenotype was the basis of a genetic screen that was used to clone the cDNA complementing the LyA defect. The gene has been named CERT, for ceramide transfer. Transfection of LyA cells with the CERT cDNA confers lysenin sensitivity and methyl-β-cyclodextrin resistance to the cells, and restores ceramide transport and SM synthesis. The CERT protein has been studied in detail. Three functional domains are present in the protein: an N-terminal pleckstrin homology (PH) domain, a C-terminal START (sterol acute regulatory protein related lipid transfer) domain, and a middle region (MR) that contains an ER-interacting domain (Fig. 13)

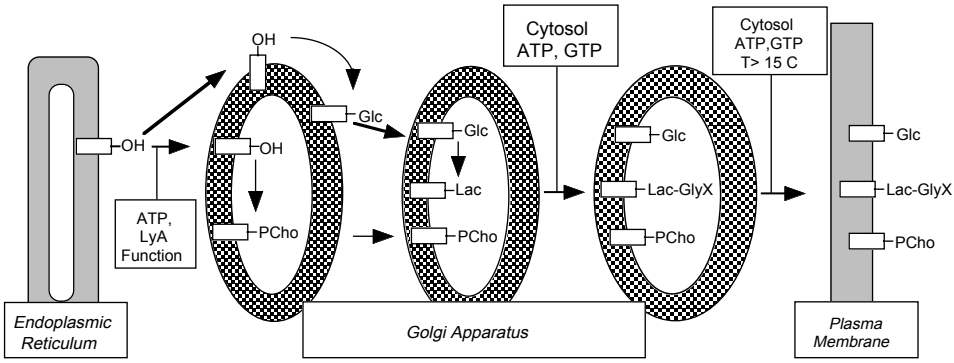


Fig. 12. Interorganelle transport of sphingolipids in eukaryotic cells from ER to plasma membrane. The structure (▭) represents the Cer portion of native, fluorescent, or short-chain synthetic sphingolipids. PCho, Glc, and Lac are the abbreviations for phosphocholine, glucose, and lactose. Ceramide is transported to the Golgi in an ATP-dependent reaction. Glucosylceramide is synthesized on the cytosolic face of the Golgi. Sphingomyelin is synthesized on the lumenal face of the Golgi. The LyA mutation selectively affects the access of ceramide to the site of synthesis. Glucosylceramide must reach the lumenal face of the Golgi for conversion to lactosylceramide and more complex glycosphingolipids (Lac–Gly X). Movement of the sphingolipids through the Golgi requires cytosol, ATP, and GTP and is inhibited by brefeldin A, monensin, GTPγS, and reduced temperature. The $t_{1/2}$ for sphingolipid transport from the Golgi to the plasma membrane is 20 min.

(K. Hanada, 2003). The PH domain binds PI-4P and enables CERT to bind to Golgi membranes containing the lipid. The original LyA mutant form of CERT contained a mutation in the PH domain that prevented interaction of the protein with PI-4P in vivo and in vitro. The START domain contains the ceramide-binding and transfer portion of the protein. Analysis of recombinant forms of the START domain, show that it is capable of transferring ceramide between different populations of liposomes in vitro. The MR domain contains an FFAT motif, which is necessary for interactions between this family of proteins and resident membrane tethering proteins such as vesicle-associated protein (VAP) in the ER (M. Kawano, 2006). A proposed cycle of events for ceramide transfer is outlined in Fig. 13. Initially, soluble CERT interacts with VAP at the ER, and the START domain becomes loaded with ceramide. Next, the CERT disengages from the ER and attaches to the Golgi via interaction of the PH domain with PI-4P. Subsequently, the Golgi-associated CERT releases ceramide that becomes available for transbilayer movement to the locus of SM synthase. In the final step, CERT disengages from the Golgi and becomes available to re-initiate the cycle.

Transport of newly synthesized sphingolipids from the Golgi to the plasma membrane. The synthesis and intracellular trafficking of SM and GlcCer have been examined using several different fluorescent ceramides and short-chain radiolabeled ceramides [9]. When fibroblasts are incubated with NBD-ceramide at 2°C, it is rapidly taken up and distributed randomly among all cell membranes (N. Lipsky, 1985). Upon warming the cells to 37°C, the fluorescent lipid concentrates in the Golgi apparatus as it is converted to NBD-SM and NBD-GlcCer. These sphingolipids are subsequently exported from the Golgi apparatus to the plasma membrane by a process that is partially monensin sensitive and brefeldin A sensitive in most cells and occurs with a $t_{1/2}$ of 20 min, a time similar to that required for the transport of many proteins from the Golgi to the plasma membrane (Fig. 12).

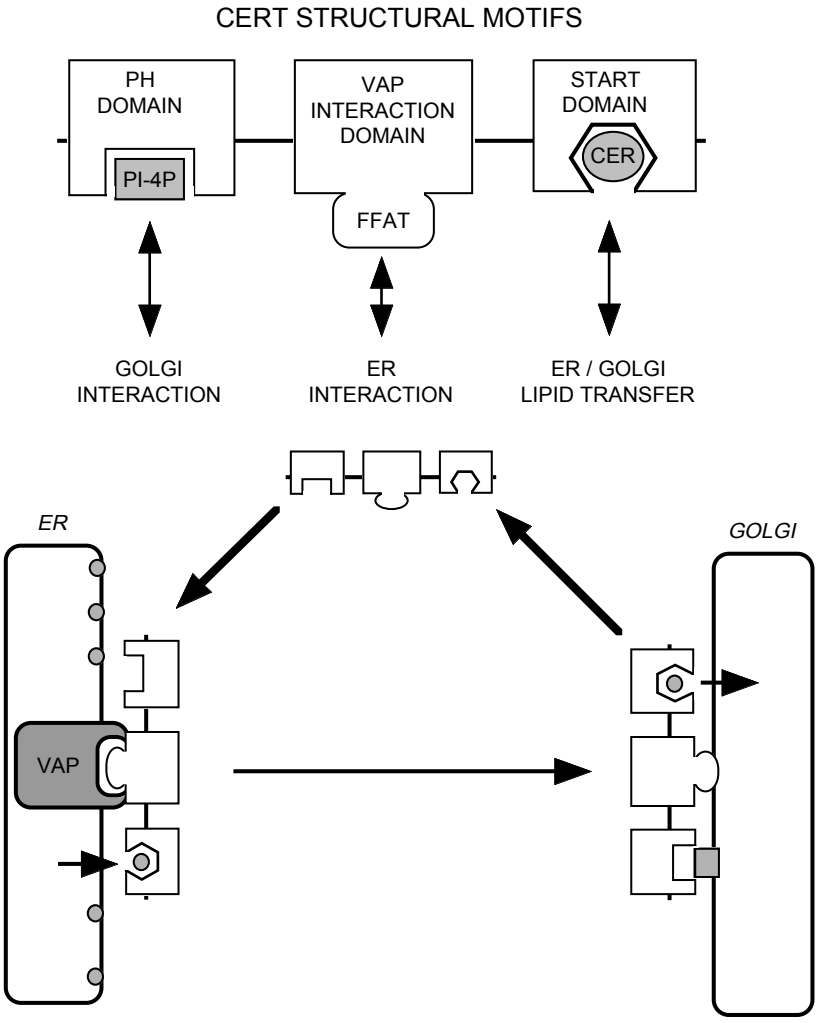


Fig. 13. Major structural domains of CERT and their role in membrane recognition and ceramide transport. CERT contains three important structural motifs consisting of a pleckstrin (PH) domain, a vesicle-associated protein (VAP) interaction domain, and sterol acute regulatory protein related (START) domain. The PH domain promotes Golgi attachment of CERT by PI-4P recognition. The VAP interaction domain contains an FFAT (two phenylalanines within an acidic tract) motif that enables ER binding. The START domain contains a hydrophobic pocket for sequestering ceramide (CER). A cycle of membrane attachment, dissociation, and diffusion is shown in the bottom part of the figure. Binding of ceramide may enable disengagement of the protein from the ER by disrupting VAP–FFAT interaction. Ligation of PI-4P may promote dissociation of ceramide at the Golgi.

However, there appears to be a pool of GlcCer that can be transported by routes insensitive to inhibitors of vesicle trafficking (G. van Meer, 2000). Vesicle-based protein transport is arrested in mitotic cells as is the transport of newly synthesized NBD-SM and NBD-GlcCer (Kobayashi, 1989). Experiments using (non-fluorescent) short-chain analogs of ceramide in permeabilized cells indicate that the export of nascent SM from the Golgi

apparatus requires ATP and cytosol and occurs via a GTP-dependent mechanism that is also consistent with vesicle budding from the organelle (J.B. Helms, 1990). Export of nascent SM from the Golgi is blocked at reduced temperatures such as 15°C and by the non-hydrolyzable GTP analog, GTPγS.

The movement of sphingolipids between elements of the Golgi has been monitored in reconstituted preparations from mutant Chinese hamster ovary cells defective in either the synthesis of lactosylceramide or the attachment of sialic acid to the latter (B. Wattenberg, 1990). In cell-free systems, donor Golgi that accumulate lactosylceramide transfer this lipid to acceptor Golgi that are devoid of the substrate. The acceptor Golgi add sialic acid to the lactosylceramide to make the ganglioside GM_3 (Chapter 13). The lipid transfer reaction between Golgi compartments requires ATP and cytosol and is inhibited by GTPγS. The properties of glycosphingolipid transport between Golgi compartments are thus identical to those for vesicular protein transport.

Import of exogenous sphingolipids. The NBD and BODIPY analogs of SM, GlcCer, lactosylceramide, and numerous other sphingolipids varying in their hydrophilic substituents can be readily inserted into the outer leaflet of the plasma membrane of fibroblasts at reduced temperature. When fibroblasts treated in such a manner are warmed to 37°C, the fluorescent sphingolipids are internalized and accumulate in the endosomal compartments of the cell. General inhibition of endocytosis by ATP depletion, or maintenance at low temperature, effectively prevents any internalization of the polar sphingolipids. Internalized NBD-SM subsequently accumulates in the perinuclear region of the cell containing the centrioles (M. Koval, 1989) and the Golgi apparatus (Fig. 14). The initial steps of BODIPY-SM internalization have been dissected using a variety of inhibitors and dominant negative structural variants of dynamin 2 (Dyn 2^{DN}) and Eps 15 (Eps 15^{DN}) (V. Puri, 2001). The Eps 15 protein has a regulatory function in clathrin-coated pit assembly. The internalization of BODIPY-SM is completely arrested by Dyn 2^{DN}, which disrupts both clathrin-dependent and clathrin-independent endocytosis. The expression of Eps 15^{DN}, treatment of cells with chlorpromazine, or K^+ depletion inhibits clathrin-dependent endocytosis to a greater extent than SM endocytosis. Conversely, inhibition of clathrin-independent endocytosis with genistein or nystatin does not fully block SM endocytosis. These results indicate that BODIPY-SM is internalized by multiple endocytic pathways.

The movement of the endocytosed fluorescent SM from the internalized pool back to the plasma membrane has also been examined in fibroblasts (M. Koval, 1989). This transport process occurs via vesicles. The properties of the recycling pool of NBD-SM are distinct from those observed for export of the newly synthesized SM out of the Golgi (Fig. 12). As stated above, monensin and brefeldin A arrest newly synthesized NBD-SM transport from the Golgi to the cell surface; but the recycling of endocytosed fluorescent SM is insensitive to monensin. The overall process of internalization of SM from the plasma membrane to the intracellular pool and transport back to the cell surface occur with a $t_{1/2}$ of ~40 min. These time constants are similar to those for membrane protein recycling processes from the plasma membrane.

The internalization of BODIPY-lactosylceramide follows a route that partially overlaps with that for fluorescent SM (V. Puri, 2001). BODIPY-lactosylceramide is internalized into endosomes and subsequently can be localized to the Golgi. The endocytosis of BODIPY-lactosylceramide is inhibited by Dyn 2^{DN}, nystatin, and genistein but not Eps 15^{DN}, chlorpromazine, or K^+ depletion. These results indicate that the fluorescent lactosylceramide

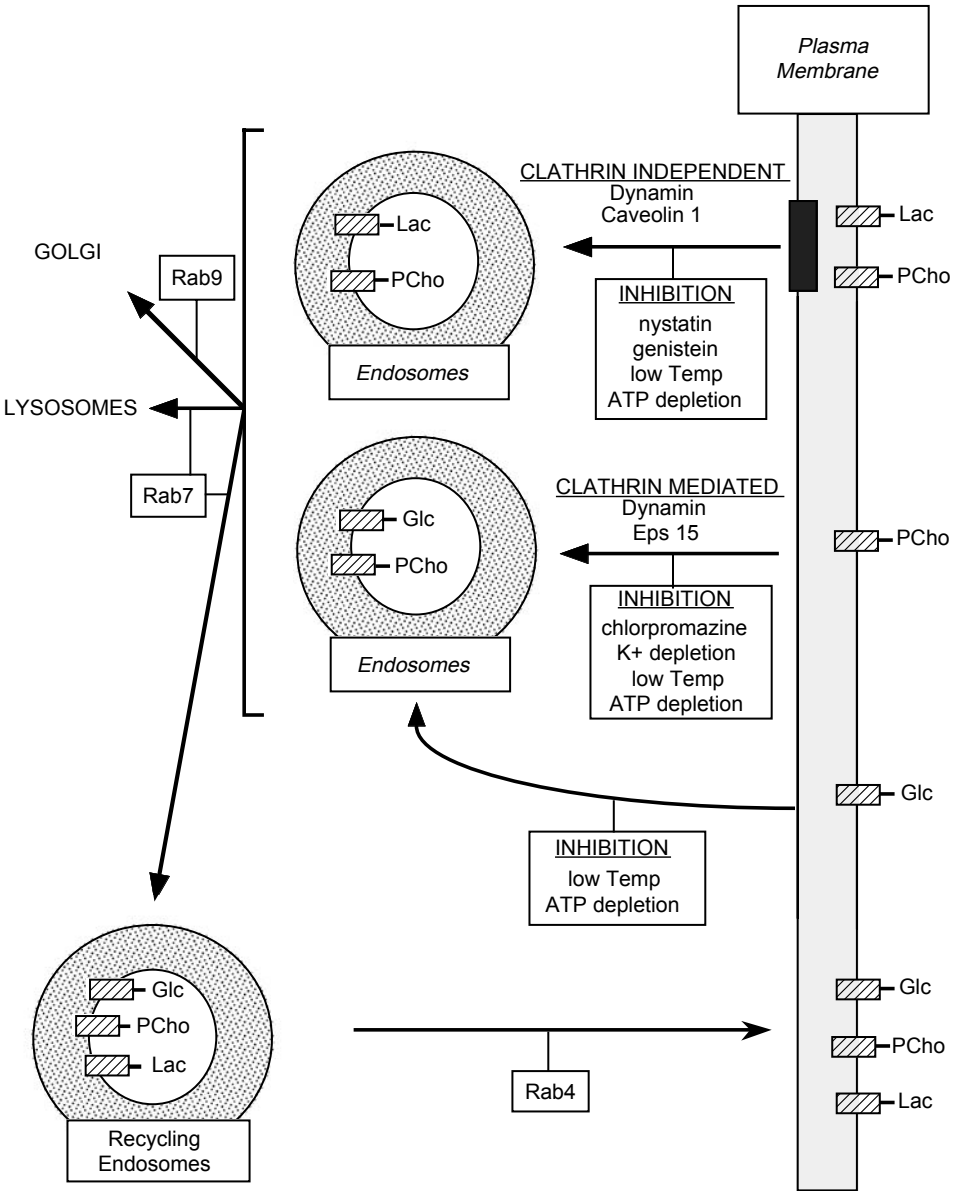


Fig. 14. Interorganelle transport of exogenously supplied sphingolipids. The structure ▨ represents the BODIPY-ceramide or NBD-ceramide portion of fluorescent sphingolipids. PCho, Lac, and Glc are the abbreviations for phosphocholine, lactose, and glucose, respectively. Following insertion into the plasma membrane at reduced temperature, SM is internalized to the endosomal compartments by both clathrin-dependent and clathrin-independent pathways that are distinguished by the inhibitors indicated. Lactosylceramide and other glycosphingolipids are endocytosed primarily by the clathrin-independent pathways involving caveolin 1. The endocytic process can be generally inhibited by ATP depletion and reduced temperature. The endocytosed lipid can recycle back to the plasma membrane and this recycling requires Rab4 activity. The endocytosed lipid can also be transported to the Golgi apparatus via Rab-9 dependent pathways, or to lysosomes and recycling endosomes via Rab7 pathways.

is endocytosed by a clathrin-independent mechanism (Z. Cheng, 2006). The specific clathrin-independent pathway followed by lactosylceramide is dependent on the presence of caveolae containing caveolin 1. Current estimates indicate that approximately 80% of the endocytosis of lactosylceramide and numerous related glycosphingolipids (including GalCer, MalCer, $Gal_2GlcCer$, SO_4-GalCer, and GM_1) occurs via the clathrin-independent pathway for endocytosis utilizing caveolae containing caveolin 1. The transport of lactosylceramide to the endosomes and Golgi and its recycling to the plasma membrane require the participation of low-molecular-weight GTPases of the Rab family of proteins [4,9,28]. Rab7 is required for assimilation of exogenous lactosylceramide into the late endosomes and lysosomes. Rab 9 is required for the lactosylceramide to reach the Golgi apparatus. Rab4 is necessary for lactosylceramide to be recycled back to the plasma membrane.

4.3.2.5. Cholesterol

Transport of cholesterol to and from the plasma membrane. Following its synthesis at the ER, cholesterol is transported throughout the cell and becomes enriched in the plasma membrane [7]. The transport of newly synthesized cholesterol to the plasma membrane has been examined in tissue culture cells using pulse-chase experiments with either the rapid plasma membrane isolation procedure (M. Kaplan, 1985), caveolae isolation (A. Uittenbogaard, 1998), oxidation of accessible cholesterol by cholesterol oxidase (Y. Lange, 1985), or desorption of newly labeled cholesterol with methyl-β-cyclodextrin (S. Heino, 2000) (Fig. 15). These lines of experimentation have revealed that the minimum transport time for cholesterol to the plasma membrane is 10 min at 37°C. The transport process can be completely blocked by reducing the temperature to 15°C or by depleting cellular ATP levels with metabolic poisons. The transport of nascent cholesterol is unaffected by treatment of the cells with cytoskeletal poisons or monensin but shows slight sensitivity to brefeldin A. When the translocation of cholesterol is inhibited by maintaining the cells at 15°C, this lipid accumulates in a low-density membrane fraction (M. Kaplan, 1985; Y. Lange, 1985). Intermediates in the transport of proteins between the ER and the Golgi apparatus accumulate at 15°C in vesicles of similar density to those containing cholesterol. However, the compartment containing the intermediates in protein transport is different from that containing cholesterol because the former is completely sensitive to brefeldin A treatment, whereas the latter is largely insensitive. This result demonstrates that a significant fraction of cholesterol travels to the plasma membrane via intermediates that are distinct from those involved in membrane protein transport. Collectively, these data suggest the presence of specialized machinery for cholesterol transport. Experiments with yeast produce similar timing for transport of ergosterol from the ER to the plasma membrane. The major route for sterol transport in yeast also appears to be largely independent of the vesicular traffic associated with membrane proteins. One mechanism that has been proposed for cholesterol transport in mammalian cells is non-vesicular and consists of a complex of caveolin with cholesterol, HSP 56, and the cyclophilins A and 40 (A. Uittenbogaard, 1998). This complex is believed to form a cytosolic cholesterol carrier that can transport the lipid from the ER to a caveolae-rich fraction of plasma membrane. Pulse-chase experiments with [^{3}H]acetate are consistent with caveolae serving as an entry point for cholesterol at the plasma membrane. Both cyclosporin A and rapamycin are predicted to disrupt the interactions of the cyclophilins and HSP 56 with caveolin. Treatment of cells with cyclosporin A and rapamycin markedly inhibited the

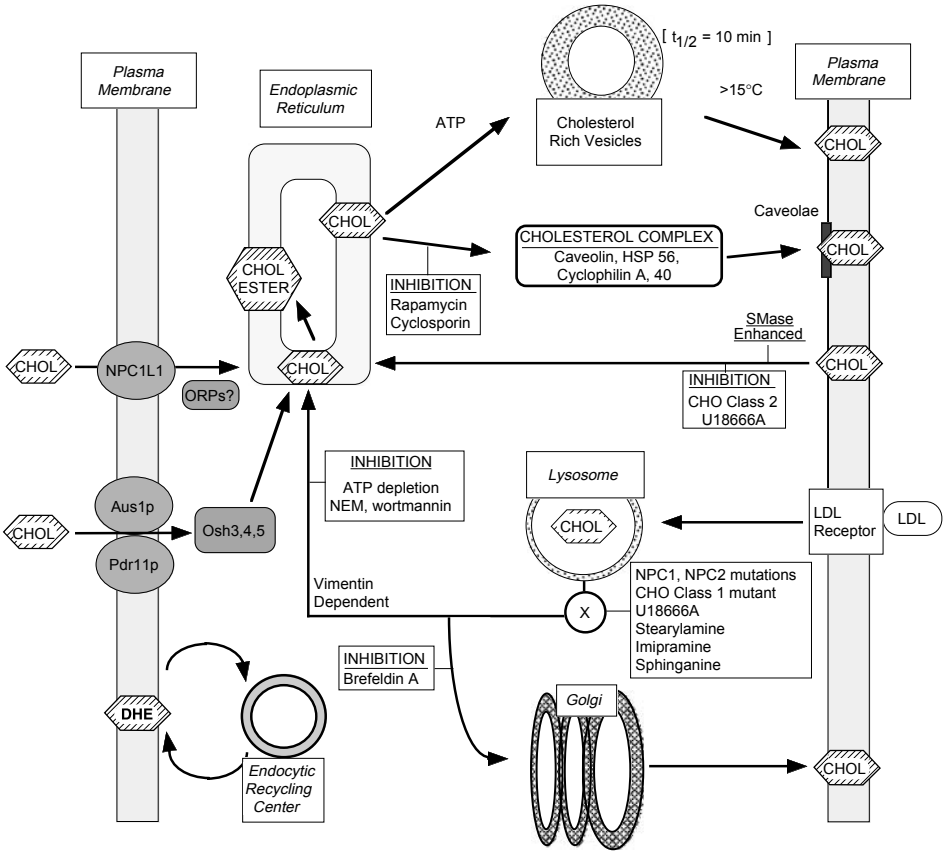


Fig. 15. Interorganelle transport of cholesterol. Newly synthesized cholesterol (CHOL) is transported from the ER to the plasma membrane in an ATP- and temperature-dependent process. One intermediate identified in this transport is a low-density cholesterol-rich fraction believed to be comprised of vesicles. A second proposed intermediate consists of a soluble cholesterol/protein complex. It is not clear if the vesicle fraction and soluble complex are the same. Cholesterol present in the plasma membrane can be induced to move to the ER by sphingomyelinase treatment of the cell surface. This latter process is inhibited by hydrophobic amines and class 2 mutations in CHO cell lines. Low-density lipoprotein (LDL) derived cholesteryl ester enters the lysosome and is cleaved to form free cholesterol. The lysosomal cholesterol is exported from the lysosomes by a process regulated by NPC1 and NPC2 gene products that is also susceptible to inhibition by hydrophobic amines. The cholesterol exported from the lysosome also traverses the Golgi en route to the plasma membrane or travels directly to the ER via a process that exhibits partial dependence on intermediate filaments and requires ATP. The lysosome–ER pathway is inhibited by *N*-ethylmaleimide (NEM) and wortmannin. Dehydroergosterol (DHE) inserted into the plasma membrane rapidly equilibrates with membranes of the endocytic recycling center by ATP- and vesicle-independent mechanisms. The yeast ABC transporters Aus1p and Pdr11p can import cholesterol that is transported to the loci of acyltransferases for the synthesis of steryl esters. This latter transport is dependent upon Osh3p, Osh4p, and Osh5p. NPC1L1 constitutes another import mechanism, which is primarily operative in enterocytes and is sensitive to ezetimibe. Mammalian oxysterol-binding protein related proteins (ORPs) constitute a class of molecules that could facilitate the routing of dietary cholesterol to the ER for packaging into chylomicrons. ORPs have been proposed to participate in other aspects of intracellular sterol transport.

appearance of nascent [^{3}H]cholesterol in caveolae and the total plasma membrane. However, there is still uncertainty about the relationship between this cytosolic complex and the low-density fraction that accumulates at 15°C.

Additional proposals for soluble sterol carriers include the oxysterol-binding protein related proteins (ORPs). Hypothetical schemes involving interactions between the PH domains of ORPs and specific organelles are attractive and are analogous to the known mechanism of CERT action (H. Yang, 2006). The crystal structure of the yeast ORP, Osh4p, reveals a cavity that can accommodate sterols, and the protein acts as a sterol transfer protein whose activity is enhanced by polyphosphoinositides in vitro (S. Raychauduri, 2006). In addition, yeast mutants harboring conditional mutations in the entire repertoire of ORPs (Osh1–Osh7 in yeast) show pleiotropic membrane defects affecting vacuole membrane homeostasis, lipid droplet formation, and endocytosis (C. Beh, 2004).

In addition to the outward movement of cholesterol to the plasma membrane, cells display a retrieval system for recovering the sterol from the plasma membrane (Fig. 15). When the plasma membrane of mammalian cells is rapidly depleted of SM by sphingomyelinase treatment, a significant fraction of the cholesterol is transported to the ER and is esterified by acyl-CoA:cholesterol acyltransferase. The cholesterol retrieval is blocked by hydrophobic amines including U18666A, and sphingosine, and steroids such as progesterone (L. Liscum, 1999) but is insensitive to ATP depletion. Liscum and coworkers isolated a cell line denoted CHO 3–6 (N. Jacobs, 1997) that is defective in recovering plasma membrane cholesterol after sphingomyelinase treatment, and this should prove useful for dissecting the transport mechanism.

Dehydroergosterol is a naturally occurring fluorescent structural homolog of cholesterol, and Maxfield and coworkers [7] have taken advantage of this property to follow the dynamic sorting of the lipid after its introduction into the plasma membrane. The dehydroergosterol loading of the plasma membrane is accomplished using methyl-β-cyclodextrin. Subsequent to its introduction into the plasma membrane, the lipid is rapidly transported, by an ATP-independent process, to the endocytic recycling center (Fig. 15). The relationship between the endocytic recycling center and the plasma membrane is dynamic with the dehydroergosterol rapidly equilibrating between the two compartments by a process that is ATP-independent. Two current models for this equilibration have been proposed. One favors a role for a soluble carrier such as an ORP with access mostly limited to the two compartments. A second model proposes that the lipid compositions of the plasma and endocytic membranes have an intrinsic chemical potential that is favorable for the free diffusion and accretion of sterols at these loci. These divergent views are unresolved and will require additional critical testing.

Dynamics of exogenous cholesterol transport. Eukaryotic cells also possess specific transport systems for the acquisition of exogenous sterols (Fig. 15). One prominent pathway is especially relevant in the intestine (but is also found in the liver) for absorption of dietary cholesterol. The transporter responsible for the bulk of this sterol uptake is related to the Niemann–Pick type C1 (NPC1) protein and is named NPC1L1 (for NPC1 like protein 1) [29]. The protein is presumed to function as a permease. Mice harboring null alleles for NPC1L1 are markedly resistant to diet-induced hyperlipidemia and exhibit significant reductions in the uptake of plant sterols (e.g., sitosterol) as well as cholesterol. Human NCP1L1 protein is the target of the drug ezetimibe, which reduces dietary sterol uptake by 70%.

In certain genetic backgrounds, yeast strains readily take up ergosterol and cholesterol. Cholesterol can substitute for the structural requirements of sterols in cell membranes and is also assimilated into lipid droplets as cholesteryl ester. Two yeast transporters for exogenous sterols have been identified. The ABC transporters Pdr11p and Aus1p transport ergosterol and cholesterol into the yeast cell [21]. Either protein is sufficient for the transport process. Subsequent to transport, the sterols can either be incorporated into the plasma membrane or esterified at the ER and incorporated into lipid droplets. Transport of the sterols to the locus of the acyltransferase occurs independently of vesicular traffic, and is primarily dependent upon the yeast ORP, Osh4p. Two other yeast proteins, Osh3p and Osh5p, appear to make minor contributions to the transport (S. Raychaudhuri, 2006).

Low-density lipoprotein (LDL) derived cholesterol. Exogenous cholesterol imported into the cell via the LDL receptor can be utilized for membrane biogenesis and regulation of sterol metabolism (Chapter 20). The mechanisms whereby lipoprotein-derived cholesterol (generated from cholesteryl esters within lysosomes) is disseminated throughout the cell are being understood with increasing detail [30]. The current view indicates that approximately 70% of the lysosomal cholesterol pool is directed to the plasma membrane, whereas 30% is directed to the ER by a separate pathway (Fig. 15). One set of inhibitors or mutations appears to affect the export of cholesterol from the lysosomes before the bifurcation of the transport between the plasma membrane and the ER. These early acting conditions include treatment with U18666A, or imipramine, and the presence of Niemann–Pick C (1 and 2) mutations. Subsequent to the bifurcation in the pathway, the routing to the plasma membrane is sensitive to brefeldin A disruption of the Golgi. After the bifurcation, the transport to the ER is sensitive to disruption of actin filaments, ATP depletion, *N*-ethylmaleimide treatment, and (weakly) wortmannin intoxication.

Important insights into the mechanism of cholesterol transport have come from LDL metabolism in cells from individuals with NPC disease [30]. In NPC fibroblasts, cholesterol transport from the lysosomal compartment to the plasma membrane is markedly retarded compared to that in normal fibroblasts (L. Liscum, 1999; E. Blanchette-Mackie, 2000). The transport defect results in the accumulation of cholesterol in lysosomes and endosomes. NPC cells also have impaired regulation of acyl-CoA:cholesterol acyltransferase, 3-hydroxy-3-methylglutaryl-Co A reductase, and LDL receptor levels, in response to LDL (Chapter 14). In contrast, the transport of newly synthesized cholesterol from the ER to the plasma membrane of NPC fibroblasts is essentially identical to that found for normal cells. These findings localize one abnormality of NPC disease to cholesterol export from the lysosomes to other organelles.

Mutations in two genes, *NPC1* and *NPC2*, are responsible for the NPC phenotype (E.D. Carstea, 1997; S. Naureckiene, 2000). The *NPC1* gene product shows significant homology to the morphogen receptor, *patched*; the major enterocyte plasma membrane transporter, NPC1L1; and members of the RND (resistance-nodulation-division) family of prokaryotic permeases. NPC1 contains 13 membrane spanning (TM) domains of which TM 3–7 have sequence homology to proteins containing sterol-sensing domains (3-hydroxy-3-methylglutaryl-CoA reductase and SREBP cleavage-activating protein, Chapter 14). NPC1 is also closely related to bacterial permeases that transport hydrophobic compounds including acriflavine (J. Davies, 2000).

The *NPC2* gene encodes a soluble lysosomal protein that was identified in a global proteomics screen of lysosomal constituents (S. Naureckiene, 2000). The protein is found in secreted and intralysosomal forms, and retrieval of the secreted protein is mediated by the mannose-6-P receptor. Incubation of NPC2-defective cells with medium containing the secreted form of wild-type NPC2 leads to protein uptake and rectification of the cholesterol accumulation seen in the mutant cells. In addition, individuals with the NPC2 phenotype (which is identical to that for NPC1) show specific mutations in the *NPC2* gene, thereby confirming the gene–mutation–disease relationship. Examination of NPC2 function in vitro reveals that it is a cholesterol-binding protein that can transfer cholesterol to phospholipid-containing liposomes (S. Cheruku, 2006). The preferred acceptor membranes are liposomes containing acidic phospholipids especially the lysosomal phospholipid, lyso-bis-phosphatidic acid. The emerging picture is that soluble NPC2 and membrane-bound NPC1 cooperate in the recognition and translocation of cholesterol out of the lysosome. Just how the cholesterol moves from the lysosome to other organelles is not entirely clear, although ORPs have been proposed as potential carriers for the sterol between organelles.

Cholesterol import into mitochondria. In steroidogenic tissues, cholesteryl esters are hydrolyzed in response to hormonal stimuli, and cholesterol is imported into mitochondria for the synthesis of pregnenolone, the precursor of all steroid hormones (Fig. 16). The transit of cholesterol between the outer and inner mitochondrial membranes is regulated by steroidogenic acute regulatory protein (StAR) [31]. Individuals with lipoid congenital adrenal hyperplasia lack functional StAR and are unable to make pregnenolone. Analysis of the StAR sequences in these individuals reveals that the mutations accumulate in the carboxy terminal region of the protein. The carboxy terminus of StAR and a structural homolog MLN-64 that binds cholesterol are now recognized to define a large protein family

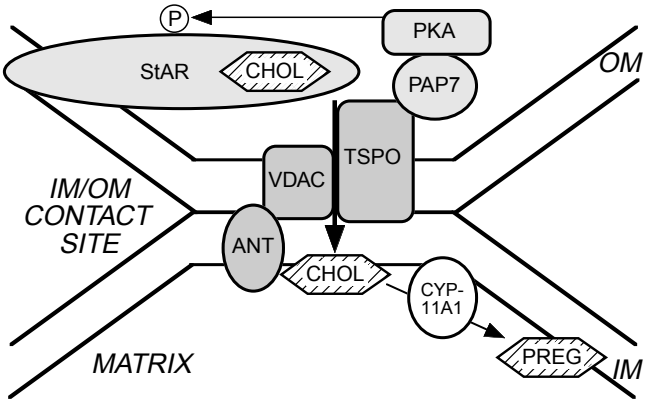


Fig. 16. Mitochondrial import of cholesterol. The StAR protein is the major cholesterol (CHOL) carrier bringing the lipid to import sites. The StAR protein is phosphorylated by cyclic AMP-dependent protein kinase (PKA) that is recruited to the mitochondria by the protein PAP7. PAP7 is a binding partner of the peripheral benzodiazepine receptor/translocator protein (TSPO), which forms a complex with the voltage-dependent anion channel (VDAC) and an adenine nucleotide transporter (ANT) at contact sites between the inner and outer mitochondrial membranes. The multiprotein complex constitutes a cholesterol transporter that moves cholesterol from StAR to the inner mitochondrial membrane where the side-chain cleavage enzyme (CYP-11A1) converts it to pregnenolone (PREG).

capable of binding hydrophobic molecules. StAR is rapidly synthesized in response to hormonal stimuli and is targeted to the mitochondria by N-terminal sequences. The StAR protein is imported into the mitochondria and becomes associated with the matrix and inner membrane. Initially, transit intermediates of StAR were proposed to be the cholesterol carriers between the outer and inner membranes. However, current data demonstrate that mitochondrial import of StAR is not essential for cholesterol import, and that the association of the C-terminus of StAR with the outer mitochondrial membrane is the critical interaction required for promoting lipid transfer. The interaction of the C-terminus of StAR with cholesterol and outer membrane proteins is now recognized to play an important role in assembling a transport complex that moves the lipid to the inner membrane.

The principal StAR-interacting protein is the peripheral benzodiazepine receptor (formerly named PBR but recently renamed translocator protein, TSPO) [8]. Biochemical experiments demonstrate direct interactions between the C-terminus of StAR and a protein complex consisting of TSPO, an interacting protein (PAP7), and a cAMP protein kinase regulatory subunit (RIα). The TSPO also interacts with the voltage-dependent anion channel located at outer mitochondrial membrane/inner mitochondrial membrane contact sites. Hormonal activation of steroidogenesis promotes direct interaction of PAP7 with TSPO. The PAP7 in turn binds the RIα and recruits the protein kinase to the complex. The active kinase can phosphorylate newly synthesized StAR, which carries cholesterol and binds TSPO. In the current model, the cholesterol is passed from StAR to TSPO. The TSPO in conjunction with the voltage-dependent anion channel subsequently acts as a transporter for moving the sterol between the outer and inner mitochondrial membranes. Physical associations between the voltage-dependent anion channel and an inner mitochondrial membrane adenine nucleotide transporter provide further physical links from the outer to the inner membrane. Upon reaching the inner membrane, cholesterol serves as a substrate for the side-chain cleavage enzyme CYP11A1, to generate pregnenolone.

4.3.2.6. Perspectives on lipid transfer proteins and membrane biogenesis. Since their discovery almost 50 years ago, lipid transfer proteins have been attractive candidates for acting as soluble lipid carriers between membranes in vivo [5]. However, there have been two major points of debate about a lipid trafficking role for the lipid transfer proteins. The first point questions how the one-for-one exchange of lipid between model membranes observed in vitro corresponds to the requirement for net transfer of lipid molecules necessary for membrane biogenesis in vivo. A second point of concern has been whether the lipid binding and exchange activity of the proteins simply reflects a lipid-binding property that has other functions in vivo.

A significant collection of evidence presented in this chapter provides new ways to think about the function of lipid transfer proteins. The proteins PstB2p, CERT, and StAR contain canonical lipid exchange/transfer domains and exhibit classical exchange activity in vitro. However, their ability to effect net lipid transfer in vivo requires cooperation with other proteins and/or lipids. For example, the START domain of CERT can exchange lipids in vitro, but is ineffective at net lipid transfer in vivo without its PH domain and FFAT domains. Likewise, the StAR protein is ineffective at net lipid transfer in vivo unless it interacts with TSPO. In a similar situation, PstB2p requires the C2 domain of Psd2p to effect the transfer of PtdSer in vivo or in reconstituted systems. This same general

framework is reiterated in the intermembrane transfer of phosphatidic acid in the chloroplast in which the Tgd2p must interact with the Tgd1p. An additional example is the apparent cooperation among MsbA, LptA, RlpB, and Imp in the transport of Ra-LPS. Collectively, the data argue that the coupling of lipid-binding and transfer/exchange domains to additional protein and lipid recognition motifs is essential to achieve net transfer of lipid molecules. It is important to emphasize that these recognition domains may require multimolecular assemblies of proteins and lipids to achieve a mature complex capable of net transport. Many of these lipid transport events also occur at closely juxtaposed membranes. The transfer of PS to Psd1p occurs at regulated junctions between the MAM and the outer mitochondrial membrane. The transfer of cholesterol to the mitochondria occurs at regions of outer and inner membrane contact. The transfer of PS to Psd2p requires physical interactions between the ER and the Golgi. The transfer of phosphatidic acid between the chloroplast envelope membranes utilizes interactions between proteins on the inner membrane and lipids in the outer membrane. One of the current models for CERT action includes restriction of the protein to regions of ER/Golgi apposition. The lipid and protein interactions of ORPs have also led to proposals for their participation as subcomponents of multipartite lipid and protein complexes. Thus, an important emerging model for lipid transport predicts a central role for zones of organelle apposition and intermembrane recognition through specific lipid and protein motifs for assembly of functional transporters.

5. Future directions

Cellular lipid transport is a fundamental process essential to all cell growth, division, and differentiation. Our understanding of lipid transport has changed markedly in the last 5 years, during which the number of genes implicated in the processes have grown from a few to several dozen. Significant advances have followed the identification of mammalian cell lines, yeast, and bacterial strains with defects in lipid traffic. In addition to forward genetic screens, reverse genetic screens are becoming more common in both simple eukaryotes and mice. The identification of human diseases with lesions in lipid traffic continues to make additional important tools, and cell lines, available for study. Advances in reconstitution of lipid traffic in permeabilized cells and cell-free systems now allow for more precise and critical tests of protein function in transport processes. The application of fluorescent probes continues to provide new insights and real-time images of selected aspects of lipid transport. The examination of lipid transport processes is now firmly rooted in the manipulation of mutant cells, genes, and gene products. However, there remains much to be accomplished. Future studies need continued focus on the development of additional genetic tools. For many of the lipid trafficking processes described in this chapter there are still no mutants available, and a concerted effort must be made to develop novel selections and screens that attack the voids in our understanding. The current genomic information and the ease of manipulating genes in heterologous systems now also allow for intuitive approaches and reverse genetic screening to be applied freely and aggressively. The mechanisms of intracellular lipid traffic in membrane assembly have historically been difficult to elucidate, but recent advances are grounds for much optimism. This discipline now provides numerous new research opportunities and rewards for those who tackle this long-standing problem of cell biology and biochemistry.

Abbreviations

ABC	ATP-binding cassette
BODIPY	boron dipyrromethene difluoride
CERT	ceramide transfer protein
DG	diacylglycerol
ER	endoplasmic reticulum
Etn	ethanolamine
GlcCer	glucosylceramide
GTPγS	guanosine 5′-0-(-3-thiotriphosphate)
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MAM	mitochondria-associated membrane
mdr	multidrug resistance
NBD	*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl
NPC	Niemann–Pick type C
ORP	oxysterol-binding protein-related protein
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PI-4P	phosphatidylinoisitol-4 phosphate
PS	phosphatidylserine
PSD	phosphatidylserine decarboxylase
SM	sphingomyelin
StAR	steroidogenic acute regulatory protein
TNBS	trinitrobenzenesulfonate
TSPO	translocator protein

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CHAPTER 17

Lipoprotein structure

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Contents

1. Introduction

Lipoproteins are soluble complexes of proteins (apolipoproteins) and lipids that transport lipids in the circulation of all vertebrates and even insects. Lipoproteins are synthesized in the liver and the intestines, arise from metabolic changes of precursor lipoproteins, or are assembled at the cell membranes from cellular lipids and exogenous lipoproteins or apolipoproteins. In the circulation, lipoproteins are highly dynamic. They undergo enzymatic reactions of their lipid components, facilitated and spontaneous lipid transfers, transfers of soluble apolipoproteins, and conformational changes of the apolipoproteins in response to the compositional changes. Finally, lipoproteins are taken up and catabolized in the liver, kidney, and peripheral tissues via receptor-mediated endocytosis and other mechanisms. This chapter deals with the composition and structure of human lipoproteins.

1.1. Main lipoprotein classes

Although the assembly, structure, metabolism, and receptor interactions of lipoproteins are determined by their apolipoprotein components, the most common classifications of lipoproteins are based on their hydrated density or mobility on agarose gel electrophoresis.

The classification into chylomicrons (CM), very low density (VLDL), low density (LDL), and high density (HDL) lipoproteins is based on their relative contents of protein and lipid that determine the densities of these lipoprotein classes. CM have only 1–2% protein while HDL have about 50% protein by weight. The diameters of lipoproteins are inversely correlated with their densities and range from about 6000 Å for CM to 70 Å for the smallest HDL (Fig. 1).

The general structural organization is similar for all the lipoprotein classes: the apolipoproteins and amphipathic lipids (mostly phospholipids (PL) and unesterified cholesterol) form a 20-Å-thick shell on the surface of spherical particles. This shell encloses the core of neutral lipids (triacylglycerols, cholesteryl esters (CE), and small amounts of unesterified cholesterol and other dissolved lipids, e.g., lipid-soluble vitamins). The main protein components are characteristic of each lipoprotein class; they are indicated in Fig. 1, and will be described in detail in Section 3 of this chapter.

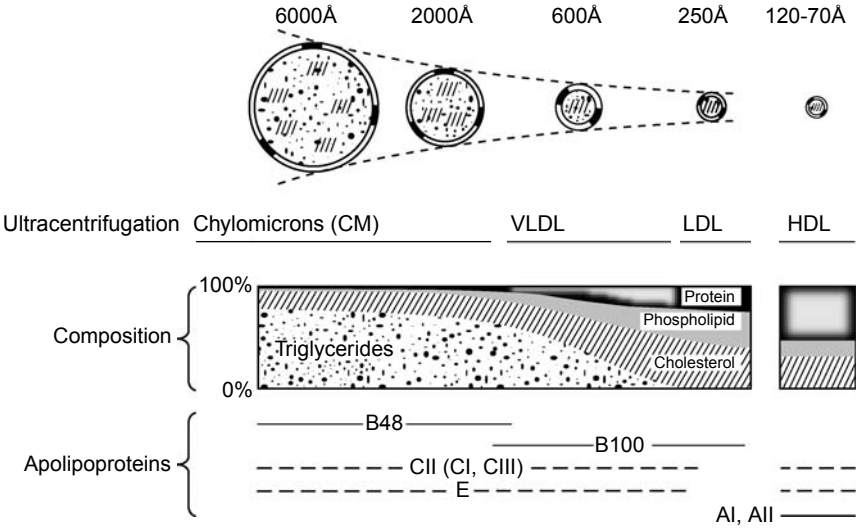

Fig. 1. Major lipoprotein classes (CM, VLDL, LDL, HDL) based on their density. Lipoprotein diameters range from about 6000 Å for CM to 70 Å for HDL. The outer shell (~20 Å) of all lipoproteins consists of apolipoproteins, unesterified cholesterol, and phospholipids; the spherical core contains triacylglycerols and cholesteryl esters. CM and VLDL have the highest contents of triacylglycerols, and 1–10% of apolipoproteins by weight; LDL and HDL contain mostly cholesteryl esters in their cores and 20–50% of apolipoproteins. The major apolipoprotein components of the various classes of lipoproteins are indicated with the solid lines; secondary or minor apolipoprotein components are indicated with the dashed lines. In this figure, 'cholesterol' refers to both esterified and unesterified cholesterol; triglycerides = triacylglycerols.

The principal functions of the lipoprotein classes are determined by their apolipoprotein (apo) and lipid components. The CM are synthesized in the intestines for the transport of dietary triacylglycerols to various tissues (Chapter 19). VLDL are synthesized in the liver for the export of endogenous triacylglycerols (Chapter 19), while LDL arise from the metabolic transformation of VLDL in circulation (Chapter 20). The function of LDL is to deliver CE to peripheral tissues and to the liver. HDL are synthesized and assembled in the liver and intestine or are formed from metabolic transformations of other lipoproteins in circulation, and from cellular lipids at the cell membranes (see Chapter 20). HDL remove excess cholesterol from cells and transport it to liver and steroidogenic tissues for metabolism and excretion.

Lipoproteins are also classified by their electrophoretic mobility on agarose gels into α, pre-β, and β lipoproteins, corresponding to HDL, VLDL, and LDL density classes, respectively; CM, when present, remain at the electrophoretic origin.

Although lipoprotein concentrations in blood plasma are highly variable, depending on age, sex, feeding state, metabolic/hormonal state, and disease state of individuals, representative concentrations of the lipoproteins (by total weight) for a fasting, healthy, adult male in plasma are approximately 0 mg/dl for CM, 150 mg/dl for VLDL, 410 mg/dl for LDL, and 280 mg/dl for HDL [1].

1.2. Lipoprotein subclasses

The lipoproteins within each class are heterogeneous in terms of their density, size, and lipid and apolipoprotein contents and compositions, as well as in their functional properties. They can be separated into subclasses by ultracentrifugation, gel filtration, electrophoresis, or affinity chromatography methods.

Based on ultracentrifugal and gel-filtration separations, HDL have been subdivided into HDL_1, HDL_2, and HDL_3 subclasses, from the largest and least dense to the smallest and most dense particles. The HDL_1 subclass is enriched in apo E and is least abundant and often disregarded. Non-denaturing gel electrophoresis further separates the main HDL_2 and HDL_3 subclasses into HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, and HDL_{3c} species spanning a density range from 1.085 to 1.171 g/ml and size (diameter) range from 106 to 76 Å, respectively [2]. Using anti-apolipoprotein immunoaffinity columns two major subclasses of HDL can be separated: one containing apo A1 but no apo A2 (LpA-I) and another containing both apo A1 and apo A2 (LpA-I/A-II). Minor proteins (apo E, apo Cs) may or may not be present in significant amounts in these HDL subclasses. On average, human HDL contain about 70% by weight of apo A1, 20% of apo A2, and 10% of the minor apolipoproteins.

Two-dimensional separations of HDL (agarose gel electrophoresis in one dimension and non-denaturing polyacrylamide gel electrophoresis in the second dimension) have yielded α-migrating, pre-β-migrating, and γ-migrating HDL subclasses of various sizes and compositions. The pre-β-migrating subclasses are present in low concentrations in plasma (in contrast to the abundant α species which are spherical HDL_2 and HDL_3 particles), and in somewhat higher concentrations in interstitial fluid, but are metabolically very important as they represent the nascent forms of HDL that are especially active in lipid uptake from cells and cholesterol esterification by lecithin cholesterol acyltransferase

(LCAT) [3,4]. The pre-β HDL fraction in human plasma contains discoidal particles containing two or three molecules of apo A1 (cf. Section 4.3) with molecular masses in excess of 300 kDa. A smaller component of the pre-β HDL fraction comprises lipid-free/poor apo A1 molecules, perhaps present in the monomeric state.

Other lipoprotein classes can also be separated into subclasses of varying density and size by the same separation methods. Subclasses of LDL in the density range from 1.027 to 1.060 g/ml and size range from 270 to 210 Å have been obtained and shown to have different metabolic properties [5]. Small dense LDL, containing high amounts of triacylglycerols, appear to be the most proatherogenic LDL species. CM and VLDL undergo continuous density, size, and composition changes due to the hydrolysis of their triacylglycerols by lipoprotein lipase and exchanges of soluble apolipoproteins; therefore, these lipoprotein classes consist of a continuous spectrum of particles.

2. *Lipid components*

2.1. Lipid composition

The lipid content and composition of the major lipoprotein classes are listed in Table 1 [6], which shows that the total lipid content is inversely correlated with the density of the lipoproteins. Glycerolipids, mainly triacylglycerols, are the major lipid components of CM and VLDL, but constitute less than 11% of the lipids of LDL and HDL, which are enriched in CE (24–51%). Unesterified cholesterol is found in all the lipoprotein classes in relatively low proportions because it is actively esterified by LCAT on HDL and then redistributed to LDL and VLDL by CE transfer protein. The total PL content of lipoproteins increases with increasing density and is directly related to the surface area of the

Table 1
Lipid composition of lipoprotein classes

	CM[a]	VLDL[b]	LDL[b]	HDL[b]
Density (g/ml)	<0.94	0.94–1.006	1.006–1.063	1.063–1.210
Total lipid (% wt)	98–99	90–92	75–80	40–48
Glycerolipids (% wt lipid)[c]	81–89	50–58	7–11	6–7
Cholesteryl esters (% wt lipid)	2–4	15–23	47–51	24–45
Unesterified cholesterol (% wt lipid)	1–3	4–9	10–12	6–8
Phospholipids (% wt lipid)[d]	7–9	19–21	28–30	42–51
PC (% wt PL)	57–80	60–74	64–69	70–81
SM (% wt PL)	12–26	15–23	25–26	12–14
Lyso PC (% wt PL)	4–10	~5	3–4	~3
Other (% wt PL)	6–7	6–10	2–10	5–10

[a]Chylomicrons (CM) were isolated during absorption of fat meals from plasma or lymph.
[b]VLDL, LDL, and HDL were isolated from fasting plasma or serum.
[c]Most glycerolipids are triacylglycerols, only about 4% are diacylglycerols or monoacylglycerols.
[d]Phospholipid (PL), phosphatidylcholine (PC), sphingomyelin (SM).
Source: Adapted from Ref. [6].

lipoprotein particles, as the surface of lipoproteins is covered by a monolayer of PL and apolipoproteins. By far, the main PL constituents are phosphatidylcholines (PC) (57–81% wt PL) followed by sphingomyelins (12–26% wt PL). Lyso-PC and other PL (phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol) constitute 5–15% wt PL. Glycolipids are present only in trace amounts and free fatty acids contribute less than 3% to the total mass of lipids.

2.2. Fatty acid composition

In the fasted state, the fatty acid composition of lipoprotein lipids reflects their biosynthetic origins and metabolic transformations in circulation (Table 2) [6].

The glycerolipids (predominantly triacylglycerols) have high proportions of 18:1 and 16:0 fatty acids, reflecting synthesis in the liver. The bulk of CE in human lipoproteins is formed in the circulation by the action of LCAT. This enzyme acts on HDL PC and unesterified cholesterol to form lyso-PC and CE. In this reaction, LCAT uses preferentially PC species with 18:2 or 18:1 fatty acids in the *sn-2* position, thus enriching CE in these fatty acids. In contrast, PC containing 18:0 or 20:4 fatty acids is a poor substrate for LCAT, explaining the decreased contents of these fatty acids in the CE. The fatty acid composition of the PL found in lipoproteins is similar to the PL fatty acid composition of cell membranes, especially of hepatic and intestinal cells, including relatively high contents of the fatty acids 18:2 and 20:4.

Table 2
Fatty acid composition of glycerolipids, cholesteryl esters, and phospholipids present in lipoprotein classes

Fatty acid	VLDL	LDL	HDL
Glycerolipids			
16:0	26.9	23.1	23.4
18:0	2.9	3.3	3.5
18:1	45.4	46.6	43.8
18:2	15.7	15.7	15.9
20:4	2.5	5.4	7.6
Cholesteryl esters			
16:0	11.5	11.3	11.4
18:0	1.4	1.0	1.1
18:1	25.6	22.4	22.4
18:2	51.8	59.9	54.8
20:4	6.1	6.9	6.3
Phospholipids			
16:0	33.8	36.0	31.8
18:0	14.7	14.3	14.5
18:1	12.2	11.6	12.3
18:2	20.3	18.9	20.6
20:4	13.6	13.2	15.7

Lipoproteins were isolated from fasting plasma. Composition is in wt% of total fatty acids.
Source: Adapted from Skipski [6].

In general, in the fasting state, the fatty acid compositions across the lipoprotein classes for specific types of lipids are fairly similar due to the transfers of lipids among all the lipoproteins by CE transfer protein and the PL transfer protein. Postprandially, the fatty acid composition of VLDL glycerolipids reflects to some extent the fatty acid composition of dietary fat, but the fatty acid compositions of LDL and HDL are hardly affected. In CM, the fatty acid compositions reflect the fatty acid composition of the meal, especially 8–10 h after the meal when CM concentrations in lymph and plasma are maximal.

2.3. Lipid organization

The surface of all lipoproteins consists of a lipid monolayer containing all the PL, and about 2/3 of all unesterified cholesterol, plus the corresponding apolipoproteins.

The dynamic properties of the lipid monolayers depend largely on the nature of the constituent lipids. For example, the surface lipids of LDL are more condensed and rigid than those of HDL due to the presence of more saturated fatty acids in the PL of LDL, a higher sphingomyelin-to-PC ratio, and a higher unesterified cholesterol-to-PL ratio in LDL [7]. The surface monolayer of VLDL is even more fluid than that of HDL due to differences in the monolayer lipids. Apolipoproteins exert little or no effect on the average molecular packing of the surface lipids especially in LDL and VLDL. In fact, isolated surface lipids, reconstituted into vesicles or microemulsions, have fluidity, diffusion rates, and mobility similar to those of the lipid monolayer components in the intact lipoproteins. The core lipids (triacylglycerols and CE) partition poorly into the surface lipid monolayer and they account for approximately 3 and 1 mol%, respectively, of the surface lipids (D.M. Small, 1983).

Under physiological conditions, the interior of lipoproteins is a fluid spherical droplet of the neutral lipids, including small amounts of dissolved unesterified cholesterol (about 1/3 of the total) and other lipophilic molecules. In HDL and VLDL, the core lipids do not appear to be organized because of the small volume available in HDL, and because of the high content of fluid triacylglycerols in VLDL. However, the physical state of the CE and triacylglycerol molecules in the core of LDL is more complex in that these lipids exhibit a reversible, broad phase transition at around 30°C [8]. The precise temperature of this phase change is sensitive to the acyl chain composition of the CE and the triacylglycerol/CE ratio. The core lipids are in a liquid state above the phase transition and in an ordered smectic liquid–crystal phase below it. Phase transitions in the core lipids of LDL apparently result in changes in the secondary structures of apo B100 on the surface and can distort the shape of the LDL particle. This indicates some coupling of hydrophobic regions of the apolipoprotein with adjacent neutral lipids.

3. Apolipoproteins

3.1. Classes and general properties

The apolipoproteins found in plasma are classified into two broad types: the non-exchangeable and the exchangeable (or soluble) apolipoproteins. Apo B100 and apo B48,

the principal protein components of LDL, VLDL, lipoprotein (a) (Lp (a)), and CM are non-exchangeable apolipoproteins. They are very large and water-insoluble proteins that are assembled with lipids at their site of synthesis in the endoplasmic reticulum of liver or intestinal cells (Chapter 19). These non-exchangeable apolipoproteins circulate bound to the same lipoprotein particle through various metabolic transformations in plasma, until they are cleared, as lipoproteins, via specific receptors (Chapter 20). In contrast, the exchangeable apolipoproteins (e.g., apo A1, apo A2, apo C, apo E) have much smaller molecular masses than apo B100 or apo B48, have limited solubilities in water in their delipidated states, can transfer between lipoprotein particles, and can acquire lipids while in circulation (Table 3) [9].

The common function of all apolipoproteins is to help solubilize neutral lipids in the circulation. The apolipoproteins bind readily to PL–water interfaces and, under appropriate conditions, can spontaneously form discrete particles with PL. In vivo, the assembly of apolipoproteins with lipids to form lipoproteins may require the assistance of cellular proteins such as the microsomal lipid transfer protein or the ABCA1 transporter.

Table 3
Major human apolipoproteins

Apolipoprotein[a]	Molecular weight[b]	Lipoprotein class[c]	Concentration in plasma (mg/dl)
Apo A1	28,100	**HDL**, *CM*	130
Apo A2[d]	17,400	HDL	40
Apo A4[e]	44,500	CM	15
Apo(a)[f]	3–8 $\times$ 10^5	Lp (a)	0.1–40
Apo B100[g]	512,000	LDL, VLDL	100
Apo B48[g]	242,000	CM	
Apo C1	6600	**VLDL, CM,** *HDL*	3
Apo C2	9000	**VLDL, CM,** *HDL*	12
Apo C3	9000	**VLDL, CM,** *HDL*	12
Apo D[h]	22,000	HDL	12
Apo E[i]	34,200	VLDL, CM, HDL	7

[a]Other, minor apolipoproteins isolated from lipoprotein fractions include apo A5, apo F, apo H, apo J, apo L, and apo M. They are present in plasma in low concentrations and their functions in lipoprotein metabolism are less well defined.
[b]Polypeptide molecular weights do not include carbohydrate contributions.
[c]In bold are the lipoprotein classes containing the highest proportion of the apolipoprotein; in italics are secondary lipoprotein classes.
[d]Human apo A2 is a disulfide-linked dimer of two identical monomers of 8.7 kDa; other mammalian apo A2 are monomeric.
[e]Apo A4 is a glycoprotein found in lymph CM and is mostly (90%) lipid-free in plasma. It exists in several polymorphic states.
[f]Apo(a) is a highly polymorphic protein, containing variable numbers of kringle structural units and glycan chains. Homologous to plasminogen, apo(a) is bound to apo B100 in LDL by a disulfide linkage forming Lp (a).
[g]Apo B48 contains 48% of the N-terminal sequence of apo B100. Both are glycoproteins. Apo B48 is present in variable amounts depending on feeding state and CM concentration.
[h]Apo D belongs to the lipocalin protein family, it may bind progesterone, but its role in lipoprotein metabolism is unknown.
[i]Apo E is glycosylated and consists of three polymorphic forms (apo E2, E3, and E4) that have cysteine–arginine interchanges at positions 112 and 158.

Intimately related to their ability to bind PL, and to solubilize neutral lipids within lipoprotein particles, the apolipoproteins have the ability to change conformation to adjust to changing lipid contents, compositions, and metabolic states of the lipoproteins. The existence of flexible regions in apolipoproteins is reflected in the fact that epitope recognition by specific antibodies and susceptibility to proteolytic digestion change with changing lipid compositions of various lipoproteins. Several of the exchangeable apolipoproteins in their respective lipoproteins are known to activate or inhibit plasma enzymes. Apo A1 and to a lesser extent apo E, apo A4, and apo C1 activate LCAT; apo C2 and perhaps apo A5 activate lipoprotein lipase; and apo C3 and apo A2 may act as inhibitors of hepatic lipase.

Apolipoproteins also have roles in receptor recognition. The best-characterized interaction is the recognition of the LDL receptor by apo B100- and apo E-containing lipoproteins. A receptor for HDL (SR-BI) has been described which binds various apolipoproteins, including apo A1, apo A2, apo B100, and apo E.

In addition to the well-known functions of apolipoproteins in lipid binding and solubilization, modulation of enzymatic activities, and receptor recognition, other functions have been described for apolipoproteins. For example, apo E has been implicated in nerve repair and regeneration as well as in plaque formation in Alzheimer's disease. Apo (a) may have a function in the blood clotting process, while apo A4, produced in the intestine and the hypothalamus, may have a role in signaling satiety in the fed state.

Evidently, the multiple functions of apolipoproteins are determined by their unique, modular structures encoded by families of genes.

3.2. Gene organization

The gene structures of the major exchangeable apolipoproteins are very similar to each other, whereas the apo B gene is distinct, as are the gene structures of apo (a) and apo D. Most of the genes of the exchangeable apolipoproteins contain four exons and three introns, with similar locations of intron–exon boundaries, and similar intron and exon lengths for the first three exons (see Fig. 2) [10]. The differences in the total length of the mRNA are due to the differences in the length of the 4th exon. The mRNAs encompass a 5′-untranslated region, a region encoding the signal sequence, a short pro-segment, the mature sequence of the protein, and a 3′-untranslated region. Exons 3 and 4 of the genes encode the entire mature sequence. For the apo A4 gene, the only difference from the other exchangeable apolipoprotein genes is the absence of the first exon and intron in the 5′-untranslated region. The homologies in the gene and protein sequences of the exchangeable apolipoproteins indicate that these genes evolved by gene duplication from a primordial gene resembling the gene of apo C1. In contrast to the genes of the exchangeable apolipoproteins, the apo B gene is short with respect to the length of its 29 exons. It is also quite asymmetric: 19 introns are concentrated within the first 1000 codons, and two very long exons (exons 26 and 29) occur in the 3′-third of the gene sequence. No homology is found between the apo B gene and other known genes [9]. It encodes one of the longest known polypeptide chains containing 4536 amino acid residues. The same gene codes for apo B100 and apo B48 but the mRNA is edited by a specific enzyme in human intestine (Chapter 18). The enzyme changes codon 2153 from a Gln to a stop

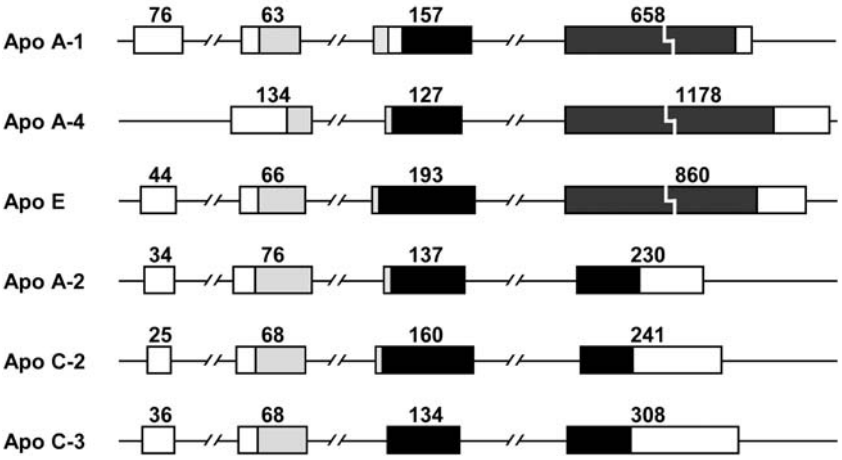

Fig. 2. Organization of the genes of the exchangeable apolipoproteins [10]. The boxes represent the exons joined by introns (broken lines). The open portions of the boxes correspond to the 5′- and 3′-untranslated regions, the hatched portions are the signal peptide regions, and the filled parts represent the regions that code the mature protein sequences. The narrow open portions in exon 3 of the apo A1 and apo A2 genes represent their pro-segments. Numbers above the exons indicate the number of nucleotides in each exon.

codon, resulting in the production of apo B48, a protein of 242 kDa, lacking the LDL receptor-binding region of apo B100 (D. Driscoll, 1990).

3.3. Primary sequences

The exchangeable apolipoproteins, in addition to having similar genes, have similar amino acid sequences. Their sequences contain 11 and 22 amino acid homologous repeats, the latter consisting of two 11-mers [11]. The last 33 amino acids encoded by exon 3 can be aligned into three 11-mers, while the sequences encoded by exon 4 fit, in general, into 22-mer segments that often start with a Pro (J.I. Gordon, 1986). The significance of these repeated sequences is that they are predicted to form amphipathic α-helices. These helices in apolipoproteins and synthetic peptides have been shown experimentally to bind to PL surfaces and to be effective in solubilizing lipids. Thus, the 22 amino acid repeats in α-helical organization are the lipid-binding units of the exchangeable apolipoproteins. Furthermore, specific repeats in the sequences encoded by exon 4 have other functional roles such as LCAT activation by the amino acid 143–165 repeat of apo A1, LDL receptor-binding by the 136–150 region of apo E, and lipoprotein lipase activation by residues 44–79 of apo C2.

In contrast, the apo B100 sequence contains many internally repeated sequences that bear little resemblance to the sequences of the exchangeable apolipoproteins (L. Chan, 1986). There are unique, Pro-rich repeats of 25 and 52 residues that contain high proportions of hydrophobic amino acids. These regions could be in contact with the core lipids in LDL and VLDL. Other features of the apo B 100 sequence include (i) 25 cysteine

residues (16 in disulfide linkages), (ii) at least 4 heparin-binding regions, and (iii) 19 potential glycosylation sites (16 of which are occupied by glycan chains, contributing about 9% of the molecular mass of apo B 100). Most of the disulfide linkages and free *cys* residues cluster in the N-terminal region of the apolipoprotein. The receptor-binding domain of apo B100, between residues 3353 and 3371, has some homology with the corresponding receptor-binding region of apo E.

Apo (a) is a distinct, highly polymorphic glycoprotein that is covalently linked to apo B100 of LDL by a disulfide linkage, to form Lp (a) [12]. Apo (a) is homologous to plasminogen, containing variable kinds and numbers of kringle sequences. Each kringle repeat contains about 80 amino acids and three internal disulfide bridges. While apo (a) has sequences homologous to those forming the plasminogen catalytic, serine protease site, the peptide sequence corresponding to the cleavage site required for activation of plasminogen is modified in apo (a), so that the serine protease activity is not expressed. In fact, the function of apo (a) in plasma is not known but an elevated Lp (a) level is a risk factor for vascular disease.

3.4. Secondary structures

The exchangeable apolipoproteins, devoid of lipids, have substantial amounts of α-helical structure in physiological aqueous solutions and their α-helix content increases markedly upon lipid binding. For example, apo A1 is about 50% helical in the lipid-free state, and becomes 60–85% helical when bound to lipids, as measured by circular dichroism spectroscopy.

Various computer algorithms have predicted the existence of amphipathic α-helix segments, coinciding quite well with the 22-mer repeats of the exchangeable apolipoproteins.

The predicted helical structures have a non-polar face and a larger polar face. Depending on the relative distribution of the charged residues on the polar face, the amphipathic helices of the exchangeable apolipoproteins fall into three classes: A, G*, and Y helices [13].

In the A-type helices (Fig. 3) the basic residues are found at the boundary between the polar and non-polar helix faces. The hydrophobic chains of the basic amino acids can contribute to the hydrophobicity of the non-polar face of the helix. The acidic residues, on the other hand, are located along the middle ridge of the polar face and are fully exposed to solvent. Such A helices in specifically designed synthetic peptides bind effectively to PL and lipoproteins, and readily solubilize lipids. The A-type helices are indeed the fundamental lipid-binding units of apolipoproteins. Two 22 amino acid synthetic A helices in tandem, joined by a Pro, are even more effective in binding lipids, suggesting inter-helix cooperativity. The G* helices are amphipathic helices typically found in globular proteins. Their basic and acidic residues are randomly distributed on the polar face of the helix. The G* helices usually participate in protein–protein interactions rather than protein–lipid interactions. G*-type helices are mostly found in the N-terminal regions of exchangeable apolipoproteins, encompassing the 11-mer repeats encoded by exon 3. Amphipathic helices of the Y-type have alternating clusters of basic and acidic residues on the polar face. Their distinction from the A-type helices in terms of lipid binding is not clear, as synthetic peptides representing A- and Y-types of helical segments of apo A1 have comparable lipid-binding properties.

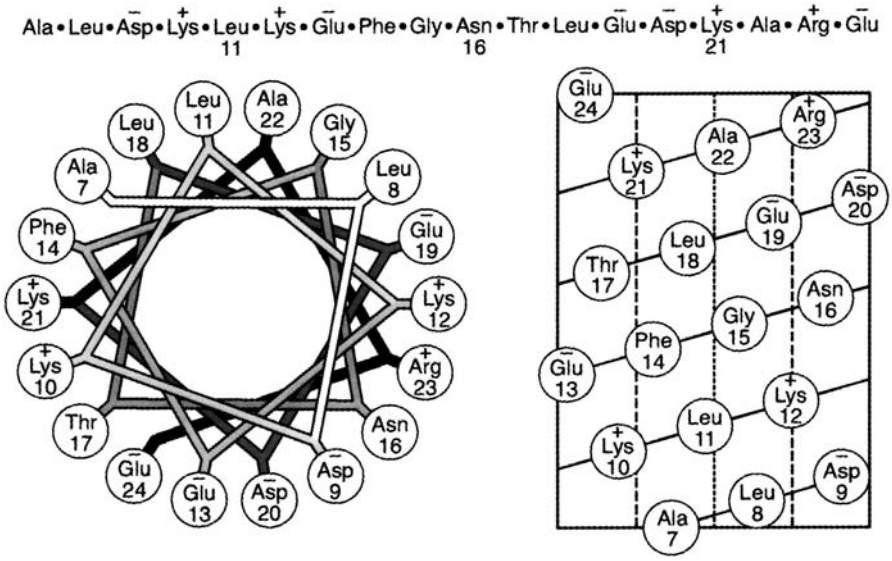

Fig. 3. Type-A amphipathic helix corresponding to residues 7–24 of apo C1. (Left) Helical wheel representation with the α-helix axis in the center of the wheel and consecutive amino acids at 100° from each other. The non-polar residues are at the top of the wheel, basic residues occur at the interface between the non-polar and polar sides of the helix, and negatively charged residues appear in the middle of the polar face (at the bottom of the wheel). (Right) The helix is represented as a flattened cylinder cut along the center of the polar face.

Because of the large size and insolubility of apo B100 in water, secondary structure measurements have been conducted on intact LDL particles and proteolytic fragments solubilized in detergents or reassembled with lipids. Reported α-helix contents in LDL range from 20 to 43%, and for β-structure from 12 to 41%, as determined by circular dichroism and infrared spectroscopic measurements. Computer analysis of the sequence of apo B100 suggests the presence of five regions of clustered secondary structures: (i) an N-terminal sequence containing G-type helices (residues 58–476); (ii) an amphipathic β-strand region (residues 827–1961); (iii) a region with α-helices resembling A- and Y-type amphipathic helices and including other kinds of helices (residues 2103–2560); (iv) a second β-strand region (residues 2611–3867); and (v) a C-terminal, third region of amphipathic α-helices (residues 4061–4338) [14].

3.5. Three-dimensional structures in solution

While most of the known functions of apolipoproteins are associated with their lipid-bound states, lipid-free or lipid-poor exchangeable apolipoproteins do exist in plasma and interstitial fluid, and have important metabolic roles in lipid uptake from cells, transfers between lipoproteins, structural remodeling of lipoproteins, and apolipoprotein catabolism.

Elucidation of the structures of apolipoproteins in the lipid-free state is difficult because they do not have a unique native structure and are conformationally plastic. Spectroscopic

studies of the smaller members of the exchangeable apolipoprotein family such as apo C1 have shown that the minimal folding unit is a helix–turn–helix motif formed of four 11-residue repeats (O. Gursky, 2001; R.J. Cushley, 2002). For larger members of the family, such as apo A1 and apo E, it is now apparent that they fold into a two-domain structure [15]. The N-terminal amphipathic α-helices form a long anti-parallel four-helix bundle with a hydrophobic core while the C-terminal region folds into a separate domain. An X-ray crystal structure for lipid-free apo A1 (Fig. 4A) reveals the N-terminal four-helix bundle domain and the two-helix C-terminal domain. Approximately 80% of the residues in this structure are in α-helices, characteristic of the level found in the lipid-associated state. Apparently, the conditions used for crystallization induced helix formation and formation of the highly ordered structure depicted in Fig. 4A. In dilute solution, the apo A1 molecule is more disordered (about 50% α-helix) although still folded into two domains (H. Saito, 2003; W.S. Davidson, 2005). The relatively loose packing in the

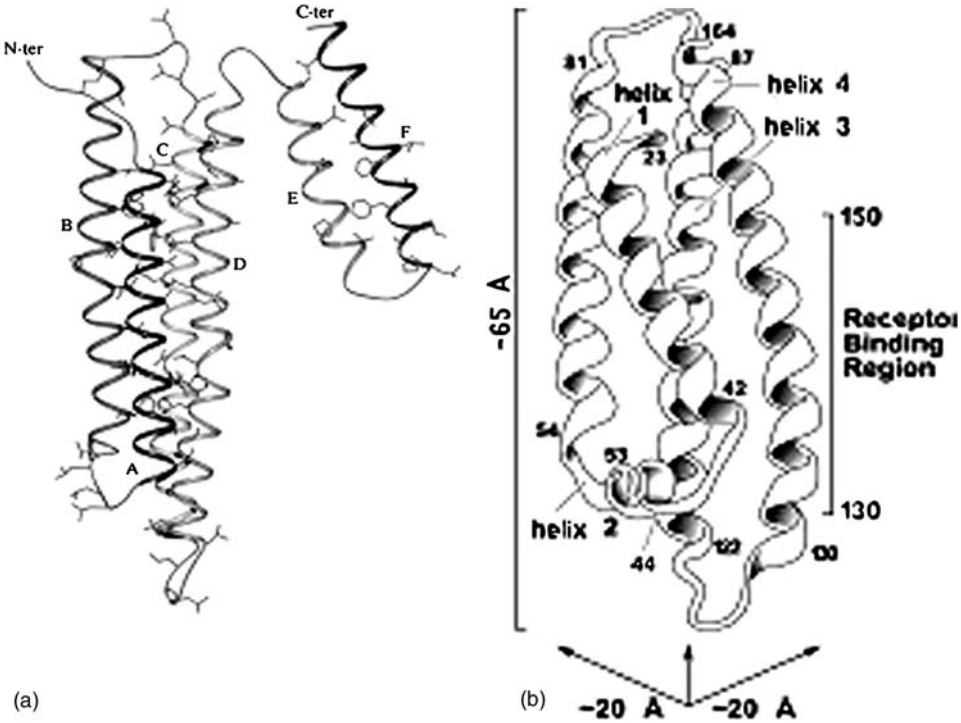

Fig. 4. Crystal structures of human apolipoproteins in the lipid-free state. (A) The six α-helices in human apo A1 are shown (H.M.K. Murthy, 2006). The N-terminal anti-parallel four-helix bundle contains helices A (residues 10–39), B (50–84), C (97–137), and D (146–187). The C-terminal domain is formed by the two α-helices E (residues 196–213) and F (219–242). Hydrophobic residues located in the interior of the helix bundles are shown as sticks. (B) Ribbon model of the structure of the 22-kDa N-terminal domain fragment of human apo E3 (D.A. Agard, 1991). Four of the five helices are arranged in an anti-parallel four-helix bundle. The residues spanned by each helix, together with the region in helix 4 recognized by the LDL receptor, are indicated.

monomeric apo A1 molecule confers flexibility enabling it to readily conform to the size and shape of different lipid surfaces.

The apo E (22-kDa N-terminal fragment) structure (Fig. 4B) consists of an elongated (65 Å) anti-parallel four-helix bundle; this helix bundle is similar to that formed by apo A1 (Fig. 4A) although it is some 20 Å shorter. The structure is stabilized by hydrophobic interactions, salt bridges, and leucine zipper interactions. The receptor-binding region contains a cluster of basic residues spanning residues 136–150 on the surface of one long helix (helix 4). This structure confirms the importance of amphipathic α-helices as a fundamental structural motif of apolipoproteins. Indeed, the four main helices contain 19, 28, 36, and 35 amino acids and encompass several of the predicted 11-mer and 22-mer repeats. However, as valuable as this partial structure of apo E is for our understanding of apolipoprotein folding in solution, it does not represent accurately the configuration of the receptor-binding region because the lipid-free form of this apo E fragment binds to the LDL receptor with very poor affinity. Only in the lipidated form does apo E adopt the correct structure for high-affinity binding to the receptor (cf. Section 4.1). The N-terminal domains of all three common apo E isoforms (Table 3) adopt a four-helix bundle conformation with subtle differences in their side-chain conformations and salt-bridge arrangements. These variations lead to differences in the conformational stability of the N-terminal domains of the apo E isoforms, with apo E4 being the least stable [16]. This conformational destabilization has been proposed to underlie the increased risk of developing atherosclerosis and neurodegenerative disorders associated with the apo E4 isoform.

It is important to note that while human apo A1 and apo E have similar, two-domain, tertiary structures, the closely related apo A4 molecule does not. In contrast, the amphipathic α-helices in apo A4 fold into a single helix-bundle domain so that the overall localization of functional domains within the sequence is quite different from apo A1 and apo E (W.S. Davidson, 2004).

It should be noted that exchangeable apolipoproteins have an extraordinary capacity for structural adaptation in response to solution conditions or to different lipid environments. In solution, high salt conditions, pH changes, or inclusion of organic solvents can lead to distinct structural states, with abnormally high α-helix contents, and specific degrees of oligomerization.

4. Complexes of apolipoproteins with lipids

4.1. Binding of apolipoproteins to phospholipid surfaces

Interactions of apolipoproteins with PL are essential for the assembly of lipoproteins, stabilization of lipoprotein structures, and expression and modulation of apolipoprotein functions. The main experimental approaches for the study of apolipoprotein interactions with PL have used isolated, exchangeable apolipoproteins in conjunction with aggregated lipids dispersed in water or spread at the air–water interface. The aggregated lipid states include lipid monolayers, various types of liposomes (small unilamellar vesicles, large unilamellar vesicles, multilamellar vesicles), and emulsions. All these lipid systems consist of or include PL, especially PC.

Apolipoproteins bind or adsorb readily to PL surfaces [7,17]. For fluid egg PC surfaces, in vesicle or emulsion form, the binding affinities (K_d) for exchangeable apolipoproteins range from about 0.1 to 10 μM, depending on the apolipoprotein, presence or absence of cholesterol in the surface, and curvature of the surface. Apo A4 has the lowest affinity for egg PC surfaces, in agreement with the observation that most of the apo A4 in plasma is lipid-free. The other exchangeable apolipoproteins have affinities comparable to one another, and similar calculated stoichiometries of amino acids bound per PC. The fraction of PL surface occupied by the apolipoproteins, at saturation, is typically around 10%. In the absence of cholesterol, this corresponds to the maximal compression of the surface PC required to accommodate the amphipathic helices of the apolipoproteins. Cholesterol added to the surface initially increases the available surface area for apolipoprotein binding, but then decreases it at higher cholesterol contents (e.g., 30 mol%).

Studies using engineered apo A1 variants have shown that the C-terminal domain is critical for lipid binding and that the binding is modulated by reorganization of the N-terminal helix bundle. This information led to the two-step mechanism of lipid binding depicted in Fig. 5. In this model, apo A1 initially binds to a lipid surface through amphipathic helices in the C-terminal domain; this process is accompanied by an increase of α-helicity in the C-terminal domain (M.N. Oda, 2003; H. Saito, 2003). Subsequently, in a slower rate-limiting step, the helix bundle in the N-terminal domain undergoes a conformational opening, converting hydrophobic helix–helix interactions to helix–lipid interactions. The binding of apo A1 and apo E to lipid is often accompanied by a large exothermic heat which is consistent with the process being enthalpically driven. Much of this enthalpy originates from a random coil to α-helix transition in the apolipoprotein, indicating that this conformational change plays a critical role in promoting the high affinity binding to the lipid surface (A. Jonas, 2004; M.C. Phillips, 2004).

The conformations of apo A1 and apo E molecules in spherical lipoprotein particles are governed by the size and lipid composition of the particle. Thus, the two-domain structure of apo E has been shown to lead to two different lipid-bound conformations on

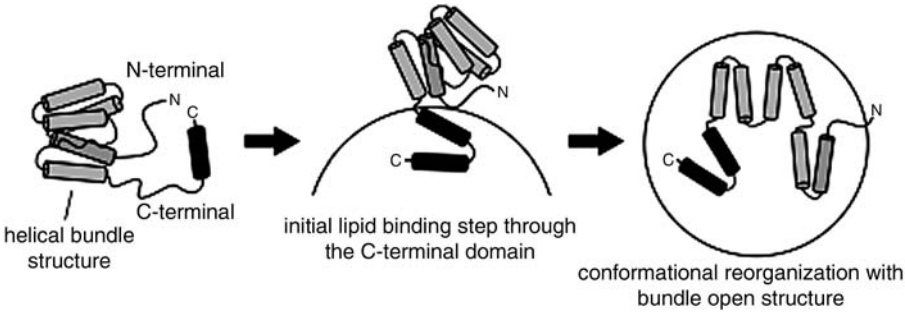

Fig. 5. Model of the two-step lipid binding mechanism of apo A1on a spherical particle [15]. In the lipid-free state in dilute solution, apo A1 is organized into two structural domains in which the N-terminal domain forms a helix bundle whereas the C-terminal domain forms a separate, less organized structure. Initial lipid binding occurs through amphipathic α-helices in the C-terminal domain accompanied by an increase in α-helicity probably in the region including residues 187–220. Subsequently, the helix bundle in the N-terminal domain undergoes a conformational opening, converting hydrophobic helix–helix interactions to helix–lipid interactions.

lipoprotein-like spherical particles. The N-terminal four-helix bundle can adopt either open or closed conformations resulting from binding competition with the C-terminal domain that has higher lipid affinity, or from other types of apolipoprotein molecules on the particle surface [15,18]. The displacement of the N-terminal domain from the lipid surface at high apo E surface concentrations causes the four-helix bundle to adopt a closed conformation (Fig. 4B), which is not recognized by the LDL receptor. At low surface concentrations, all α-helices are in contact with the lipid surface which increases the exposure of the basic residues in the receptor-binding region to the aqueous phase and allows binding to the LDL receptor (S. Lund-Katz, 2000).

It is clear from the above considerations that amphipathic α-helices can be displaced from a lipid–water interface, leading to the exchangeability of apolipoproteins such as apo A1 and apo E. In marked contrast, apo B100 on either a VLDL or LDL particle is not exchangeable in that the protein does not desorb when the available surface area is decreased. The reason for this behavior is that apo B100 contains amphipathic β-strand-rich domains (Section 3.4) which, when absorbed at the lipid–water interface, are elastic and not pushed off the interface when the surface is compressed. Thus, the amphipathic β-strands are a non-exchangeable motif and anchor apo B to the surface. In contrast, the helical domains in apo B100 are forced off the interface by compression and re-adsorb when the interface is expanded. In sum, the conformational flexibility of apo B arises from the elasticity of the β-strand domains and the exchangeability of the α-helical domains (D.M. Small, 2006).

4.2. Lipoprotein-like complexes

Although PL liposomes are favored systems for the study of apolipoprotein binding to PL surfaces, vesicle–apolipoprotein complexes are not the ideal models for lipoproteins. Vesicles have an interior water compartment not present in lipoproteins, are incapable of solubilizing large amounts of neutral lipids within the PL bilayer, and are too large to mimic the surface curvature of HDL. Thus, methods have been developed to prepare small, micellar complexes of exchangeable apolipoproteins (in particular apo A1) with lipids that mimic discoidal and spherical HDL in shape, composition, and functional properties. For LDL and VLDL, microemulsions and emulsions of lipids of selected diameter and composition, with added apo B 100, make good models of the native lipoproteins.

Several methods are known for the reconstitution of HDL-like complexes from pure components: (i) spontaneous formation of HDL discs from dimyristoylphosphatidylcholine liposomes; (ii) detergent-mediated reconstitution of HDL discs with various PL; and (iii) co-sonication of apolipoproteins and lipids to form either discoidal or spherical HDL analogs [19].

Dimyristoyl-PC liposomes can bind apolipoproteins reversibly, as described in the preceding section; however, at the transition temperature (T_m) of the lipid (24°C) and at sufficiently high proportions of apolipoprotein to dimyristoyl-PC (1/3 or greater, wt/wt), the apolipoproteins can solubilize the liposomes to give rise to small discs analogous to nascent HDL. The rate of the liposome disruption and solubilization depends on the temperature of the reaction. It is highest at the onset of the main phase transition of dimyristoyl-PC (when lattice defects in the PC bilayer, into which apolipoprotein amphipathic helices can penetrate, are maximal) and decreases a thousand-fold on either

side of T_m. While the same reaction does occur with dipalmitoyl-PC liposomes at the T_m of 41°C, the rate is much slower than for dimyristoyl-PC. For long-chain, unsaturated PC, such as palmitoyloleoyl-PC, the rates of reaction are too slow to be measured at accessible temperatures. Therefore, for practical purposes, under ordinary conditions, only dimyristoyl-PC can be used effectively to reconstitute HDL discs by this method. In this system, smaller apolipoproteins and peptide analogs solubilize dimyristoyl-PC liposomes at higher rates than larger apolipoproteins. More flexible apolipoproteins also react more rapidly (S. Lund-Katz, 2002). Thus, under some circumstances synthetic peptide mimics of A-type amphipathic helices may lyse even egg PC or palmitoyloleoyl-PC vesicles.

The mechanism of the disruption of dimyristoyl-PC liposomes into discoidal reconstituted HDL particles requires binding of the apolipoprotein to the liposome surface, followed by penetration of the bilayer by apolipoprotein, and breakdown of the liposome into bilayer discs. The discs are surrounded and stabilized on the periphery by the amphipathic helices of the apolipoproteins (Section 4.3).

A more universal method of producing discoidal reconstituted HDL particles uses detergent (usually Na cholate) to solubilize the PL into mixed micelles, followed by addition of exchangeable apolipoprotein and removal of detergent by dialysis or column chromatography. Depending on the proportions of PC to apolipoprotein, in the range from 1/1 to 4/1 (wt/wt), reconstituted HDL discs of different diameters containing different numbers of apolipoprotein molecules with varied conformations can be produced. Cholesterol (up to 15 mol%) or small amounts of other lipids can be readily incorporated during the lipid solubilization step.

The third method for making HDL-like particles is extensive co-sonication of apolipoproteins with PL, in the absence or presence of neutral lipids. Usually, the components are mixed in the proportions found in native HDL and yield discoidal or spherical analogs of HDL depending on the absence or presence of neutral lipids. While the ability to synthesize spherical reconstituted HDL particles by this method is very attractive, controlling particle homogeneity and yield is difficult.

To produce models of LDL, VLDL, and CM, lipid mixtures of PC and a neutral lipid are first sonicated to give metastable emulsion particles of the general size of the desired lipoproteins, and then the apo B component is added from a detergent dispersion, or exchangeable apolipoproteins are added in solution [17].

The advantage of reconstituted lipoproteins over the native lipoproteins is that the model particles can be made with a single apolipoprotein and one or a few defined lipid components to study each component individually. In addition, particles of uniform size can be isolated where high-resolution structural analysis is potentially possible. Also the reconstituted lipoproteins lend themselves for the study of structure–function relationships. In fact, reconstituted lipoproteins display all the known functions ascribed to native lipoproteins, including enzyme activation, receptor binding, and uptake and transfer of lipids.

4.3. Reconstituted HDL

The most thoroughly studied reconstituted HDL are the discoidal particles containing apo A1 and a single type of PC, with or without added small amounts (<20 mol%) of

other PL or cholesterol [20]. The discoidal shape of the particles has been confirmed by the methods of negative-stain electron microscopy, small-angle X-ray scattering, and atomic force microscopy. All methods indicate a disc thickness of 45–55 Å that corresponds to the PL bilayer thickness, and diameters ranging from 70 to 180 Å. The main phase transition of the PC is preserved in the particles, but T_m is shifted about 3°C to higher temperatures, indicating a greater ordering and restriction of the PC in the particles than in corresponding liposomes.

When analyzed by non-denaturing gradient gel electrophoresis the particles display discrete diameters and size distributions that vary depending on the initial PC/apo A1 ratios of the preparations. Isolated particles of a specific size have reproducible, characteristic physical and functional properties. For example, some particles containing palmitoyloleoyl-PC (78 and 109 Å) are poor substrates for LCAT while others, particularly the 96 Å particles, are very good LCAT substrates (A. Jonas, 1989). In addition, there is evidence of different binding affinities of the particles for the scavenger receptor BI (SR-BI) receptor (D.R. van der Westhuyzen, 2001; M.C. Phillips, 2004). Thus reconstituted HDL subclasses, with different lipid contents, have different apolipoprotein conformations that result in dramatically altered functional properties. In vivo, this conformational adaptability of apolipoproteins probably leads to metabolic switching for the diverse functions of HDL subclasses. Apo A1 conformation is also regulated by the saturation or unsaturation of the PC acyl chains and by high contents of cholesterol (>15 mol%) or polyunsaturated PC. Other exchangeable apolipoproteins (apo A2, apo E, apo A4) also form discoidal reconstituted HDL with diameters that roughly correspond to their contents of amphipathic α-helices and molecular weights.

Reconstituted spherical HDL can be made by co-sonication of selected HDL components (e.g., apo A1, PC, CE) or by extensively reacting discoidal reconstituted HDL with LCAT in the presence of an exogenous source of cholesterol. The products are spheroidal and, like the discoidal precursor particles, contain two, three, or four apo A1 molecules per particle, PC, cholesterol, and a CE core. The diameters of the particles range from 80 to 120 Å. Although not well studied, the conformation of apo A1 appears distinct from that in the discoidal particles and is variable depending on the particle diameter (A. Jonas, 1990; M.G. Sorci-Thomas, 2002).

In reconstituted discoidal HDL, the apolipoproteins form a protective shell, one-helix thick, around the periphery of the discs. The organization of apo A1 molecules in such particles has been studied extensively using various spectroscopic techniques, as well as chemical cross-linking and mass spectrometry methods [21,22]. There is general agreement that the α-helices of apo A1 are aligned perpendicular to the PL acyl chains (Fig. 6A). The precise registry of the apolipoprotein helices is dependent upon the size of the disc, and various models to account for this effect have been proposed [21,22]. The nascent HDL particles created by ABCA1-mediated efflux of cellular PL and cholesterol to apo A1 are discoidal with structures like those depicted in Fig. 6B (M.C. Phillips, 2006).

4.4. Structures of native lipoproteins

Electron microscopic images of lipoproteins show predominantly spherical shapes. Only nascent HDL appear as stacks of discs by negative-stain transmission electron microscopy. The stacks are artifacts of the method, because in solution nascent HDL

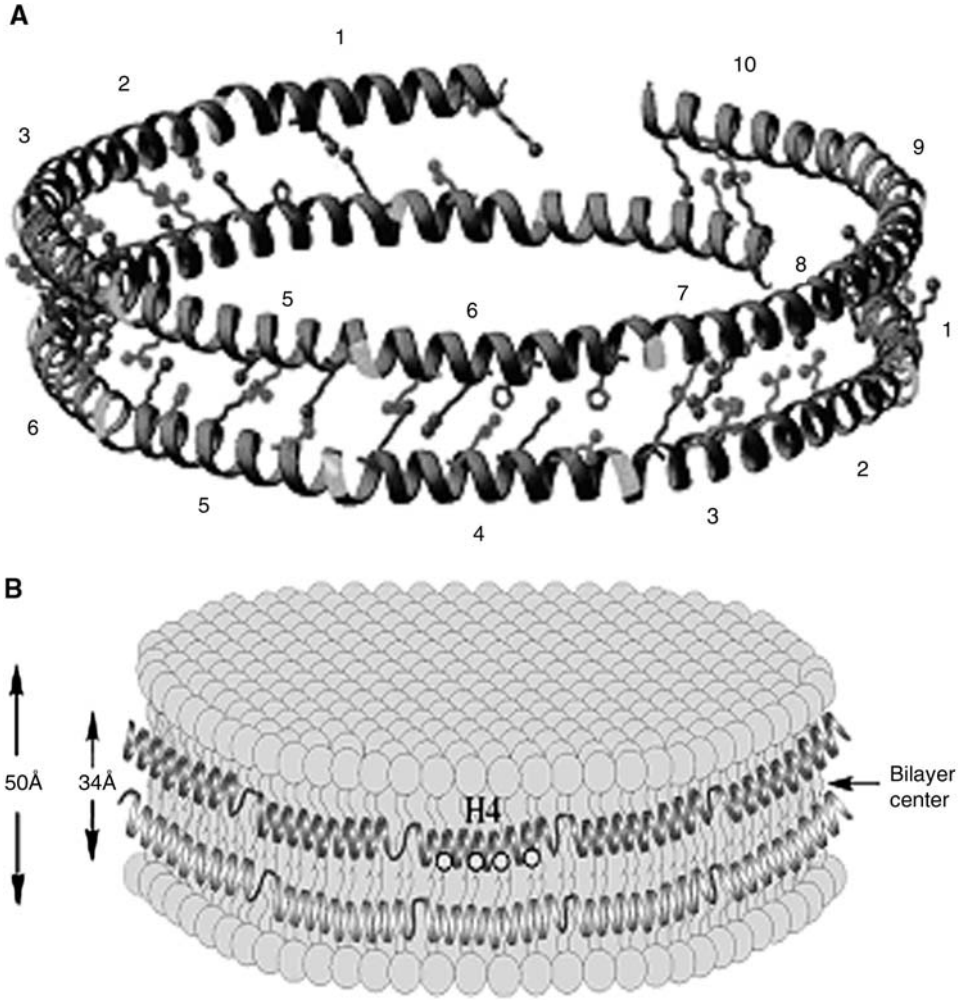

Fig. 6. Organization of apolipoprotein molecules in discoidal HDL particles. (A) 'Double belt' model for apo A1 structure at the edge of discoidal HDL complex. Two ring-shaped molecules of apo A1 are stacked on top of each other with both molecules in an anti-parallel orientation, allowing the helix registry to maximize intermolecular salt-bridge interactions. Only the charged residues at selected positions are explicitly displayed. (B) Model of apo E in discoidal HDL complex depicting the locations of engineered tryptophan residues on helix 4. Fluorescence from these amino acids was monitored to determine helix orientation. Two out of a total of about four molecules/particles of apo E are depicted in which the helical axes are oriented perpendicular to the PL acyl chains.

and their reconstituted HDL analogs are freestanding discs. Regarding the structure of native HDL, their electron microscopic images do not have enough resolution to show any significant surface features, and other methods have not yet been successful in providing high-resolution structural information.

Fig. 7 shows the LCAT-induced conversion of a discoidal apo A1-PC HDL particle, in which the apo A1 molecules are organized according to the model in Fig. 6A, to a spherical

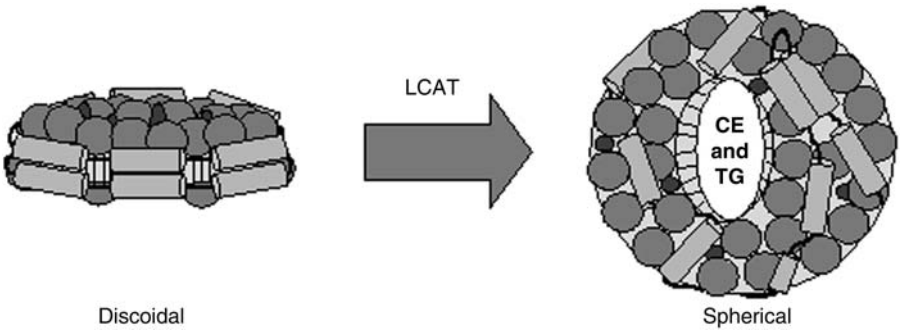

Fig. 7. Influence of neutral lipids (cholesteryl ester (CE) and triacylglycerol (TG)) on the shape of HDL particles. Cholesterol molecules in discoidal apo A1/PL particles are converted to CE by the action of LCAT. As CE molecules are relatively insoluble in the PL bilayer of the discoidal particle, they form a separate phase in the core of the particle. The 'oil-drop' model of spherical HDL comprises a non-polar core of CE and TG molecules encapsulated by a surface monolayer of amphipathic α-helical apolipoprotein and PL molecules. The apo A1 amphipathic α-helices, FC and PL molecules are depicted as cylinders, oblongs, and circles with two tails, respectively. The picture is not drawn to scale.

HDL particle. Apo A1 is a cofactor for LCAT (A. Jonas, 2000) and the conversion of cholesterol to CE (Section 2.2) induces the formation of a neutral lipid core and the concomitant change in particle shape. Triacylglycerol molecules are introduced into the core of spherical HDL as a consequence of CE transfer protein activity. The apo A1 amphipathic α-helices are embedded among the PL molecules on the surface of spherical HDL (Fig. 7) but the detailed conformations of the apo A1 molecules are not known. Spherical HDL in plasma can be remodeled via a fusion event into large and small particles by PL transfer protein (K.A. Rye, 2001). Some HDL particles contain both apo A1 and apo A2 molecules and the interactions between these two proteins need to be understood. The presence of apo A2 inhibits HDL remodeling by CE transfer protein and the dissociation of apo A1 molecules to create pre-β-HDL. It seems that apo A2 interacts with apo A1 and reduces the ability of the latter to desorb from the HDL particle surface (K.A. Rye, 2003).

Given the limited understanding of the surface structure of HDL with its relatively small apolipoproteins, it is perhaps not surprising that the conformation of the large apo B100 molecule (4536 residues) on LDL is also not known at high resolution. However, studies using imaging methods and computer modeling have provided some insights into the organization of apo B100 on LDL. Recently, small-angle neutron scattering has been used to generate a three-dimensional model of lipid-free apo B100 and apo B100 on an LDL particle (R. Prassl, 2006). The protein is composed of distinct domains connected by flexible regions and it adopts a curved shape with a central cavity. This model for apo B100 structure was accommodated on a 250 Å sphere to reconstruct an LDL particle. This analysis and that conducted by Segrest and colleagues of their pentapartite model of apo B100 secondary structure [14] have yielded hypothetical models for the organization of apo B100 on an LDL particle (Fig. 8). It is of note that this model shows the protrusion from the LDL surface seen by cryo-electron microscopy

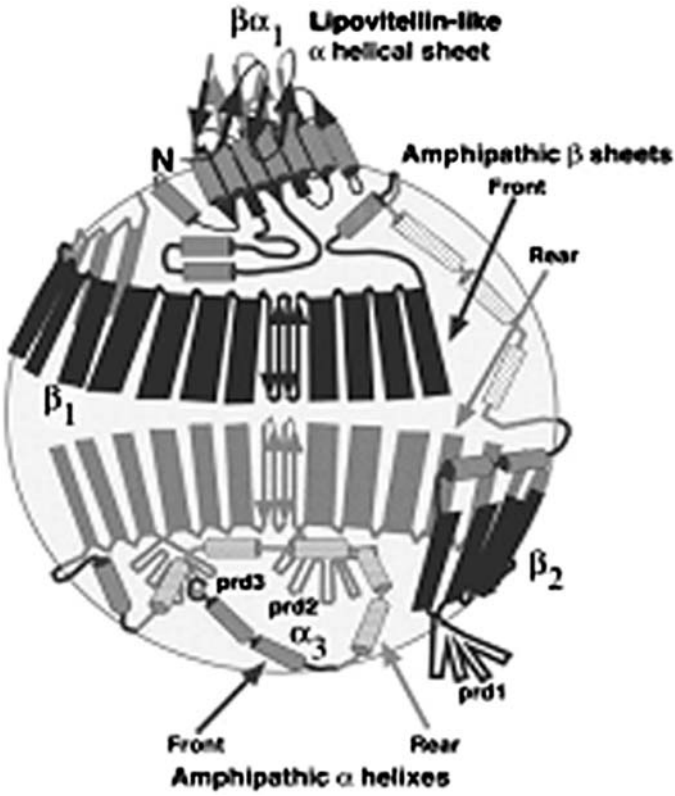

Fig. 8. Schematic representation of the apo B100 molecule on an LDL particle [14]. The lipids are organized in an oil-drop model (cf. Fig. 7) and the elements of apo B100 secondary structure on the particle are depicted. The N-terminal globular domain formed by lipovitellin-like structure is shown at the top of the diagram. The remaining secondary structure is a cartoon representation of the pentapartite model of apo B100 (prd = proline rich domain).

(D. Atkinson, 1994) that is formed by the compact globular structure of the lipovitellin-like N-terminal domain. The flexible α-helical domains, and elastic and irreversibly bound β-sheet regions discussed in Section 4.1, are also apparent in this model.

5. Future directions

In spite of the great advances made during the last three decades in the elucidation of the structures and structure–function relationships of lipoproteins, much remains to be accomplished in this field of research.

Key areas of future research include

(1) Structural and functional studies of variant forms of the apolipoproteins as well as the roles of minor lipid and apolipoprotein components of lipoproteins, including the products of oxidative reactions that occur in vivo and in vitro.

(2) Investigation of the conformational adaptability of apolipoproteins during assembly with lipids and during metabolic transformations of lipoproteins in circulation. Elucidation of the conformational changes at the atomic level will be a major challenge dependent on the success of high-resolution analysis of apolipoprotein and lipoprotein structures.
(3) High-resolution determination of the three-dimensional structure of native lipoproteins under physiologic conditions. Accomplishing this goal will require not only major advances in the crystallization, X-ray, and NMR methodologies, but also the isolation or preparation of highly homogeneous lipoproteins.
(4) Further developments in computer modeling of reconstituted or native lipoproteins will require powerful molecular dynamics algorithms and much more extensive experimental information on the folding and topology of the apolipoproteins.

Abbreviations

apo	apolipoprotein
CE	cholesteryl ester
CM	chylomicrons
HDL	high density lipoproteins
K_d	equilibrium dissociation constant
LCAT	lecithin cholesterol acyltransferase
LDL	low density lipoproteins
Lp (a)	lipoprotein (a)
PC	phosphatidylcholine
PL	phospholipid
SR-BI	scavenger receptor BI
T_m	main phase transition temperature
VLDL	very low density lipoproteins

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CHAPTER 18

Assembly and secretion of triacylglycerol-rich lipoproteins

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Contents

1. Overview of secretion of triacylglycerol-rich lipoproteins

The triacylglycerol (TG)-rich lipoproteins (very low density lipoproteins (VLDLs) and chylomicrons (CM)) are secreted into the circulation by hepatocytes of the liver and enterocytes of the intestine. All plasma lipoproteins share a common structure with a neutral lipid core, consisting of TGs and cholesteryl esters, surrounded by a surface monolayer of

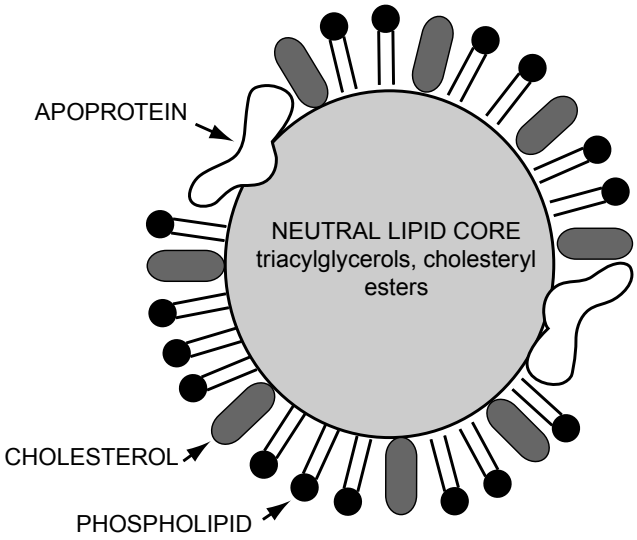

Fig. 1. Generalized structure of a lipoprotein particle. Lipoproteins are approximately spherical particles that consist of a neutral lipid core (triacylglycerols and cholesteryl esters) surrounded by a surface monolayer of amphipathic lipids (unesterified cholesterol and phospholipids) and specific apoproteins.

phospholipids, unesterified cholesterol, and specific proteins, known as apolipoproteins (apos) (Fig. 1 and Chapter 17). The primary function of plasma lipoproteins is to transport hydrophobic, water-insoluble lipids within the circulation to other tissues. The TG-rich lipoproteins contain apo B and deliver TGs made in the liver and intestine to other tissues in the body for either storage or utilization as an energy source. VLDLs and CM can be converted into low density lipoproteins (LDLs) in the circulation. As discussed in Chapter 19, high-density lipoproteins (HDLs) remove cholesterol from tissues for delivery to the liver and excretion into bile in a process termed 'reverse cholesterol transport'. In general, a high level of plasma LDL is a strong risk factor for the development of atherosclerosis and cardiovascular disease whereas a high level of HDL is protective (Chapter 21). The steady-state level of lipoproteins in the circulation reflects a balance between the rate of lipoprotein secretion/formation and the rate of lipoprotein removal from plasma. This chapter focuses on the mechanisms by which TG-rich lipoproteins are assembled and secreted from the liver and intestine. The synthesis and metabolism of the major lipid constituents of lipoproteins are described in Chapter 8 (phospholipids), Chapter 10 (TGs), and Chapter 14 (cholesterol).

Plasma lipoproteins are usually classified according to their density (Chapter 17). Since the buoyant density of lipids is lower than that of proteins, lipoproteins with a high ratio of lipid to protein have a lower density than lipoproteins with a low ratio of lipid to protein. Electron micrographs of the major classes of plasma lipoproteins are shown in Fig. 2. CM the largest and most lipid-rich particles, whose major lipid component is TG, are secreted by the intestine and are abundant in plasma only after a meal. VLDLs are also rich in TG and are secreted mainly by the liver, although some are of intestinal origin.

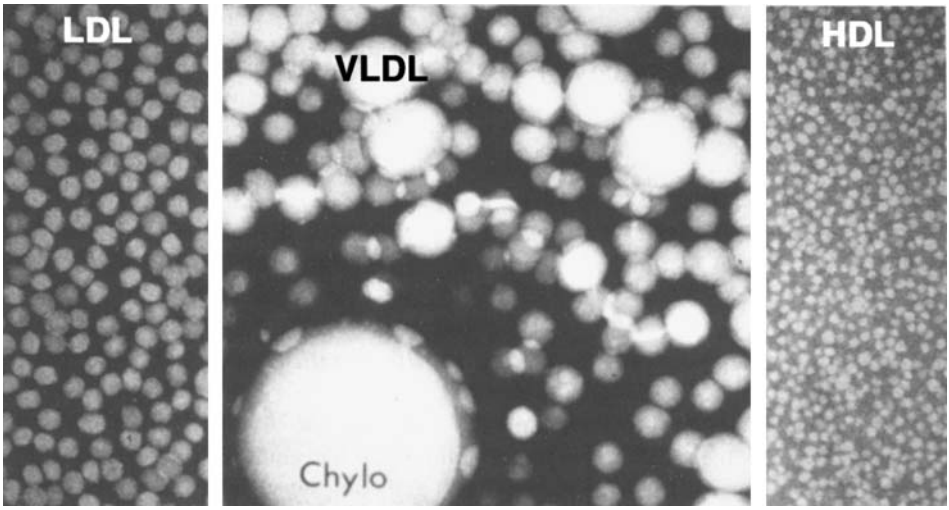

Fig. 2. Negative-staining electron micrographs of human plasma lipoproteins (diameters 10–1000 nm). The largest particles [chylomicrons (Chylo) and VLDLs] contain a higher ratio of lipid to protein, and are therefore less dense, than low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) that contain relatively more protein. Photograph courtesy of Dr. R. Hamilton, University of California at San Francisco, with permission.

Intermediate-density lipoproteins and LDLs are generated in the circulation by lipolysis of TGs within CM and VLDLs (Chapter 19). Apo B is an essential component of CM, VLDLs, and LDLs. Unlike the 'exchangeable' plasma apolipoproteins (apo E, apo A1, apo A2, and apo C), apo B does not exchange among lipoproteins and is present in plasma only in association with lipid. In addition to apo B, VLDLs and CM contain apo E and apo C; CM contain small amounts of apo A1 and apo A2. In contrast, apo B is the only apolipoprotein of LDLs. HDLs are particles of diverse composition that are generated in the circulation by complex lipid transport processes (Chapter 19).

The synthesis and secretion of apo B follows essentially the same pathway as that used by typical secretory proteins. Apo B mRNA is translated on ribosomes bound to the endoplasmic reticulum (ER) and the protein is co-translationally translocated across the ER membrane and into the ER lumen. Unlike other secretory proteins, however, apo B cannot be secreted unless it is non-covalently associated with lipids. The intracellular site at which TG is assembled with apo B is still controversial. Several studies have concluded that the bulk of TG is added to the particles in the ER lumen (J.E. Vance, 1993; H.N. Ginsberg, 2003), whereas other studies have indicated that the majority of TG is assembled with apo B in the lumen of the Golgi (R.J. Havel, 1976; E.A. Fisher, 2003). The reasons for these divergent conclusions remain to be unraveled. Nascent apo B-containing particles are transported through the Golgi and are subsequently secreted from the cell [1]. In cultured rat hepatocytes, the synthesis of an apo B molecule takes 7–15 min, and ~30 min later the apo B is secreted into the culture medium. Pulse-chase radiolabeling experiments indicate that the slow step in movement of apo B through the secretory pathway is transport of apo B out of the ER (R.A. Davis, 1987).

2. Structural features of apo B

Full-length human apo B (apo B100) contains 4536 amino acids (M_r 513,000) and is one of the largest single polypeptide chains known (L. Chan, 1986). The form of apo B secreted by human liver is exclusively apo B100 whereas the intestine secretes apo B48 (M_r 264,000), a truncated variant of apo B100, along with smaller amounts of apo B100 (J.P. Kane, 1983). Rodent livers, on the other hand, secrete both apo B100 and apo B48. A convenient 'centile' nomenclature for apo B variants is commonly used in which the apo B species is designated by a number that indicates the size of the molecule relative to full-length apo B100. In this shorthand scheme, apo B48 is the N-terminal 48% of apo B100, whereas apo B15 refers to the N-terminal 15% of apo B100.

Apo B is an amphipathic protein and is the only known protein that requires association with lipids for its secretion. Each VLDL particle contains a single molecule of apo B (J. Elovson, 1988). Computer-based structural analysis predicts that apo B100 has a unique pentapartite domain structure (H_2N-$\beta\alpha_1$-β_1-α_2-β_2-α_3-COOH) consisting of an N-terminal globular domain followed by alternating amphipathic β-sheets and α-helices (Fig. 3). As much as 50% of the apo B100 molecule is composed of β-sheet structure that is concentrated in two large regions. At least 25% of apo B100 consists of α-helices that are too short to span a membrane. Translation of the first 22–29% of apo B100 is required for initiation of assembly with lipids. A 'lipid pocket' within the first 1000 amino acid residues of apo B has been proposed to initiate VLDL assembly (Fig. 4) [2]. The β-strands are thought to be the primary motif that mediates the association of apo B with lipids. It has been suggested that a sequential accumulation of TGs within the cavity of the lipid pocket occurs by unfolding of the β-strands in the β1-domain. Since the β-sheets have both polar and non-polar faces, the non-polar face is predicted to interact with lipids whereas the polar face would be exposed to the aqueous environment. The five alternating α-helices and β-sheets (Fig. 3) are common features of apo B100 in all vertebrate species examined. Similar amphipathic β-strands are present in the egg yolk protein lipovitellin. The X-ray crystal structure of lamprey lipovitellin reveals that this protein contains a lipid pocket joined by three anti-parallel β-sheets. A low-resolution model of lipid-free apo B100 has recently been reported from small-angle neutron scattering studies. The model depicts apo B100 with a curved shape and a central cavity (A. Johs, 2006).

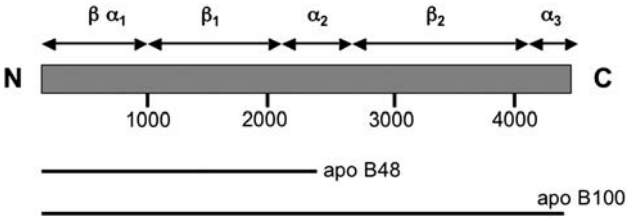

Fig. 3. Schematic representation of the pentapartite structure of apo B100. The N-terminal globular domain ($\beta\alpha_1$) and the alternating α and β domains are indicated above the representation of the polypeptide. Numerical values below the polypeptide indicate an amino acid residue scale. Human apo B100 contains 4536 amino acids; the size of apo B48 is indicated.

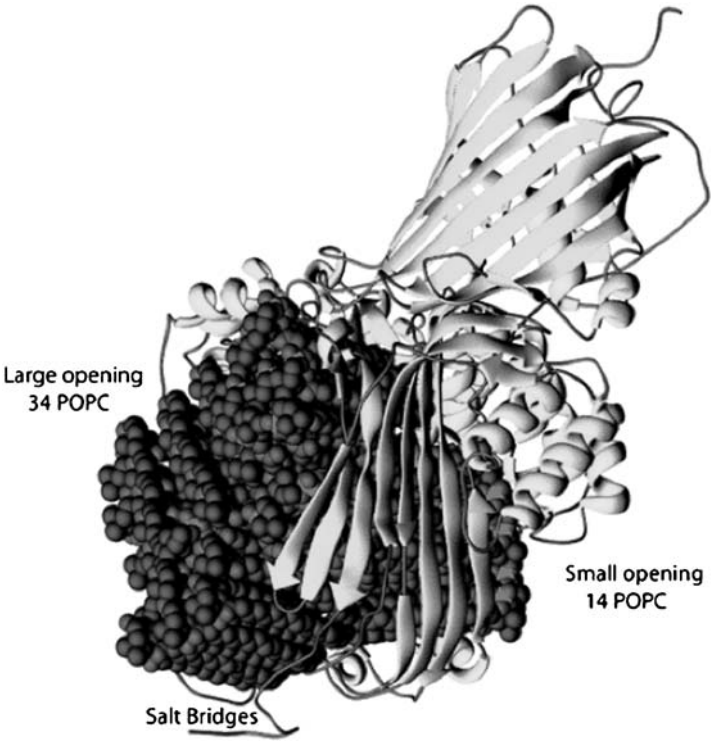

Fig. 4. The lipid pocket of apo B. Apo B22.5 is represented with β-strands shown as green arrows and α-helices shown as turquoise tubes. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) is shown with carbon atoms in gray and oxygens in red. There are 34 POPC molecules in the large opening and 14 POPC molecules in the small opening. Thus, a total of 48 POPC molecules fit into the lipid pocket. From Ref. [24], with permission. (See color plate section, plate no. 16.)

In general, the size and density of the secreted lipoproteins depend on the length of the apo B molecule. Thus, a correlation exists between the length of apo B and its ability to associate with lipids. Naturally occurring mutations in the human apo B gene, in the group of disorders known as hypobetalipoproteinemia, have shown that the secretion of lipoprotein particles containing certain truncated forms of apo B (e.g., apo B38.9) is severely compromised (G. Schonfeld, 2003). The relationship between the size of apo B and the degree of lipid association was demonstrated in cultured rat hepatoma cells that were transfected with cDNAs encoding C-terminally truncated variants of human apo B100 (apo B15 to apo B94). Apo B18 was secreted independently of neutral lipid, whereas apo B28 was secreted in particles that contained some neutral lipid and were the size and density of HDLs. As the size of the apo B molecule increased — from B37 to B48 to B53 to B72 to B94 — progressively more lipid was associated with the apo B and the particles were increasingly large and less dense (Z. Yao, 1991). From these and other studies, it is apparent that the sequence of apo B between B17 and B41 is crucial for assembly of TG-rich lipoproteins. Further experiments have shown that the sequence of apo B that lies between B32.5 and B41 is responsible for binding most of the TG. In this region,

approximately one molecule of TG binds per two amino acids of apo B (D.M. Small, 2000). An intriguing question, however, is why does apo B48 assemble into intestinal CM, the largest and most TG-rich lipoprotein particles, whereas apo B100, which is approximately twice as large as apo B48, forms the smaller VLDLs.

3. Transcriptional regulation of apo B synthesis

Under most metabolic conditions, changes in the level of apo B mRNA do not modulate apo B secretion. In addition, changes in the amount of apo B secreted can occur over at least a 7-fold range without any alteration in the amount of apo B mRNA (J. Scott, 1989). Thus, apo B secretion is regulated, for the most part, co- and post-transcriptionally. Apo B is synthesized primarily in the liver and intestine but small amounts of apo B are also produced in hearts of humans and mice (S.G. Young, 1998). The presence of apo B and the microsomal TG transfer protein (which is also required for lipoprotein assembly — Section 6.4) in the heart facilitates the secretion of apo B100-containing lipoproteins by this tissue. The secretion of TG-rich lipoproteins by the heart is thought to provide a mechanism by which the amount of TGs stored in the heart is regulated, thereby protecting the heart from a detrimental accumulation of lipids [3]. Apo B is also made in the egg yolk sac. In mice, the production of apo B in the yolk sac is essential during fetal development because in mice, but not humans, apo B is required for delivery of lipid nutrients to the developing embryo (R.V. Farese, 1999). Consequently, elimination of apo B production in mice is lethal during embryonic development.

3.1. DNA elements that regulate apo B transcription

The human apo B gene spans 43 kb, is located on chromosome 2p, and consists of 28 introns and 29 exons. More than half of the apo B coding sequence is encoded by a single, exceptionally large (7572 bp) exon — exon 26. The apo B mRNA transcript is 14 kb and is remarkably stable with a half-life of 16 h (J. Scott, 1989). Several elements responsible for expression of the apo B gene have been defined. Positive and negative *cis*-acting elements that are required for transcription of apo B have been identified in cultured human hepatoma (HepG2) cells and intestinal (CaCo-2) cells. The proximal promoter (bp −898 to +1) contains a TATA box as well as sequences that bind the liver-specific transcription factors HNF-3 and NF-1. The majority of enhancer activity resides within intron 2 and involves the binding of two nuclear transcription factors, HNF-1 and C/EBp. Intron 3 contains a weaker enhancer element, and a negative element was identified in the 5′-flanking region between bp −3211 and −1802. When these promoter elements were linked to reporter genes and expressed in transgenic mice, a complex picture of transcriptional regulation of apo B emerged. Although in mouse liver both the proximal promoter (bp −898 to +1) and the enhancer in intron 2 were required for expression of a β-galactosidase reporter, no expression occurred in the intestine although the same constructs induced expression in the intestinal cell line, CaCo-2. Furthermore, the inhibitory element that was identified between bp −3200 and −1802 in transient transfection studies in HepG2 cells did not decrease expression in livers of transgenic mice. Consequently, while

CaCo-2 cells and HepG2 cells are frequently used as experimental models of the intestine and liver, respectively, results obtained from these cultured cells are not recapitulated in intact animals. These observations highlight the need for verification of the in vivo relevance of in vitro studies by using intact animals.

Factors that control the intestinal expression of apo B have now been elucidated. A high level of intestinal expression of human apo B was achieved in transgenic mice with a construct containing ~80 kb of the 5′-flanking sequence. The DNA element that drove expression of apo B transgenes in the mouse intestine was located in a 3-kb segment more than 55 kb upstream of the transcriptional start site [4]. This region of the gene contains putative binding sites for the transcription factors HNF-3β, HNF-4, and C/EBp. Elements of the apo B gene that confer in vivo expression in the liver (intron 2 enhancer and 5′-upstream enhancer) do not play a role in the intestinal expression of apo B transgenes in mice.

3.2. Apo B mRNA editing

Apo B100 contains 4536 amino acid residues and is the only isoform of apo B produced by the human liver. In contrast, the small intestine of all mammals and the liver of some species synthesize apo B48, the N-terminal 2152 residues of apo B100. Apo B100 and apo B48 are produced from a single gene. The mechanism by which apo B48 is generated involves an mRNA editing process in which a single cytidine at position 6666 of apo B100 mRNA is deaminated to a uridine (J. Scott, 1987). A stop codon is thereby introduced and the truncated variant, apo B48, is generated. The *cis*-acting elements that direct this site-specific deamination of the mRNA are located in an AU-rich region flanking the target cytidine. A region of 11 nucleotides, 5 bases downstream of this cytidine, is particularly important for the editing process and is called the ‘mooring sequence’. A 27-kDa protein, apobec-1 (apo B editing complex-1), which is present in enterocytes is required for apo B mRNA editing [5]. Apobec-1 is highly homologous to other cytidine deaminases but does not by itself edit the RNA. Rather, apobec-1 is the catalytic subunit of a multi-protein complex. Apo B48 is generated only in tissues that express apobec-1 mRNA. However, apobec-1 is also expressed in some tissues that do not synthesize apo B mRNA, suggesting additional substrates for this deaminase. Interestingly, transgenic mice that over-express apobec-1 develop hepatocellular carcinomas and liver dysplasia (T.L. Innerarity, 1997).

Apo B48-containing lipoproteins are removed from plasma more rapidly than are apo B100-containing lipoproteins because apo E, which associates with apo B48-containing lipoproteins after secretion, is a high-affinity ligand for the LDL receptor (Chapter 20). It was reasoned, therefore, that if apo B100 secretion were prevented, by inducing the complete editing of apo B mRNA, all apo B would be in the form of apo B48, fewer apo B-containing lipoproteins would be present in plasma, and the animals would be less susceptible to diet-induced hypercholesterolemia and atherosclerosis. The *Apobec-1* gene was, therefore, over-expressed in livers of atherosclerosis-prone mice via adenovirus-mediated gene transfer. As predicted, the amount of apo B-containing lipoproteins in plasma was strikingly decreased and the extent of atherosclerosis was reduced (B. Teng, 1994). On the other hand, targeted disruption of the *Apobec-1* gene in mice abolished apo B mRNA editing and resulted in complete elimination of plasma apo B48 (N.O. Davidson, 1996), demonstrating that there is no functional duplication

of apobec-1 activity. The *Apobec-1* gene is not essential for viability. *Apobec-1*$^{-/-}$ mice are fertile and healthy, and serum levels of cholesterol and TG are normal. Furthermore, mice that express apo B48 without apo B100 (by replacement of the apo B48 editing codon with a stop codon) show no obvious defects in growth, reproduction, or function (S.G. Young, 1996).

3.3. Apo B mRNA translational control

The acute regulation of apo B secretion occurs largely post-translationally. The translational regulation by insulin has been well documented (J.D. Sparks, 1994). In addition, inhibition of neutral lipid transfer to apo B via microsomal triacylglycerol transfer protein (MTP) (Section 6.4) slows apo B translation (H.N. Ginsberg, 2000). Despite evidence for translational control of apo B production, the molecular mechanisms that govern this process are unknown. Recent evidence, based on both experimental data and thermodynamic modeling, suggests that translational regulation of apo B production is governed by *cis–trans* interactions at the 3′- and 5′-untranslated regions of the apo B gene. The 5′-untranslated sequence of apo B mRNA is GC rich with 76% (G + C) content; GC-rich regions have a high potential for forming stable secondary structures. Analysis of the 5′-untranslated region identified two GC boxes located at positions −20 and −81 upstream of the translational start site. Between these two GC boxes is a GAGGCC doublet; the role of such elements in mRNA translation is not known. The 3′-untranslated region of apo B mRNA has the potential to form secondary structures and includes sequence elements such as AUUUA and AUUUUUA. AU-rich regions are known to play roles in determining mRNA stability. Mfold analysis of the 5′- and 3′-untranslated sequences of apo B mRNA revealed RNA elements with the potential to form stable secondary structure and to mediate the translational control of apo B mRNA (L. Pontrelli, 2004). Chimeric mRNAs containing the 5′- and/or 3′-untranslated regions of apo B linked to a luciferase reporter gene, or to the apo B15 sequence, were used to investigate the biological activity of these motifs. The data suggest that the 5′-untranslated motifs are important for optimal translation of the apo B message, whereas the 3′-untranslated region attenuates mRNA expression. Potential *cis–trans* interactions of these motifs with RNA binding proteins/translational factors are likely to govern apo B mRNA translation and protein synthesis. More recently, a 110-kDa insulin-sensitive factor was identified that binds to the 5′-untranslated region of apo B mRNA and increases apo B translation. Insulin-mediated alterations in the binding of this 110-kDa factor to the 5′-untranslated region appear to modulate interactions at the 5′-untranslated region and alter the rate of apo B synthesis (K. Adeli, 2007).

4. Models used for studying the secretion of apo B and VLDL

Hepatoma cell lines, such as human HepG2 hepatoma cells and McArdle 7777 rat hepatoma cells, are frequently used for studying VLDL secretion. In addition, primary hepatocytes isolated from livers of rats, mice, or hamsters are commonly used. Each model has advantages and limitations. Hepatoma cells are convenient laboratory models since they can be

easily maintained and grown under defined conditions. However, although hepatoma cells have retained many properties of hepatocytes, several important liver functions, particularly some aspects of lipid metabolism, are defective. An advantage of HepG2 cells is that they are of human origin and, like human liver, secrete apo B100 but not apo B48. Nevertheless, a significant drawback to the use of HepG2 cells for studying VLDL secretion is that the secreted apo B-containing lipoproteins are not true VLDLs but contain less lipid and are smaller than VLDLs. McArdle rat hepatoma cells, like rat liver but unlike human liver, secrete both apo B100- and apo B48-containing lipoproteins. In the absence of fatty acid supplementation, these cells also secrete lipoproteins that are relatively lipid poor and denser than VLDLs, but when supplemented with oleic acid, a significant proportion of the secreted apo B is in the form of VLDLs. An advantage of McArdle hepatoma cells is that they are very amenable to transfection with cDNAs such as those encoding truncated and mutated forms of apo B.

Primary hepatocytes isolated from livers of rats, mice, and hamsters are also frequently used for studying VLDL assembly and secretion. A disadvantage of primary hepatocytes for studying VLDL secretion is that these cells cannot readily be stably transfected with cDNAs or gene-silencing siRNAs. Although primary hepatocytes must be freshly isolated for each experiment, they retain most properties of native hepatocytes, at least for ~24 h. However, unlike human hepatocytes, rat and mouse hepatocytes secrete apo B48 in addition to apo B100. Nevertheless, the apo B-containing particles have the composition, size, and density of nascent VLDLs. An excellent alternative model for studying VLDL secretion is hamster primary hepatocytes. These hepatocytes secrete VLDLs that contain apo B100 but not apo B48 (K. Adeli, 2000).

Hepatocytes isolated from genetically modified mice provide many opportunities for studying the role of specific genes in VLDL secretion. For example, a line of transgenic mice was generated in which Arg-3500 (the site that binds to the LDL receptor) in apo B100 was mutated. These mice developed severe hypercholesterolemia because they were unable to clear apo B-containing lipoproteins from plasma. Another useful mouse model is that in which the endogenous apo B gene has been disrupted, but which express human apo B100. As indicated above (Section 3), targeted disruption of the apo B gene in mice is embryonically lethal. Although the mice expressing human apo B100 were indistinguishable from wild-type mice during the suckling period, they did not produce CM and exhibited fat malabsorption and retarded growth [6] because the human apo B gene was not expressed in the intestine (Section 3.1). Other interesting mouse models with altered apo B metabolism include strains of transgenic mice that express apo B with point mutations that result in the secretion of low levels of poorly lipidated, truncated apos B (such as apo B70, apo B81, and apo B83). As in humans with hypobetalipoproteinemia, these mice have low levels of plasma apo B-containing lipoproteins and develop severe neurodevelopmental abnormalities [7].

VLDL secretion can also be studied in vivo by injection of mice or rats with the detergent Triton WR1339 or poloxmer. These compounds block the lipolysis of TG-rich lipoproteins in plasma so that the amount of plasma apo B-containing lipoproteins reflects only the secretion of apo B, in the absence of lipolysis. This methodology has some limitations, primarily because neither detergent is specific for blocking VLDL lipolysis (J.T. Billheimer, 2005).

5. Covalent modification of apo B

Some co- and post-translational modifications of apo B have been identified but, for the most part, their relevance for apo B secretion and function is not known. Apo B100 contains at least 20 putative glycosylation sites, and plasma apo B contains 8–10% carbohydrate (by weight) with the carbohydrates attached at both *N*- and *O*-linked glycosylation sites. Treatment of chicken hepatocytes with tunicamycin, which inhibits *N*-linked glycosylation, did not reduce the amount of apo B secreted (M.D. Lane, 1982). In contrast, incubation of HepG2 cells with tunicamycin reduced apo B100 secretion and increased apo B degradation (Section 7). Nevertheless, the apo B-containing particles that were secreted had normal buoyant density (L. Chan, 2001).

Several studies have shown that secreted apo B is phosphorylated at multiple serine residues (R.A. Davis, 1984). The extent of phosphorylation is greater in diabetic than in non-diabetic rats (J.D. Sparks, 1990). The phosphorylation of apo B was reported to occur in the Golgi, consistent with the finding that the Golgi contains kinases that can phosphorylate secretory proteins (L.L. Swift, 1996).

Apo B100 secreted by HepG2 cells is covalently palmitoylated on at least one cysteine residue (J.M. Hoeg, 1988). The palmitoyltransferase involved has not been identified. Mutation of Cys-1085 of apo B29 expressed in McArdle hepatoma cells abolished palmitoylation at this site and the secreted apo B29-containing lipoproteins were less lipidated than those in which Cys-1085 of apo B was palmitoylated (Y. Zhao, 2000). However, whether apo B palmitoylation is important for determining the amount and/or degree of lipidation of secreted apo B remains controversial because another study reported that palmitoylation of apo B does not play a role in apo B secretion by McArdle hepatoma cells (Z. Yao, 2003).

6. Regulation of apo B secretion by lipid supply

Under most metabolic conditions, apo B is synthesized constitutively and in excess of the amount required for secretion. Thus, the rate of apo B synthesis does not usually determine how much apo B is secreted. The assembly of VLDLs is a complex process in which several types of lipids — including TG, cholesterol, cholesteryl esters, and phospholipids — become non-covalently associated with newly synthesized apo B. When insufficient lipid is available for formation of stable apo B-containing particles, excess apo B, and apo B that has been incompletely assembled with lipids, is not secreted but is targeted for intracellular degradation (Section 7). An increased supply of lipid promotes apo B translocation across the ER membrane and into the secretory pathway, resulting in a larger proportion of the newly synthesized apo B being secreted.

Sterol-response element-binding proteins (SREBPs) are transcription factors that coordinately increase the synthesis of fatty acids and cholesterol (Chapters 6, 7, and 14). The transcriptionally active forms of SREBPs are generated from precursor proteins by a sterol-dependent proteolytic cleavage [8]. Several studies have indicated that an increased production of transcriptionally active SREBPs stimulates VLDL secretion.

6.1. Fatty acids and triacylglycerols

Although an increased supply of fatty acids and TG to hepatocytes increases the amount of lipid secreted in association with apo B, the rate of apo B secretion is not solely a function of the rate of TG synthesis. Supplementation of HepG2 hepatoma cells with oleic acid increases the synthesis of TG and concomitantly increases the amount of secreted TG and apo B [9]. Furthermore, the oleic acid-induced increase in apo B secretion is blocked by Triacsin D, an inhibitor of TG synthesis. The situation is, however, different in primary rat and mouse hepatocytes in which the synthesis and secretion of TG are increased by oleate supplementation whereas apo B secretion is not. Furthermore, glucose stimulates the synthesis and secretion of TG but does not increase apo B secretion. Thus, enhanced lipogenesis is not necessarily reflected in increased apo B secretion. The type of fatty acid supplied to hepatocytes also influences the secretion of apo B-containing lipoproteins. Compared to oleic acid, the (n-3) fatty acids, eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6), which are particularly abundant in fish oils, decrease plasma TG levels in humans and decrease the secretion of apo B-containing lipoproteins from rat hepatocytes and hepatoma cells (E.A. Fisher, 1993; Z. Yao, 2006). Consistent with these findings, (n-3) fatty acids increase the intracellular degradation of apo B (Section 7).

The source of TG used for assembly with apo B has been proposed to originate primarily (~70%) from the cytosolic TG storage pool rather than from the pool of TG made by de novo synthesis in the ER [10]. One model for the assembly of TG with apo B is that cytosolic TG is hydrolyzed, perhaps by the microsomal TG hydrolase (R. Lehner, 1999), to diacylglycerol/monoacylglycerol, which are subsequently re-esterified in the ER lumen to TG which assembles with apo B. However, many questions remain regarding the topology of this proposed lipolysis/re-esterification cycle and the molecular identity of the players. For example, it is not known if the active site of the enzyme that makes TG for assembly with apo B resides on the lumenal or cytosolic side of the ER membrane. If the formation of TG by re-esterification occurred within the ER lumen, one would also need to explain how fatty acids entered the ER lumen.

Several experiments have indicated that the ER is the site of addition of the bulk of TG to apo B. First, the size distribution of lipoproteins is the same in the lumen of the ER and Golgi of rat liver, and in newly secreted lipoproteins (J.E. Vance, 1993). Furthermore, a series of pulse-chase radiolabeling experiments in rat hepatoma cells indicate that the majority of TG associates with apo B in the ER (H.N. Ginsberg, 2003). On the other hand, immuno-electron microscopy studies in rat liver (R.J. Havel, 1976), and pulse-chase radiolabeling experiments in chicken hepatocytes (M.D. Lane, 1990) and rat hepatoma cells (E.A. Fisher, 2003), have suggested that the majority of TG assembles with apo B in the Golgi apparatus. The reasons for these conflicting conclusions remain unclear.

The synthesis of TG in mice is mediated by two diacylglycerol acetyltransferases, DGAT1 and DGAT2 (Chapter 10). *Dgat1* knockout mice (R.V. Farese, 2002) retain the ability to synthesize TG, and the plasma of fasted *Dgat1*$^{-/-}$ mice contains normal amounts of TG. However, intestinal TG absorption is severely impaired. Subsequently, a second diacylglycerol acyltransferase gene (*Dgat2*), which is highly expressed in the liver, was identified and *Dgat2*$^{-/-}$ mice were generated (R.V. Farese, 2004). Disruption of the *Dgat2*

gene decreased the plasma level of TG by ~75% and markedly reduced the TG content of the liver. However, *Dgat2*$^{-/-}$ mice died shortly after birth because the availability of substrates for energy metabolism was reduced. These mice also exhibited defects in the permeability barrier of their skin. The catalytic site of DGAT2 resides on the cytosolic side of the ER membrane (S.J. Stone, 2006). However, the identity and topology of the diacylglycerol acyltransferase activity that produces the pool of TG for VLDL assembly is not yet clear.

6.2. Phospholipids

Phosphatidylcholine is the major phospholipid on the surface monolayer of all lipoproteins, including VLDLs. In the liver, phosphatidylcholine is synthesized by two biosynthetic pathways: the CDP-choline pathway and the phosphatidylethanolamine *N*-methyltransferase pathway (Chapter 8). Choline is an essential biosynthetic precursor of phosphatidylcholine via the CDP-choline pathway. When cells or animals are deprived of choline, plasma levels of TG and apo B are markedly reduced and TG accumulates in the liver, resulting in fatty liver. These observations led to the widely held view that the fatty liver caused by choline deficiency is due to inhibition of PC synthesis, which in turn would decrease VLDL secretion. This hypothesis was tested in primary rat hepatocytes cultured in medium lacking choline. Upon removal of choline/methionine from culture medium, the TG content of hepatocytes was increased ~6-fold, and the secretion of TG and apo B in VLDL was markedly reduced. The interpretation of these experiments was that hepatic VLDL secretion requires the synthesis of phosphatidylcholine from either the CDP-choline or methylation pathways which require choline or methionine, respectively, as precursors (D.E. Vance, 1988). However, since choline deprivation was induced in a background of methionine insufficiency, it was not clear whether the lack of choline per se, and inhibition of the choline pathway for phosphatidylcholine synthesis, decreased VLDL secretion. More recent experiments have shown, surprisingly, that deficiency of choline in primary mouse hepatocytes does not *reduce*, but *increases*, phosphatidylcholine synthesis via the CDP-choline pathway, and does not decrease VLDL secretion (J.E. Vance, 2004). Thus, a deficiency of dietary choline reduces plasma TG and apo B levels by a mechanism that does not involve reduction of phosphatidylcholine synthesis.

To determine the role of the CDP-choline pathway of phosphatidylcholine synthesis for VLDL secretion, knockout mice were generated in which the gene encoding CTP:phosphocholine cytidylyltransferase-α (*Pcyt1a*), a key enzyme in phosphatidylcholine synthesis via the CDP-choline pathway (Chapter 8), was disrupted only in the liver. TG accumulated in livers of these mice and the secretion of TG and apo B was decreased (D.E. Vance, 2004). Thus, elimination of the CDP-choline pathway in the liver inhibits VLDL secretion. In addition, disruption of the gene encoding the liver-specific enzyme, phosphatidylethanolamine *N*-methyltransferase, in mice fed a high-fat/high-cholesterol diet, also markedly reduces the secretion of TG and apo B in VLDLs (D.E. Vance, 2003). Thus, the hepatic synthesis of phosphatidylcholine via *both* the CDP-choline and methylation pathways appears to be required for normal VLDL secretion.

In addition to phosphatidylcholine, smaller amounts of other phospholipids such as phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin

are components of VLDL, but the physiological relevance of these phospholipids is unknown. Inhibition of sphingomyelin synthesis in rat hepatocytes by >90% by fumonisin B did not reduce VLDL secretion (A.H. Merrill, 1995). Thus, normal amounts of hepatic sphingomyelin are apparently not required for VLDL secretion. Interestingly, the phosphatidylethanolamine content of newly secreted VLDLs and VLDLs isolated from the Golgi of rat liver is several-fold higher than that in circulating VLDLs (P.E. Fielding, 1989; A. Kuksis, 2005), suggesting that either the initial assembly of phosphatidylethanolamine into VLDLs or the specific hydrolysis of this phospholipid in newly secreted VLDLs might have functional significance.

6.3. Cholesterol and cholesteryl esters

There are conflicting data on whether the availability of cholesterol and/or cholesteryl esters directly influences apo B secretion. Several studies have suggested that cholesterol supply can regulate VLDL secretion. For example, VLDL production in animals and man is decreased by statin treatment, and inhibition of cholesterol synthesis by a statin, an inhibitor of the rate-limiting step of cholesterol biosynthesis (Chapter 14), reduced VLDL secretion in perfused rat livers (M. Heimberg, 1990) and primary hepatocytes. However, this effect of statins can perhaps be ascribed to increased expression of LDL receptors rather than to a reduction in cholesterol synthesis (Section 7.1). Depletion of cholesterol in rodent hepatocytes by the ABCA1-dependent lipidation of apo A1 (Chapter 19) also decreases VLDL secretion (R. Lehner, 2004). Furthermore, the secretion of apo B100-containing VLDLs is increased in primary hepatocytes derived from Niemann–Pick C1-deficient mice. Niemann–Pick C1-deficiency causes a severe defect in trafficking of unesterified cholesterol out of the lysosomal/endosomal pathway and consequently, Niemann–Pick C1-deficient hepatocytes accumulate 5- to 10-fold more unesterified cholesterol than do wild-type hepatocytes. In hepatocytes from Niemann–Pick C1-deficient mice, cholesterol synthesis is increased and the rate of cholesterol esterification and the amount of the transcriptionally active form of SREBP-1 are also increased (J.E. Vance, 2007). However, because of multiple alterations in lipid metabolism in these hepatocytes, increased VLDL secretion cannot be attributed specifically to increased synthesis of cholesterol or cholesteryl esters.

Cholesteryl esters are quantitatively minor constituents (5–15% of total lipids) of VLDLs but the amount of cholesteryl esters relative to TG in VLDLs increases when rats are fed a high cholesterol diet. The esterification of cholesterol is mediated by two distinct acyl-CoA:cholesterol acyltransferases (ACATs) [11]. Inhibition of cholesterol esterification with an ACAT inhibitor in hepatocytes decreased apo B secretion in some studies but not in others. For example, severe reduction in cholesteryl ester content of hepatoma cells decreased apo B secretion, whereas increased cholesteryl ester content did not stimulate apo B secretion. In mouse liver and intestine, the majority of cholesteryl esters are made by ACAT2. Nevertheless, normal quantities of apo B-containing lipoproteins are produced in mice lacking ACAT2 despite the absence of essentially all hepatic ACAT activity. However, ACAT2-deficient mice exhibit reduced intestinal absorption of cholesterol and are resistant to diet-induced hypercholesterolemia (R.V. Farese, 2000). Thus, the observed reduction of plasma cholesterol in response to ACAT inhibitors is probably due to decreased cholesterol absorption rather than decreased VLDL secretion.

In mice with targeted disruption of the gene encoding ACAT1, cholesterol esterification in the liver and plasma cholesterol levels are normal (R.V. Farese, 2001). On the basis of experiments with ACAT-deficient mice, it has been suggested that ACAT1 provides cholesteryl esters primarily for storage in cytosolic lipid droplets, whereas ACAT2 is linked to the secretion of cholesteryl esters into VLDLs. Consistent with this concept, the two ACATs have been proposed to have distinct membrane topologies so that the active site of ACAT1 faces the cytosol whereas the active site of ACAT2 faces the ER lumen (L. Rudel, 2000). It is not yet clear, however, how these findings apply to humans since ACAT1, not ACAT2, accounts for the majority of ACAT activity in human hepatocytes, whereas ACAT2 is the major ACAT isoform in human intestine.

6.4. Microsomal triacylglycerol transfer protein

The process by which TG molecules are concentrated within the core of VLDLs requires a 97-kDa microsomal protein, the microsomal triacylglycerol transfer protein (MTP) (J.R. Wetterau, 2000). Individuals with the rare genetic disease abetalipoproteinemia have mutations in the *MTP* gene and, despite a lack of defects in the apo B gene, have barely detectable levels of apo B in their plasma (J.R. Wetterau, 1992). MTP is present in the ER lumen of liver and intestine as a soluble heterodimer associated with protein disulfide isomerase (J.R. Wetterau, 1990), a ubiquitous ER lumenal protein that catalyzes disulfide bond formation during protein folding. Although MTP lacks a C-terminal KDEL sequence (a motif that retains soluble proteins in the ER lumen), the association of MTP with protein disulfide isomerase, which does contain a KDEL sequence, is thought to retain MTP within the ER lumen. In in vitro assays, MTP transfers lipids between donor and acceptor liposomes with the substrate preference being: TG > cholesteryl ester > diacylglycerol > phosphatidylcholine (H. Jamil, 1995). Lipid transfer proceeds with ping-pong, bi-bi kinetics, implying that MTP transfers lipids via a shuttle mechanism. The tissue and subcellular distribution of MTP, and its preference for transferring neutral lipids, suggested that MTP is involved in loading nascent apo B with TG [12].

Specific inhibitors of the lipid transfer activity of MTP have been developed as a potential treatment for atherosclerosis based on the premise that inhibition of MTP reduces VLDL secretion. Indeed, MTP inhibitors effectively reduce plasma cholesterol levels by up to 80% in rats, hamsters, and rabbits [13]. However, heterozygosity for MTP deficiency in humans does not diminish plasma lipids or lipoproteins, indicating that a modest reduction in MTP activity does not limit VLDL production. On the other hand, adenovirus-mediated over-expression of MTP in mouse liver increased the secretion, and plasma levels, of TG, apo B100, and apo B48 (D.J. Rader, 1999). MTP inhibitors are not currently used therapeutically, probably because they induce some storage of TG in the liver (steatosis) as a result of the blockage in VLDL secretion.

Complete elimination of MTP is embryonically lethal in mice because, unlike the situation in humans, apo B-containing lipoproteins are required for transferring lipids from the yolk sac to the developing mouse embryo [14]. However, when the *Mtp* gene was inactivated specifically in the liver, the mice were viable. In addition, the amount of plasma apo B100 was reduced by >90% compared to that in wild-type mice whereas, surprisingly, apo B48 was only slightly reduced [14]. Consistent with these observations, apo B100

secretion by $Mtp^{-/-}$ mouse hepatocytes was eliminated, whereas the amounts of apo B48 secreted were essentially normal. Similarly, treatment of mouse hepatocytes with an MTP inhibitor markedly reduced the secretion of apo B100 but only slightly reduced the secretion of apo B48 (J.E. Vance, 2002). In contrast, in another line of liver-specific MTP-deficient mice, the secretion of both apo B100 and apo B48 was undetectable (L. Chan, 1999). Thus, apo B100 secretion clearly requires MTP, whereas the secretion of apo B48 appears to be less dependent upon MTP. One cannot discount the possibility that different mechanisms are involved in the assembly of these two classes of VLDLs.

6.4.1. Role of MTP in lipid assembly with apo B

The precise function of MTP in the assembly of lipids with apo B has not yet been elucidated [12]. Short apo B variants, such as apo B18, are secreted from hepatoma cells in poorly lipidated form, independent of MTP whereas the secretion of longer (>apo B23), more highly lipidated apo B variants is enhanced by MTP. MTP has been suggested to play a role in several steps along the VLDL assembly pathway including: a chaperone role in facilitating the translocation of apo B across the ER membrane; a role in the co-translational transfer of lipids, particularly phospholipids, to apo B; a role in facilitating the movement of TG into the ER lumen; and a role in transferring the bulk of TG into the VLDL core.

MTP has been suggested to function as a chaperone for promoting the translocation of apo B across the ER membrane and into the ER lumen for VLDL assembly (H.N. Ginsberg, 2001). Co-immunoprecipitation experiments demonstrated that apo B and MTP interact via specific sites on each protein (C.C. Shoulders, 1999). The interaction is transient and increases when VLDL assembly is stimulated by oleic acid, and decreases when TG synthesis is inhibited. Apo B also physically interacts with well-known ER lumenal chaperone proteins such as calnexin, calreticulin, GRP94, Erp72, and BiP (H. Herscovitz, 1998). Presumably these chaperones ensure the correct folding of apo B.

Studies with $Mtp^{-/-}$ mice and MTP inhibitors suggest that MTP promotes the movement of TG from cytosolic lipid droplets, or from a TG pool within the ER membrane, across the ER membrane and into a lumenal, apo B-free TG pool that can subsequently be used for VLDL assembly. The existence of such a pool of apo B-free TG in the ER lumen was demonstrated in mouse hepatocytes in which inhibition of MTP decreased the pool of apo B-free TG within the microsomal lumen (J.E. Vance, 2002). Furthermore, ultrastructural analysis of $Mtp^{-/-}$ hepatocytes compared to wild-type hepatocytes showed that the ER and Golgi lumina of cells lacking MTP contained very few lipid-staining particles of the size of VLDLs. Consistent with these observations, $Mtp^{-/-}$ mouse livers contain an increased number of cytosolic lipid droplets (S.G. Young, 1998). An apo B-free lumenal TG pool was also observed in enterocytes from mice that lacked apo B synthesis in the intestine (R.L Hamilton, 1998).

MTP has the ability to transfer TG, cholesteryl esters, and phospholipids between membranes in vitro (H. Jamil, 1995). In experiments designed to define the importance of transfer of each type of lipid by MTP for VLDL assembly, human MTP and its *Drosophila* ortholog were compared (M. Hussain, 2006). *Drosophila* MTP lacks the ability to transfer neutral lipids in vitro yet possesses phospholipid transfer activity and, when heterologously expressed in COS cells, enhanced apo B secretion. These observations demonstrate

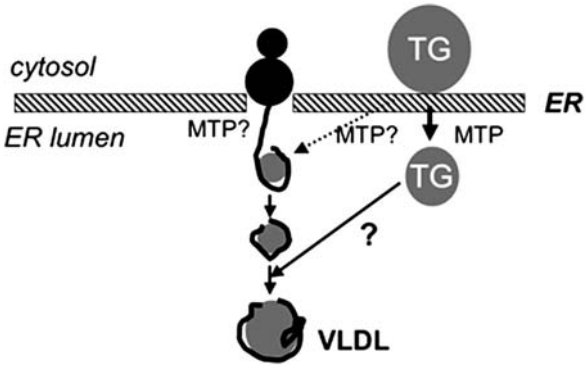

Fig. 5. A model for the role of MTP in VLDL assembly. As newly synthesized apo B (soild black line) translocates across the ER membrane, a small, lipid-poor apo B-containing particle (small gray circle surrounded by solid line) is formed by the addition of some lipid, particularly phospholipid and unesterified cholesterol; a role has been proposed for MTP in this process. Large, fully lipidated VLDL particles are formed by addition of more TG to the small apo B-containing particles in an MTP-independent process. The TG used in this step is proposed to be derived in an unidentified process from an apo B-free TG droplet that resides in the ER lumen. MTP is required for transfer of TG to this lumenal TG droplet, likely from the cytosolic TG pool.

that the phospholipid transfer activity of MTP is sufficient for promoting the secretion of apo B-containing lipoproteins. Although it was originally thought that MTP directly transfers TG to apo B, either by shuttling TG monomers to apo B or by fusion of a poorly lipidated apo B particle with a lumenal TG droplet, pulse-chase experiments in several experimental models indicate that MTP is not required for direct transfer of the bulk of TG to apo B (H. Jamil, 2000; J.E. Vance, 2002). Moreover, the biophysical mechanism by which a TG droplet would fuse with a small apo B-containing particle is unclear.

Based on these observations, one model for the role played by MTP in VLDL assembly is shown in Fig. 5. In this model, MTP transfers TG across the ER membrane to generate a pool of apo B-free TG in the ER lumen. During the co-translational translocation of newly synthesized apo B across the ER membrane, some lipids (phospholipids, cholesterol, and small amounts of TG) assemble with the nascent apo B to form a small, lipid-poor particle. The phospholipid transfer activity of MTP might be involved in promoting the movement of TG across the membrane and/or might induce the budding of the small apo B-containing particle into the ER lumen, or might transfer phospholipids to apo B. As discussed above, a function for MTP in translocating apo B across the ER membrane has also been suggested. According to the model shown in Fig. 5, the small apo B-containing particle would be converted into a fully lipidated VLDL by the addition of TG in a process that is independent of MTP. Although in Fig. 5, VLDL assembly is depicted as occurring within the aqueous lumen of the ER, experiments performed to date do not rule out the possibility that VLDL is completely assembled on the inner surface of the ER membrane.

6.4.2. Regulation of MTP gene expression

MTP gene expression is transcriptionally regulated by dietary intake of fat, carbohydrate, and alcohol, as well as by insulin. The promoter region of the *MTP* gene contains elements

that are predicted to bind transcription factors such as HNF-1, HNF-4, and AP-1. These elements are thought to dictate the cell type-specific expression of MTP. The human *MTP* promoter activity is positively regulated by cholesterol and negatively regulated by insulin. In general, the level of hepatic MTP mRNA expression is increased by metabolic conditions that increase lipogenesis and increase VLDL secretion [12]. Hepatic MTP mRNA is increased in hamsters fed a high-fat diet. The activity of the human *MTP* promoter is increased by cholesterol in an SREBP-dependent manner (Chapter 14). Insulin decreases MTP mRNA levels in HepG2 cells mainly by transcriptional regulation through the MAPK-ERK cascade, not through the phosphatidylinositol-3 kinase pathway (C.S. Au, 2004). Cellular MAPK-ERK and MAPK-p38 activities play a counterbalancing role in regulating transcription of the *MTP* gene. Interestingly, oleic acid was shown to stimulate *MTP* gene transcription in an SREBP-independent manner, suggesting a possible additional mechanism by which fatty acids enhance the assembly and secretion of apo B-containing lipoproteins. Oleate might act partially by inhibiting MAPK-ERK activity leading to increased expression of MTP mRNA.

7. Intracellular apo B quality control and degradation

Apo B mRNA is translated on ER-associated ribosomes, and the apo B nascent chain is co-translationally translocated across the ER membrane for targeting to the pathways of lipoprotein assembly and secretion. The availability of lipids at the site of apo B synthesis in the ER appears to dictate the amount of apo B that is secreted. Under lipid-poor conditions, a major proportion of newly synthesized apo B is rapidly degraded by proteasomal and non-proteasomal pathways. The reason why apo B100 is apparently synthesized in such a large excess of its needs for VLDL secretion is not clear. In addition to the availability of lipids, apo B structural elements dictate the assembly of apo B into VLDLs. Studies using truncated mutants of apo B have shown a positive correlation between apo B length and VLDL secretion efficiency [15]. Specific regions of apo B100 that lie between the C-terminal 22% and 43% of apo B100 induce a rapid proteasomal degradation of apo B, with the shorter apo B variants being more stable (R.S. McLeod, 2004).

7.1. Proteasomal degradation of apo B

It is now widely recognized that the intracellular degradation of apo B is mediated mainly by the cytosolic ubiquitin–proteasome pathway. Newly synthesized apo B is ubiquitinated and intracellularly degraded in a co-translational manner [16]. Degradation via the ubiquitin–proteasome pathway involves two steps: covalent attachment of multiple ubiquitin molecules to the substrate and degradation of the tagged protein by the 26S proteasome. Ubiquitinated apo B has been detected in association with factors that are involved in protein translocation across the ER membrane, such as the Sec61 complex and calnexin. Over-expression of MTP stimulates apo B secretion through decreased ubiquitin–proteasome-mediated degradation of apo B. An in vitro rabbit reticulocyte lysate system and permeabilized HepG2 cells were used to demonstrate that cytosolic components are required for the proteasomal degradation of newly synthesized apo B (J.L. Dixon, 1999). An ER-associated ubiquitin ligase, Grp78,

was identified as the E3 enzyme that mediates the targeted ubiquitination and proteasomal degradation of apo B [17]. Over-expression of this RING finger-dependent ubiquitin ligase induces the rapid ubiquitination and proteasomal degradation of apo B.

Despite the identification of the ubiquitin–proteasome pathway as the major pathway responsible for the degradation of apo B, the mechanism by which apo B is targeted to the cytosolic proteasomal system is poorly understood and controversial. Some evidence supports the notion that an inefficient and incomplete translocation of newly synthesized apo B in lipid-poor states results in a bitropic orientation, or bulging, of the apo B protein during translocation, thereby exposing domains of apo B to the cytosol where the protein is subsequently ubiquitinated and degraded [9]. Another model is based on a complete and efficient translocation of the apo B molecule. According to this 'retrograde translocation' model, full-length apo B is retracted from the ER lumen into the cytosol through a Sec 61p translocation channel, after which the apo B is ubiquitinated and targeted for proteasomal degradation (G.S. Shelness, 1999). A third model involves a co-translational retrograde translocation of the N-terminus of apo B into the cytosol via a second nearby translocon (H.N. Ginsberg, 2000).

7.2. Non-proteasomal degradation of apo B

Early evidence from a number of laboratories suggested that newly synthesized apo B is subject to multiple degradative pathways involving ER-associated proteases. For example, the ER-localized protein, ER-60, that possesses both chaperone and protease activity, has been implicated in apo B degradation. Newly synthesized apo B associates with ER-60 leading to apo B degradation via a proteasome-independent mechanism [18]. Over-expression of ER-60 in HepG2 cells increases apo B100 degradation in a process that is inhibitable by the thiol protease inhibitor *p*-chloromercuribenzoate (K. Adeli, 2004). Over-expression of ER-60 also induces the accumulation of a unique 50-kDa degradation intermediate of apo B100. These data suggest the existence of an ER-60-mediated degradative process for apo B in the ER. However, it is not clear whether ER-60 acts directly as a protease or indirectly as a chaperone.

There is also intriguing evidence that (ω-3) fatty acids induce apo B degradation via mechanisms that do not involve the conventional proteasomal or lysosomal degradation pathways. In the presence of (ω-3) fatty acids, apo B degradation was shown to occur in a post-ER, pre-secretion process (E.A. Fisher, 2004). Lipid peroxidation and oxidative stress mechanisms were implicated in the increased degradation and decreased secretion of apo B. Addition of antioxidants to cells treated with (ω-3) fatty acids restored normal apo B secretion. Interestingly, the induction of ER stress, with glucosamine or over-expression of Grp78, also increased apo B degradation and decreased apo B secretion (K. Adeli, 2005). Indeed, ER stress mechanisms appear to lead to both post-ER pre-secretory proteolysis of apo B as well as proteasome-mediated ER-associated degradation (K. Adeli, 2006).

The LDL receptor has also been implicated in the pre-secretory degradation of apo B [19]. The rate of secretion of apo B in LDL receptor knockout mice is higher than in their wild-type counterparts, and adenovirus-mediated over-expression of the LDL receptor restored normal levels of apo B secretion. Naturally occurring point mutations were identified within the LDL receptor that caused retention of both the mutant LDL receptor,

as well as apo B, within the ER resulting in enhanced degradation of apo B within the secretory pathway. Furthermore, LDL receptor/MTP double knockout mice secrete small LDL- and HDL-sized apo B-containing lipoproteins, whereas MTP knockout mice expressing the LDL receptor do not secrete any apo B. These observations suggest that hepatic LDL receptors can target apo B for degradation, but the mechanism of proteolysis has not been defined.

8. Metabolic regulation of VLDL secretion: overproduction in insulin-resistant states

Insulin resistance is a central pathophysiological feature of type 2 diabetes and abdominal obesity. Insulin resistance, or insulin insensitivity, is associated with reduced suppression of lipolysis in adipose tissue, high fatty acid flux, enhanced hepatic lipogenesis, and increased VLDL secretion (G.F. Lewis, 2002). Increased circulating levels of VLDLs lead to hypertriglyceridemia, the presence of small, dense LDLs, and reduced levels of HDL cholesterol. Thus, hepatic VLDL overproduction appears to be a key metabolic defect in insulin-resistant states, underlying many of the other lipid and lipoprotein abnormalities characteristic of insulin resistance. Evidence for a strong link between insulin resistance and increased VLDL production comes from studies in both genetic and dietary models of insulin resistance. Genetically modified animal models of insulin resistance, such as the *ob/ob* mouse, the *db/db* mouse, the Zucker *fa/fa* rat, and the *ZDF/Drt fa* rat, exhibit phenotypes of insulin resistance and/or diabetes, accompanied by metabolic dyslipidemia. JCR:LA-cp rats, in which there is an enhanced secretion of VLDLs (J.E. Vance, 1990), also exhibit obesity, insulin resistance, and hypertriglyceridemia. In another model of insulin resistance, the apo B/BAT-less mice develop obesity, hypertriglyceridemia, hypercholesterolemia, and hyperinsulinemia when fed a high-fat diet (H.N. Ginsberg, 2001). In these mice, apo B secretion is increased without alterations in the levels of mRNAs encoding MTP or apo B. In the 'sand rat' model of insulin resistance, plasma fatty acids, as well as hepatic lipogenesis and MTP activity, are increased. Fructose-induced insulin resistance in the hamster is accompanied by substantially increased in vivo hepatic secretion of apo B and TG into VLDLs (K. Adeli, 2000). Chronic fructose feeding enhances hepatic VLDL assembly and secretion via increased intracellular stability and availability of apo B, increased TG availability, and increased MTP mass and activity. In addition, hepatic VLDL overproduction has been reported in insulin-resistant humans.

Molecular mechanisms underlying the increased production of hepatic VLDLs in insulin resistance are beginning to be unraveled. Studies in animal models suggest a complex relationship among hepatic insulin resistance, hepatic fatty acid flux, SREBP-1 expression (Chapter 14), de novo lipogenesis, MTP gene expression, and VLDL overproduction in insulin-resistant states. Increased secretion of VLDLs appears to result predominantly from decreased sensitivity of the liver to the inhibitory effects of insulin on VLDL secretion (G.M. Reaven, 1998). Insulin could potentially control the rate of hepatic VLDL secretion directly by influencing the rate of apo B synthesis and degradation (J.D. Sparks, 1994) and/or by modulating *MTP* gene expression and activity. Development of insulin resistance in hamsters is associated with higher levels of MTP mRNA, protein,

and activity (K. Adeli, 2005), and augmented MTP expression and activity correlates with increased secretion of apo B-containing lipoproteins. Increased MTP expression is also increased in livers of obese diabetic mice and in a rat model of type 2 diabetes with visceral fat obesity, the Otsuka Long-Evans Tokushima fatty rat. The mechanism by which *MTP* gene expression is enhanced in insulin resistance and type 2 diabetes is currently unknown. Presumably, the increase in MTP expression in insulin resistance is due to an impaired insulin-regulatory system because insulin is a negative regulator of *MTP* gene expression (R. Sato, 1999).

Regulation of expression of the *MTP* gene appears to occur via activation of SREBPs which bind a putative negative sterol-response element within the *MTP* gene promoter. The MTP promoter contains overlapping insulin- and sterol-response elements. Emerging evidence suggests that MTP gene expression is also controlled by the MAP kinase cascade (C.S. Au, 2003). While ERK signaling inhibits MTP promoter activity, p38 kinase activates the promoter. Since MAPK is known to activate SREBP-1, MAPK activation would be expected to increase the level of SREBP-1c, thereby attenuating *MTP* gene expression. However, the opposite was observed: *MTP* gene expression is *enhanced* in the fructose-fed hamster liver in the setting of elevated levels of SREBP-1c. A similar observation was made in obese diabetic mice in which MTP mRNA and activity were enhanced in the presence of elevated levels of SREBP-1a and SREBP-1c. As an explanation for these findings, it has been suggested that increased hepatic flux of fatty acids and/or increased TG stores stimulate *MTP* gene expression whereas altered hepatic SREBP-1 levels play a minor role. Elevated levels of SREBP-1c in transgenic mice over-expressing cholesterol 7α-hydroxylase were associated with accelerated lipogenesis as well as increased *MTP* gene expression and increased apo B secretion. These findings indicate that MTP expression is promoted in insulin resistance by an unknown factor that blocks the SREBP-mediated inhibition of MTP promoter activity. Although earlier studies suggest that fatty acids do not affect MTP expression, more recent studies using MTP gene promoter constructs clearly show that oleic acid enhances the activity of the MTP promoter (K. Adeli, 2005). Interestingly, oleate-mediated stimulation of MTP promoter activity appears to be independent of the sterol-response element. Since MAP kinases regulate gene expression of both SREBP-1c and MTP, a potential link might exist among chronic, basal activation of the MAP kinase cascade, induction of de novo lipogenesis, and increased expression of MTP, which together could facilitate hepatic VLDL secretion.

9. Assembly and secretion of chylomicrons

The intestinal assembly of CM is essential for the absorption of dietary fat and fat-soluble vitamins. Intestinal enterocytes rapidly and efficiently absorb fatty acids released from emulsification and hydrolysis of dietary fat, and re-synthesize TG to form a heterogeneous population of lipoproteins referred to as CM. Available evidence suggests that the intestine tightly regulates its intracellular handling of newly synthesized TG and its output of TG into CM. Intestinally derived lipoproteins have a range of sizes that include LDL-sized CM (CM_{LDL}),

VLDL-sized CM (CM_{VLDL}), small CM (CM_s), and large CM (CM_L). It is generally assumed that CM carry predominantly TG derived from dietary sources, while VLDLs transport TG produced endogenously from synthesis in the liver. There is evidence that the intestine constitutively synthesizes VLDL-like particles during fasting and post-absorptive states [20], whereas the assembly of large CM is characteristic of enterocytes during the postprandial state. Overall, it has been proposed that the major function of CM is to transport dietary fat, whereas the constitutive synthesis of VLDLs provides a mechanism for re-absorption of endogenously synthesized lipids.

Apo B48 is an essential structural component of intestinally derived lipoproteins. Available evidence indicates that apo B48 secretion is primarily regulated post-transcriptionally. However, whether apo B48 is degraded by the proteasome in intestinal cells remains controversial. Apo B48 and apo B100 both form large CM. Thus, CM formation is a characteristic of enterocytes and not a unique property of apo B48 (R.V. Farese 1996; M. Hussain, 1997). An important factor required for assembly of intestinal lipoproteins is MTP. Putative roles for MTP in CM assembly include the rescue of apo B from degradation as well as the lipidation of small apo B48-containing particles by facilitating the association of apo B with TG droplets.

Two models have been proposed for CM assembly [20]. In the first model, the assembly of VLDLs and CM occurs by two independent pathways. The second model is based on the concept of 'core expansion' that generates lipoproteins of various sizes. This model proposes the initial formation of small lipoprotein particles containing apo B48 and phospholipids, followed by release of these particles from the ER membrane, and subsequent addition of lipids to form lipoproteins of different sizes depending upon the extent to which expansion of the core of the small particle with TG occurs. Small 'primordial' lipoproteins have been detected in enterocytes and recent studies support the second model.

A number of recent advances have enhanced our understanding of the process by which intestinal CM assembly occurs, including (i) isolation of pre-CM transport vesicles that traffic from the ER to Golgi in rat proximal intestines (C.M. Mansbach, 2003), (ii) detection of MTP in the Golgi of intestinal enterocytes (E. Levy, 2002), and (iii) discovery that Sar1 GTPase of COPII vesicles is critical for CM trafficking and secretion [21]. Mutations in the gene encoding Sar1 are responsible for CM retention disease (also known as Anderson's disease) [21] in which CM production is impaired despite the presence of normal genes encoding apo B48 and MTP. Interestingly, in CM retention disease, VLDL production by the liver is unaffected. These findings emphasize a key, and specific, role of Sar1 GTPase in intracellular CM transport and secretion.

There is evidence that intestinal lipoprotein production is increased in diabetes and insulin-resistant states. Intestinal lipoprotein overproduction has been suggested to be a major contributor to the fasting and postprandial lipemia observed in insulin-resistant states (M.R. Taskinen, 2003). Evidence for increased formation of intestinal apo B48-containing lipoproteins as a result of insulin resistance comes from studies in animal models (K. Adeli, 2006) as well as humans. The underlying mechanisms are currently unknown, but increased de novo lipogenesis, reduced apo B48 degradation, and higher MTP expression have been implicated.

10. Assembly of lipoprotein(a)

In addition to its presence on plasma LDLs and VLDLs, apo B100 is also present in the plasma of humans, some primates and hedgehogs (but not rodents) covalently bound via a single disulfide linkage to the glycoprotein apo(a) (Fig. 6). Apo(a) is synthesized in significant quantities only in the liver and binds to apo B100 in LDLs to form lipoprotein(a). In human plasma, the concentration of lipoprotein(a) varies from <1 to >100 mg/dl. A high level of plasma lipoprotein(a) is an independent risk factor for the development of coronary artery disease (A.M. Scanu, 1992; J. Danesh, 2000). The correlation between high plasma levels of apo(a) and coronary artery disease has been ascribed to the concomitant high plasma levels of LDLs.

Significant amino acid sequence homology between apo(a) and plasminogen has suggested a potential pathogenic role for lipoprotein(a) in which this lipoprotein contributes to thrombosis, although the protease-like region of apo(a) is catalytically inactive. Apo(a) contains tandem repeats of sequences that are similar to the 'kringle' motifs, particularly kringle-4, of plasminogen, suggesting that apo(a) arose by duplication of the plasminogen gene. Kringles occur in the non-catalytic domain of several proteases involved in the blood coagulation and fibrinolytic pathways. Apo(a) is highly glycosylated (~28% carbohydrate by weight) but the function of glycosylation is not known. Apo B and apo(a) are present in a 1:1 molar ratio in lipoprotein(a). A cysteine residue (Cys-4057) in one of the kringles of apo(a) forms a disulfide linkage with Cys-4326 of apo B100. Apo B100 of many mammals is unable to form a disulfide linkage with human apo(a). Consequently, in transgenic mice expressing human apo(a), apo(a) circulates in plasma free of LDLs. Most data support a model for lipoprotein(a) assembly that occurs in two steps in the plasma after the

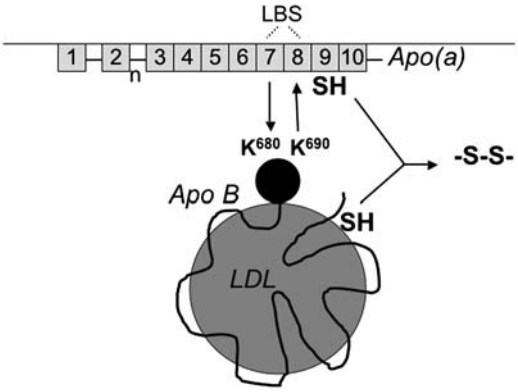

Fig. 6. A model of lipoprotein(a) assembly. Lipoprotein(a) assembly proceeds through a two-step model in which an initial non-covalent interaction between apo(a) and apo B precedes specific disulfide bond formation. The initial non-covalent interaction is mediated by interaction between two lysine residues (K^{680} and K^{690}) within the N-terminal 18% of apo B100 and the weak lysine-binding sites (LBS) in apo(a) kringle IV types 7 and 8. A disulfide bond is subsequently formed between a cysteine in the C-terminal region of apo B100 and apo(a). The types of kringle IV are indicated by boxed numbers. From Dr. J.P. Segrest, University of Alabama at Birmingham Medical Center, with premission.

LDLs and apo(a) have been secreted. Initially, two lysine residues (Lys-680 and Lys-690) in the N-terminal 18% of apo B100 interact non-covalently with a lysine-binding site in apo(a) (Fig. 6) (M.L. Koschinsky, 2001). Next, the C-terminal region of apo B100 interacts non-covalently with amino acids 4330–4397 of apo(a), and a disulfide bond is subsequently formed between apo B100 and apo(a). The kringle-4 domain of apo(a) can be present in a variable number of copies (3 to >40), resulting in size heterogeneity of lipoprotein(a). Generally, the plasma level of lipoprotein(a) is inversely correlated with apo(a) isoform size (i.e., the number of kringles). An inverse relationship has also been observed between apo(a) isoform size and the efficiency of covalent association of apo(a) with apo B. Consequently, it has been proposed that this relationship contributes to the inverse correlation between apo(a) isoform size and plasma lipoprotein(a) levels [22].

11. Future directions

The past few years have seen great advances in understanding the physiological implications of lipoprotein metabolism and the mechanisms by which lipoprotein classes are produced. Studies with genetically modified mice have been particularly useful for elucidating mechanisms of VLDL secretion. Nevertheless, some limitations are inherent in using mice as models of human lipoprotein production since lipoprotein metabolism in mice is distinct in several respects from that in humans.

Despite major advances in understanding the mechanisms of assembly of VLDLs, important questions remain. Although MTP has been extensively studied, and although we know that an absence of MTP severely impairs the secretion of apo B-containing lipoproteins, the precise role of MTP in VLDL assembly remains unclear. It is widely assumed that MTP is involved in transferring lipids to apo B, but a lipid transfer function for MTP has not been directly demonstrated in vivo. One possible function of MTP is the transport of TG to a pool (perhaps lumenal TG apo B-free droplets) that is subsequently used for assembly with apo B. The composition and physiological importance of these lipid droplets are unknown. The phospholipid transfer function of MTP for VLDL assembly also requires further study. Moreover, it is not clear why the hepatic synthesis of phosphatidylcholine via *both* the CDP-choline pathway and the methylation pathway is required for VLDL secretion. The intracellular site of addition of TG to apo B has not been unambiguously established and almost no information is available on the intracellular site of addition of phospholipids to VLDLs.

Another unresolved question is: why do animals make lipoproteins containing apo B100 since mice that express only apo 48 appear normal? We also do not understand why animals make apo B in large excess of its requirement for lipoprotein secretion and why hepatocytes degrade such a large fraction of newly synthesized apo B intracellularly in an apparently wasteful process. The importance and identity of the proteases that degrade apo B within the lumen of the secretory pathway remain unclear. More information is also required on how VLDL secretion is modulated by transcription factors that regulate the expression of genes that globally alter hepatic lipid metabolism. Great strides have been made in understanding the regulation of VLDL secretion under metabolic conditions such as insulin resistance and diabetes. Understanding the molecular factors responsible for

hepatic VLDL overproduction in insulin resistance will increase our knowledge of the basic mechanisms of VLDL assembly and help to identify potential new targets for therapeutic interventions. Inhibition of VLDL secretion is an attractive therapeutic target for atherosclerosis. So far, however, it appears that whenever VLDL secretion is blocked, fat accumulates in the liver, an unacceptable side effect. Thus, the development of agents that attenuate hepatic apo B secretion without causing hepatic TG accumulation or liver damage is a challenge for the future [23].

Abbreviations

ACAT	acyl-CoA:cholesterol acyltransferase
Apo	apolipoprotein
CM	chylomicrons
DGAT	diacylglycerol acyltransferase
ER	endoplasmic reticulum
HDL	high-density lipoprotein
LDL	low-density lipoprotein
MTP	microsomal triacylglycerol transfer protein
SREBP	sterol-response element-binding protein
TG	triacylglycerol
VLDL	very low density lipoprotein

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CHAPTER 19

Dynamics of lipoprotein transport in the circulatory system

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Contents

1. Overview

The major classes of plasma lipoproteins were defined about 50 years ago. It is only in the last 25 years that their individual roles in lipid transport have been made clear. Identification of the individual enzymes, receptors, transporters, and transfer proteins has continued up to the present time. Indeed a number of such factors now recognized to play key roles in plasma lipid transport had not even been identified when the last edition of this book appeared. As will be clear from this chapter, there are still many areas where research, and controversy, continue. Nevertheless it seems likely that the major outlines of these complex reactions have emerged.

1.1. Functions of the major apolipoproteins

Plasma lipoproteins are soluble complexes of lipids with specialized proteins (apolipoproteins, apo). The function of lipoproteins is to deliver insoluble lipids from the tissues where they are synthesized to those that utilize, degrade, or store them. The apolipoproteins solubilize and stabilize the lipids in the lipoprotein particles, and prevent the formation of aggregates that could promote the formation of blood clots. Many apolipoproteins have additional functions. Some are receptor ligands which can promote the binding of lipoprotein particles to particular tissues. Some apolipoproteins are cofactor proteins that stimulate the metabolism of lipoprotein lipids. Others are negative regulators, opposing the activity of cofactors and receptors. The protein component of lipoproteins largely determines the distribution of the lipids through the circulation to different tissues. The protein composition of lipoproteins changes as their lipid cargo is unloaded or catabolized. The result of these complex changes is that while there are two major classes of plasma lipoproteins, each is made up of a spectrum of particles of different size, and apoprotein and lipid composition (Chapter 17).

While lipoproteins are the products of many different genes, the major apolipoproteins share properties distinguishing them from most lipid-free and membrane-associated proteins. For example, apolipoproteins consist of a single polypeptide chain that has relatively little tertiary structure. Most apolipoproteins contain stretches of amphipathic alpha-helix, whose hydrophobic face can be turned to the lipid surface of the particle. The apolipoproteins are flexible, as is reflected in their unusually small free energy of unfolding. As these apolipoproteins expand and contract at the cell surface, different protein domains are exposed that are detectable with monoclonal antibodies. These properties reflect the role of apolipoproteins at the surface of lipoprotein particles whose size changes as they circulate.

Trace amounts of many other proteins are recovered in purified fractions of plasma lipoproteins. Some, like albumin, have plausible functions both at the lipoprotein surface [binding unesterified fatty acids newly generated from lipoprotein triacylglycerol (TG)] and in free solution to ensure osmotic balance between cells and their surroundings. Blood-clotting proteins are present in low amounts in TG-rich lipoproteins, but it is not clear that they play a unique biological role in lipoproteins. Many lipoprotein-associated proteins have no known function in lipid transport.

1.2. 'Forward' lipid transport

This process is the delivery of lipids to peripheral tissues from lipoprotein particles secreted by the liver or intestine. These particles are TG-rich, and all contain a single copy of apo B, as well as cholesteryl esters (CE) that are needed for proper apo B folding. Lipoprotein particles secreted by the human liver contain full-length apo B100; particles secreted from the intestine contain a truncated form, apo B48 (Chapter 18).

The protein moiety of newly synthesized TG-rich lipoproteins consists only of apo B. After secretion, other apolipoproteins are exchanged on to the particles from high-density lipoprotein (HDL) particles in the circulation. As TG is hydrolyzed by endothelial lipases (ELs) (Section 2), these extra proteins are displaced from the particle surface.

Consequently, the apo B particle gradually becomes smaller, and denser. Functionally, very low-density lipoproteins (VLDLs, density <1.006 g/ml), intermediate density lipoproteins (IDLs, density 1.006–1.019 g/ml), and low-density lipoproteins (LDLs, density 1.019–1.063) represent a continuum created by the lipolysis of TG. Some IDL and all of LDL are cleared from the circulation by the liver. Residual lipids may be recycled in the form of new VLDL particles, or be catabolized and secreted into bile. While there are differences in the metabolism in the plasma of chylomicrons from the intestine, and VLDL from the liver, the same lipases are involved in both cases.

1.3. 'Reverse' lipid transport

Reverse lipid transport is the movement of lipids, mainly cholesterol and phospholipids, from peripheral tissues, through the extracellular compartment, to the liver for catabolism. Unlike the forward transport of lipids, that involves mainly TG packaged into lipoproteins inside hepatic and intestinal cells, the cholesterol and phospholipids contributing to reverse transport are assembled into lipoproteins extracellularly, as the result of events in the plasma.

Reverse cholesterol transport (RCT) plays a key role in maintaining whole body cholesterol homeostasis. RCT involves plasma lipoprotein particles containing one to four copies of apo A1, the major protein of HDLs. HDLs also include several other apolipoproteins that are involved in the metabolism of its lipids and its delivery to the liver.

Several sources of cellular cholesterol contribute to RCT. Part of the process of RCT reflects peripheral (extra-hepatic) cholesterol synthesis. Despite the down-regulation of cholesterol synthesis mediated by the LDL receptor via the delivery of LDL, a considerable amount of sterol is made in peripheral tissues. The importance of this source of cholesterol to homeostasis may be as great as that of dietary cholesterol in many individuals. After hydrolysis of LDL-CE by cellular cholesterol esterases, this cholesterol is made available for recycling to the cell surface and can be recovered there by apo A1 for incorporation into HDLs. Cholesterol is also available from VLDLs, LDLs, and chylomicrons directly internalized by peripheral cells. Cholesterol from HDLs bypasses the lysosomal pathway and becomes part of recycling endosomes that return to the cell surface. Some of the cholesterol recovered on HDLs originates from blood cells. Finally, some cholesterol is transferred directly to other lipoproteins from chylomicrons, VLDLs, and LDLs, without entering the cell.

Apo A1, like apo B, is secreted mainly from liver and intestinal cells. Unlike apo B, however, apo A1 is secreted in lipid-free or lipid-poor from. The cholesterol and phospholipids that are transferred to HDLs move down concentration gradients driven by the plasma enzyme lecithin:cholesterol acyltransferase (LCAT). LCAT, which is bound to HDL, converts cholesterol and phosphatidylcholine to insoluble CE and lysophosphatidylcholine (Section 3.4), which is soluble and is transferred to albumin in the plasma. Cholesterol has a small but significant solubility and, as a result, can be transferred spontaneously from cell and lipoprotein surfaces to apo A1. Cholesterol may also be transferred as a result of molecular collision between lipoprotein particles. The LCAT reaction consumes equal amounts of cholesterol and phospholipids, but the rate at which phospholipids are transferred spontaneously between cells and lipoproteins is much lower

than for cholesterol. Two mechanisms have been proposed to accelerate the transfer of phospholipids to apo A1. First, ATP-binding cassette (ABC) transporters are transmembrane proteins that promote the movement of phospholipids across the cell-surface bilayer, and facilitate phospholipid transfer to apo A1. Second, a circulating protein, phospholipid transfer protein, catalyzes the movement of PL from the surface of chylomicrons, VLDLs and LDLs to HDLs. The end-result of these reactions is that apo A1 becomes lipid-loaded, and is transformed into a spectrum of HDLs of decreasing density. Lipid-poor HDL has a density >1.21 g/ml. The classical subfractions of HDL, HDL-3 (density 1.12–1.21 g/ml), HDL-2 (density 1.063–1.12 g/ml), and HDL-1 (density <1.063 g/ml), are a continuum reflecting the balance between HDL synthesis and removal, and the rate of RCT.

Part of the CE formed in HDL is transferred to apo B lipoproteins, particularly VLDL and LDL, by a CE transfer protein (CETP), prior to removal of these lipoproteins by the liver. Another part of the CE in HDL is selectively internalized by the liver and by tissues synthesizing steroid hormones, by a scavenger receptor protein (SR-BI) (Chapter 20). Finally some CE is internalized as part of intact HDL by hepatic HDL receptors/binding proteins.

The main fluxes of plasma lipids are depicted in Fig. 1.

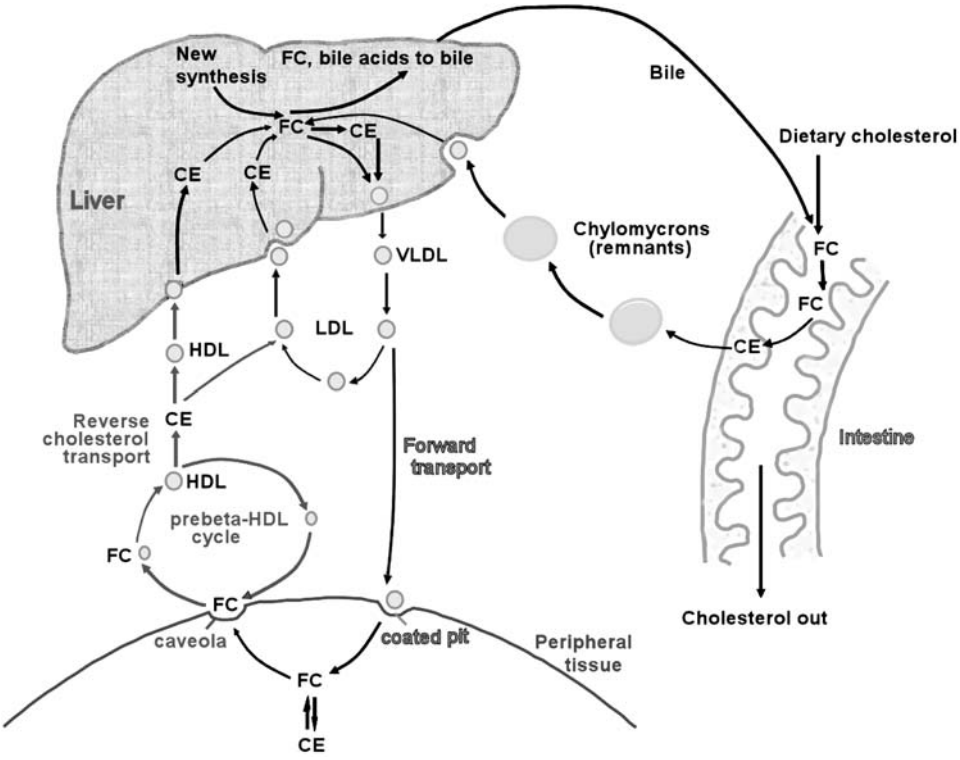

Fig. 1. Summary of major 'forward' and 'reverse' lipid transport pathways through the extracellular compartment that link the liver and intestine with peripheral tissues. FC, unesterified cholesterol. For other abbreviations see list of abbreviations. (See color plate section, plate no. 17.)

2. Lipoprotein TGs and hydrolysis

2.1. Initial events

Each newly synthesized intestinal apo B-containing lipoprotein particle (chylomicron) consists of a core lipid droplet rich in TG, surrounded by a monolayer made up mainly of protein, phospholipids, and cholesterol. The chylomicron lipid core contains a small amount of CE. The lipids of TG-rich particles are in a dynamic equilibrium where each lipid can exchange rapidly between the surface and core. As a result, while the great majority of TG is in the core, the surface contains a small but rapidly replenished pool of TG which is the direct substrate of the plasma lipases responsible for chylomicron metabolism in the circulation.

Chylomicron TG is derived almost entirely from dietary fatty acids. Chylomicron CE is derived almost entirely from dietary cholesterol internalized by the intestinal epithelial cells and esterified with dietary fatty acids. On the other hand, chylomicron phospholipids contain a considerable amount of fatty acids that are synthesized in the intestine. The cholesterol of chylomicrons is added extracellularly, mainly by transfer from HDLs. In addition, chylomicrons contain small but significant amounts of lipid vitamins.

Chylomicrons are secreted into the intestinal lymph with a single molecule of the truncated (2152 aa residues) form of apo B, apo B48 (Chapter 18). Since apo B does not exchange between lipoprotein particles, the presence of apo B48, which is diagnostic for intestinal TG-rich lipoproteins, can be used to monitor the metabolism of chylomicrons during their subsequent metabolism in the plasma compartment. Newly secreted chylomicrons also contain small amounts of other apolipoproteins. However most of the non-apo B proteins on chylomicrons in plasma have been transferred from other plasma lipoproteins, mainly HDLs (Fig. 2). These proteins include apo C2, the cofactor required for the activity of lipoprotein lipase (LPL), which is the major plasma TG lipase (Section 2). This process, which occurs over ~5 min in the circulation, probably reflects the need for these particles to be fully distributed in the plasma compartment prior to the inception of lipolysis. Since most fatty acids derived from TG are used for oxidative metabolism (mainly by muscle cells) or storage (mainly by adipose tissue) and the rate at which these tissues clear fatty acids from TG-rich lipoproteins is under hormonal control, lipolysis rates are generally determined by the level of lipase activity at the capillary endothelial surface. Intestinal lymph chylomicrons also contain some apo A1. About 10% of plasma apo A1 is thought to be intestinally derived. However, apo A1 is rapidly transferred away from the chylomicron, independent of TG hydrolysis. It seems unlikely that this apo A1 plays any special role in TG metabolism or that its association with the chylomicrons is other than casual. At the same time as apo A1 is lost from the chylomicrons, apo C and apo E are transferred to the chylomicron surface from circulating HDLs.

VLDLs secreted from human liver contain a single molecular of apo B100 which contains 4536 amino acids. (Rodent liver secretes apo B48 in addition to apo B100 — Chapter 18.) The TG-rich core of newly secreted VLDL contains significant amounts of CE which is required for its secretion from rat hepatocytes [1]. Although phosphatidylcholine is the major phospholipid of VLDLs, the phospholipid moiety of newly synthesized VLDL is enriched in phosphatidylethanolamine [2]. This property can be used

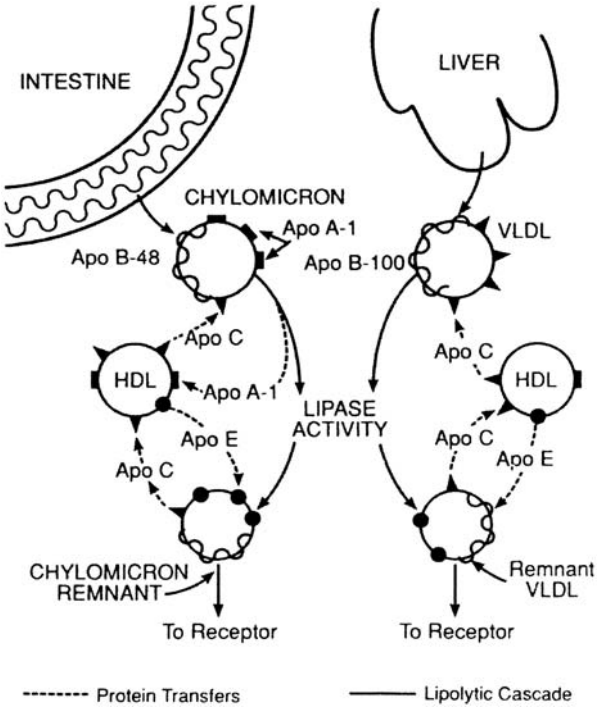

Fig. 2. Transfer of plasma apolipoproteins to newly secreted chylomicrons and VLDLs. These small proteins play a critical role in optimizing the reaction rate of triacylglycerol-rich particles with peripheral lipases and receptor proteins.

to distinguish newly synthesized from partially metabolized VLDL in the plasma compartment. Like chylomicrons, VLDLs are secreted with little apo C and no apo E. As in the case of chylomicrons, the delayed modification of their protein content, which occurs over 5–10 min in plasma, probably reflects the need to distribute VLDL in plasma prior to lipolysis. Since apo E is a ligand that promotes VLDL uptake by hepatic LDL receptors (Chapter 20), the absence of apo E would minimize a futile secretion/reabsorption cycle of newly secreted VLDL at the hepatocyte surface.

A chylomicron or VLDL whose composition is optimized for lipolysis by LPL contains 10–20 molecules of apo C2/molecule of apo B. Titration of apo C2 levels against the rate of lipolysis has shown that no more than two or three apo C2 molecules per particle are needed for maximal TG hydrolysis rates. As lipolysis begins, and the surface area of the VLDL or chylomicron decreases, apo C proteins are displaced. This feature was probably developed to ensure that constant lipolysis rates of TG-rich lipoproteins are maintained until only small amounts of substrate remain, along with its apo B, apo E, and a few apo C proteins. The end-products of chylomicron and VLDL lipolysis (‘remnants’) are removed by the liver via LDL receptors and other receptors (Chapter 20).

2.2. Regulation, structure, and activation of LPL

LPL hydrolyzes sequentially the *sn*-1(3) ester linkages of TG in chylomicrons and VLDLs whose surface contains apo C2. The primary product of LPL-mediated hydrolysis is 2-monoacylglycerol. Since this lipid isomerizes spontaneously, some 1-monoglycerol is formed which is also a substrate for LPL, albeit less effectively than diacylglycerols or TGs. Plasma and platelets contain low but significant levels of monoacylglycerol hydrolase. Monoacylglycerols are also cleared rapidly by vascular cells. As a result the end-products of LPL-mediated TG hydrolysis are unesterified fatty acids, monoacylglycerol, and glycerol. LPL also has significant phospholipase activity, generating 2-lysophosphatides from surface phospholipids. However as TG-rich lipoproteins become smaller under the influence of LPL, the excess of surface phospholipids and cholesterol are, for the most part, transferred to other lipoprotein particles, particularly HDLs.

Owing to the large size of chylomicrons and VLDLs, lipolysis takes place at the vascular endothelial surface although LPL is synthesized in the parenchymal cells of adipose and muscle tissues [3]. These tissues use TG-derived fatty acids for storage and oxidative metabolism, respectively. LPL gene transcription in adipose tissue is enhanced by sterol response element binding protein-1 and is also stimulated by the transcription factor Sp1, and inhibited by Sp3. LPL transcription in adipose tissue is upregulated by peroxisome proliferator activator receptor gamma. A second nuclear receptor, COUP-TFII also activates LPL transcription. Transcription of LPL is down-regulated by the corepressor SMRT. In muscle cells, LPL transcription is more responsive to the transcription factors liver X receptor (LXR) and retinoid X receptor (RXR). These nuclear receptors probably bind directly to the LPL promoter. An additional regulatory mechanism depends on the long, 3′-untranslated tail of LPL mRNA which, in muscle cells, extends for 3.6 kb (rather than 3.2 kb as in adipocytes). The longer transcripts are significantly more active than the shorter transcripts [4]. However, the most important factors regulating the activity of LPL at the vascular endothelial surface of adipose and muscle tissues are post-translational. In particular, the distribution of LPL between parenchymal cell and endothelial cell fractions is highly regulated in response to nutritional and other physiological factors.

The secreted LPL protein contains 448 amino acids [2] and is functional only as a dimer, following Ca^{2+}-dependent self-association. LPL is a member of a lipase family that also includes hepatic lipase (HL), EL, and pancreatic lipase. The latter has been crystallized, and most of what is known about the structure of the serum lipases has been extrapolated from the coordinates of the pancreatic enzyme. Other information has been obtained by site-directed mutagenesis of key residues in the predicted catalytic site, and the receptor- and heparin-binding sites. A major structural feature of LPL and other lipases in this family is the presence of a well-defined ‘lid’ (residues 239–394 in LPL) which opens when the lipase binds to its TG-rich substrate. Heparin binding, a characteristic of LPL, is mediated by the interaction of heparin with two clusters of positively charged residues of LPL (R_{279}–R_{297}, K_{403}–K_{407}) together with additional residues in the C-terminus [5].

2.3. Transport of LPL to its endothelial site

LPL is transported out of parenchymal cells, through the pericyte layer, then through or between the cells of the vascular endothelial monolayer (Fig. 3). Effective secretion and transport of LPL depends on its N-glycosylation. All potential sites are N-glycosylated. The oligosaccharide chain linked to Asn43 appears to be of particular importance. Parenchymal cells take up and degrade part of the LPL they secrete. Two distinct pathways operate for the uptake and degradation of LPL. One pathway is proteoglycan-dependent, the other involves the 39 kDa receptor-associated protein, which binds to members of the LDL receptor family (Chapter 20).

2.4. Structure of the LPL–substrate complex at the vascular surface

Given the very large size of LPL substrate lipoproteins (chylomicrons and VLDLs) and the complex series of events involved in their remodeling in the course of lipolysis, it is

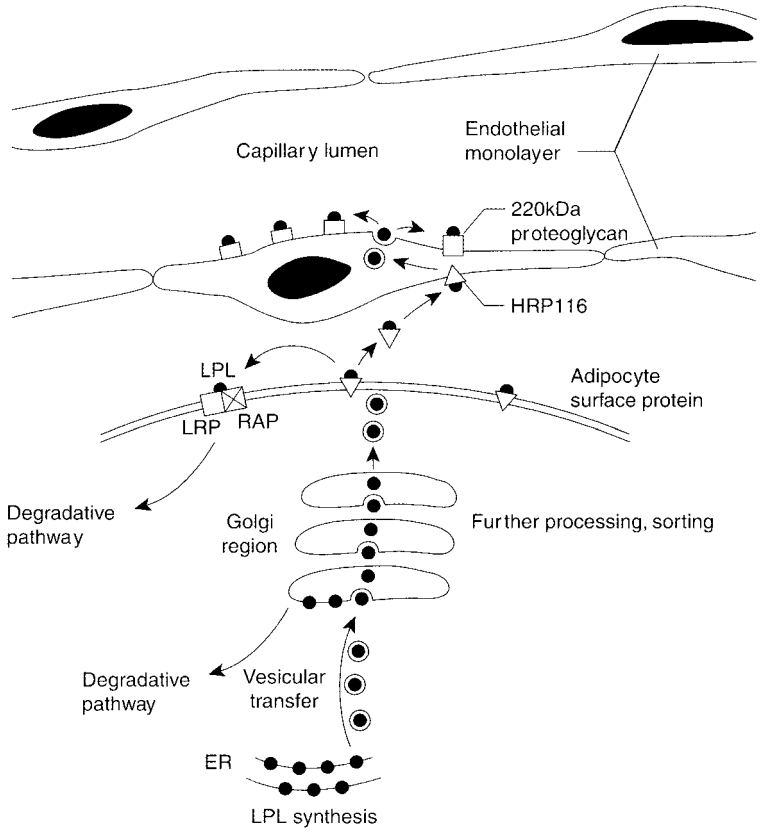

Fig. 3. Synthesis, secretion, and transport of lipoprotein lipase (LPL) from the adipocyte to the vascular endothelial surface of adipose tissue. Degradative pathways from the Golgi compartment and cell surface are illustrated. ER, endoplasmic reticulum; RAP, receptor-associated protein; LRP, LDL receptor-related protein.

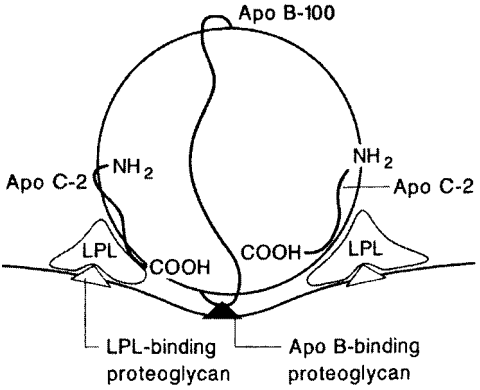

Fig. 4. Model showing the interactions between the endothelial cell surface, triacylglycerol-rich lipoproteins, apo C2, and lipoprotein lipase (LPL). Two LPL molecules are shown reacting with the same VLDL particle. These are representative of the multiple LPLs that probably react with each triacylglycerol-rich lipoprotein. The location of the recently identified glycosylphosphatidylinositol-anchored HDL binding protein-1 within the substrate–lipase complex has not yet been identified.

not surprising that multiple endothelial-surface proteins are implicated in anchoring the substrate to the endothelium, and to LPL (Fig. 4). It is not clear that LPL binds to apo B directly since TG-rich emulsion particles containing apo B and activated by apo C2 are excellent substrates for the enzyme. However, in view of the key regulatory role of apo C2 in lipolysis, direct binding of this cofactor to endothelial LPL is highly likely. From the known catalytic rate of LPL, and the rate of degradation of TG from chylomicrons and VLDLs, it seems likely that multiple LPL molecules are simultaneously active with a single substrate lipoprotein particle. LPL molecules are probably organized into a binding site that includes glycosaminoglycan anchors for both apo B and LPL. LPL also binds to several members of the LDL receptor protein family, though this reaction may be mainly involved in the endocytosis and degradation of LPL. Very recently, an additional endothelial surface protein, glycosylphosphatidylinositol-anchored HDL-binding protein-1, has been implicated as a factor that plays a critical role in the lipolysis of chylomicrons [6]. Since glycosylphosphatidylinositol-anchored proteins are concentrated in sterol-rich lipid-raft domains of the cell surface (Chapter 2), it is possible that the entire LPL binding/functional site is raft-associated. However further research will be needed to establish this.

2.5. Kinetics of the LPL reaction

A newly secreted chylomicron particle contains ~3×10^5 TG molecules. The catalytic rate of TG hydrolysis by LPL is about 10/s. Even if chylomicron TGs were hydrolyzed simultaneously by several LPL molecules, conversion of a chylomicron to a TG-depleted remnant would take 10–15 min, and for a VLDL would take >30 min. A question that has been raised is: does the substrate particle remain bound to LPL at the vascular endothelial surface, or is it released back into the circulation, then rebound and released again,

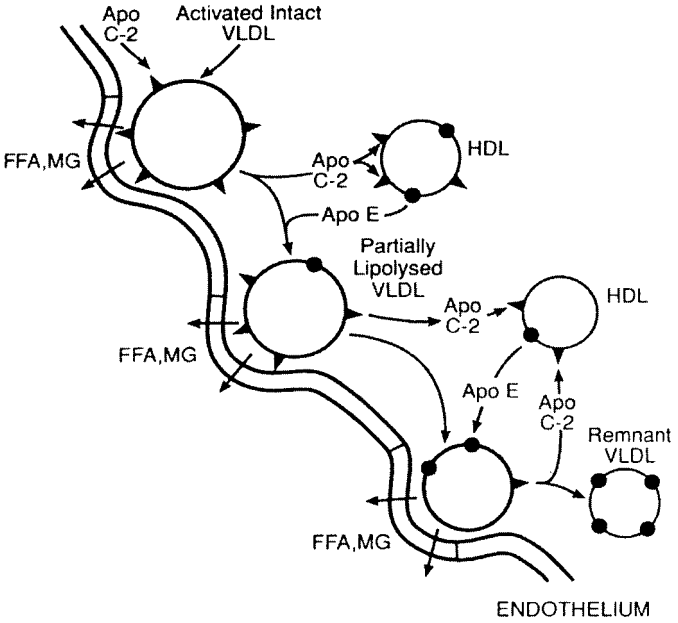

Fig. 5. Mechanism of remnant lipoprotein formation at the endothelial surface. Apo B is not illustrated. FFA, unesterified fatty acid; MG, monoacylglycerol; closed triangles, apo C2; closed circles, apo E. This model reflects the appearance of partially lipolyzed lipoprotein particles in the circulation during lipoprotein lipase-mediated lipolysis of triacylglycerol-rich lipoproteins.

its TG fatty acid load released in quanta at many different hydrolysis sites? Studies with isolated perfused heart and adipose tissues showed convincingly that lipolysis of chylomicrons and VLDLs by LPL does not result from a single binding event, and that as part of a delipidation cascade, partially hydrolyzed particles are detectable in the perfusate (Fig. 5). There has been considerable discussion about the mechanism by which these events occur. During lipolysis, end-products (unesterified fatty acids, monoacylglycerols, and lysophospholipids) are recovered as part of chylomicrons and VLDLs. Each of these lipids is a potential lysogen. Their accumulation could lead to dissociation of the substrate lipoprotein thereby terminating lipolysis by LPL, and the substrate lipoprotein could rebind to the endothelial surface. It seems possible that a mechanism of this kind would be protective for the endothelium. The perfusion experiments also showed that only part of the unesterified fatty acids generated by LPL in a tissue bed is retained there. In vivo, perhaps as much as 50% of the fatty acid product bound to albumin is carried away for uptake at other sites.

LPL plays an important role in directing TG-derived fatty acids for utilization (muscle) or storage (adipose). In addition to the transcriptional regulation of LPL (Section 2.2), the rate of LPL activity is regulated post-translationally and kinetically. In adipocytes, clearance of fatty acids released from TG is decreased during fasting. In fasting adipocytes LPL is secreted in a high-mannose form, which has low specific activity compared to the mannose-trimmed enzyme which is the major product in the fed state. Insulin is an

important determinant of polysaccharide chain processing in adipocytes. Studies with perfused adipose and heart tissues also showed that the apparent K_m of LPL in adipose tissue is significantly higher than that in cardiac tissue. Thus, while the uptake of fatty acids via LPL is always proportional to substrate concentration in adipose tissue, it is saturated in heart tissue even at the low chylomicron and VLDL TG concentrations characteristic of the fasting state.

2.6. Metabolism of TG in chylomicron and VLDL remnants

Though chylomicrons and VLDLs are both substrates for LPL, the processing of the end-products of their metabolism is quite different. Chylomicron remnants recirculate until about 80% of their original TG content has been removed. These remnants retain almost the whole of their content of CE and retinyl ester. Excess surface molecules (mainly apo C proteins, cholesterol, and phospholipids) are transferred from the remnants, either spontaneously or by the activity of phospholipid transfer protein, mainly to HDLs. The chylomicron remnants, with apo E as the major ligand, are cleared quantitatively by hepatic receptors of the LDL receptor family.

The complete catabolism of VLDL is much more complex. Some VLDL remnants (IDL) are cleared by liver LDL receptors, but most (50–70% in normal human metabolism) are further modified in the circulation to form LDLs. Comparison of the composition of IDLs and LDLs indicates that the conversion of IDLs to LDLs requires the loss of 80–90% of the TG of IDLs, as well as some phospholipids, any remaining apo C proteins, and all of the remaining apo E. The unesterified cholesterol content of IDLs and LDLs is similar, while the amount of CE in a LDL particle is significantly greater than in IDL.

It was formerly considered that the conversion of IDL to LDL was mainly a function of HL. This enzyme is located in liver and although it is related structurally to LPL it has greater activity towards partial glycerides and phospholipids than does LPL. HL lacks LPL's dependence on the presence of apo C2. Animals (mice) in which HL was knocked out, and mice and rabbits over-expressing HL, have similar levels of IDL in the circulation, even post-prandially. While a few cases of congenital HL deficiency have been reported in humans, the evidence that this lipase is involved in the processing of chylomicron remnants and/or IDL has remained ambiguous. Currently, it seems likely that the major role played by this lipase is related to optimizing the surface lipid of these particles to promote CE transfer from HDL in exchange for TG in IDL, or in the selective uptake of CE from HDL by the scavenger receptor-BI SR-BI (Chapters 17 and 20), or in the hydrolysis of TG in HDL. These possibilities are evaluated in later sections.

2.7. Congenital deficiencies of lipoprotein TG metabolism

Genetic deficiency of LPL (type I hyperlipidemia) is associated with significant increases in the circulating levels of chylomicrons and VLDLs, and the absence of LPL from post-heparin plasma. VLDLs are increased to a smaller extent than are chylomicrons, probably because intact VLDLs, unlike intact chylomicrons, can be taken up — albeit inefficiently — by the liver. Apo C2 deficiency has a phenotype almost indistinguishable from that of type I hyperlipidemia, demonstrating that the major, or only, function of this apolipoprotein lies

in the promotion of LPL-mediated TG hydrolysis. Deficiency of other proteins linked to the LPL endothelial binding site, such as glycosylphosphatidylinositol-linked HDL binding protein-1 [6], also gives rise to symptoms resembling those of LPL deficiency.

While congenital HL deficiency is associated with increased levels of plasma total TG, the pattern of lipoprotein abnormalities is more variable and less clear-cut than that of LPL deficiency. It seems probable that other plasma and ELs can take over many of HL's functions.

3. HDL and plasma cholesterol metabolism

3.1. The origin of HDL

Unlike apo B lipoproteins, which are secreted in lipid-rich form from the liver and intestine to unload their cargo in the peripheral tissues, apo A1-containing lipoproteins are secreted in lipid-poor form. These lipoproteins accumulate cholesterol and phospholipids, mainly from peripheral tissues, forming HDLs. This cargo is then returned to the liver. There have been many recent developments in our understanding of the synthesis and reactions of HDLs. These findings have modified our understanding of this pathway from one that was largely passive, driven by the concentration gradients of diffusible cellular lipids to apo A1, to one highly regulated by transporters, transfer proteins and receptors, and subject to tight regulation at multiple steps [7].

Like most other plasma apolipoproteins, apo A1, the major protein of all normal HDLs, is organized into a series of amphipathic helical repeats of 11 or 22 amino acids which are linked by helix-breaking proline or glycine residues (Chapter 17). It was earlier thought that the polypeptide chain of apo A1 was flexible at each 'joint'. However, the most recent model of lipid-free apo A1, based on evidence from crystallography, favors a simpler structure, at least for lipid-free and lipid-poor apo A1, in which the primary sequence is organized into six domains, four N-terminal domains (making up about 75% of its length) and two C-terminal domains [8].

The majority of apo A1 is secreted from the liver as a pro-protein that is extended N-terminally by six amino acids. Pro-apo A1 is rapidly cleaved by a circulating metalloproteinase to generate the mature (243 aa) polypeptide. Native pro-apo A1 secreted by human liver-derived cells has physical properties of a typical globular protein and is unable to bind either phospholipids or cholesterol. In the presence of a cell-surface transporter (ABCA1) [9] the mature apo A1 polypeptide is refolded to a more extended form that can bind up to 2 mol of phospholipids/mol apo A1 (Fig. 6). Although ABCA1 is best known as a transmembrane lipid transporter, the protein refolding and phospholipid transport functions of ABCA1 appear to be distinct. Glyburide, an ABC transporter inhibitor, has no effect on folding of apo A1 but completely inhibits phospholipid transport to apo A1. The HDL end-product of pro-peptide cleavage, ABCA1-mediated refolding, and ABCA1-dependent phospholipid transfer has molecular and biological properties that are similar or identical to those of the small, lipid-poor HDL designated pre-beta$_1$-HDLs (i.e., the HDL fraction with pre-beta electrophoretic migration) that represent about 5% of total apo A1 in normal plasma [10].

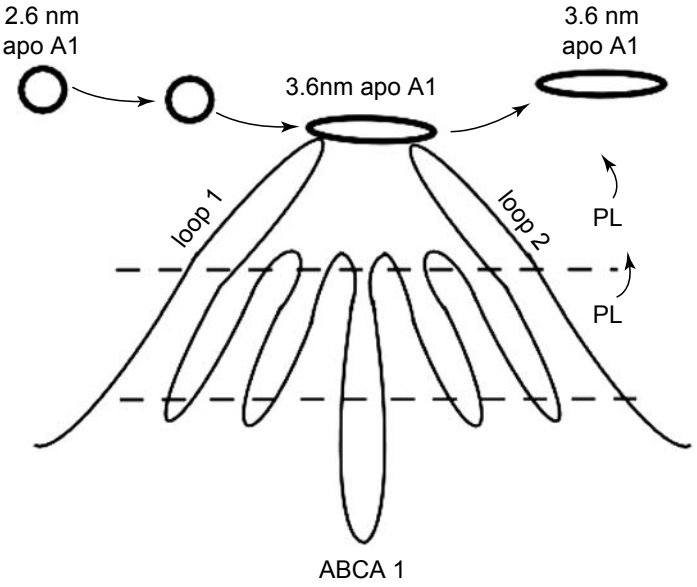

Fig. 6. Steps in the formation of pre-beta$_1$-HDL by human liver-derived cells. Lipid-free apo A1 is refolded by ABCA1 at the cell surface, prior to lipidation by phospholipids. A role for the two major extracellular loops of ABCA1 in apo A1 binding has been indicated by mutational data.

Very little is yet known about how phospholipid binds to apo A1 in native HDL (Chapter 17). Studies of lipid binding by wild type and mutant apo A1 species in vitro suggested important roles for the first (N-terminal) and last (C-terminal) amphipathic helical repeats. Until recently, mature apo A1 isolated from serum HDL by delipidation, urea-denaturation, and dialysis was widely used as a surrogate for native lipid-free apo A1 and/or pre-beta$_1$-HDL. It has become clear, however, that these molecules differ significantly in physical properties. For example, serum-derived apo A1 self-associates whereas pre-beta$_1$-HDL does not. Serum-derived apo A1 can completely unfold to seal the edge of discoidal recombinant particles containing large amounts of phospholipids and cholesterol. In contrast pre-beta$_1$-HDL is not converted to discoidal HDL by additional phospholipid transfer.

3.2. Role of the ABCA1 transporter in HDL genesis

In human Tangier Disease there is an almost complete deficiency of plasma HDL and the ABCA1 transporter protein is functionally defective [9]. Low levels of apo A1 (1–2% of normal) are present, but apo A1 synthesis rates are normal. The phospholipid associated with apo A1 in Tangier plasma is lysophosphatidylcholine rather than phosphatidylcholine. Almost all of the apo A1 in Tangier plasma is present as the pro-peptide, even though pro-peptide converting activity in Tangier plasma is normal and substrate (pro-apo A1) levels are low. In mouse *Abca1*$^{-/-}$ cells, there is a broad reduction of protein export from the

Golgi compartment. These findings suggest a complex role for ABCA1 in cellular lipid homeostasis. The low circulating level of apo A1 in Tangier Disease and the high proportion of pro-protein seem be the result, at least in part, of a clearance rate of lipid-free/lipid-poor apo A1 by the kidney that is much greater than for mature, lipid-filled HDLs.

There has been considerable research on the mechanism of ABCA1-dependent phospholipid transfer (Chapter 17). A consensus has not yet been reached. Apo A1 can bind directly to ABCA1. Studies of the distribution and properties of apo A1 in cells of Tangier Disease patients with mutations in the two large external loops of ABCA1 (Fig. 6) suggest a special role for these domains in apo A1 binding [11]. It is unclear if ABCA1 transfers lipids to apo A1 directly. ABCA1 might instead promote a dynamic imbalance of lipids across the cell surface bilayer thereby reducing the energy required for the spontaneous transfer of lipids from the cell surface to apo A1. Apo A1 is not required for cell membrane lipids to be redistributed under the influence of ABCA1. An additional point of controversy has been whether unesterified cholesterol is a direct substrate for ABCA1 or whether cholesterol is transferred secondarily down a concentration gradient maintained by the active transport of phospholipids. The rate of uncatalyzed cholesterol efflux from cell membranes normally exceeds the rate at which it is sequestered in plasma. As a result there is no obvious role for transporter-driven active cholesterol transport. However simultaneous transfer of cholesterol and phospholipids ('microsolubilization') has not been excluded.

Because of the essential role of ABCA1 in HDL formation, and the inverse correlation established between HDL cholesterol levels and atherosclerotic heart disease, there has been great interest in the relationship between the regulation of ABCA1 expression at the cell surface and the formation of HDLs. Heterodimers of the LXR (activated by oxysterols) and the RXR are major physiological activators of ABCA1 transcription. However, oxysterols strongly inhibit apo A1 transcription from human liver- and intestine-derived cells, which becomes rate-limiting for pre-beta$_1$-HDL formation [12]. This effect was not seen earlier because most studies on the regulation of ABCA1 were performed in the presence of saturating levels of serum-derived apo A1. ABCA1 activity is also regulated post-translationally by fatty acids, by self-association of ABCA1 monomers at the cell surface, and by proteolysis. The complexity of ABCA1 regulation has made it difficult to design drug candidates to stimulate transport of phospholipids and cholesterol out of peripheral cells to increase circulating HDL levels.

3.3. The origin of cellular cholesterol for RCT

There is general agreement that cholesterol transferred to lipid-free/lipid-poor apo A1 originates from the cell surface. The plasma membrane consists of a mosaic of sterol-rich and sterol-poor microdomains (Chapter 1). Sterol-rich microdomains are conveniently classified into two kinds — planar lipid-rafts and invaginated caveolae. Caveolae are stabilized by a structural protein, caveolin. Both caveolae and lipid rafts are assembly sites for transmembrane signaling complexes and other multiprotein associations that function in lipid transport and metabolism at the cell surface. The expression of both types of microdomain is strongly dependent on the cholesterol content of these microdomains. Based on the rigidity imposed on synthetic lipid bilayers by cholesterol, it was suggested

that sterol-poor microdomains, rather than caveolae or lipid rafts, would be more effective at transferring cholesterol to acceptors such as apo A1. There is now evidence that the opposite is the case [13] providing a direct link between RCT and cellular transmembrane signaling. Even transfer of cholesterol to a non-specific acceptor like cyclodextrin selectively depletes cholesterol-rich domains at the cell surface. However cholesterol distribution between cell surface microdomains is very fluid and is responsive to metabolic control. Loss of sterol from caveolae and lipid rafts is compensated in part by adjustments of lipid composition in other cellular pools. As a result, the effects of cholesterol depletion on cellular metabolism are often complex and indirect.

3.4. Role of LCAT in HDL genesis

Like ABCA1, and apo A1 itself, LCAT plays an essential role in the formation of normal HDLs. Deficiency of any of these proteins is associated with reduced circulating levels of apo A1 which is mainly in the form of lipid-free or lipid-poor particles [14].

The LCAT reaction

$$\text{Cholesterol} + \text{phosphatidylcholine} \rightarrow \text{CE} + \text{lysophosphatidylcholine}$$

is strongly dependent on the presence of apo A1. LCAT converts two diffusible substrates to an insoluble product (CE) that is retained in HDLs with apo A1, and a second (lysophosphatidylcholine) that is transferred to a binding site on albumin.

The level of LCAT in the human circulation (5 μg/ml) is such that only one of each hundred HDL particles includes bound LCAT. LCAT deficiency in humans and rodents is associated with the presence in plasma of discoidal HDL particles resembling the synthetic, discoidal apo A1-containing particles that are formed with phospholipids and cholesterol in vitro. Apo A1 discoidal complexes do not have a neutral lipid core and are excellent substrates for the LCAT reaction. These data led to the hypothesis that such discoidal particles were the 'missing link' between lipid-poor, pre-beta$_1$-HDLs, and the spheroidal HDLs with alpha-electrophoretic migration, formed by LCAT; both of these types of apo A1-containing particles are easily identified in plasma. However several pieces of evidence now argue otherwise. For example, discoidal HDLs that circulate in congenital or acquired LCAT deficiency are rich in apo E but bind little if any apo A1, even though the latter is present in pre-beta$_1$-HDLs. This observation suggests that under physiological conditions, apo A1 is poorly incorporated into discoidal particles. It was argued earlier that discoidal HDLs are formed in the extracellular space of peripheral tissues before the action of plasma LCAT. However, recent analyses have reported that few if any discs are present in normal lymph. Furthermore, lipid-poor (pre-beta$_1$) HDLs are excellent direct substrates for LCAT. The conversion of cholesterol and phospholipids to CE draws additional lipid into the HDL complexes in plasma without the intermediary appearance of any discoidal lipoproteins. Thus, the balance of evidence now indicates that discoidal apo A1-HDL is not a normal intermediate of spherical HDL formation. Additional information about how cholesterol in HDL becomes esterified by LCAT was obtained when human plasma was incubated for <1 min with cultured cells that had been labeled to equilibrium with high levels of [^{3}H]cholesterol [15]. When medium was

removed from the cells, the movement of labeled unesterified and esterified cholesterol could be monitored between lipid-poor and larger HDLs. Consistent with earlier reports, a large amount of [^{3}H]cholesterol was recovered at early time points in pre-beta$_1$-HDL, confirming the significance of this HDL fraction as a primary acceptor of cell-derived sterol. Of the LCAT in plasma, about one half was bound to lipid-poor HDLs, even though this fraction of HDL contributed only a few percent of total plasma apo A1 particles. The remainder of the LCAT was recovered with large HDLs (MW_{app} $>$ 250 kDa) even though these particles neither bound nor esterified significant amounts of [3H]cholesterol. In contrast, in the small particles that contained LCAT, cholesterol quickly became highly enriched in [^{3}H]CE. As this process continued, the size of the lipid-poor HDLs enlarged by quanta of about 30 kDa, consistent with a model (Fig. 7) in which lipid-poor HDL subunits fuse to generate HDLs containing two and then three apo A1 molecules (Y. Nakamura, 2005). The largest HDLs in native plasma, which were not labeled with cell-derived [^{3}H]cholesterol, might contain phospholipids and cholesterol originating from the surface of chylomicrons, VLDLs, and LDLs. If this were correct, a significant compartmentation of LCAT in plasma would be indicated so that esterification of newly secreted cell-derived sterol would be favored. The effect of this compartmentalization would be that the effectiveness of RCT would be multiplied. Overall, these data confirm the important role of pre-beta$_1$-HDLs as an essential building block in the synthesis of HDLs from cell-derived cholesterol and phospholipids.

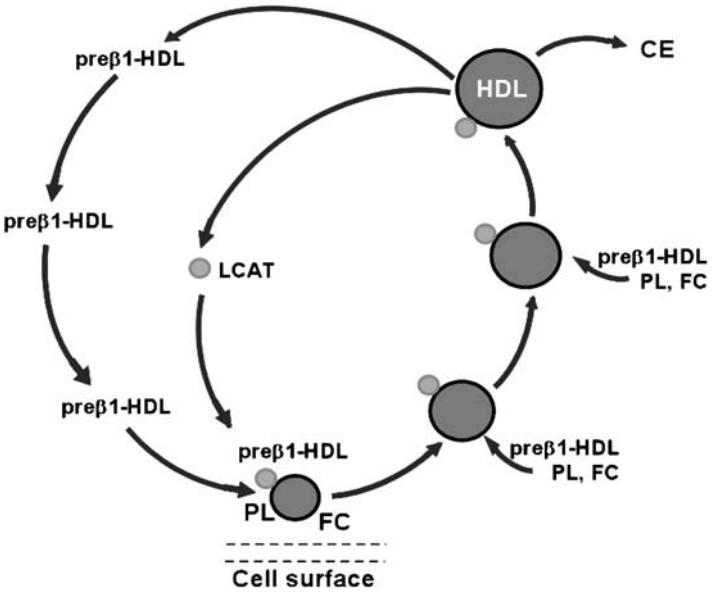

Fig. 7. A model illustrating how pre-beta$_1$-HDL, reacting directly with lecithin:cholesterol acyltransferase (LCAT), may fuse to generate spherical HDLs containing multiple apo A1 subunits. Based on experimental data in Ref. [15].

3.5. Role of ABCG1 in HDL metabolism

In addition to ABCA1, mammalian cells express a number of other ABC transporters. One of these, ABCG1, influences HDL metabolism, particularly in vitro. Over-expression of ABCG1 increased (~1.5-fold) the efflux of cholesterol to centrifugally isolated HDLs. ABCG1 also stimulates cholesterol transport to synthetic phospholipid vesicles and to phospholipid-rich apo A1-containing complexes formed by ABCA1. Nevertheless, HDL cholesterol levels in *Abcg1*$^{-/-}$ mice are within normal limits. The major phenotypic effect of ABCG1 deficiency is the accumulation of phospholipids in lung type II cells [16]. The balance of available evidence suggests that ABCG1 plays only an indirect role in HDL formation.

3.6. Apo A1 recycling and clearance of HDL lipids

Apo A1 recycles extracellularly between lipid-poor (pre-beta-migrating) and lipid-rich (spheroidal) species (Fig. 7). Each spheroidal apo A1 particle transports multiple lipid loads from peripheral tissues to the liver. In contrast other lipid-binding proteins (e.g., apo B and apo E) are cleared by hepatocytes as part of whole lipoprotein particles.

There is strong evidence that the recycling of apo A1 out of large spheroidal HDLs is linked to the selective removal or hydrolysis of its lipids. A number of lipases, lipid transfer proteins, cell-surface receptors, and apolipoproteins have been indicated as key determinants of the rate of recycling based on the hypothesis that loss of core lipids (CE and TG) from large HDL would generate 'excess surface' components (cholesterol, apo A1 and particularly, phospholipids). What is not yet clear is why apo A1 is initially released from cells as a lipid-poor pre-beta$_1$-HDL, instead of as a 'surface remnant' rich in polar lipids and protein reflecting the surface composition of the donor HDLs.

Phospholipid transfer protein is a plasma protein that promotes the transfer of phospholipids down a phospholipid concentration gradient, probably by a shuttle mechanism [17]. Under physiological conditions, this gradient is probably maintained by the phospholipid content of the surface of TG-rich lipoproteins, and by the consumption of phospholipids, along with cholesterol, by the LCAT reaction. In vitro, phospholipid transfer protein promotes the recycling of apo A1 from HDL as evidenced by a significant increase in plasma pre-beta$_1$-HDL. In vivo, an increased amount of plasma phospholipid transfer protein reduces both HDL cholesterol and total apo A1, probably by increasing the renal clearance of lipid-poor HDLs. Phospholipid transfer protein deficiency reduces foam cell formation and apo B secretion in atherosclerosis-prone mice and reduces ABCA1-mediated sterol efflux from macrophages [18].

CETP is another important plasma protein involved in lipoprotein metabolism in the circulation [19]. This protein promotes the transfer of CE down a concentration gradient from HDL, its site of synthesis via the LCAT reaction, to HDLs, VLDLs, and LDLs (in exchange for TG) and to cells expressing the surface scavenger receptor-BI (see below and Chapter 20). In normal human plasma ~60% of CE generated by LCAT is transferred to apo B lipoproteins. The CE/apo B ratio in LDLs is ~1.5–1.7-fold greater than in newly secreted VLDLs, reflecting CETP activity. When CETP activity is reduced, unusually large HDLs accumulate in the circulation without any decrease in LCAT activity or

accumulation of cholesterol in peripheral cells. Over-expression of CETP does not change pre-beta$_1$-HDL levels.

HL is secreted into plasma by the liver and reacts with a wide range of glycerolipid substrates. Unlike LPL, HL activity does not require an apolipoprotein cofactor. The main role of HL was initially thought to be in the catabolism of residual TG in IDL and chylomicron remnants, following the displacement of apo C2. More recently, however, attention has focused on the ability of HL to promote CETP activity and to increase the selective import of CE from HDL by cells via the scavenger receptor-BI on hepatocytes and steroid hormone-producing tissues (adrenal and gonadal cells). EL is a structural relation of LPL and HL that is located on the vascular surface of the endothelium. The major substrate of EL is the phospholipid of HDLs. EL increases the cholesterol/phospholipid ratio of HDLs. However incubation of EL with HDL in vitro does not generate pre-beta$_1$-HDLs.

The scavenger receptor-BI (Chapter 20) promotes the selective uptake of CE from HDLs in the absence of uptake of the whole HDL particle [20]. This receptor also promotes the facilitated exchange of cholesterol between HDLs and cell surfaces. The major sites of expression of scavenger receptor-BI are adrenal and gonadal cells. In mice the scavenger receptor-BI is expressed at a high level in the liver but levels of this receptor are much lower in human liver than in rodent liver. Increased expression of the receptor promotes RCT, as indicated by a significant increase in cholesterol clearance from the plasma compartment, and increased bilary excretion of sterols and bile acids despite decreased circulating HDL cholesterol and apo A1 levels [20]. The decrease in apo A1 probably reflects an increased production, and renal filtration, of small lipid-poor HDLs.

Another plasma protein that is involved in lipoprotein metabolism is apolipoprotein M. Apo M is a small (22 kDa) apolipoprotein mainly associated with HDLs [21]. Levels of apo M in human plasma (20–35 μg/ml or less than) are similar to those of apo A2, the second most abundant HDL protein in the circulation. In apo M knock-out mice, pre-beta-HDL is undetectable. These mice accumulated abnormally large plasma HDLs and showed increased susceptibility to atherosclerosis. Apo M over-expression in mice was associated with increased pre-beta$_1$-HDL levels in plasma. Apo M in the circulation retains an uncleaved hydrophobic leader sequence that may play a role in anchoring this protein to HDLs. The molar concentration of apo M in plasma is similar to that of apo A1 in pre-beta$_1$-HDLs. Partial displacement of apo A1 by apo M on the surface of large HDLs may be sufficient to stimulate RCT. Direct evidence for a role of apo M in human apo A1 recycling is, however, presently lacking.

Because of a rapid equilibrium between HDL 'core' and 'surface' lipids, CETP, phospholipid transfer protein, HL, EL, and scavenger receptor-BI each has the potential to modify the HDL surface lipid composition, and indirectly alter the affinity of apo A1 for HDL (Fig. 8). There is presently insufficient evidence to assess the relative significance of these factors in promoting apo A1 recycling or RCT either in human plasma or in mice. The most direct link between recycling and pre-beta$_1$-HDL is that now linked to apo M in mice.

3.7. Other functions of HDLs

The preceding sections focused on the role of HDLs in cholesterol transport and the central role of apo A1 in regulating RCT. Recently other protective functions of HDLs — against

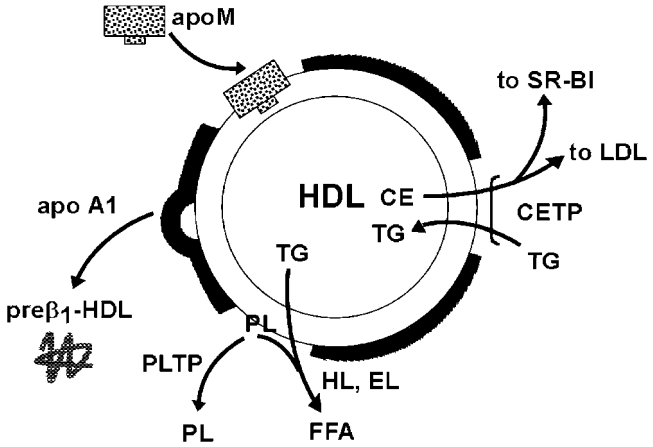

Fig. 8. A model suggesting roles for scavenger receptor-BI (SR-BI), CETP, PLTP, HL, and apo M in the recycling of pre-beta$_1$-HDL from large HDLs. Probable roles for these factors are supported by data but their quantitative contributions have not been determined. PLTP, phospholipid transfer protein; FFA, fatty acid. Other abbreviations are defined in the list of abbreviations.

oxidation, infection, and inflammation — have been documented [22,23]. These functions are of course indirectly related to RCT because without the activities of apo A1, ABCA1, and LCAT there would be no HDLs in the circulation to support these functions. Nevertheless, factors promoting apo A1 recycling may be different from those that depend on HDL cholesterol levels. Large HDLs are the preferred scaffold for enzymes such as paraoxonases and platelet activating factor hydrolase (Chapter 9) that can degrade oxidized phospholipids that promote apoptosis. As part of the inflammatory response, these protective enzymes are displaced from HDLs and significant changes occur in HDL lipid composition.

3.8. Animal models of human HDL metabolism

The availability of animal models in which individual genes have been over-expressed or knocked out has had a wide impact on our understanding of HDL metabolism because the expression of a single enzyme or transport protein can be studied in vivo against a background of interactive factors. In some cases, a mouse gene has been knocked out and replaced by its human equivalent. Many of these studies were initiated to estimate the role of individual genes in promoting or inhibiting atherosclerosis. Experimental problems include significant differences in the transcriptional regulation, expression, and physiology of lipid genes in humans and mice. Rapidly induced experimental atherosclerosis in mice (e.g., in LDL-receptor-deficient mice or apo E-deficient animals) may have modest relevance to the decades-long evolution of human vascular disease.

Apo A1$^{-/-}$, *Abca1*$^{-/-}$, and *Lcat*$^{-/-}$ mice have severe reductions in circulating HDL levels, illuminating the key roles of these factors in normal HDL genesis, and emphasizing the inability of other apolipoproteins to substitute for apo A1 in HDL, despite many overall similarities of structure. In contrast, it has been difficult to establish the roles of many of the proteins in HDL recycling. CETP is normally absent from mice which might explain

the predominant role of the scavenger receptor-BI in clearing HDL CE from the plasma of these animals. In human plasma, where CETP is active, the contribution of SR-BI to HDL CE clearance is diminished.

4. Summary and future directions

The identification of new apolipoproteins, enzymes, and receptors related to plasma lipoprotein metabolism has continued. The overall effect of these discoveries has been to significantly increase our understanding of the complex regulation metabolism of TGs, phospholipids, and cholesterol in plasma. The availability of mice over-expressing or deficient in individual lipid transport factors has provided some key insights, as has increasingly sophisticated identification of human gene defects and their effects on lipoprotein metabolism. Nevertheless, in several areas major gaps remain. Little is still known of the organization of binding proteins and glycosaminoglycans that contribute to the binding of lipases to the endothelium. The organization of cholesterol within microdomains of the cell surface, and the interaction of these domains with the RCT pathway, is not well understood. Finally the whole area of HDL recycling — its mechanism and its regulation — is under-investigated. Ample targets remain for future research.

Abbreviations

ABC	ATP-binding cassette
Apo	apolipoprotein
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
EL	endothelial lipase
HDL	high-density lipoprotein
HL	hepatic lipase
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
LPL	lipoprotein lipase
RCT	reverse cholesterol transport
TG	triacylglycerol
VLDL	very low-density lipoprotein

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CHAPTER 20

Lipoprotein receptors

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Contents

1. Introduction

The main task of lipoprotein receptors in systemic lipid metabolism is the clearance of lipoproteins from the circulation, body fluids, and interstitial spaces. There are several reasons why lipoproteins need to be cleared from extracellular fluids into cellular compartments, for instance (i) they are transport vehicles for components that are vital to the target cells, (ii) their uptake serves signaling and/or regulatory roles in cellular metabolism, (iii) they have done their job and have become dispensable, and (iv) they might have deleterious effects if allowed to remain extracellular for a prolonged time. Chapters 17, 18, and 19 have described the structures, syntheses, and interconversion pathways of lipoprotein particles in the circulation. Here, the players in and mechanisms of lipoprotein transport from the plasma compartment to various types of cells of the body are described. In addition, newly discovered functions of receptors thus far thought to be specialized exclusively for lipoprotein transport are summarized.

When considering lipoprotein transport via cell surface receptors, an important aspect is that the two major transported lipid components of lipoproteins, triacylglycerols and cholesterol (unesterified and/or esterified), have quite different fates. Triacylglycerols are delivered primarily to adipose tissue and muscle where their fatty acids are stored or oxidized for production of energy, respectively. Cholesterol, in contrast, is continuously shuttled among the liver, intestine, and other extrahepatic tissues. The major transport form of cholesterol is its esterified form; within cells the cholesteryl esters are hydrolyzed and the unesterified sterol has multiple uses. Among their many functions, sterols serve as structural components of cellular membranes, as substrates for the synthesis of steroid hormones and bile acids, and they perform several regulatory functions (a classical example is the low-density lipoprotein (LDL) receptor pathway, Section 2.2). For correct targeting of lipoproteins to sites of metabolism and removal, the lipoproteins rely heavily on the apolipoproteins (apos) associated with their surface coat. Apos mediate the interaction of lipoprotein particles with enzymes, transfer proteins, and with cell surface receptors, the topic of this chapter.

Key features of human lipoprotein metabolic pathways are schematically summarized in Fig. 1; this outline necessarily omits many of the details, which are less significant for aspects of receptor-mediated removal of lipoproteins. The interwoven complex pathways can be divided into exogenous and endogenous branches, concerned with the transport of dietary and liver-derived lipids, respectively. Both metabolic sequences start with the production and secretion of triacylglycerol-rich lipoproteins (Chapter 18). Intestinally derived chylomicrons (Fig. 1) are secreted into the lymph and from there enter the bloodstream, where they function as energy carriers by providing triacylglycerol-derived fatty acids to peripheral tissues. This lipolytic extraction of fatty acids from the triacylglycerol-core of the lipoprotein particles ('Lipolysis' in Fig. 1) is achieved mainly by the enzyme lipoprotein lipase, which is bound to the lumenal surface of the endothelial cells lining the capillary bed. Removal of triacylglycerol in extrahepatic tissues results in decreased size of the chylomicrons and produces cholesteryl ester-rich lipoprotein particles termed chylomicron remnants. During this conversion, apos C are lost from the surface of the particles; the remnants, having finished their task, are destined for catabolism by the liver, which occurs almost exclusively by receptor-mediated processes.

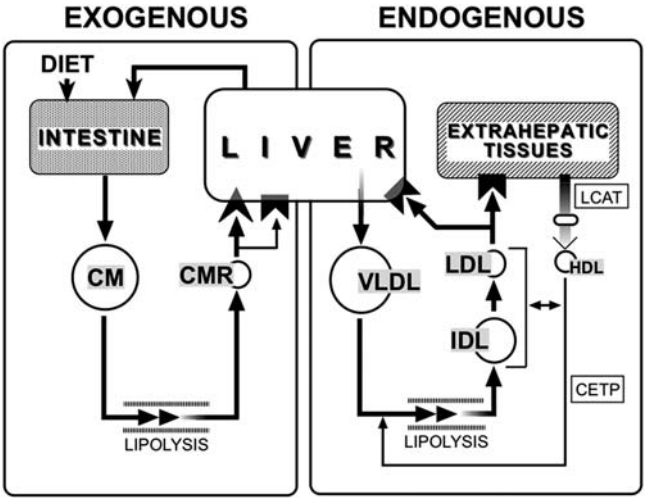

Fig. 1. Simplified schematic summary of the essential pathways for receptor-mediated human lipoprotein metabolism. The liver is the crossing point between the exogenous pathway (left-hand side), which deals with dietary lipids, and the endogenous pathway (right-hand side) that starts with the hepatic synthesis of VLDL. The endogenous metabolic branch starts with the production of chylomicrons (CM) in the intestine, which are converted to chylomicron remnants (CMR). Very-low-density lipoprotein particles (VLDL) are lipolyzed to LDL particles, which bind to the LDL receptor. IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; ⮝, LDL receptor-related protein (LRP1); and ⮟, LDL receptor. 'Lipolysis' denotes lipoprotein lipase-catalyzed triacylglycerol lipolysis in the capillary bed.

Both the so-called LDL receptor-related protein (⮟ in Fig. 1, and Section 3.1) and the LDL receptor (⮝ in Fig. 1, and Section 2) mediate their removal via recognition of apo E. The apo B48, which resides on chylomicrons throughout their life span, is not recognized by these receptors.

In analogy to the exogenous lipid transport branch, the endogenous pathway begins with the production and secretion of triacylglycerol-rich lipoprotein particles by the liver, here termed very-low-density lipoprotein (VLDL). In contrast to chylomicrons, VLDL contains apo B100, in addition to the apos C and E. Lipoprotein lipase in the capillary bed hydrolyzes triacylglycerol of secreted VLDL, but less efficiently than from chylomicrons, which is likely one of the reasons for slower plasma clearance of VLDL ($t_{1/2}$, days) compared to chylomicrons ($t_{1/2}$, minutes to a few hours). Lipolysis during the prolonged residency of VLDL particles in the plasma compartment generates intermediate-density lipoproteins (IDL) (Fig. 1) and finally, LDL. In parallel, apos and surface components (mostly phospholipids and unesterified cholesterol), and also cholesteryl esters and triacylglycerol, are subject to transfer and exchange between particles in the VLDL lipolysis pathway and certain species of high-density lipoproteins (HDLs). In addition, the so-called VLDL receptor (Section 3.2) appears to deliver fatty acids to a limited set of peripheral tissues. Enzymes involved in cholesterol loading and esterification, and in interparticle-transfer reactions are lecithin:cholesterol acyltransferase (LCAT),

cholesteryl ester transfer protein, and phospholipid transfer protein (Chapter 19). IDL particles (which still harbor some apo E) to a variable degree, and LDL as the end product of VLDL catabolism in the plasma, are catabolized via the LDL receptor. This receptor is found in the liver (which harbors 60–70% of all the LDL receptors in the body) as well as in extrahepatic tissues, and is a key regulatory element of systemic cholesterol homeostasis (Section 2.2). Thus, steady-state plasma LDL levels are not only the result of lipoprotein receptor numbers, but also are influenced by the rate of VLDL synthesis, the activity of lipoprotein lipase and other lipases, the VLDL receptor, and by other metabolic processes.

As far as HDL levels and metabolism are concerned, one result of the LCAT- and transfer protein-catalyzed reactions is the production of a dynamic spectrum of particles with a wide range of sizes and lipid compositions (Chapter 19). Nascent HDL particles contain mostly apo A1 and phospholipids, and undergo modulation and maturation in the circulation. For instance, the unesterified cholesterol incorporated into plasma HDL is converted to cholesteryl esters by LCAT, creating a concentration gradient of cholesterol between HDL and cell membranes, which is required for efficient cholesterol efflux from cells to HDL. In addition, cholesteryl ester transfer protein transfers a significant amount of HDL cholesteryl ester to VLDL, IDL, and LDL for further transport, primarily to the liver. Thus, a substantial fraction of cell-derived cholesterol is delivered as part of HDL indirectly to the liver via hepatic endocytic receptors for IDL and LDL; this process is termed ‘reverse cholesterol transport’. However, receptor-mediated delivery of HDL cholesterol to cells is fundamentally different from the classic LDL receptor-mediated endocytic pathway, as described in Section 7.3.2.

In addition to receptor-mediated metabolism of lipoproteins, which is the predominant mechanism for removal of intact lipoproteins, individual components of lipoproteins, particularly unesterified cholesterol, can diffuse into cells across the plasma membrane. Other minor uptake processes include so-called fluid-phase endocytosis, which does not involve binding of lipoproteins to specific cell surface proteins, and phagocytosis, in which lipoproteins attach to the cell surface via more or less specific forces, and are subsequently engulfed by the plasma membrane.

2. Removal of LDL from the circulation

The supply of cells with cholesterol via receptor-mediated endocytosis of LDL is one of the best characterized processes of macromolecular transport across the plasma membrane of eukaryotic cells. The following sections describe this process, provide an overview of the biochemical and physiological properties of the LDL receptor, and discuss the molecular basis for the genetic disease, familial hypercholesterolemia (FH).

2.1. Receptor-mediated endocytosis

This multistep process, originally defined as a distinct mechanism for the cellular uptake of macromolecules, emerged from studies to elucidate the normal function of LDL by M.S. Brown and their colleagues [1]. The salient features of the itinerary of an LDL

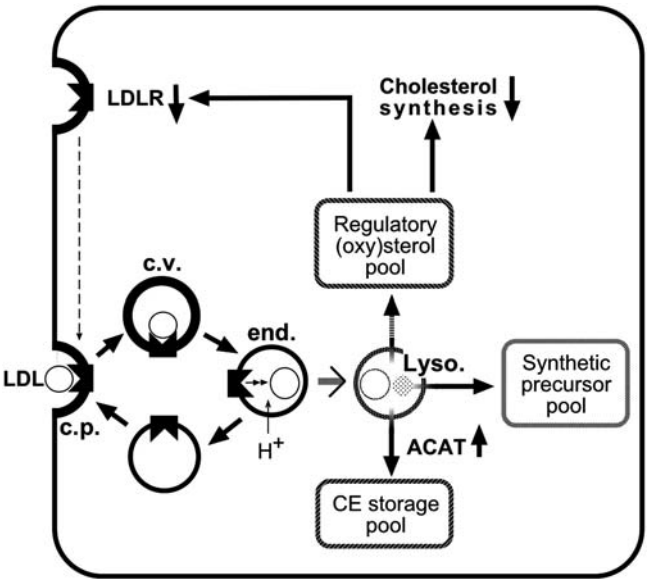

Fig. 2. The LDL receptor pathway: regulation of cellular cholesterol homeostasis. LDL receptors (LDLR) are synthesized in the endoplasmic reticulum, undergo post-translational modification in the Golgi compartment, and travel to the cell surface, where they collect in coated pits (c.p.). LDL particles bound to LDL receptors (⮝) are internalized in coated vesicles (c.v.), which become uncoated and acidified by protons (H^+) being pumped into their lumen, resulting in endosomes (end.) in which the LDLs dissociate from the receptors due to the low pH. From there, the LDLs are delivered to lysosomes (Lyso.), but almost all of the receptors travel back to the cell surface (where they become incorporated into c.p. again) within a recycling vesicle. Lysosomal degradation of LDL results in the complete breakdown of apo B100 and liberation of cholesterol via hydrolysis of cholesteryl esters. The LDL-derived cholesterol has three main fates: (a) it is reconverted to cholesteryl esters via stimulation of acyl-CoA:cholesterol acyltransferase (ACAT) for storage in droplets (CE storage pool; bottom); (b) it is used as biosynthetic precursor for bile acids, steroid hormones, and membranes (synthetic precursor pool, right-hand side); and (c) it serves, especially if converted to oxysterols (top), several regulatory functions. The most important of these are suppression of cholesterol synthetic enzymes and decrease in the production of LDL receptors.

particle (mean diameter ~22 nm) from the plasma into a normal human fibroblast are summarized in Fig. 2. First, the lipoprotein particle binds to one of the approximately 15,000 LDL receptors on the surface of the cell. LDL receptors are not evenly distributed on the cell surface; rather, up to 80% are localized to specialized regions of the plasma membrane comprising only 2% of the cell surface. These regions form pits and are lined on their cytoplasmic side with material that in electron micrographs has the appearance of a fuzzy coat. Each of these so-called 'coated pits' contains several kinds of endocytic receptors in addition to LDL receptors, but LDL particles bind only to 'their' receptors, due to their extremely high affinity and specificity. Next, the receptor/LDL complex undergoes rapid invagination of the coated pit, which eventually culminates in the release of the coated pit into the interior of the cell. At this point, the coated pit has been transformed into an endocytic 'coated vesicle,' a membrane-enclosed organelle that is coated on its exterior (cytoplasmic) surface with a polygonal network of fibrous protein(s),

the main structural component of which is a fascinating protein called clathrin [1]. Subsequently, the coat is rapidly removed, in concert with acidification of the vesicles' interior and fusion with other uncoated endocytic vesicles. Transiently, LDL and the receptor are found in smooth vesicles in which the lipoprotein particles dissociate from the receptor due to the acidic environment. LDL is then delivered to lysosomes, where it is degraded, while the receptor escapes this fate and recycles back to the cell surface, homes in on a coated pit and is ready to bind and internalize new ligand molecules [1].

There are variations to the theme, as ligand degradation and receptor recycling are not coupled in all systems of receptor-mediated endocytosis. However, all systems have in common the initial steps leading to the formation of endosomes. Then, the receptors are either degraded, recycled back to the cell surface, or are transported (for example, across polarized cells); their respective ligands can follow the same or divergent routes [1]. The reutilization of the LDL receptor via recycling constitutes an economical way to ensure efficient removal of LDL from the extracellular space.

2.2. The LDL receptor pathway

The LDL receptor is a key component in the feedback-regulated maintenance of cholesterol homeostasis [1]. In fact, as an active interface between extracellular and intracellular cholesterol pools, it is itself subject to regulation at the cellular level (Fig. 2). LDL-derived cholesterol (generated by hydrolysis of LDL-borne cholesteryl esters) and its intracellularly generated oxidized derivatives mediate a complex series of feedback control mechanisms that protect the cell from over-accumulation of cholesterol. First, (oxy)sterols suppress the activities of key enzymes that determine the rate of cellular cholesterol biosynthesis. Second, the cholesterol activates the cytoplasmic enzyme acyl-CoA:cholesterol acyltransferase, which allows the cells to store excess cholesterol in re-esterified form. Third, the synthesis of new LDL receptors is suppressed, preventing further cellular entry of LDL and thus cholesterol overloading. The coordinated regulation of LDL receptors and cholesterol synthetic enzymes relies on the sterol-modulated proteolysis of a membrane-bound transcription factor, SREBP, as described in Chapter 14.

The overall benefits from, and consequences of, this LDL receptor-mediated regulatory system are the coordinated utilization of intracellular and extracellular sources of cholesterol at the systemic level. Mammalian cells are able to subsist in the absence of lipoproteins because they can synthesize cholesterol from acetyl-CoA. When LDL is available, however, most cells primarily import LDL cholesterol and keep their own synthetic activity suppressed. Thus, a constant level of cholesterol is maintained within the cell while the external supply in the form of lipoproteins can undergo large fluctuation.

Most of these concepts have arisen from detailed studies in cultured fibroblasts from normal subjects and from patients with the disease, FH. Lack of the above-described regulatory features in FH fibroblasts led to the conclusion that the abnormal phenotype is caused by lack of LDL receptor function, and thus, disruption of the LDL receptor pathway. In particular, the balance between extracellular and intracellular cholesterol pools is disturbed. Clinically, the most important effect of LDL receptor deficiency is hypercholesterolemia with ensuing accelerated development of atherosclerosis and its complications (Chapter 21). In the following sections, a detailed description of the

LDL receptor is provided, with emphasis on the impact of mutations on its structure and function.

2.3. Relationships between structure and function of the LDL receptor

Studies at the levels of protein chemistry, molecular biology, and cell biology have led to a detailed understanding of the biology of the LDL receptor. The mature receptor is a highly conserved integral membrane glycoprotein of 839 residues consisting of five domains (Fig. 3). In order of appearance from the amino terminus, these domains are (i) the ligand-binding domain, (ii) a domain that has a high degree of homology with the epidermal growth factor (EGF) precursor, (iii) a domain that contains a cluster of O-linked carbohydrate chains, (iv) a transmembrane domain, and (v) a short cytoplasmic region. A highly schematized view of the arrangement of these domains is presented in Fig. 3.

The ligand-binding domain. This domain mediates the interaction between the receptor and lipoproteins containing apo B100 and/or apo E [2]. The domain, at the amino terminus

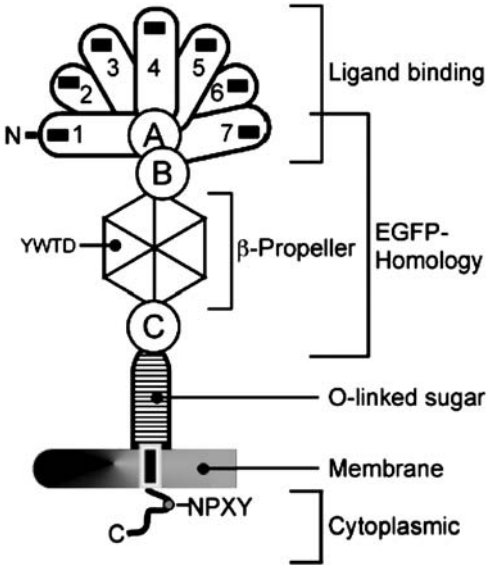

Fig. 3. Domain model of the LDL receptor. The five domains of the mature protein, from the amino terminus (N) to the carboxy terminus (C) are (i) the ligand-binding domain, characterized by seven cysteine-rich repeats containing clusters of negatively charged amino acids whose core consists of Ser–Asp–Glu repeats 2–7 cooperatively bind apo B100 and apo E; (ii) the epidermal growth factor precursor region (EGFP-Homology), consisting of approximately 400 amino acid residues; adjacent to the ligand-binding domain and at the carboxy terminus of this region, respectively, are located three repeats with high homology to repeat motifs found in the precursor to epidermal growth factor (encircled letters A, B, and C). The remaining portion of this domain, termed β-propeller, consists of six internally homologous stretches of approximately 50 amino acid residues, each of which contains the sequence Tyr–Trp–Thr–Asp (YWTD); (iii) the O-linked sugar domain, consisting of 58 amino acids with 18 serine and threonine residues containing O-linked carbohydrate chains; (iv) a single membrane-spanning domain; and (v) the cytoplasmic tail with 50 amino acid residues containing the internalization sequence Asn–Pro–Val–Tyr ('NPXY'; the Val is not absolutely conserved in all species).

of the receptor, is comprised of seven repeats of approximately 40 residues each, called ligand-binding repeats. These repeats have six cysteines each, which presumably mediate the folding of each repeat into a compact structure with clusters of negatively charged residues with the signature tripeptide Ser–Asp–Glu (SDE) on its surface. The linkers between the individual repeats provide flexibility to the domain to accommodate lipoprotein ligands of different sizes via positively charged residues on apo B100 (apo B) or apo E [3].

The epidermal growth factor precursor homology domain. This region of the LDL receptor lies adjacent to the ligand-binding site and is comprised of approximately 400 amino acids; the outstanding feature is the sequence similarity of this region to parts of the EGF precursor, i.e., three regions termed 'growth factor repeats' (also called type B repeats). Two of these repeats are located in tandem at the amino terminus, and the third is at the carboxy terminus of the precursor homology region of the LDL receptor. The remainder consists of modules of about 40 residues with a consensus tetrapeptide, Tyr–Trp–Thr–Asp (YWTD); six of these modules, tandemly arranged, form a so-called six-bladed beta-propeller. In turn, the beta-propeller and the type B repeats constitute the EGF precursor homology domain. X-ray crystallographic studies suggest that the acidic conditions in the endosome (pH 5.0) cause a conformational change that expels LDL from the binding domain via close apposition of binding repeats 4 and 5 and the beta-propeller [4].

The O-linked sugar domain. This domain of the human LDL receptor is a 58-amino acid stretch enriched in serine and threonine residues, located just outside the plasma membrane. Most, if not all, of the 18 hydroxylated amino acid side chains are glycosylated. The O-linked oligosaccharides undergo elongation in the course of receptor synthesis and maturation. When leaving the endoplasmic reticulum, *N*-acetylgalactosamine is the sole O-linked sugar present, and upon processing in the Golgi, galactosyl and sialyl residues are added. Despite the detailed knowledge about the structure of this region, its functional importance remains unclear [5].

The membrane-anchoring domain. This domain lies carboxyterminally to the O-linked carbohydrate cluster and consists of ~20 hydrophobic amino acids. As expected, deletion of this domain in certain naturally occurring mutations, or by site-directed mutagenesis, leads to secretion of truncated receptors from the cells.

The cytoplasmic tail. This domain of the LDL receptor constitutes a short stretch of 50 amino acid residues involved in the targeting of LDL receptors to coated pits. Naturally occurring mutations and site-specific mutagenesis (M.A. Lehrman, 1987) have identified an 'internalization signal', Asn–Pro–Xxx–Tyr (NPxY in Fig. 3, where Xxx denotes any amino acid). Recently, the cytoplasmic domains of the LDL receptor and structural relatives have come into new focus, since they hold the key to the involvement of these receptors in signal transduction (Sections 5.2., 5.3., and 6.1). For further details on these aspects, see Ref. [6].

2.4. The human LDL receptor gene: organization and naturally occurring mutations

The ~48-kb human LDL receptor gene contains 18 exons and is localized on the distal short arm of chromosome 19. There is a strong correlation between the functional domains

of the protein and the exon organization of the gene. For instance, the seven ligand-binding repeats of the domain are encoded by exons 2 (repeat 1), 3 (repeat 2), 4 (repeats 3–5), 5 (repeat 6), and 6 (repeat 7). The EGF precursor homology domain is encoded by eight exons, organized in a manner very similar to the gene for the EGF precursor itself. The O-linked sugar domain is translated from a single exon between introns 14 and 15. Thus, the LDL receptor gene is a compound of shared coding sequences; in fact, many more molecules containing all or some of these elements have been discovered. Membrane proteins with clusters of ligand-binding repeats in their extracellular domains are now recognized as relatives of the so-called LDL receptor gene family. Fig. 4 depicts a summary of the simplified schematized structures of these membrane proteins.

Molecular genetic studies in FH patients have identified close to 700 different mutations in the LDL receptor gene. Comprehensive information about LDL receptor mutations in FH patients can be found at http://www.ucl.ac.uk/fh/, and a useful reference for LDL receptor mutations in Europe is Ref. [7]. In order to gain insight into the nature of these mutations, they are grouped into five classes according to their effects on the protein as follows.

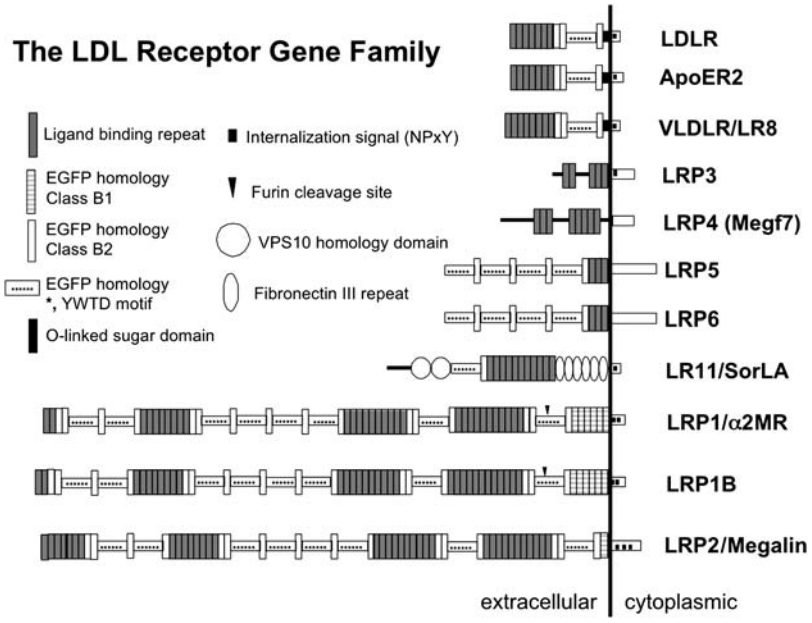

Fig. 4. The LDL receptor gene family. The structural building blocks making up these proteins are listed in the left-hand top part (for more details, see Fig. 3). Presumed extracellular domains are depicted to the left of the plasma membrane (black vertical line). The standard modules are negatively charged ligand-binding repeats with six cysteines each; epidermal growth factor precursor (EGFP) homology repeats (in the entire family, two subclasses with slightly different consensus sequences are distinguished, termed B1 and B2; these repeats also contain six cysteines each); the 'YWTD' motifs within the β-propeller structure of EGFP homology domains; the O-linked sugar domains, just outside the plasma membrane, typical for LDL receptor, apoER2, and VLDL receptor/LR8; and the consensus or presumed internalization signals, (FD)NPxY. Several large members of the gene family harbor consensus furin cleavage sites; LR11 (Section 6.2) contains two VPS10 domains and six fibronectin type III repeats, domains not found in other relatives.

2.4.1. Class 1: null alleles — no detectable receptor
These mutant alleles fail to produce receptor proteins; cells carrying these mutations do not bind any LDL in saturable fashion. This phenotype arises from point mutations causing premature termination codons early in the protein-coding region, mutations in the promoter region that block transcription, mutations that lead to abnormal splicing and/or instability of the mRNA, and large deletions.

2.4.2. Class 2: slow or absent processing of the precursor
These alleles, accounting for over half of all mutant LDL receptor alleles, specify transport-deficient receptor precursors that fail to move with normal rates from the endoplasmic reticulum to and through the Golgi apparatus and on to the cell surface. While some mutations merely attenuate processing, most of these mutations are complete in that transport from the endoplasmic reticulum fails and the mutant receptors never reach the cell surface.

2.4.3. Class 3: defective ligand binding
These receptors in general reach the cell surface at normal rates, but are unable to bind LDL efficiently due to subtle structural changes in or close to the ligand-binding domain. By definition, these mutant receptors undergo the normal maturation process.

2.4.4. Class 4: internalization-defective
One of the pre-requisites for effective ligand internalization — localization of LDL receptors to coated pits — is not met. The failure of these 'internalization-defective' receptors to localize to coated structures results from mutations that alter the carboxy-terminal domain of the receptor. Variants of class 4 mutations have large deletions that lead to a lack of both the cytoplasmic and transmembrane domains. The majority of these mutant truncated proteins are, as expected, secreted.

2.4.5. Class 5: recycling-defective
The classification of these mutations into a separate class is based on the observation that deletion of the first two EGF precursor domains of the human LDL receptor allows the truncated receptor to bind and internalize ligand. However, as suggested by recent observations [4], release of LDL in the acidic environment of the endosome is blocked, and the mutant receptor is rapidly degraded. All class 5 mutants affect the EGF precursor homology domain, and most often the YWTD modules thereof.

In summary, to a large extent through the delineation of natural mutations in the LDL receptor gene, structural as well as regulatory features of receptor-mediated metabolism of the major cholesterol-carrying lipoprotein in human plasma are now thought to be well understood. Nevertheless, very recent studies have revealed that there are additional mechanisms for control of LDL receptor activity; two of these modulatory mechanisms and their key components are outlined in the next section.

2.5. Disease-related modulators of LDL receptor activity

Both molecules described below have been identified in studies of FH proven not to be caused by mutations in the LDL receptor gene itself.

2.5.1. Autosomal recessive hypercholesterolemia (ARH)

ARH is characterized by defects in LDL receptor endocytosis, which manifest in specific cell types such as lymphocytes and liver cells but not in fibroblasts (H.H. Hobbs, 2005). The phenotype is caused by homozygous mutation in *ARH*, which encodes an adapter protein that interacts with the cytosolic domain of the LDL receptor, phosphoinositides, and two important components of the endocytosis machinery, i.e., clathrin and adapter protein-2 (AP-2). ARH must bind, via its phosphotyrosine-binding domain, to LDL receptor tail and either clathrin or AP-2 to facilitate normal LDL receptor clustering and internalization. In fibroblasts, ARH deficiency appears to be compensated by a mechanism likely mediated by disabled-2 (Dab-2), which may not operate in lymphocytes and hepatocytes. ARH homozygotes have LDL cholesterol levels that in general are in the range of receptor-defective FH homozygotes and are lower than receptor-negative FH homozygotes.

2.5.2. Proprotein convertase subtilisin-like kexin-type 9 (PCSK9)

PCSK9 accelerates the degradation of hepatic LDL receptors. The initial surprising finding was that gain-of-function mutations cause hypercholesterolemia, while loss-of-function mutations in *PCSK9* lead to hypocholesterolemia and protect from heart disease. The current model for PCSK9 action [8] holds that the protein, following maturation and secretion from cells, binds to the LDL receptor on the hepatocyte surface. Subsequently, PCSK9 is internalized, and mediates re-routing of the LDL receptor from endosomes to lysosomes where the receptor is degraded [9]. This property of PCSK9 is based on its sequence-specific interaction with the receptor's first EGF precursor repeat (Section 2.3), which apparently interferes with the acid-dependent conformational change essential for receptor recycling. Furthermore, this property explains the effects of both loss- and gain-of-function mutations in this LDL receptor modulator gene.

In the following sections, our still incomplete knowledge about the events involved in receptor-mediated plasma clearance of triacylglycerol-rich lipoproteins is outlined.

3. Removal of triacylglycerol-rich lipoproteins from the plasma

3.1. Catabolism of chylomicrons by LRP1

Chylomicrons are too large to cross the endothelial barrier; thus, their prior lipolysis to remnants serves a dual function: transport of energy to tissues, and decrease in size to facilitate terminal catabolism. The triacylglycerols in chylomicrons are hydrolyzed by lipoprotein lipase (Chapter 19) along the lumenal surface of the capillaries, and this process is dependent on molecules that anchor the enzyme on the endothelial surface. A protein termed glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (Gpihbp1) has been proposed to play a critical role in the lipolytic processing of chylomicrons. Gpihbp1 is expressed highly in heart and adipose tissue, the same tissues that express high levels of lipoprotein lipase, in agreement with Gpihbp1 being an important platform for the processing of chylomicrons in capillaries [10]. The role of hepatic heparan sulfates in triacylglycerol-rich lipoprotein metabolism was studied by inactivating in murine hepatocytes the gene for GlcNAc *N*-deacetylase/*N*-sulfotransferase 1,

an important enzyme in heparin sulfate biosynthesis. These mice were viable and healthy, but they accumulated triacylglycerol-rich lipoprotein particles containing apo B100, apo B48, apo E, and apos C1-4, suggesting that heparan sulfate participates in the clearance of both intestinally derived and hepatic lipoprotein particles [11].

While these recent studies indicate the requirement for non-receptor-mediated steps in the overall clearance pathways for triacylglycerol-rich lipoproteins, the ultimate uptake occurs by endocytotic mechanisms involving specific receptors. Studies in a variety of experimental systems predicted that the removal of chylomicron remnants occurs independent of the LDL receptor, despite the presence of apo E on these particles. Accordingly, individuals with homozygous FH, who lack functional LDL receptors, show no signs of delayed clearance of chylomicron remnants.

Since the LDL receptor and the chylomicron remnant receptor must share at least the ability to bind apo E, attempts to isolate this receptor were based on the presumed similarity of its ligand-binding region to that of the LDL receptor. Indeed, homology cloning resulted in the characterization of an unusually large membrane protein, composed exclusively of structural elements found in the LDL receptor molecule; it has therefore been termed LDL receptor-related protein, or LRP, later designated LRP1 [12]. As shown in Fig. 4, LRP1 (LRP/α_2MR), a 4526-amino acid integral membrane glycoprotein, contains 31 ligand-binding repeats and 22 repeats of the growth factor type. LRP1 binds lipoproteins in an apo E-dependent fashion.

Soon after its cloning, LRP1 was shown to be identical to the receptor for α_2-macroglobulin, a major plasma protein that functions in 'trapping', and thereby inactivating, cellular proteinases that have entered the plasma compartment. Since then, many more plasma proteins and protein complexes have been identified, which at least in vitro bind to LRP1 [13]. Importantly, α_2-macroglobulin–proteinase complexes are cleared by the liver with the same kinetics as chylomicron remnants, indicating that LRP1 may indeed perform multiple functions in the removal of spent vehicles of intestinal lipid transport and of potentially harmful proteinases. In addition, recent findings also support roles of LRP1 in signal transduction; these are described in Section 5.3. Another LDL receptor relative with an even broader range of functions is introduced in the following section, and further properties of this protein are described in detail in Sections 4 and 5.

3.2. The so-called VLDL receptor: a role in catabolism of VLDL?

The name VLDL receptor was coined for a protein discovered in 1992 by T. Yamamoto and colleagues (S. Takahashi, 1992). The overall modular structure of the VLDL receptor is virtually superimposable with that of the LDL receptor, except that the ligand-recognition domain contains an additional binding repeat located at the amino terminus (Fig. 4). The VLDL receptor shows an amazing degree of conservation among different species; there is 95% identity between the corresponding mammalian proteins. Even the VLDL receptor homologs of more distant species such as the chicken and frog share 84 and 73%, respectively, of identical residues with the human VLDL receptor. In addition, VLDL receptors exist in variant forms, arising from differential splicing of exon 16 that specifies an O-linked sugar domain.

However, uptake of significant amounts of VLDL by this receptor has not been conclusively shown, despite the fact that its tissue distribution is highly suggestive of a role in

triacylglycerol transport into metabolically active tissues. In contrast to the LDL receptor, and as expected from a receptor implicated in triacylglycerol transport, the VLDL receptor is not regulated by cellular sterols, but its level appears to be influenced by hormones such as estrogen and thyroid hormone. On the other hand, its expression pattern is different from that of lipoprotein lipase, with which the VLDL receptor would be expected to act in concert. Nevertheless, numerous studies suggest that the VLDL receptor is, at least in part, involved in the delivery of fatty acids derived from VLDL-triacylglycerols to peripheral tissues, mainly adipose tissue [14].

Subsequent to the realization that the VLDL receptor likely has only a limited role in lipoprotein metabolism, novel VLDL receptor functions have been uncovered. Elegant experimentation showed that it is involved in neuronal migration in the developing brain via binding of a ligand quite distinct from lipoproteins [6]. These important results are described in Section 5. However, there is a VLDL receptor, described in the following section, which indeed does deserve this name, since it has a very well defined function in lipoprotein metabolism.

4. Multifunctional receptors in the chicken

A particularly interesting VLDL receptor homolog is that of the chicken (termed LDL receptor relative with eight binding repeats, or LR8; Fig. 4), because its functions are documented by both biochemical and genetic evidence. LR8 mediates a key step in the reproductive effort of the hen, that is, oocyte growth via deposition of yolk lipoproteins [15]. This conclusion is based on studies of a non-laying chicken strain carrying a single mutation at the *lr8* locus that disrupts LR8 function (the 'restricted ovulator', R/O, strain) [16]. As a consequence of the mutation, the hens fail to deposit into their oocytes VLDL and the lipophosphoglycoprotein vitellogenin, which are produced at normal levels in the liver, and the mutant females develop severe hyperlipidemia and features of atherosclerosis. The phenotypic consequences of the single-gene mutation in R/O hens revealed the extraordinary multifunctionality of LR8, that is, it recognizes over 95% of all the yolk precursors that eventually constitute the mass of the fully grown oocyte. Obviously, R/O hens, which represent a unique animal model for an oocyte-specific receptor defect leading to FH (Section 2.2), are sterile due to non-laying.

LR8, like all VLDL receptors, exists in isoforms that arise by differential splicing of the exon encoding the O-linked sugar domain; the larger form is termed LR8+ and the smaller one LR8–. In mammals, the predominant form is LR8+. In chicken, the somatic cells and tissues, in particular the granulosa cells surrounding the oocytes, heart, and skeletal muscle, express predominantly LR8+ (albeit at very low levels), while the oocyte is by far the major site of LR8– expression. In the male gonad, the same expression dichotomy exists: somatic cells express the larger, and spermatocytes the shorter, form of LR8 [17].

The properties of LR8 and its central role in reproduction strengthen the hypothesis that the avian receptor is the product of an ancient gene with the ability to interact with many, if not all, ligands of more recent additions to the LDL receptor gene family (Fig. 4). In this context, vitellogenin, which is absent from mammals, and apo E, which is not found in birds, possess certain common biochemical properties and regions of sequence similarities, and have been suggested to be functional analogs (W.J. Schneider, 1990).

Even high-density lipophorin, an abundant lipoprotein in the circulatory compartment of insects, is endocytosed in a variety of tissues via an LR8 homolog with very high similarity to the VLDL receptor/LR8 group [18] (N.P. Dantuma, 1999). Presumably, binding of lipophorin to this receptor is mediated by apolipophorins I and II, which share sequence homology with mammalian apo B, and thus may behave similarly to the major yolk precursor proteins.

In summary, studies in the chicken have revealed that members of the LDL receptor gene family from different animal kingdoms have common structures, and share a growing list of physiological roles, including the most recently discovered function(s) in signal transduction, as described in the following section.

5. VLDL receptor, apo E receptor type 2 (apoER2), and LRP1 in signal transduction

5.1. ApoER2: a close relative of the VLDL receptor

The structure of apoER2, which was discovered by homology cloning, is highly reminiscent of that of the VLDL receptor (Fig. 4). However, the proteins produced from differentially spliced mRNAs harbor clusters of either three, four, five, seven, or eight binding repeats, dependent on the species and organ expressing the gene [15]. In addition, a murine apoER2 splice variant gives rise to a receptor containing a furin consensus cleavage site at the carboxy terminal end of the ligand-binding domain. The receptor fragment constituting the ligand-binding domain could potentially act as a dominant negative extracellular ligand trap. ApoER2 is predominantly found in brain, placenta, and testis, in contrast to other members of the LDL receptor family, which are all expressed to a small extent in the brain, but most prominently in other organs. Besides the liver, the brain is also the most prevalent site of mammalian apo E expression, and it is widely believed that apo E serves a role in local lipid transport in the central nervous system (R.W. Mahley, 1999). Ligand-binding studies with human apoER2 demonstrated high affinity of the receptor for β-VLDL, indicating that the receptor might be involved in apo E-mediated transport processes in the brain. In addition, the apoER2 variant containing eight binding repeats can act as receptor for α_2-macroglobulin (also a ligand of LRP1, Section 3.1) in brain, which suggests a role in the clearance of α_2-macroglobulin–proteinase complexes from the cerebrospinal fluid and from the surface of neurons. However, despite these intriguing possibilities, the exciting discovery of the involvement of apoER2 and the VLDL receptor in signal transduction has re-focused interest in these receptors.

5.2. Genetic models reveal new roles for apoER2 and VLDL receptor in signal transduction

Surprisingly, targeted disruption of both the VLDL receptor and the apoER2 genes in mice (double-knockout mice) elicits a dramatic phenotype, which is essentially identical to that of mice lacking the extracellular matrix glycoprotein reelin [19]; single-knockout mice of either receptor gene show only very subtle phenotypes. Reelin, secreted by cells

in the outermost layer of the developing cerebral cortex, orchestrates the migration of neurons along radial fibers, thus forming distinct cortical layers in the cerebrum.

The reason for the grossly abnormal phenotype of the double-knockout mice (disturbed foliation of the cortical layers) is that reelin normally interacts with the extracellular domains of both the VLDL receptor and apoER2 [6], but the ensuing vital signal cascade remains inactivated when the receptors are missing. This cascade is triggered upon reelin binding to the VLDL receptor and apoER2, leading to phosphorylation of the cytoplasmic adapter protein disabled-1 (mDab-1) bound to the NPxY motifs present in the receptors' tails. Reelin-stimulated tyrosine-phosphorylation of mDab-1 then likely starts kinase cascade(s) controlling cell motility and shape by acting on the neuronal cytoskeleton. Since Dab-1 binds to the intracellular domains of VLDL receptor, apoER2, LDL receptor, and LRP1, it is possible that besides endocytosis of macromolecules, signal transduction might be a general function common to many members of the LDL receptor family. In such a scenario, adapter molecules like Dab-1 might be part of a machinery that defines the actual function of a particular member of the receptor family. In any case, the specificity of the reelin signaling via the apoER2 and VLDL receptor is achieved by selective binding of reelin to these receptors and not to other members of the LDL receptor family.

5.3. Signaling through LRP1

As described in Section 3.1, LRP1 is one of the largest members of the LDL receptor family. In contrast to the main function of apoER2, endocytosis of a broad range of ligands that bind to the extracellular domain of LRP1 is an undisputed and probably the most important task performed by this receptor [13]. LRP1 has an intracellular domain, which is significantly larger than those of LDL receptor, VLDL receptor, and apoER2, and mediates ligand uptake with very high endocytosis rates. Interestingly, an LRP1-tail-specific YxxL motif, and not the NPxY motif, appears to be the dominant determinant for internalization (M.P. Marzolo, 2000). In addition, cAMP-dependent phosphorylation of a serine residue within the cytoplasmic tail of LRP1 modulates endocytosis efficiency, indicating a possible regulation of ligand uptake by external signals.

Phosphorylation of tyrosine(s) in the intracellular domain of LRP1 by v-Src, however, might be involved in cellular transformation. By generating a binding site on phospho-LRP1 for the protein tyrosine-binding domain of Shc, Shc comes in close proximity to v-Src, which also becomes tyrosine-phosphorylated. Thus, in this case, LRP1 acts as an anchor rather than as bona fide signaling receptor. On the other hand, platelet-derived growth factor (PDGF) binds to LRP1 and together with the PDGF-receptor induces the tyrosine-phosphorylation of the intracellular domain of LRP1 (H. Barnes, 2001). Interestingly, PDGF induces phosphorylation of only LRP1 that is present in caveolae, suggesting that PDGF heterodimerizes LRP1 and PDGF-receptor in the caveolae fraction of the plasma membrane. Identification of LRP1 as part of a signaling pathway involving the PDGF-receptor confirms results that show that apo E inhibits PDGF-induced cell migration in an LRP1-dependent manner.

Another signaling system based on LRP1 heterodimerization appears to involve the *N*-methyl-D-aspartate receptor, which mediates calcium flux in cultured primary neurons.

Postsynaptic density protein-95, which binds to the LRP1 tail, might be a candidate adapter protein mediating the interaction of LRP1 with the *N*-methyl-D-aspartate receptor [20]. Finally, LRP1 can be proteolytically processed by a γ-secretase-like activity within the transmembrane domain leading to the release of the entire cytoplasmic domain of the receptor into the cytosol [21]. Whether the released LRP tail acts as cofactor of a putative transcription factor complex and/or as a modulator for the localization of LRP1-targeted adapter proteins is currently under investigation.

6. Other relatives of the LDL receptor family

In addition to the VLDL receptor, apoER2, and LRP1, several additional LDL receptor gene family members have been identified at the molecular level in the past few years. These receptor proteins are often referred to as 'LDL receptor relatives'. As several of them are better known under their originally proposed names, these will also be mentioned where appropriate. The most prominent members of the family are, listed in the order of their discovery, LDL receptor, LDL receptor-related protein (LRP1), megalin (also called LRP2, and originally named gp330), VLDL receptor (in chicken termed LR8), LR11 (also named SorLA), apolipoprotein E (apo E) receptor type 2 (apoER2), LRPs 3–6, and LRP1B (also termed LRP1-DIT).

Common features of these proteins are the structurally and functionally defined domains described in Section 2.3 for the human LDL receptor (i.e., the ligand-binding domain, the EGF precursor domain consisting of type B repeats, YWTD-motifs, the beta-propeller domain, a facultatively present O-linked sugar domain, a single membrane-spanning stretch, and the cytoplasmic domain). Since all of these, by definition, contain LDL receptor ligand-binding repeat clusters and may therefore play roles in lipid-related metabolism, they are also described in this chapter.

6.1. Small and mid-sized LDL receptor relatives: LRPs 3–6

These members of the gene family were discovered more or less serendipitously. Degenerative probes corresponding to the highly conserved amino acid sequence WRCDGD, found in LDL receptor ligand repeats, were used to screen a rat liver cDNA library, resulting first in the cloning of LRP3, and then of its human homolog from a HepG2 cDNA library [22]. The same approach, using a murine heart cDNA library, resulted in the cloning of LRP4 (also called Megf7). LRP3 is a 770-residue membrane protein with clusters of two and three binding repeats, respectively. Murine LRP4 contains two clusters of LDL receptor-binding repeats with three and five modules, respectively. Defects in LRP4 lead to abnormal limb development (syndactylies; fusion of digits). Future studies will help to clarify whether these proteins are capable of binding ligands that have been shown to interact with other LDL receptor relatives, whether they are endocytically competent, and of course, what is their spectrum of in vivo functions is.

Two other new members of the LDL receptor family, LRP5 and LRP6 (reviewed in Ref. [23]), have been discovered in the course of attempts to identify the nature of the insulin-dependent diabetes mellitus locus IDDM4 on chromosome 11q13. Human and

mouse LRP5 and LRP6 are type I membrane proteins, approximately 1600 residues long (about twice as large as the LDL receptor), and their extracellular domains are organized exactly as the central portion of LRP1 (Fig. 4). The cytoplasmic domains of LRP5 and LRP6 contain motifs (dileucine and aromatic-X-X-aromatic/large hydrophobic) similar to those known to function in endocytosis of other receptors. Furthermore, they harbor serine- and proline-rich stretches that can interact with Src homology 3 and WW (a variant of Src homology 3) domains, properties that relate these receptors to signal transduction pathways, albeit different ones from those of apoER2 and the VLDL receptor described above. For instance, LRP6 has been shown to be a co-receptor for Wnt proteins, which trigger signaling pathways important for correct development of anterior structures. LRP6 also interacts with Dickkopf, which inhibits Wnt signaling by releasing receptor-bound Wnt, and with Axin, a component in the cascade that regulates the activation of gene expression in the nucleus of target cells (R. Nusse, 2001). The interplay of Wnt-, Dickkopf-, and Axin-binding to LRP5/6 may hold the key to important developmental signals, similar to the role of VLDL receptor and apoER2 in neuronal migration (Section 5.2). Indeed, following reports that mutations in LRP5 are causally linked to alterations in human bone mass, considerable progress has been made towards understanding the molecular links between Wnt signaling and bone development and remodeling [24].

6.2. The unusual one: LR11

This membrane protein, a structurally very complex member of the LDL receptor gene family (Fig. 4), was discovered first in rabbit, and subsequently in man, mouse, and chicken [25]. Significantly, overall sequence identities between the ~250-kDa proteins range from 80% (man vs. chicken) to 94% (man vs. rabbit). LR11 (also termed SorLA; gene locus, *SORL1*) is made up of 7 distinct domains (Fig. 4) including a cluster of 11 LDL receptor ligand repeats. Unusual features are a ~50-kDa domain highly homologous to a yeast receptor for vacuolar protein sorting, VPS10p, and six tandem fibronectin type III repeats. The membrane-spanning and cytoplasmic domains are extremely highly conserved. Interestingly, as other related VPS10p-containing membrane proteins, LR11 can be cleaved within the membrane-spanning domain by γ-secretase following shedding of the extracellular domain. The intracellular domain appears to enter the cell where it may perform specific, but yet unidentified, signaling functions.

LR11 is found mainly in the nervous system and, depending on the species, also in testis, ovary, adrenal glands, and kidney. LR11 levels are increased during proliferation, but decrease following differentiation of neuroblastoma cells. A genetic association of LR11/SorLA with Alzheimer disease has been shown [26], in agreement with the finding that decreased expression of LR11 in the brain increases β-amyloid production, a hallmark of the degenerative disease. LR11 also is markedly increased in arterial intimal smooth muscle cells and stimulates their migration, which is related to intimal thickening during atherogenesis. Thus, to date, available information suggests that LR11 is involved in cellular proliferation during development as well as in the pathological processes underlying atherosclerosis and Alzheimer disease. Despite its demonstrated ability to bind apo E-containing ligands, a direct involvement in lipoprotein metabolism has not been demonstrated unequivocally to date.

6.3. Large LDL receptor relatives: LRP2 and LRP1B

6.3.1. LRP2/megalin, a true transport receptor

This 600-kDa protein is another large member of the LDL receptor gene family, which like LRP1 contains four clusters of LDL receptor ligand-binding repeats. Although many proteins that bind to LRP1 are also ligands of LRP2, its expression pattern and specificity for certain ligands account for physiological roles distinct from those of LRP1. LRP2 is expressed mainly in polarized epithelial cells of the kidney, lung, eye, intestine, uterus, oviduct, and male reproductive tract. Its roles in embryogenesis are well established; for instance, LRP2 is required for development of the forebrain by taking up apo B-containing lipoproteins into the embryonic neuroepithelium [27]. Another important function of LRP2 is its involvement in the metabolism of certain lipophilic vitamins. For instance, in the kidney, vitamin B12/transcobalamin complexes are recaptured from the ultrafiltrate directly by binding to megalin expressed on proximal tubule cells (S.K. Moestrup, 1998). Furthermore, megalin mediates the reabsorbtion from the proximal tubules of 25-(OH) vitamin D3/vitamin D binding protein complexes, which constitutes a key step in converting the precursor into active vitamin D3 in the kidney (reviewed in Ref. [27]).

6.3.2. LRP1B

This 4599-residue type I membrane protein contains 32 LDL receptor ligand-binding repeats in its extracellular portion (Fig. 4). Among all of its relatives, LRP1B shows the highest homology to LRP1. Compared to LRP1, it contains one additional ligand-binding repeat and an insertion of 33 amino acids in the cytoplasmic domain (Fig. 4). These structural properties suggest roles of LRP1B in lipoprotein metabolism, but such activities have not yet been demonstrated directly. The receptor is expressed mainly in brain and skeletal muscle, and its regulation has been studied in smooth muscle cells derived from rabbit arteries and in an established smooth muscle cell line. In both systems, LRP1B expression is induced during the exponential phases of cellular proliferation, similar to LR11 (Section 6.2) [28]. However, peaks of expression seem to occur at later time points than those observed for LR11 induction, consistent with different physiological roles of the two proteins. In any case, LRP1B-deficiency in smooth muscle cells causes atherosclerosis via modulation of intracellular PDGF signaling.

7. Scavenger receptors: lipid uptake and beyond

In addition to the type of scavenger receptors (SRs) described in this chapter 19, there is a growing list of hepatic and extrahepatic SRs with potential disease-related functions. For many of these, the criterion for being classified as SR is their broad spectrum of ligands, which include diverse polyanionic compounds and modified lipoproteins such as oxidized LDL. The currently known SRs are classified according to their primary structure, tissue distribution, and proposed function(s) into six groups (SR-A, -B, -D, -E, -F, and -G). The most prominent and probably best understood SRs in the context of lipoprotein metabolism are the SR class A (SR-A), class B (SR-BI/II and CD36), and

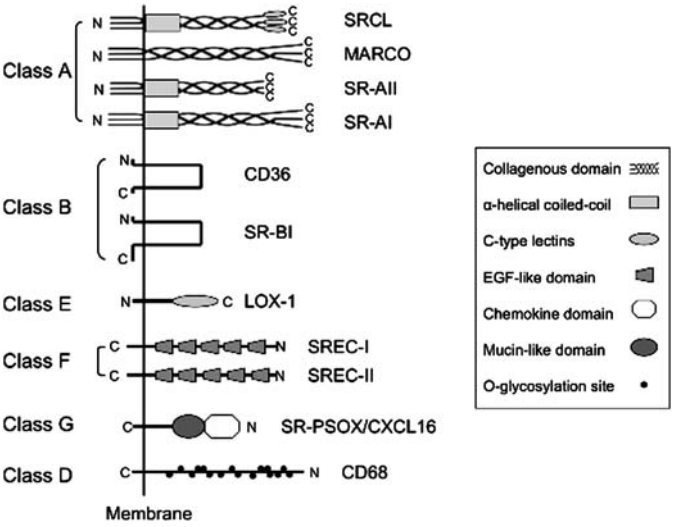

Fig. 5. Scavenger receptors (SRs). Structural features of scavenger receptors with proposed roles in atherosclerosis. The plasma membrane or the endosomal membrane (for CD68, which is mainly found in the endosomal compartment) is indicated by the vertical line, with the cytoplasmic domains to the left. The individual structural domains are described in the box. SRCL, SR with C-type lectin; MARCO, macrophage receptor with collagenous structure; SREC, SR expressed by endothelial cells; SR-PSOX/CXCL16, membrane-bound chemokine L16; other SRs are described in the text (Section 7). Not drawn to scale.

the class E SR, LOX-1 (for a concise review of SRs, see Ref. [29]). Fig. 5 summarizes the structural features of the SRs that are currently under study for their possible involvement in atherosclerosis and/or as co-regulators of the balance between lipid accumulation and inflammation in the arterial wall.

7.1. Class A SRs

The class A receptors (SR-A, types I–III) are trimeric membrane proteins characterized structurally by a small amino-terminal intracellular region, an extracellular coiled-coil collagen-like stalk, and a cysteine-rich carboxy-terminal domain. Two more receptors are classified as A-types, termed MARCO and SR with C-type lectin, respectively (Fig. 5). The three SR-A isoforms are produced from the same gene by differential splicing and are expressed at different levels in tissue macrophages, Kupffer cells, and various extrahepatic endothelial cells. SR-AI expression is induced by some of its ligands, which include, in addition to modified lipoproteins and polyanions, gram-positive bacteria, heparin, lipoteichoic acid, and a precursor of lipid A from lipopolysaccharide of gram-negative bacteria.

A potential role of SR-As in atherosclerotic plaque development was initially reported in a study on apo E and SR-A double-knockout mice. Mice deficient in apo E developed severe plaques, but simultaneous absence of SR-A led to a reduction in plaque size by 58%. This reduction may be related to the greatly reduced uptake of acetylated LDL and

oxidized LDL that can be observed in in vitro uptake studies using macrophages and liver cells of SR-A knockout mice. However, these early results could not be confirmed subsequently. The discrepancy arising from conflicting results may be reconciled by SR-A having different roles in early versus advanced atherosclerotic lesions or by different genetic backgrounds of the mice used [29].

7.2. Class E SR: lectin-like oxidized LDL receptor (LOX)-1

This 50-kDa transmembrane protein shows no structural similarity to other SRs (Fig. 5). The amino-terminal cytoplasmic tail of the lectin-like molecule contains several potential phosphorylation sites (T. Kita, 2001). LOX-1 can act as endocytic receptor for atherogenic oxidized LDL, but in contrast to SR-A, it interacts only weakly, if at all, with negatively charged LDL, which can be produced in vitro by extensive acetylation of lysine residues of apo B (so-called acetylated LDL). Also, LOX-1 differs from other SRs in that binding of oxidized LDL is inhibited by polyinosinic acid and delipidated oxidized LDL, but not by acetylated LDL, maleylated bovine serum albumin, or fucoidin. LOX-1 is found in thoracic and carotid vessels, and highly vascularized tissues such as placenta, lungs, brain, and liver. Its expression is apparently not constitutive, but can be induced by pro-inflammatory stimuli; it is then detectable in cultured macrophages and in activated smooth muscle cells. LOX-1 also mediates the recognition of aged red blood cells and apoptotic cells. However, little is known about whether LOX-1 functions in clearance of damaged cells in vivo.

7.3. Class B SRs

7.3.1. CD36: role in lipid uptake

CD36 (also termed fatty acid translocase, FAT) belongs to a gene family specifying proteins with two internal hydrophobic, putative membrane-spanning domains adjacent to short cytoplasmic amino- and carboxy-terminal tails. This molecule was originally identified as a platelet receptor for thrombospondin, and only later was recognized as a macrophage receptor for moderately oxidized LDL, but not for extensively oxidized LDL or acetylated LDL. CD36 can also bind native LDL, HDL and VLDL, collagen, and fatty acids and has been proposed to mediate the uptake of fatty acids from the circulation (R. Ehehalt, 2006). The most significant role of CD36 in lipoprotein metabolism is the clearance of oxidized LDL, contributing over 60% of the cholesteryl esters accumulating in macrophages exposed to oxidized LDL. In agreement with these in vitro data, apo $E^{-/-}$/$CD36^{-/-}$ double-knockout mice show marked reductions in atherosclerotic lesion area compared to apo $E^{-/-}$ mice. CD36 has also been reported to promote pro-inflammatory signaling that may lead to chronic inflammation in the artery wall, and also in Alzheimer disease (J.B. El Khoury, 2003). Yet, the signaling pathway(s) triggered by CD36 activation have not been delineated.

7.3.2. Removal of HDL: SR-BI/II

HDL binding to SR class B type I (SR-BI) on the cell surface mediates the transfer of its cholesteryl esters to the cell and subsequent release of the lipid-depleted HDL into

the extracellular fluid, rather than the particle's lysosomal degradation. This novel cellular mechanism, called selective lipid uptake, can extract cholesteryl esters from LDL as well as HDL. The mechanism is based on the reversible incorporation of cholesteryl esters into the plasma membrane that may be followed by irreversible internalization and subsequent hydrolysis through a non-lysosomal pathway [30]. In rats, the liver removes 60–70% of HDL cholesteryl esters from plasma via the selective lipid uptake pathway. Selective uptake of HDL cholesterol by steroidogenic tissues can also provide cholesterol as substrate for steroid hormone synthesis. For example, in the rodent adrenal gland, selective uptake accounts for 90% or more of the cholesterol destined for steroid hormone production [30]. The main pathway for cholesterol removal via HDL into the liver is essential for cholesterol homeostasis, because peripheral cells are unable to degrade cholesterol. This pathway, known as reverse cholesterol transport, is considered to be partly responsible for the anti-atherogenicity of HDL. Furthermore, recent observations suggest that HDL may play roles in regulating the complement system, and that the protein cargo of HDL contributes to its anti-inflammatory and anti-atherogenic properties (A. Chait, 2005; J.W. Heinecke, 2007).

The predicted sequences of SR-BI proteins (509 amino acids) from different mammalian species share approximately 80% sequence identity. As for CD36, the bulk of the protein lies between the two hydrophobic plasma membrane-anchoring domains on the extracellular aspect of cells and contains a set of conserved cysteines. An alternatively spliced mRNA of SR-BI and its corresponding protein have been identified and designated SR-BII. SR-BII differs from SR-BI only in that the C-terminal 42 amino acids in the C-terminal cytoplasmic domain of SR-BI are replaced by 39 residues encoded by an alternatively spliced exon [31].

In summary, SRs are a widely expressed and highly diverse group of proteins that are appropriately named for their recognition of a broad array of ligands. At least some members of this intriguing group of receptors, as indicated here, may play roles in the metabolism of modified lipoproteins, and consequently have been implicated in lipid disorders, atherosclerosis, and inflammatory processes.

8. Future directions

The multifunctionality of LDL receptor gene family members is not limited to their extracellular moiety. The original concepts regarding their functions in lipoprotein metabolism remain valid, but have been extended significantly by the discovery that these membrane proteins also play roles in important signal transduction pathways. Given the wealth of signaling pathways, future efforts will have to develop concepts that define the contributions of the individual intracellular domains of LDL receptor relatives. These efforts are also expected to add alternative aspects to the ongoing quest to delineate the evolutionary history of the LDL receptor family. In fact, based on the most recently gained knowledge, an evolutionary theory must consider the combinatorial events arising from the multitude of both intracellular and extracellular domains of these proteins in the generation of signaling and/or transport scaffolds.

Another point to take into account is the apparent functional redundancy of receptors involved in a multitude of metabolic pathways and events. This aspect has been addressed here not only for the LDL receptor gene family, but also for the group of SRs, which show broad overlapping ligand specificities (Section 7). In the case of the well-understood LDL receptor gene family, when need arises (for example, when one or more receptors are dysfunctional), certain members can substitute for others, or are at least sufficiently active for preserving life. For example, VLDL receptor$^{-/-}$ mice, and apoER2$^{-/-}$ mice, have phenotypes that are at first sight indistinguishable from normal. However, the double-knockout mice are grossly abnormal (Section 5.2). Thus, functional redundancy of LDL receptor relatives can be due to their simultaneous expression in the same cells in a given organ. In turn, different receptor functions despite similar ligand spectra may arise from their expression in different cell types within a tissue or organism. In vivo, receptors presumably have access to different ligands in different environments, and in addition, their cytoplasmic domains would interact with cell-type-specific adapter proteins in order to mediate a spectrum of signal transduction pathways.

Many of these considerations likely apply to the SRs as well. However, an added complication is the difficulty in determining the in vivo ligand spectrum for these receptors, as there may be enormous overlaps under physiological and/or pathophysiological conditions, and the affinities of SRs for ligands may vary greatly in different settings. As a consequence, specific functions of lipoprotein receptor gene families must be defined at two levels: the cellular level, in order to delineate molecular events, and the physiological level, where state-of-the-art genetic manipulations will continue to reveal the functional relevance of receptor redundancy.

Finally, increasing our knowledge about modulators of the activity of the LDL receptor family (such as ARH [32] and PCSK9 [8], Section 2.5) will be important for the characterization of modifier genes of lipoprotein metabolic pathways in the general population.

Abbreviations

Apo	apolipoprotein
ApoER2	apo E receptor type 2
ARH	autosomal recessive hypercholesterolemia
Dab-1	disabled-1
EGF	epidermal growth factor
FH	familial hypercholesterolemia
HDL	high-density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LOX-1	lectin-like oxidized LDL receptor
LRP	LDL receptor-related protein
PCSK9	proprotein convertase subtilisin-like kexin-type 9
PDGF	platelet-derived growth factor
SR	scavenger receptor
SR-BI	scavenger receptor class B type I
(V)LDL	(very)-low-density lipoprotein

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CHAPTER 21

Lipids and atherosclerosis

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Contents

1. Introduction

Atherosclerotic vascular disease is the cause of heart attacks, stroke, aortic aneurysms, and peripheral vascular disease, which together represent the most frequent causes of death in the industrialized world. Indeed, the aging of the population and the 'westernization' of world diet is predicted to increase the impact of atherosclerosis worldwide over the next few decades despite continuing advances in plasma lipid-lowering therapy (E. Braunwald, 1997).

Atherosclerosis progresses in a series of stages, although some lesions at each stage may not progress further or may even regress if inciting events, such as hypercholesterolemia,

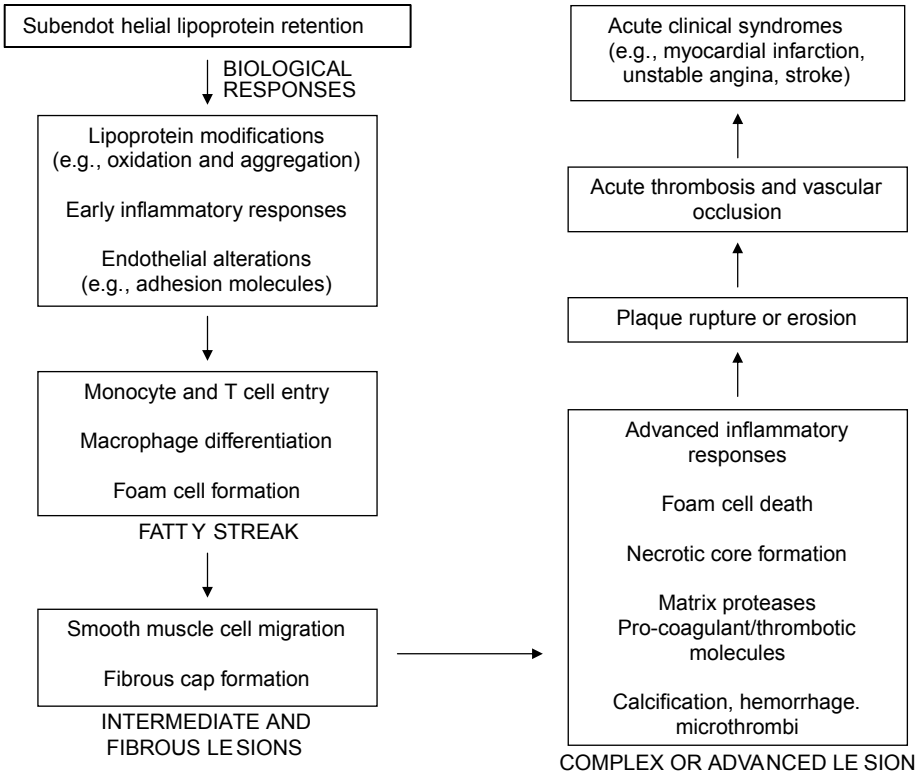


Fig. 1. Progression of atherosclerotic lesions. As noted in the text, only a portion of lesions at any stage progress and, under the proper conditions, lesion regression may occur.

diabetes, smoking, or hypertension, are controlled [1–4] (Fig. 1). The initial stage involves the accumulation of subendothelial lipoproteins in focal areas of the arterial tree, usually at branch points with disturbed laminar flow. In response to this retention, a series of biological responses ensue, including lipoprotein oxidation, endothelial alterations, inflammatory responses including T cell recruitment, cytokine secretion, monocyte chemotaxis, and subendothelial macrophage accumulation, and intracellular cholesterol accumulation in macrophages [5]. Much of the cholesterol is stored as cholesteryl fatty acid esters (CE) in cytoplasmic lipid droplets surrounded by a monolayer of phospholipid. These cytoplasmic droplets give the macrophages a foamy appearance when viewed by microscopy, and thus these cells are referred to as 'foam cells' (Fig. 2C). The presence of macrophage foam cells defines the earliest pathological lesion, referred to as the 'fatty streak.'

Although sensitive tests of endothelial function show abnormalities in vasodilation in the very earliest phases of atherosclerosis (R.A. Vogel, 1998), fatty streaks are not occlusive and cause no overt symptoms. However, some fatty streaks may progress over years to more complex lesions that can give rise to chronic symptoms or, more importantly, acute events. An important event in the progression of fatty streaks involves the migration of smooth muscle cells from the media to the intima and the secretion of large amounts of

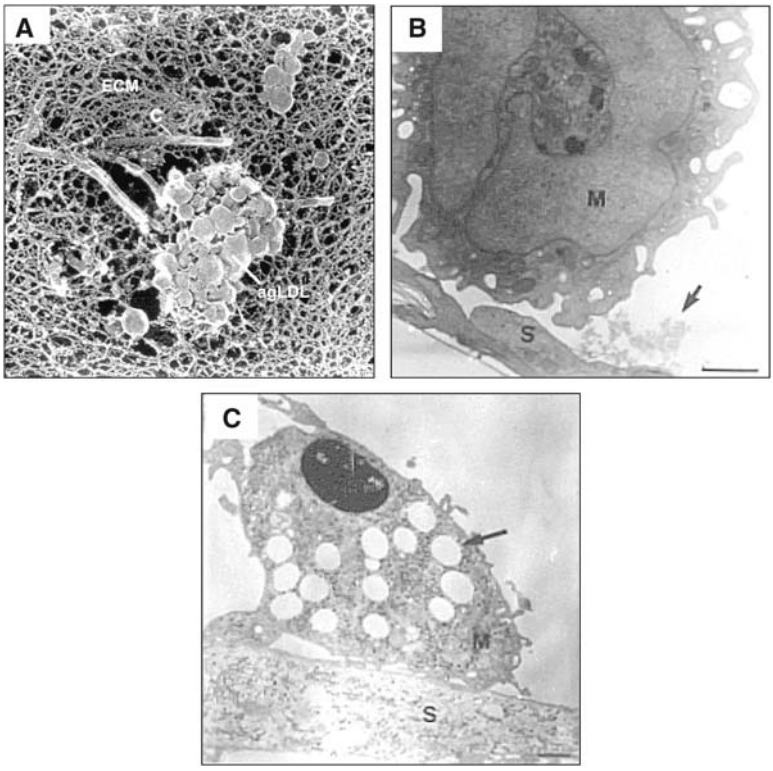


Fig. 2. Lipoprotein aggregation and macrophage foam cell formation. (A) Freeze-etch replica-plated electron micrograph of rabbit aorta subendothelium 2 h after intravenous injection of LDL [from [31]. *Arterio. Thromb.* **11**: 1795–1805]. The figure shows aggregated LDL particles (agLDL) bound to extracellular matrix (ECM) in the subendothelial space. 'c', collagen. (B) A J774 murine macrophage (M) immediately after plating on sphingomyelinase-induced LDL aggregates (arrow) formed on the surface matrix of smooth muscle cells (S). (C) After 24 h of incubation, the aggregates have been internalized by the macrophage, which now has large cytoplasmic neutral lipid droplets consisting mostly of cholesteryl ester (arrow). The cytoplasmic droplets are characteristic of lesional foam cells. Bars in (B) and (C): 1 μm. Panels B and C are from [32]. *J. Biol. Chem.* **268**: 20419–20432. Reproduced with permission from the publisher.

collagen and other matrix proteins by these cells. In addition, macrophages proliferate and continue to accumulate lipid. Smooth muscle cells can also accumulate lipid and become foam cells. These events give rise to so-called fibrous lesions, which are eccentric lesions consisting of lipid-loaded macrophages and smooth muscle cells covered by a fibrous cap. Further progression to complex lesions involves the accumulation of extracellular lipid, which results from a combination of aggregation and fusion of matrix-retained lipoproteins and release of lipid droplets from dying foam cells. Calcification, hemorrhage, and microthrombi can also be observed in these complex lesions [1].

At this stage, several fates of the lesion are possible [1,6,7]. Plasma cholesterol lowering can result in lesion regression, particularly of the foam cells. In this case, the macrophages begin to lose their cholesterol via the process of cholesterol efflux (Chapter 20), and the

number of macrophages decreases, probably through a combination of decreased monocyte entry, decreased macrophage proliferation, and increased macrophage egress and apoptosis. Alternatively, the complex lesions can progress. If arterial occlusion increases gradually, the patient may experience exercise-induced ischemia, but collateral vessel formation often prevents additional clinical symptoms. However, if the lesions rupture or erode before they become large and occlusive, acute vascular events such as unstable angina, heart attack, sudden death, or stroke can occur. Rupture involves the abrupt disruption of the fibrous cap, followed by exposure of thrombogenic material and acute thrombosis. Importantly, rupture mostly occurs in lipid-rich and macrophage-rich 'shoulder' regions of the plaque and is probably triggered by the degradation of the fibrous cap by proteases secreted by macrophages or released from dying foam cells, perhaps coupled with decreased collagen synthesis due to death of collagen-synthesizing smooth muscle cells in the intima. Physical stresses related to pools of soft lipid underneath a thin fibrous cap also contribute to plaque rupture. These pools of lipid and cellular debris, often referred to as 'necrotic' or 'lipid' cores, result from the death of macrophages (M.J. Mitchinson, 1995).

As is evident from this overview, lipids are the sine qua non of atherosclerosis. Most of the atherogenic lipids are initially transported into the intima of certain regions of the arterial tree via plasma lipoproteins, although atherogenic modification of these lipoprotein-derived lipids may occur after entry into the intima. Atherogenesis will not occur when atherogenic lipoproteins in the plasma, and hence in the arterial wall, fall below a certain threshold level. However, the absolute value for this threshold level varies among individuals and may be very low in subjects with genetic and/or environmental factors that render their arteries highly susceptible to the atherogenic process [5]. The major types of lipids that accumulate during the various stages of atherosclerosis are shown in Table 1. In addition, there are many lipids that are minor in quantity but, because of their biological activities, are thought to have a major impact on atherogenesis. This chapter will cover the properties and activities of many of the lipids that occur in atherosclerotic lesions, with an emphasis on their roles in lesion development and progression.

Table 1
Percent weight of major lesions in four progressive stages of atherosclerotic lesions

Lipid	Fatty streak (weight %)	Intermediate lesion (weight %)	Fibrous lesion (weight %)	Advanced lesion (weight %)
Cholesterol	9.6	21.1	22.5	31.5
Triacylglycerol	2.8	4.4	5.2	6.0
Cholesteryl ester	77.0	55.0	55.5	47.2
Phospholipid	10.1	19.6	16.8	15.3
Phosphatidylcholine	4.8	7.6	4.5	4.3
Sphingomyelin	5.6	11.0	11.7	10.1
Lysophosphatidylcholine	0.3	1.0	0.6	0.9

Source: Adapted from Ref. [17]. Reproduced with permission from the publisher.

2. Cholesterol and atherosclerosis

2.1. Cholesterol deposition in the arterial wall

As alluded to in the Section 1, the primary event in atherogenesis is cholesterol deposition in the arterial wall. The cholesterol originates from circulating plasma lipoproteins, which contain both unesterified ('free') cholesterol and cholesteryl ester (CE) (Chapter 18). The two classes of lipoproteins that contribute most to atherogenesis are low-density lipoprotein (LDL) and so-called remnant lipoproteins, which are the lipolytic products of chylomicrons and very low-density lipoprotein (VLDL). Plasma lipoproteins continually enter the subendothelial space of vessels via 'leakage' through transient gaps between endothelial cells and probably also via endothelial transcytosis. Under normal conditions, lipoproteins are not retained in the subendothelium and simply re-enter the circulation. In certain focal areas of the arterial tree, however, lipoprotein retention by subendothelial extracellular matrix is increased, leading to their net accumulation in the arterial wall. This retained material elicits a series of biological responses, leading to the cellular and extracellular processes that constitute atherosclerotic lesion formation (Section 1 and Refs. [4,5]). Because a high concentration of circulating atherogenic lipoproteins promotes the accumulation of these lipoproteins in the arterial wall, this model explains the well-established relationship between plasma cholesterol levels and atherosclerosis in both experimental animal models and humans.

The fate of the FC and CE moieties of retained lipoproteins includes both extracellular and intracellular processes. Extracellular matrix-retained lipoproteins are modified by lipases, proteases, and oxidation reactions (P.T. Kovanen, 2000) [8]. These reactions can lead to the generation of lipid vesicles that are rich in FC but poor in protein and CE (H. Kruth, 1985). The biological and pathological significance of these FC-rich vesicles is not known. Other reactions lead to the generation of modified lipoproteins that act as extracellular signaling molecules on lesional cells, especially endothelial cells, or that are avidly internalized by macrophages and smooth muscle cells. Thus, these modified lipoproteins are responsible for foam cell formation and a variety of cell-signaling events.

2.2. Cholesterol accumulation in lesional macrophages: lipoprotein internalization

The major cell type that internalizes subendothelial lipoproteins is the macrophage [2,3]. Lesional macrophages are derived from circulating monocytes that enter the arterial wall in response to chemokines; the chemokines are secreted by endothelial cells in response to both underlying retained lipoproteins and T cell-derived cytokines. Under the influence of other molecules secreted by endothelial cells, notably macrophage colony stimulating factor, subendothelial monocytes differentiate into macrophages. The differentiated macrophages then engage and internalize subendothelial lipoproteins and thus accumulate lipoprotein-derived cholesterol in the form of intracellular CE droplets (foam cell formation). As outlined in Section 1, this cellular event is the hallmark of early lesion development and also contributes to late lesional complications.

Two key issues in the area of macrophage foam cell formation include the cell-surface processes and receptors involved in lipoprotein internalization and the metabolic fate of lipoprotein-derived cholesterol following internalization [9]. Most studies examining macrophage–lipoprotein interactions use an experimental system in which monolayers of cultured macrophages are incubated with soluble, monomeric lipoproteins dissolved in tissue culture media. Most of these studies have revealed that native LDL is poorly internalized by macrophages, suggesting that LDL undergoes modification in the arterial wall. However, Kruth and colleagues have shown that human monocyte-derived macrophages can accumulate large amounts of native LDL via the cellular internalization process known as macropinocytosis [10]. Regarding LDL modifications, two types, namely oxidation and aggregation, have received the most attention [2,8].

LDL particles with oxidative modifications of both its protein and lipid moieties are known to exist in atherosclerotic lesions and are readily internalized by macrophages. A number of receptors have been implicated in oxidized LDL uptake by macrophages, including class A and B scavenger receptors (e.g., CD36) and lectin-like oxidized LDL receptor-1 (LOX-1). While internalization of oxidized LDL by macrophages may have important implications in atherogenesis, it is unlikely that all of the hallmarks of macrophage intracellular cholesterol metabolism that are known occur in lesions can be explained by this process alone [8,11].

As stated above, lipoproteins in the subendothelium are also known to be aggregated and fused, a process that might result from oxidation, lipolysis, or proteolysis [8,12] (Fig. 2A). For example, hydrolysis of the sphingomyelin on LDL particles to ceramide by sphingomyelinase leads to LDL aggregates that appear similar to those that exist in lesions. There is also evidence that LDL in the arterial wall is hydrolyzed by a form of sphingomyelinase secreted by arterial-wall cells [13] (Fig. 2B). Aggregated lipoproteins, like oxidized LDL, are readily internalized by macrophages. When aggregated LDL is added in tissue culture medium to monolayers of cultured macrophages, the LDL receptor seems to participate in a phagocytic-like process to internalize these particles. In vivo, however, most of the aggregated lipoproteins are bound to extracellular matrix, and newer experimental systems that attempt to mimic the uptake of retained and aggregated LDL have revealed that multiple receptors in addition to, or instead of, the LDL receptor are involved. Most importantly, macrophage internalization of aggregated lipoproteins leads to massive CE accumulation, which is the key intracellular cholesterol metabolic event that is known to occur in macrophage foam cells in early lesions [9] (Fig. 2C). As noted above, subendothelial lipoproteins that are not aggregated, retained, or oxidized may still have the potential to cause macrophage foam cell formation through the process of macropinocytosis [10].

Remnant lipoproteins are also important in atherogenesis (R.W. Mahley, 1985; R.J. Havel, 2000). These particles can be internalized by macrophages in their native form, although both oxidation and aggregation of these particles occur and probably further enhance macrophage uptake. The receptor or receptors involved in the uptake of remnant particles is not definitively known, but the likely candidates are the LDL receptor and the LDL receptor-related protein (LRP), which interact with the apolipoprotein E moiety of the remnant lipoproteins. Remnant lipoproteins, like aggregated lipoproteins, lead to massive CE accumulation in macrophages. Finally, it is worth mentioning that another lipoprotein called lipoprotein(a), in which a large glycoprotein called apolipoprotein(a) is covalently attached to the apolipoprotein B100 moiety of LDL (Chapter 19), has been implicated in

atherogenesis (A.M. Scanu, 1998). Although macrophage receptors for lipoprotein(a) have been described, neither the mechanism of atherogenicity nor the role of lipoprotein(a) lipids in macrophage cholesterol loading and lesion development are known.

2.3. Cholesterol accumulation in lesional macrophages: intracellular trafficking of lipoprotein-derived cholesterol

The fate of lipoprotein cholesterol after internalization is a key issue in understanding the biology and pathology of lesional macrophages. After internalization by receptor-mediated endocytosis or phagocytosis, the lipoproteins are delivered to late endosomes or lysosomes, where hydrolysis of proteins and lipids occurs. Most importantly, the large lipoprotein-CE stores are hydrolyzed by a lysosomal enzyme called lysosomal acid lipase. The liberated FC then trafficks to the plasma membrane and other cellular sites [14].

The trafficking of lipoprotein-derived cholesterol from lysosomes has been a major area of focus in the field of intracellular cholesterol metabolism, and many of the cellular and molecular events are not known (Chapter 17). By analyzing cells with mutations in cholesterol transport, investigators have identified roles for two proteins, called NPC1 and NPC2 (HE1), in lysosomal and/or endosomal cholesterol transport (E.J. Blanchette-Mackie, 2000; P. Lobel, 2000). In addition, the lipid lysobisphosphatidic acid and certain GTPases called Rab proteins may also play roles in these processes (J. Gruenberg, 1999; E. Ikonen, 2006). The mechanisms by which these molecules are involved in cholesterol transport, however, are poorly understood [14].

From the point of view of atherosclerosis, the two most important peripheral trafficking pathways are those to the endoplasmic reticulum (ER), where cholesterol is esterified by acyl-CoA:cholesterol acyltransferase (ACAT), and to the plasma membrane, where cholesterol can be transferred to extracellular acceptors in a process known as cholesterol efflux (Chapter 20). The former process leads to the massive CE accumulation seen in foam cells [14–16]. The ACAT reaction utilizes primarily oleoyl-CoA, thus ACAT-derived CE is rich in oleate. In contrast, plasma lipoprotein-CE tends to be rich in linoleate. As expected, therefore, the cholesteryl oleate:cholesteryl linoleate ratio in foam cell-rich fatty streak lesions — 1.9 — is relatively high [17]. However, the ratio in advanced lesions is only 1.1, suggesting an increase in lipoprotein-CE in advanced atheromata due to poor cellular uptake of lipoproteins or to defective lysosomal hydrolysis following uptake by lesional cells. Further discussion of the cholesterol esterification pathway appears in Chapter 15, and cholesterol efflux, which is an important mechanism that may prevent or reverse foam cell formation, is covered in Chapter 20.

2.4. Accumulation of unesterified cholesterol in lesional macrophages

Interestingly, foam cells in advanced atherosclerotic lesions accumulate large amounts of unesterified cholesterol [9,17], some of which is in crystalline form and may be deposited in the extracellular space when foam cells die (Fig. 3). For example, while 2 of 13 abdominal aortic and femoral artery fatty streak lesions contained cholesterol crystals, all of 24 advanced lesions had these structures [17]. The mechanism of unesterified cholesterol accumulation is not known, but could involve either defects in cholesterol trafficking to

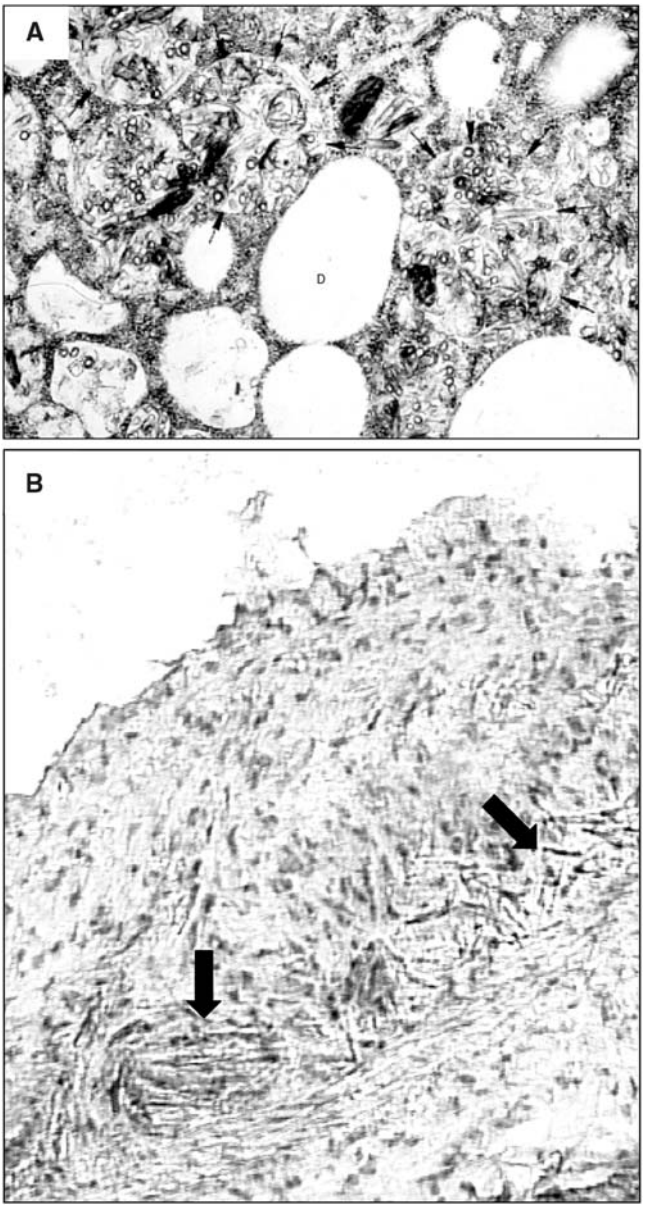


Fig. 3. (A) Intracellular unesterified cholesterol accumulation in a lesional foam cell. Electron micrograph of the cytoplasm of a foam cell isolated from an advanced aortic atherosclerotic lesion in a cholesterol-fed rabbit. The cell was treated with filipin, which forms spicules with unesterified cholesterol. Multiple spicules are observed in vesicles, shown to be lysosomes (depicted by arrows). 'D', neutral lipid droplet. Bar: 0.5 μm. From [34]. *Lab. Invest.* **41**: 160–167. (B) Extracellular cholesterol crystals in an advanced atherosclerotic lesion. The section is from the proximal aorta of a fat-fed apolipoprotein E knockout mouse. This mouse model is often used to study atherosclerosis in vivo because the high plasma levels of remnant lipoproteins resulting from absence of apolipoprotein E leads to a much greater degree of atherosclerosis lesion development than observed in wild-type mice. The arrows depict the areas of cholesterol crystals. Reproduced with permission from the publisher.

ACAT or a decrease in ACAT activity itself. Because much of the cholesterol accumulating in the cells appears to be associated with lysosomes, it is tempting to speculate that defects in lysosomal cholesterol transport arise in advanced foam cells. In this context, macrophages exposed to oxidized LDL can internalize a substantial amount of cholesterol, but there is relatively little stimulation of ACAT-mediated cholesterol esterification [8]. According to one model, oxysterol-induced inhibition of lysosomal sphingomyelinase leads to accumulation of lysosomal sphingomyelin, which binds cholesterol and thus inhibits transport of the cholesterol out of lysosomes (M. Aviram, 1995).

Unesterified cholesterol accumulation in macrophages may be an important cause of macrophage death in advanced atherosclerotic lesions [9] (Fig. 4A–C). Cholesterol-induced apoptosis in cultured macrophages requires an increase in the cholesterol:phospholipid ratio of the ER membrane. This process, which in essence 'stiffens' the ER membrane by promoting the packing of membrane phospholipids, triggers a well-known ER stress signal transduction pathway called the unfolded protein response (UPR) [9] (B. Feng, 2003; Y. Li, 2004). The UPR effector CHOP (C/EBP-homologous protein), in

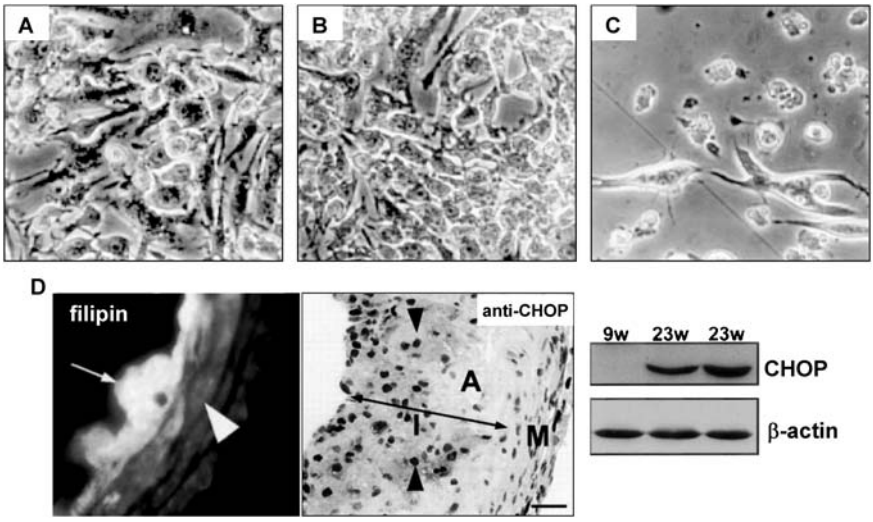


Fig. 4. Cholesterol-induced death in macrophages and evidence for unesterified cholesterol accumulation and activation of the unfolded protein response in advanced atherosclerotic plaques. (A–C) Macrophages were cultured under control conditions (A) or conditions leading primarily to cholesteryl ester loading (B) or unesterified cholesterol loading (C). Note that cholesterol-loaded macrophages, but not those loaded mostly with cholesteryl esters, have obvious signs of cytotoxicity, including detachment from the dish and altered morphology. From [33]. *J. Biol. Chem.* **271**: 22773–22781. (D) Shows an aortic root lesion from a chow-fed *Apoe*$^{-/-}$ mouse stained with filipin to depict unesterified cholesterol; the arrow shows a macrophage-rich area, and the arrowhead is in the smooth muscle cell-rich media. The middle panel shows evidence that the unfolded protein response (UPR) is activated in the macrophage-rich region of an aortic root lesion from an *Apoe*$^{-/-}$ mouse. In particular, immunostaining of the UPR effector CHOP (C/EBP-homologous protein) is shown in the nuclei of macrophages in the intima (arrowheads); I, intima; M, media; A, acellular necrotic core. Bar: 50 μm. The lower right panel depicts an immunoblot showing the induction of CHOP in aortic tissue lysates from 9-week-old (9w) or duplicate 23-week-old (23w) chow-fed *Apoe*$^{-/-}$ mice; the blot for β-actin is a control for protein loading. From [35]. *Circulation*. **111**: 1814–1821. Reproduced with permission from the publisher.

conjunction with the activation of apoptosis pathways and suppression of cell survival pathways, triggers macrophage apoptosis through both mitochondrial and cell-surface (i.e., Fas receptor) pathways [9]. Importantly, recent studies have shown that macrophages in advanced atherosclerotic lesions have both increased cholesterol accumulation and activation of the UPR and expression of CHOP (B. Feng, 2003; R. Austin, 2005; A.J. Lusis, 2006) (Fig. 4D).

2.5. Cholesterol accumulation in lesional smooth muscle cells

Smooth muscle cells in atherosclerotic lesions also accumulate large amounts of CE, although the mechanisms involved are poorly understood [3]. As with macrophages, native LDL is a poor inducer of foam cell formation, but substantial cholesterol accumulation has been induced in cultured smooth muscle cells by aggregated LDL (L. Badimon, 2004). Cytokine treatment of cultured smooth muscle cells leads to the induction of the type A scavenger receptor, but no data specifically show that oxidized LDL can cause foam cell formation in smooth muscle cells either in vitro or in vivo. Finally, remnant lipoproteins, including β-VLDL, cationized LDL, and CE emulsions, can induce cholesterol accumulation in cultured smooth muscle cells, but their roles in vivo are not known.

2.6. The fate of foam cell cholesterol in atheromata

Cholesteryl esters, which exist in membrane-bound droplets in the macrophage cytoplasm, undergo a continuous cycle of hydrolysis by neutral CE hydrolase and re-esterification by ACAT (M.S. Brown and J.L. Goldstein, 1980). If extracellular cholesterol acceptors, like high-density lipoproteins or apolipoprotein A1, are available, some of this cholesterol can leave the cell via the action of cell-surface ATP-binding cassette (ABC) transporter proteins, enter the circulation, and be transported to the liver in a process known as reverse cholesterol transport (Chapter 20). The fatty acyl and neutral lipid composition of foam cell droplets may influence this process by affecting the fluidity of the droplets. It is also possible that the foam cells themselves can leave lesions, perhaps after being transformed into dendritic-like cells [18]. Finally, as described above, foam cells in atherosclerotic lesions die, and thus cellular stores of CE and unesterified cholesterol, including cholesterol crystals, can be released into the lesions. This process undoubtedly contributes to the formation of the necrotic, or lipid, core of advanced atheromata, because such areas contain macrophage debris [9] (M.J. Mitchinson, 1995). As described in Section 1, necrotic cores have important pathophysiologic significance because they predispose lesions to plaque rupture, the proximate cause of acute vascular clinical syndromes.

3. Oxysterols and atherosclerosis

3.1. Origins of oxysterols

Oxysterols arise from dietary sources, non-enzymatic oxidation, and enzymatic oxidation reactions [19]. The structure of some of the oxysterols that may be involved in atherosclerosis are shown in Fig. 5. Dietary oxysterols are incorporated into chylomicrons and

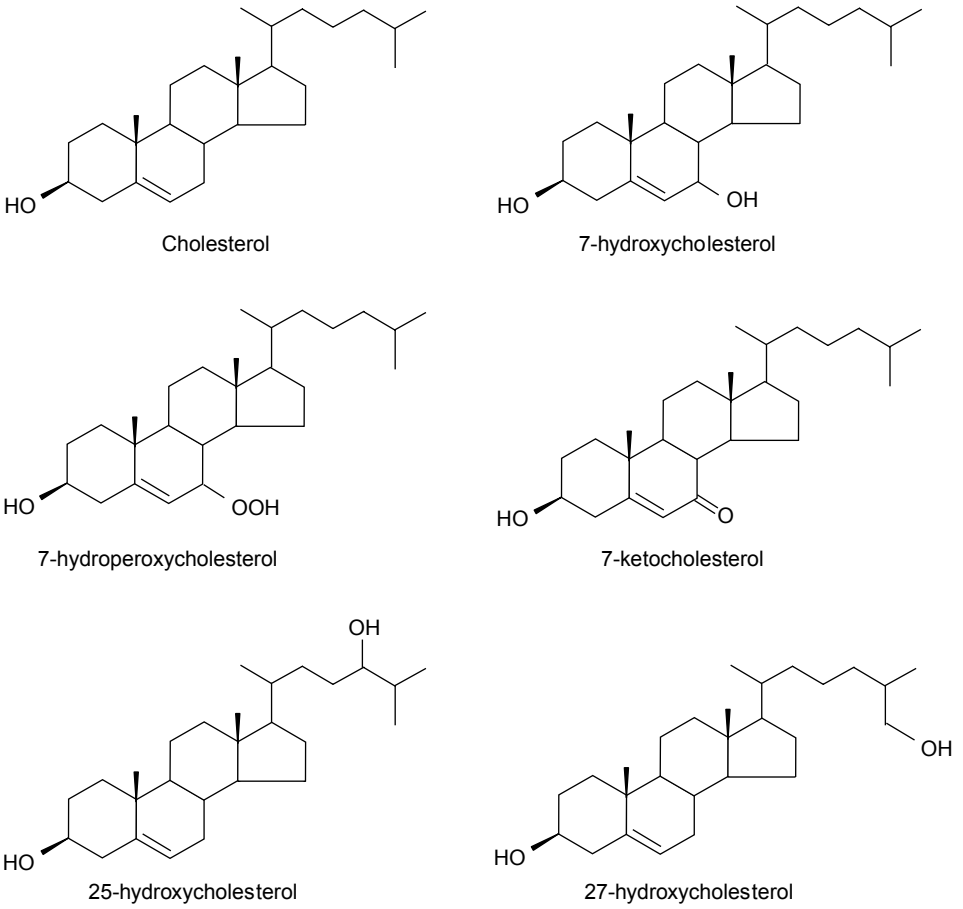


Fig. 5. Structures of some oxysterols that have been implicated in atherogenesis. Adapted from Ref. [19]. Reproduced with permission from the publisher.

include 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, and α- and β-5,6-epoxycholesterol. 7-hydroxycholesterol, 7-ketocholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol can be formed in vivo, but it is not clear whether non-enzymatic or enzymatic mechanism are involved. Specific enzymatic reactions include the formation of 7α-hydroxycholesterol by cholesterol 7α-hydroxylase in liver (Chapter 16) and 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid by 27-hydroxylase in liver and macrophages.

3.2. Oxysterols in plasma, lipoproteins, and atherosclerotic lesions

The most abundant oxysterols in human plasma are 27-, 24-, and 7α-hydroxycholesterol (Fig. 5), and most of these are esterified to fatty acids at the 3β position by lecithin:cholesterol acyltransferase [19]. Both unesterified and esterified oxysterols partition in

lipoproteins, similar to cholesterol and CE, respectively, although 27-hydroxycholesterol is generally not found in VLDL, and unesterified 25-hydroxycholesterol can be associated with albumin. With the possible exception of 7β-hydroxycholesterol, there is no clear relationship between plasma levels of oxysterols and atherosclerosis.

Oxysterols are also found in copper-oxidized LDL and consist predominantly of 7-ketocholesterol, 7-hydroperoxy-cholesterol, 7-hydroxycholesterol, and epoxycholesterol. LDL oxidized by more physiologic means, such as during contact with macrophages or by incubation with lipoxygenase, accumulates 7-hydroperoxyxycholesterol (Fig. 5). Myeloperoxidase-treated cholesterol leads to the formation of unique chlorinated cholesterol derivatives, which can give rise to cholesterol epoxides [19].

The predominant oxysterols in atherosclerotic lesions include 27-hydroxycholesterol and 7-ketocholesterol, where the levels are approximately 1% of cholesterol, with smaller amounts of 7-hydroxycholesterol; as in plasma, the vast majority of the oxysterols are esterified [19]. These oxysterols are found mostly in macrophage foam cells, which probably reflects the abundance of 27-hydroxycholesterol (Section 3.3). Moreover, 27-hydroxylated 7-ketocholesterol is found in human atherosclerotic lesions, probably via the action of macrophage sterol 27-hydroxylase on 7-ketocholesterol internalized by macrophages (W. Jessup, 2000). When cultured macrophages are incubated with copper-oxidized LDL, 50% of the accumulated sterols are oxysterols, many of which accumulate in lysosomes as non-ACAT-derived oxidized fatty acid esters of oxysterols (W. Jessup, 2000). Although copper-oxidized LDL contains substantial amounts of 7OOH, macrophages accumulate very little of this lipid probably due to conversion to 7OH by phospholipid hydroperoxide glutathione peroxidase [19].

3.3. Physiologic significance of oxysterols in atherosclerosis

The proposed roles of oxysterols in atherosclerosis are based primarily on the results of cell-culture experiments. There are a number of in vivo studies in which investigators have exposed animals to oxysterols through diet or injection, but the overall results are not conclusive. For example, in a review by Brown and Jessup [19] of 13 oxysterol dietary studies, 6 showed an increase in atherosclerosis, but 4 demonstrated a decrease in lesion size and 3 showed no effect. Differences in animal models and types and doses of oxysterols undoubtedly account for some of these differences.

There are four major areas of oxysterol biology that have emerged from cell-culture studies. These include oxysterol effects on the regulation of intracellular cholesterol metabolism, cellular cytotoxicity, sterol efflux from macrophages, and activation of nuclear transcription factors. Issues related to oxysterols and intracellular cholesterol metabolism are covered in detail in Chapters 15 and 16. In brief, both cholesterol and certain oxysterols, such as 25-hydroxycholesteol and 7-ketocholesterol, suppress the proteolytic activation of sterol response element-binding protein. This, in turn, leads to transcriptional down-regulation of the LDL receptor and certain enzymes in the cholesterol biosynthetic and fatty acid synthesis and metabolism pathways. 25-Hydroxycholesterol and 7-ketocholesterol, like cholesterol, can also promote the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, a rate-limiting enzyme in isoprenoid and cholesterol biosynthesis, and 25-hydroxycholesterol can activate ACAT and suppress neutral CE hydrolase activity. Cells possess

a protein that binds oxysterols, called oxysterol-binding protein, but its role in cellular responses to oxysterols is not known. Importantly, the physiologic role of oxysterols in cellular cholesterol metabolism as it relates to atherosclerosis is far from certain, particularly because the concentrations of oxysterols used in most macrophage cell-culture studies far exceeds those found in macrophage foam cells in vivo [19].

Oxysterols have diverse roles in cholesterol efflux, a critical topic in foam cell biology. On the one hand, cells incubated with 7-ketocholesterol and 25-hydroxycholesterol have decreased cholesterol efflux. Possible mechanisms include inhibition of membrane desorption of cholesterol or phospholipids or, as mentioned above, inhibition of lysosomal sphingomyelinase leading to lysosomal sequestration of cholesterol (M. Aviram, 1995). On the other hand, the conversion of cholesterol by macrophage sterol 27-hydroxylase to 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, which are efficiently effluxed from cells, has been proposed to promote sterol efflux from foam cells (I. Björkhem, 1994). Indeed, 27-hydroxycholesterol is a prominent oxysterol found in lesions. Rare patients lacking 27-hydroxylase activity have xanthomas and premature coronary artery disease, and some studies have shown an inverse correlation between 27-hydroxylase levels and atherosclerosis in subjects without enzyme deficiency per se (N.R. Cary, 2001). However, 27-hydroxylase-deficient mice on a non-atherogenic background or diet do not develop xanthomas or spontaneous atherosclerotic lesions (E. Leitersdorf, 1998).

A major area of investigation has been on the cytotoxic effects of certain oxysterols on cultured endothelial cells, macrophages, and smooth muscle cells [19]. The most potent cytotoxic oxysterols include 27OH, 7βOOH, 7αOH, and 7K (G.M. Chisolm, 1996). These sterols damage cells through a variety of mechanisms including cholesterol starvation, membrane perturbation, cellular lipid peroxidation, and activation of apoptotic pathways (M. Sinensky, 2004). Although death of endothelial cells and macrophages occurs in atherosclerotic lesions and might be expected to promote complications in advanced lesions, the role of isolated oxysterols or oxysterols in oxidized LDL in these events is far from certain. The conditions used in many cell-culture studies, notably high concentrations of oxysterols and/or serum-free medium, may not reflect the situation in vivo.

Exciting recent work has revealed that certain oxysterols are activators of nuclear transcription factors [20] (D.W. Russell, 2007). In particular, several oxysterols found in vivo, including 24,25-epoxycholesterol, 24-, 22-, 25-, and 27-hydroxycholesterol, but not cholesterol, activate the liver X receptor (LXR)α, LXRβ, and the farnesoid X receptor (Chapter 16). Once activated, these receptors heterodimerize with activated retinoid X receptor, forming active transcription factors which translocate to the nucleus and induce several genes important in atherosclerosis. In particular, a set of genes important in the reverse cholesterol transport pathway is activated by this pathway [21]. The proteins encoded by these genes include macrophage ABCA1 and apolipoprotein E, which promote cholesterol efflux from foam cells; plasma CE transfer protein, which transfers high-density lipoprotein-cholesterol to lipoproteins that can be internalized by hepatocytes; and hepatic cholesterol 7α-hydroxylase, which is the key enzyme that converts cholesterol into bile acids for excretion. Studies with genetically manipulated mice have demonstrated the importance of the LXR pathway in vivo. For example, activation of the retinoid X receptor reduces atherosclerosis in apolipoprotein E knockout mice (J. Auwerx, 2001), and the livers of cholesterol-fed LXRα knockout mice accumulate very large

amounts of cholesterol (D.J. Mangelsdorf, 1998). Moreover, LXR activators promote reverse cholesterol transport and suppress atherosclerosis progression in mice (P. Tontonoz, 2002; D. Rader, 2006).

In summary, oxysterols are known to exist in atherosclerotic lesions and have been demonstrated in cell-culture experiments to have profound cellular effects that could influence the development, progression, and reversal of atherosclerosis. The key question in this field of research, however, is whether the concentrations of oxysterols in vivo are high enough to influence atherogenesis. Thus far, only the oxysterol-activated nuclear transcription pathway has been directly supported by in vivo data, and even in this case the precise roles and identification of the activating oxysterols in vivo have not yet been elucidated. Moreover, to the extent that oxysterols are generated in vivo and not just obtained from the diet, their role in human atherosclerosis has been questioned by clinical trials showing little or no protective effect of antioxidants on atherosclerotic coronary artery disease (K.J. Williams and E.A. Fisher, 2005).

4. Triacylglycerols and atherosclerosis

There are two major issues that arise when considering the role of triglycerides in atherosclerosis: the effect of triacylglycerol-containing lipoproteins in the plasma on atherosclerotic lesion development, and the direct role of arterial-wall triacylglycerols in atherogenesis. The association between triacylglycerol-rich lipoproteins and atherosclerotic vascular disease is often difficult to assess due to complex metabolic relationships between these lipoproteins and other risk factors for atherosclerosis, including low plasma high-density lipoproteins, insulin resistance, and hyperfibrinoginemia [22]. Certainly, those triacylglycerol-containing lipoproteins that also have a high content of cholesterol, such as remnant lipoproteins, have been shown to be associated with atherosclerotic disease and probably function largely by delivering large amounts of cholesterol to the subendothelial space. The possibility that triacylglycerols and triacylglycerol-derived fatty acids also contribute to the atherogenicity of these lipoproteins, however, must also be considered (Section 5). Interestingly, metabolic disorders resulting in severe increases in plasma triacylglycerol, such as lipoprotein lipase deficiency, are not associated with increased risk of atherosclerosis. In these disorders, the triacylglycerol-rich lipoproteins are so large that they cannot enter the arterial wall (D.B. Zilversmit, 1989).

Triacylglycerols constitute a measurable proportion of the lipid content of atherosclerotic lesions, although considerably less than that of cholesterol. In one study, for example, the weight-percentages of triacylglycerols in fatty streaks and advanced lipid-rich lesions were 2.8 and 6.0%, respectively; the corresponding total cholesterol percentages were 9.6 and 31.5%, respectively [17]. However, both extracellular and intracellular triacylglycerols could play an important role in atherogenesis by serving as a source of fatty acids following hydrolysis by extracellular and intracellular lipases. Indeed, the relatively low content of lesional triacylglycerol may be partially due to triacylglycerol hydrolysis in lesions. As discussed in Section 5, fatty acids are precursors of potentially important bioactive lipids.

Lesional cells in general, and macrophages in particular, can accumulate triacylglycerols via the uptake of triacylglycerol-rich lipoproteins and by intracellular triacylglycerol synthesis. Although triacylglycerols are a relatively minor component of neutral lipid droplets in lesional foam cells, even small percentages can lower the melting temperature of these droplets [17]. Polyunsaturated fatty acids in the CE and triacylglycerols of lipid droplets also lower their melting temperature. Liquid neutral lipid droplets in foam cells are hydrolyzed at a more rapid rate than liquid crystalline droplets, and thus foam cells with a higher content of triacylglycerols may have an increased rate of cholesterol efflux (J.M. Glick, 1989).

5. Fatty acids and atherosclerosis

5.1. Direct effects of fatty acids

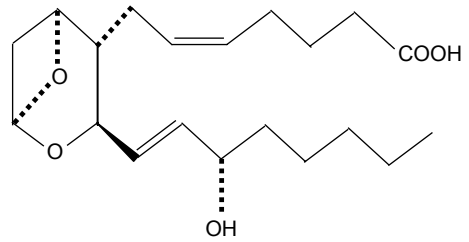
Fatty acids may have direct effects on atherogenesis and are also the precursors of specific bioactive lipids that may have important roles in lesion development. Lesions contain up to 0.4 mg of unesterified fatty acids per gram of wet tissue (L. Robert, 1976). In terms of direct effects, high concentrations of extracellular fatty acids could, in theory, lower the pH of focal areas in lesions, thus enabling the action of certain enzymes, such as lysosomal hydrolases that are secreted or leak from cells. When taken up by cells, fatty acids stimulate the synthesis of CE, phospholipid, and triacylglycerol. Individual types of fatty acids can have specific effects. For example, oleate, but not linoleate, is a potent stimulator of the ACAT reaction [16], and neutral lipids esterified to polyunsaturated fatty acids have a lower melting temperature, which tends to promote neutral lipid hydrolysis and lipid efflux.

5.2. Oxidation of long-chain polyunsaturated fatty acids: introduction

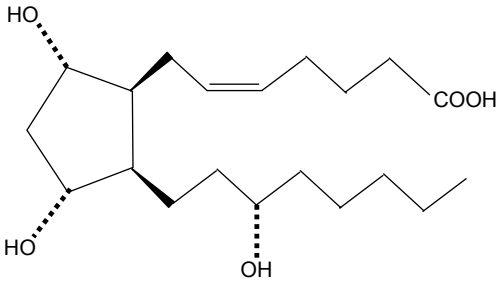
The major bioactive products of fatty acid metabolism relevant to atherosclerosis are those that result from enzymatic or non-enzymatic oxidation of polyunsaturated long-chain fatty acids. In most cases, these fatty acids are derived from phospholipase A_2-mediated hydrolysis of phospholipids (Chapter 11) in cellular membranes or lipoproteins, or from lysosomal hydrolysis of lipoproteins after internalization by lesional cells. In particular, arachidonic acid is released from cellular membrane phospholipids by arachidonic acid-selective cytosolic phospholipase A_2. In addition, there is evidence that group II secretory phospholipase A_2 (Chapter 11) hydrolyzes extracellular lesional lipoproteins, and lysosomal phospholipases and cholesterol esterase release fatty acids from the phospholipids and CE of internalized lipoproteins. Indeed, Goldstein and Brown surmised that at least one aspect of the atherogenicity of LDL may lie in its ability to deliver unsaturated fatty acids, in the form of phospholipids and CE, to lesions (J.L. Goldstein and M.S. Brown, 2001).

5.3. Oxidative metabolites of arachidonic acid

An important fate of arachidonic acid is enzymatic conversion to prostaglandins by one of two prostaglandin G/H synthases [23]. As described in Chapter 12, these enzymes have



Thromboxane A_2



8-iso-$PGF_{2\alpha}$

Fig. 6. Structures of two arachidonic acid derivatives proposed to play important roles in thrombosis and atherogenesis. Thromboxane A_2 is a potent inducer of platelet aggregation that contributes to acute thrombosis in advanced atherosclerosis in vivo. The isoprostane 8-*iso*-$PGF_{2\alpha}$ is being investigated as a marker of oxidative stress in atherosclerosis and may also have direct atherogenic effects on platelets and smooth muscle cells.

both cyclooxygenase (COX) and hydroperoxidase activities, and are often referred to as COX-1 and -2. Although atherosclerotic lesions express both isoforms, mature human platelets express only COX-1. In this regard, the most well-documented role of a COX product on atherothrombotic vascular disease is platelet-derived thromboxane A_2 (Fig. 6). Thromboxane A_2 is a potent inducer of platelet aggregation and vasoconstriction, and aspirin-induced inhibition of platelet COX-1 accounts for its benefit in the secondary prevention of strokes and myocardial infarction. Moreover, selective inhibition of COX-2 appears to promote atherothrombotic coronary artery disease in humans (G.A. FitzGerald, 2006). This finding may be related to endothelial COX-2-induced synthesis of prostacyclin, which blocks platelet aggregation, cellular interactions, and vascular smooth muscle cell proliferation in vitro and in vivo. Indeed, studies in $Apoe^{-/-}$ mice have shown that thromboxane A_2 promotes the initiation and progression of atherosclerosis by enhancing platelet activation, and that prostacyclin has opposite effects by suppressing the interaction of leukocytes with endothelial cells (S. Narumiya, 2004). A third COX-derived prostaglandin that has received recent interest is 15-deoxy-D12,14-prostaglandin J_2 (15d-PGJ_2), which, at least in vitro, is an agonist of peroxisomal proliferator-activated receptor-γ (PPARγ) (C.K. Glass, 1998). PPARγ is expressed in atherosclerotic lesions and

in cultured endothelial cells, vascular smooth muscle cells, and monocyte/macrophages (B. Staels, 2000). Although cell-culture studies have revealed a variety of biological effects that may be pro- or anti-atherogenic, in vivo studies suggest that PPARγ plays an anti-atherogenic role (C.K. Glass, 2004). The physiologic significance of 15d-PGJ_2 in PPARγ biology remains to be determined.

A relatively new class of oxidized arachidonic acid derivatives with potential relevance to atherosclerosis are F_2 isoprostanes [24] (Fig. 6) (Chapter 12). These compounds form as a result of non-enzymatic, free-radical attack of the fatty acid moieties of cellular or lipoprotein phospholipids, followed by release of the isoprostanes from the phospholipids by a phospholipase. 8-*iso*-prostaglandin-F_2 may also be formed by the action of COX-1 or -2 in platelets or monocytes, respectively, but the significance of COX-dependent 8-*iso*-PGF_2 formation in vivo is unproven. F_2 isoprostanes circulate in the plasma and appear in the urine as free compounds or esterified to phospholipids, and 8-*iso*-prostaglandin-F_2 is found in atherosclerotic lesions in association with macrophages and smooth muscle cells. The potential significance of isoprostanes to atherosclerosis are their effects on platelets and vascular cells, as demonstrated in cell-culture studies, and their potential usefulness as a non-invasive marker of oxidant stress. 8-*iso*-PGF_2 induces platelet aggregation, DNA synthesis in vascular smooth muscle cells, and vasoconstriction. Moreover, elevated levels of F_2 isoprostanes are found in cigarette smokers, diabetics, and subjects with hypercholesterolemia, where they may serve as an indicator of increased lipid peroxidation.

Arachidonic acid can also be oxidized by 5-, 12-, and/or 15-lipoxygenases to various mono-, di-, and tri-hydroxyderivatives called leukotrienes (Chapter 12), some of which are present in atherosclerotic lesions [25]. The monohydroxylated leukotrienes 12(*S*)- and 15(*S*)-HETEs can be produced by human arterial endothelial cells and can promote monocyte adherence, an important early event in atherogenesis. Although 15(*S*)-HETE is found at relatively high levels in human atherosclerotic lesions, its role in vivo is not known. Atheromatous tissue has the capacity to synthesize the dihydroxylated leukotrienes LTC_4 and LTB_4 (R. De Caterina, 1988; C. Patrono, 1992). Leukotriene-C_4 can be made by monocytes, macrophages, and endothelial cells, and leukotriene-B_4 is synthesized by activated monocytes. In theory, leukotriene-C_4 could promote vasoconstriction, and leukotriene-B_4 could contribute to atherosclerosis-related endothelial alterations, such as increased permeability and adhesiveness. Moreover, leukotriene-B_4 is also an activator of PPARα, which appears to promote atherogenesis in vivo (C.F. Semenkovich, 2001). Finally, the trihydroxylated derivatives of arachidonic acid, the lipoxins, possess some anti-inflammatory properties, but their overall effect on atherogenesis and atherothrombotic vascular disease remains to be determined.

Another fate of arachidonic acid with potential relevance to atherosclerosis is cytochrome P450 monooxygenase-derived metabolism to epoxyeicosatrienoic acids (EETs) (Chapter 12), which may also be formed non-enzymatically by the interaction of arachidonic acid with free radicals (D.D. Gutterman, 2006). EET synthesis in cultured endothelial cells can be induced by LDL, and EETs are found both in LDL and in human atherosclerotic lesions. Biological effects of EETs include potentially anti-atherogenic effects, such as vasodilatation and prevention of platelet aggregation, and atherogenic responses, such as increased monocyte adhesion.

5.4. Atherogenic and anti-atherogenic effects of other long-chain polyunsaturated acids

Dietary intake of n-6 fatty acids such as linoleic acid, and n-3 fatty acids, such as the fish oils eicosapentanoic acid and docosahexaenoic acid, lowers plasma cholesterol and antagonizes platelet activation, but the fish oils are much more potent in this regard [26]. In particular, n-3 fatty acids competitively inhibit thromboxane synthesis in platelets but not prostacyclin synthesis in endothelial cells. These fatty acids have also been shown to have other potentially anti-atherogenic effects, such as inhibition of monocyte cytokine synthesis, smooth muscle cell proliferation, and monocyte adhesion to endothelial cells. While dietary intake of n-3 fatty acid-rich fish oils appears to be atheroprotective, human and animal dietary studies with the n-6 fatty acid linoleic acid have yielded conflicting results in terms of effects on both plasma lipoproteins and atherosclerosis. Indeed, excess amounts of both n-3 and n-6 fatty acids may actually promote oxidation, inflammation, and possibly atherogenesis (M. Toberek, 1998). In this context, enzymatic and non-enzymatic oxidation of linoleic acid in the *sn*-2 position of LDL phospholipids to 9- and 13-hydroxy derivatives is a key event in LDL oxidation (Section 6.2).

6. Phospholipids and related lipids

6.1. Introduction

Phospholipids comprise the outer monolayer of lesional lipoproteins and the membranes of lesional cells. In lipoproteins, the phospholipid monolayer provides an amphipathic interface between the neutral lipid core and the aqueous external environment, and provides the structural foundation for the various apolipoproteins (Chapter 18). In the specific context of atherosclerosis, the phospholipids of lesional lipoproteins are modified by various oxidative reactions that could have important pathological consequences. In lesional cells, membrane phospholipids not only play structural roles but also are precursors to important phospholipase-generated signaling molecules that may participate in atherogenesis.

6.2. Oxidative modification of phosphatidylcholine in lesional lipoproteins

Oxidation of LDL, and probably other lesional lipoproteins, occurs in atherosclerotic lesions and may contribute to lesion pathology at various stages of atherogenesis [2]. Based on in vitro studies and, in some cases, genetically altered mutant mouse models, LDL oxidation may be triggered by oxidative enzymes secreted by lesional cells, including myeloperoxidase, inducible nitric oxide synthase, and, 15-lipoxygenase. In the above sections, oxidative modifications of cholesterol, CE, and fatty acids in LDL were discussed. The phospholipids of LDL also undergo oxidative modification, and the products of these reactions are found in atherosclerotic lesions and have potentially important atherogenic effects on lesional cells.

The most abundant and important oxidative changes in LDL phospholipids are those that occur to the unsaturated fatty acids in the *sn*-2 position. Berliner and colleagues described several products of phospholipid oxidation that result from this process [27] (Fig. 7). An

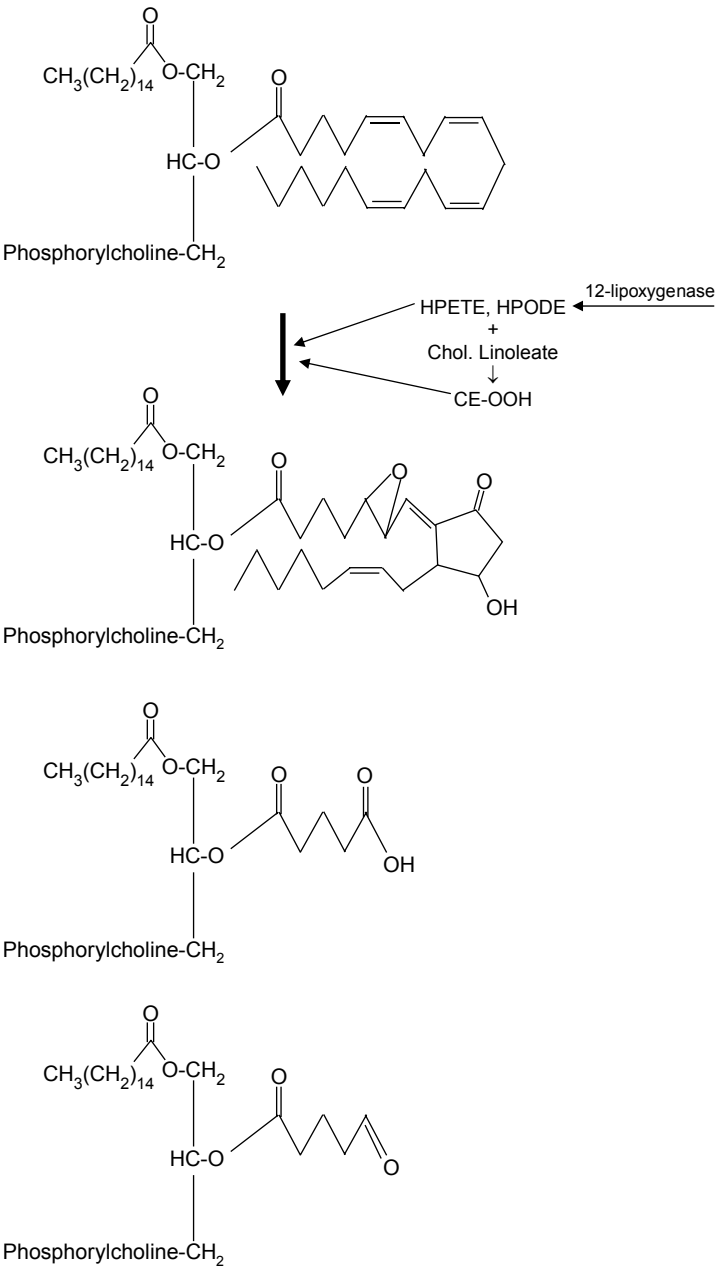


Fig. 7. Oxidation of LDL phospholipids in the generation of minimally modified LDL. 'Seeding' molecules like HPETE, HPODE, and cholesteryl linoleate hydroperoxide (CE-OOH) are proposed to trigger the oxidation of 1-palmitoyl-2-arachidonoyl phosphatidylcholine in LDL, leading to the generation of three oxidized phosphatidylcholine species that confer atherogenic activity to minimally modified LDL. 12-LO, 12-lipoxygenase. Adapted from Ref. [28]. Reproduced with permission from the publisher.

early event is the addition of oxygens to these fatty acids, resulting in the generation of hydroxy fatty acids, hydroperoxy fatty acids, and isoprostanes. In one model, lipoxygenase-generated hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE) in LDL surface phospholipids act as 'seeding' molecules. These hydroperoxy fatty acids then trigger the oxidation of arachidonate-containing phospholipids, notably 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine. This early series of events occurs before any oxidative modification of apolipoprotein B100 and results in the generation of so-called 'minimally modified' LDL. Minimally modified LDL is found in atherosclerotic lesions and promotes monocyte binding to endothelial cells and monocyte chemotaxis in cultured cell studies [28]. The oxidized arachidonate-containing phospholipids, which can account for much of the biological activity of minimally modified LDL, include those in which the arachidonoyl group is modified to 5-oxovaleroyl, glutaroyl, or 5,6-epoxyisoprostane phosphatidylcholine. The potentially atherogenic molecules induced in endothelial cells and smooth muscle cells by these oxidized phospholipids include E-selectin, vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, macrophage colony stimulating factor, P-selectin, and interleukin-8. These cellular effects, like those of leukotriene-B_4 (Section 5.3), likely involve the activation of PPARα.

Another possible consequence of oxidation of *sn*-2 unsaturated fatty acids in phospholipids of oxidized LDL is fragmentation of the fatty acid, resulting in a phospholipid with a short acyl group in the *sn*-2 position [27]. If the *sn*-2 fatty acid were arachidonate, the truncated acyl group would be a 5-carbon aldehyde or a 5-carbon carboxylic acid. If the *sn*-2 group were linoleate, the products would be a 9-carbon aldehyde or carboxylic acid. Phospholipids containing these shortened *sn*-2 acyl chains have biological activity similar to that of platelet-activating factors, where an ether-linked fatty acyl group occupies the *sn*-1 position and an acetyl group is in the *sn*-2 position (Chapter 9). By interaction with a G-protein-coupled receptor on a variety of cell types, platelet-activating factor activates both platelets and leukocytes and increases vascular permeability. In terms of atherosclerosis, studies with platelet-activating factor receptor antagonists have suggested that the chemotactic activity of minimally modified LDL may be mediated through platelet-activating factor-like phospholipids acting directly on monocytes (P.D. Reaven, 1997). Similarly, the smooth muscle cell mitogenic activity of oxidized LDL can be mimicked by phosphatidylcholine containing a 5-carbon carboxylic acid and can be blocked by a platelet-activating factor receptor antagonist (S.M. Prescott, 1995).

Phospholipase A_2-mediated hydrolysis of oxidized phospholipids can result in the release of either intact oxygenated free fatty acids by phospholipase A_2 (Section 5), or fragmented fatty acids, such as malondialdehyde, which can lead to protein modification. The other product of this reaction is lysophosphatidylcholine (D. Steinberg, 1988) [29]. In vitro studies have revealed multiple effects of lysophosphatidylcholine on lesional cells, including expression of adhesion molecules on endothelial cells, monocyte chemotaxis and macrophage scavenger receptor expression, growth factor expression by smooth muscle cells and macrophages, cellular cytotoxicity, and inhibition of T cell activation. Although it is not known whether sufficient lysophosphatidylcholine exists in lesions to cause these effects in vivo, a receptor for lysophospholipids, called G2A, was identified, and studies with G2A-deficient mice suggest a possible role for lysophospholipids in the regulation of T cell activation in vivo (O.N. Witte, 2001). Finally, adducts of oxidized

phospholipids and apolipoprotein B100 can result from LDL oxidation. These adducts are recognized by macrophages and thus can mediate cellular uptake of oxidized LDL. These adducts form a potent epitope that can elicit cellular and humoral immune responses that may play important roles in atherogenesis (J.L. Witztum, 2003).

Fogelman and colleagues have suggested that one of the anti-atherogenic mechanisms of high-density lipoprotein may be its ability to prevent LDL oxidation or reduce its atherogenic activity (A.M. Fogelman, 2006). Multiple mechanisms may be involved, including removal of the 'seeding' molecules HPODE and HPETE and degradation of the oxidized phospholipids themselves by paraoxonase, an esterase/peroxidase, and platelet-activating factor-acetylhydrolase, a lipoprotein-bound phospholipase A_2-like enzyme that can cleave oxidized acyl groups from the *sn*-2 position of oxidized phospholipids. In apolipoprotein E knockout mice, targeted disruption of the paraoxonase gene increases lipoprotein oxidation and atherosclerosis (A.J. Lusis, 2000).

6.3. The phospholipids of lesional cells

Phosphatidylcholine is the major phospholipid of lesional cells and, as mentioned above, serves both structural and signaling functions (Chapter 8). In terms of cellular membrane structure, the cholesterol:phospholipid ratio in lesional cells must be kept within a certain limit in order for the proper functioning of membrane proteins [14]. Cholesterol-rich foam cells isolated from atherosclerotic lesions have intracellular phospholipid whorl-like structures, and phosphatidylcholine biosynthesis is increased in lesional areas of the arterial wall (Fig. 8). Cell-culture studies have revealed that cholesterol loading of macrophages directly leads to the activation of CTP:phosphocholine cytidylyltransferase and an increase in phosphatidylcholine biosynthesis and mass. Proof that this is an adaptive response to FC excess came from a study in which the cytidylyltransferase-α gene was disrupted in macrophages, which resulted in

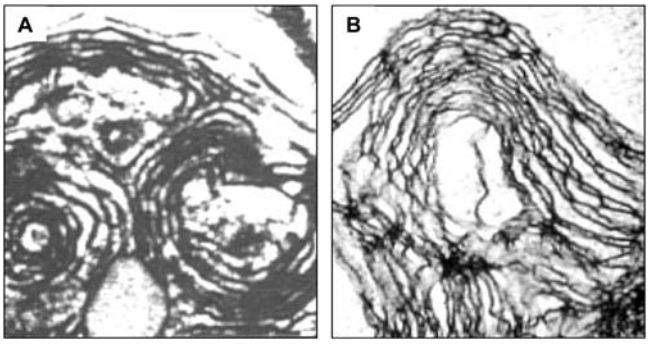


Fig. 8. Phospholipid whorls in cholesterol-loaded macrophages. (A) Electron micrograph showing a membrane whorl in the cytoplasm of a foam cell. The cell was isolated from an advanced aortic atherosclerotic lesion in a cholesterol-fed rabbit. From [34]. *Lab. Invest.* **41**: 160–167. (B) Electron micrograph showing a membrane whorl in the cytoplasm of a cholesterol-loaded J774 macrophage. The cell was in the 'adaptive' stage, i.e., before the onset of unesterified cholesterol-induced death. From Shiratori et al. (1994). *J. Biol. Chem.* **269**: 11337–11348. Reproduced with permission from the publisher.

accelerated unesterified cholesterol-induced death (I. Tabas, 2000). Thus, activation of phosphatidylcholine biosynthesis in cholesterol-loaded lesional macrophages may help to protect these cells from the toxicity of cholesterol excess.

Cellular phospholipids, particularly phosphatidylinositol and phospholipids containing unsaturated fatty acids in the *sn*-2 position, are precursors to a variety of signaling molecules (Chapters 8 and 12). These include diacylglycerol and inositol tris-phosphate that are generated by phosphatidylinositol-specific phospholipase C-induced hydrolysis of phosphatidylinositol; phosphatidic acid produced by phospholipase D; fatty acids and lysophosphatidylcholine generated by phospholipase A_2; and platelet-activating factor-like molecules produced by oxidation. Diacylglycerol activates protein kinase C, and inositol tris-phosphate leads to intracellular calcium release. Both of these reactions are involved in a variety of signaling processes that occur in lesional smooth muscle cells, macrophages, and endothelial cells, including responses to cytokines and growth factors. Oxidized LDL has been shown to activate phospholipase D in cultured vascular smooth muscle cells by a tyrosine kinase-mediated mechanism, and phosphatidic acid could mimic the proliferative effects of oxidized LDL in these cells (S. Parthasarathy, 1995). Finally, given the potential importance of apoptosis of macrophages and smooth muscle cells in atherosclerosis, phosphatidylserine is an important phospholipid in lesional cells. Phosphatidylserine is normally a component of the inner leaflet of the plasma membrane, but it becomes externalized and oxidized during apoptosis and acts as a recognition signal and ligand for phagocytes (Chapter 8). Interestingly, the macrophage CD36 receptor recognizes oxidized phosphatidylserine on apoptotic cell as well as oxidized LDL (S.L. Hazen, 2006). Thus, it is possible that phagocytosis and clearance of apoptotic cells in lesions is competitively inhibited by oxidized LDL (D. Steinberg, 1999). This is a potentially important issue, because there is evidence that suppression of phagocytic clearance of apoptotic macrophages in advanced atherosclerotic lesions leads to post-apoptotic necrosis and contributes to the generation of the necrotic core of vulnerable plaques (I. Tabas, 2005).

6.4. Sphingomyelin and ceramide

Sphingomyelin is an important component of both the phospholipid monolayer of lesional lipoproteins and of membranes of lesional cells (Chapter 8). In atherogenic lipoproteins like LDL, hydrolysis of sphingomyelin to ceramide results in lipoprotein aggregation and fusion, resulting in the formation of large aggregates that appear similar to those that occur in extracellular regions of the subendothelium of atherosclerotic lesions [8]. The mechanism of sphingomyelinase-induced aggregation and fusion, which is dependent on lipoprotein ceramide content, probably lies in both the physical effects of ceramide on lipoprotein structure and on hydrogen bonding between ceramide on one particle and phospholipids on a neighboring particle. Extracellular hydrolysis of LDL-sphingomyelin by sphingomyelinase likely occurs in the subendothelium of atherosclerotic lesions and may be catalyzed by a form of acid sphingomyelinase, called *S*-sphingomyelinase, that is secreted by endothelial cells and macrophages [13]. Although the overall importance of this reaction in vivo remains to be determined, its potential importance is that subendothelial lipoprotein retention

promotes lipoprotein retention in the arterial wall and is a potent substrate for macrophage, and possibly smooth muscle cell, foam cell formation. Lipoproteins with a high sphingomyelin:phospholipid ratio are particularly good substrates for *S*-sphingomyelinase. In this context, lipoproteins isolated from atherosclerotic lesions have a very high sphingomyelin (as well as ceramide) content. Moreover, a recent analysis of plasma samples from a case:control study showed that a high sphingomyelin:phospholipid ratio in plasma lipoproteins was an independent risk factor for coronary artery disease in humans (X.C. Jiang, 2006).

High-density lipoproteins also contains sphingomyelin. Because sphingomyelin avidly binds cholesterol, sphingomyelin may increase the ability of high-density lipoproteins to act as an extracellular acceptor for cholesterol effluxed from cells (G. Rothblat, 1997). However, high density lipoprotein-sphingomyelin has also been shown to inhibit the binding of lethicin:cholesterol acyltransferase to the lipoprotein, and so this effect may balance the effect of high density lipoprotein-sphingomyelin-induced cholesterol efflux on reverse cholesterol transport (A. Jonas, 1996). Application of this latter principle has potentially critical implications for humans: high-density lipoprotein-2 from subjects with CE transfer protein deficiency has a very high cholesterol efflux potential, and the mechanism may be related to the low sphingomyelin:phospholipid ratio and high lecithin:cholesterol acyltransferase activity in this high-density lipoprotein (A. Tall, 2006) (Chapter 20).

Sphingomyelin in cellular membranes may have several important roles related to atherogenesis. A number of findings suggest that excess sphingomyelin in late endosomes suppresses cholesterol trafficking from that organelle. For example, the defective intracellular trafficking and ACAT-mediated esterification of oxidized LDL-derived cholesterol in macrophages may be due to the inhibition of acid sphingomyelinase by oxidized LDL lipids (M. Aviram, 1995). Moreover, the sphingomyelin accumulation that occurs in acid sphingomyelinase-deficient macrophages leads to defective cholesterol trafficking and efflux (I. Tabas, 2001). In the past, the mechanism has been ascribed to the ability of sphingomyelin to interact strongly with cholesterol and thus retard its removal. However, recent work has raised an alternative mechanism related to a very early step in late endosomal cholesterol trafficking, namely, defective transfer of cholesterol derived from newly hydrolyzed CE from the late endosomal lumen to the late endosomal membrane. This concept is based on data showing that membranes enriched in sphingomyelin are relatively poor acceptors of cholesterol complexed with the Niemann Pick C2 protein, which is thought to be the mediator of lumen-to-membrane cholesterol transport in late endosomes (J. Storch, 2006) (Chapter 17).

Another area of sphingomyelin biology with potential relevance to atherosclerosis is related to cell signaling [29]. Hydrolysis of cellular sphingomyelin by either neutral or acid sphingomyelinase results in the generation of intracellular ceramide, which is involved in a variety of cell-signaling reactions (Chapter 14). In terms of atherosclerosis, ceramide-mediated signaling may play roles in smooth muscle cell proliferation and apoptosis and macrophage apoptosis [29]. Alterations in ceramide synthesis and ceramide hydrolysis by cellular ceramidases may also influence these events. In this context, ceramidase-generated sphingosine can be phosphorylated to sphingosine-1-phosphate, which is another signaling molecule that has been implicated in atherosclerosis [30].

6.5. Glycosphingolipids

Sugar transferases convert ceramide to a variety of glycosphingolipids, including neutral glycosphingolipids such as glucosylceramide and lactosylceramide, and polar glycosphingolipids such as gangliosides, which contain ceramide, sugars, and sialic acid and/or *N*-glycolylneuraminic acid (Chapter 14). Glycosphingolipids are found both in plasma lipoproteins and in the cells and extracellular regions of atherosclerotic lesions [29]. Chatterjee and colleagues have proposed that lactosylceramide, synthesized from glucosylceramide by the enzyme UDP-galactose:glucosylceramide-β1-4 galactosyltransferase-2, is a lipid second messenger that is involved in the proliferation of vascular smooth muscle cells by oxidized LDL [29]. In cultured smooth muscle cells, oxidized LDL stimulates the galactosyltransferase-2 activity and lactosylceramide synthesis. Proliferation induced by oxidized LDL in these cells was blocked by an inhibitor of the galactosyltransferase, and exogenous lactosylceramide was able to stimulate proliferation in the absence of oxidized LDL. The mechanism may involve 5-oxovaleroyl phosphatidylcholine-mediated stimulation of NADPH oxidase by lactosylceramide, leading to a signaling cascade triggered by superoxide radicals and involving Ras activation and p44-mitogen-activated protein kinase. Interestingly, native LDL was shown to decrease GalT-2 activity and lactosylceramide synthesis in smooth muscle cells in an LDL receptor-dependent manner. While these cell culture studies have provided a potentially interesting role for the galactosyltransferase-2 activity and lactosylceramide in atherosclerosis, the physiologic significance of these findings overall awaits future in vivo studies.

7. Future directions

The potential roles of the many types of lesional and lipoprotein lipids in atherogenesis is staggering (Table 2). Not surprisingly, most studies investigating these roles have used cultured cells where the concentrations of the lipids and the overall state of cells may be very different from those in atherosclerotic lesions. Thus, one of the most important, and difficult, areas in future studies will be to sort out these effects in vivo through the use of inhibitory compounds or genetic manipulations in mice. In some cases, such studies have already provided impressive results, such as the decrease in atherosclerosis observed in 15-lipoxygenase knockout mice (C.D. Funk, 1999). On the other hand, the effects of antioxidants in both humans and animal models have yielded conflicting results (R. Stocker, 2001). Further understanding of the molecular basis of lipid synthesis and catabolism, and of the action of bioactive lipids in cells, will help in the design of improved in vivo models. The most important of these lipids include cholesterol, oxidized phospholipids, fatty acids, eicosanoids, oxysterols, and sphingolipid derivatives. Key areas for investigating the cellular effects of bioactive lipids include inflammatory responses in endothelial cells, T cells, and macrophages; secretion of atherogenic and anti-atherogenic molecules by lesional cells; proliferation of macrophages and smooth muscle cells; and apoptotic and necrotic death in lesional

Table 2
Summary of proposed roles of lesional lipids in atherosclerosis

Lipid	Overall effects in atherosclerosis	Specific examples
Cholesterol	Accumulation in and alteration of macrophages and SMCs, including gene regulation and, in excess, death	Stimulation of ACAT
		Repress transcription of LDL receptor gene
		FC-induced macrophage death
Cholesteryl ester	Accumulation in macrophages and SMCs	Foam cell formation
	Substrate for oxidation	Cholesterol linoleate hydroperoxide as a pro-oxidant
Oxysterols	Regulation of cellular cholesterol metabolism	Stimulation of ACAT
	Cytotoxicity	7K-induced macrophage death
	Sterol efflux pathways	Efflux of 27-OH
	Activation of nuclear transcription factors	Activation of LXR by 22-OH
Triacylglycerols	Source of fatty acids	Liquid crystalline → liquid neutral transformation of foam cell droplets
	Affect neutral lipid droplet fluidity in foam cells	
Fatty acids	Stimulate CE, TG, and PL synthesis	Thromboxane A_2 → platelet aggregation
	Polyunsaturated FAs are sources of bioactive eicosanoids	Isoprostanes → SMC proliferation
Phospholipids (other than sphingolipids)	Structural roles in lipoproteins and lesional cells	Part of adaptive response to FC-induced cytotoxicity
	Source of signaling molecules	Lyso-PC → monocyte chemotaxis
	Substrate for oxidative modification into bioactive molecules	PGPC, PGPC, PEIPC → induction of endothelial adhesion molecules
Sphingolipids	Source of signaling molecules	Ceramide → lesional cell death and proliferation
	Involve on lipoprotein aggregation	SMase-induced LDL aggregation
	Influence intracellular cholesterol trafficking	LacCer → SMC proliferation

cells. Moreover, the mechanism and consequences of macrophage and smooth muscle cell lipid accumulation, particularly of CE and unesterified cholesterol, represent fundamental areas in lesional cell biology that require further investigation. New advances in genomics and proteomics have already begun to aid in these efforts and will increasingly do so. Ultimately, the goal of these studies is to elucidate novel targets for drug or gene therapy that can complement plasma cholesterol-lowering therapy in the fight against the leading cause of mortality worldwide.

Abbreviations

ABC	ATP-binding cassette
ACAT	acyl-CoA:cholesterol acyltransferase
CE	cholesteryl ester
CHOP	C/EBP-homologous protein
COX	Cyclooxygenase
EET	epoxyeicosatrienoic acid
ER	endoplasmic reticulum
HPETE	hydroperoxyeicosatetraenoic acid
HPODE	hydroperoxyoctadecadienoic acid
LDL	low-density lipoprotein
LXR	liver X receptor
PPAR	peroxisome proliferator-activated receptor
UPR	unfolded protein response
VLDL	very low-density lipoprotein

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Subject index

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Color plate section

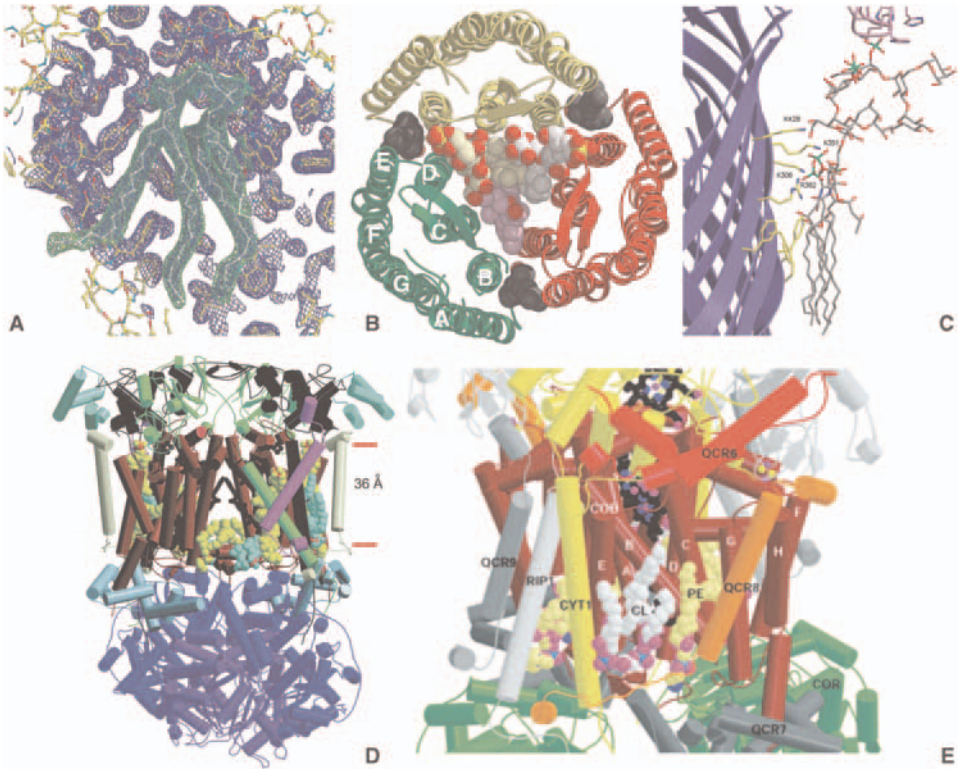

Plate 1. Atomic structure of protein-bound lipids. (A) Model of CL (green) tightly bound to the surface of the photosynthetic reaction center (blue) from *R. sphaeroides*. The space-filling model was derived from X-ray crystallographic data that resolved between 9 and 15 carbons of the acyl chains of CL. Figure adapted from McAuley et al. [15]. Copyright 1999 National Academy of Sciences, USA. (B) Lipid packing in crystals of bacteriorhodopsin. Top view of the trimer in three different colors (domains A–E noted in one of the trimers) in complex with lipid (space-filling models) viewed from the extracellular side. Three phytanyl chains of lipid (gray) lie in the crevices formed between the A–B domain of one monomer and the D–E domain of the adjacent monomer. The central core of the trimer is filled with a sulfated triglycoside attached to archaeol. Red denotes the oxygen atoms of the sugars in white. Figure adapted from Essen et al. [16]. Copyright 1998 National Academy of Sciences, USA. (C) Crystal structure of FhuA complexed with lipopolysaccharide. The ribbon structure (blue) represents the outside surface of the β barrel of FhuA with extended chains (yellow) of amino acids. The amino acids of the aromatic belt interact with the acyl chains (gray) and the basic amino acids interact with the phosphate (green and red) groups of Lipid A. The remainder of the lipopolysaccharide structure extends upward into the periplasm. Adapted from Ferguson et al. [17]. Copyright 2000 Elsevier Science, Ltd. (D) Crystal structure of yeast Complex III dimer with the interface between monomers in the center and the putative interface with Complex IV (shown in (E)) that lies within the transmembrane region between red lines on either side. The cyan CL on the right of (D) is the white CL in the center of (E). Bottom faces the mitochondrial matrix. (D) is adapted from Pfeiffer et al. [18]. Copyright 2003 The American Society for Biochemistry and Molecular Biology. (E) is adapted from Hunte [19]. Copyright 2005 Biochemical Society, London. (See page 21 in this volume.)

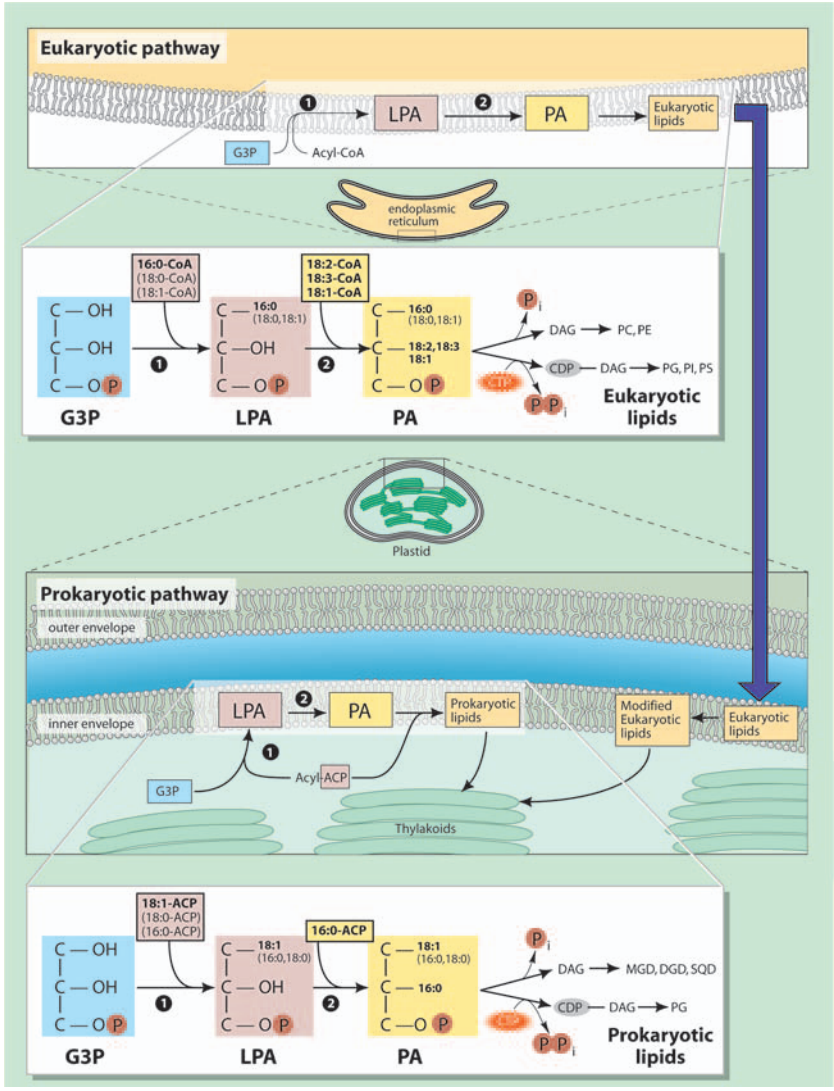

Plate 2. The prokaryotic and eukaryotic pathways of plant glycerolipid synthesis. The prokaryotic pathway takes place in plastids and esterifies mainly palmitate to the *sn*-2 position of lysophosphatidate (LPA). The eukaryotic pathway occurs outside the plastid, primarily in the ER and results in 18-carbon fatty acids esterified to the *sn*-2 position of glycerolipids. In the prokaryotic pathway, acyl-ACP is condensed with glycerol-3-phosphate (G3P) by a soluble enzyme, G3P acyltransferase (reaction 1). The product, LPA, partitions into the membranes where LPA is converted to phosphatidic acid (PA) by a membrane localized LPA-acyltransferase (reaction 2). PA is then converted to the other lipids found in chloroplasts. It is thought that most of the reactions of lipid synthesis by the prokaryotic pathway take place in the inner envelope of the plastids. The initial reactions of the eukaryotic pathway are similar except that acyl-CoA substrates are used and the G3P acyltransferase is thought to be associated with the ER. After desaturation of 18:1 to 18:2, lipids move from the ER to the other organelles, including the outer envelope of plastids. Eukaryotic lipids in the outer envelope are transferred into the inner membranes and modified by the replacement of headgroups and by the action of additional desaturases. CDP-DG, cytidine diphosphate-diacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidyserine; DGD, digalactosyl diacylglycerol; MDG, monogalactosyl diacylglycerol; SQD, sulfoquinovosyl diacylglycerol. (See page 105 in this volume.)

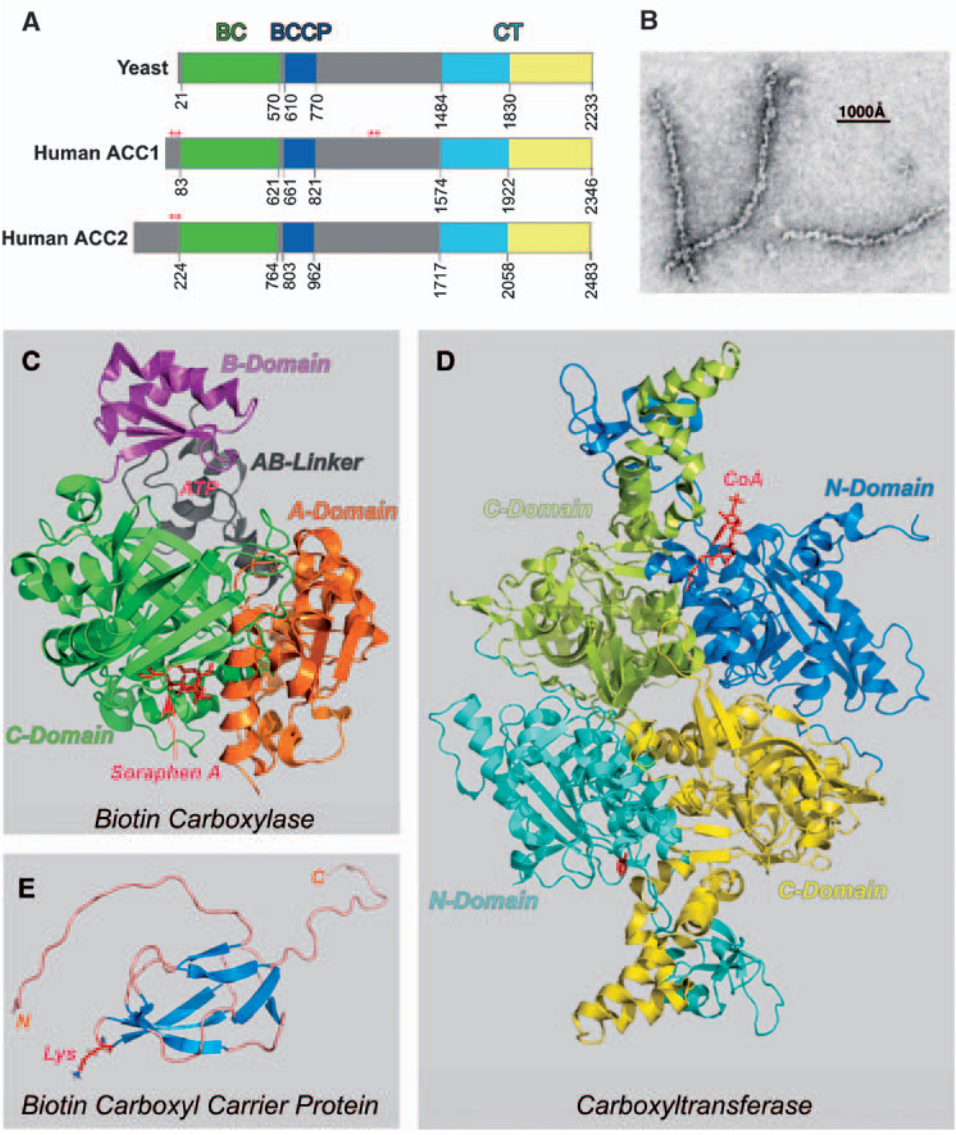

Plate 3. Acetyl-CoA carboxylase. (A) Eukaryotic ACCs contain ~2300 residues organized into three functional domains — biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT). The role of the region between the biotin carboxyl carrier and carboxyltransferase domains is unknown. The biotin carboxyl carrier protein contains a typical conserved biotin attachment-site motif, VM**K**MV. The sites of phosphorylation are indicated by asterisks. (B) Electron micrograph of polymerized rat acetyl-CoA carboxylase (F. Ahmad, 1978). (C) Crystal structure of the biotin carboxylase domain of the yeast enzyme. In the presence of soraphen A, the biotin carboxyl carrier protein domain forms an inactive monomer; the likely position of the modeled ATP-binding site is shown (adapted from Ref. [2]). (D) Crystal structure of the dimeric carboxyltransferase domain of the yeast enzyme. Although acetyl-CoA was included in the crystallization, density was observed only for CoA at one site and adenine at the other (adapted from Ref. [2]). (E) NMR structure of the biotin carboxyl carrier apoprotein domain of the human ACC2 The lysine attachment site for biotin is shown (RIKEN Structural Genomics/Proteomics Initiative, 2006). (See page 159 in this volume.)

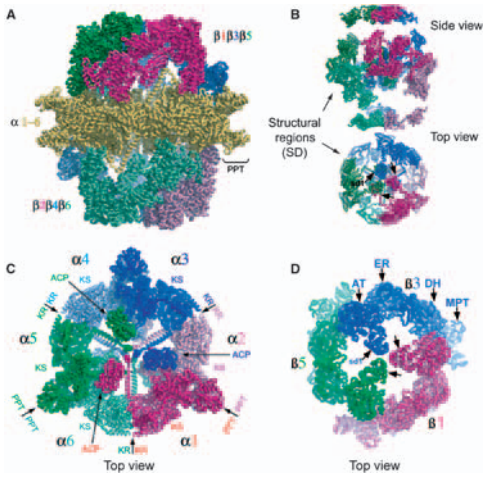

Plate 4. Architecture of the fungal FAS. (A) Structural overview of the barrel-shaped molecule showing the location of the equatorial wheel composed of the six alpha subunits flanked by two domes, each composed of a β subunit trimer. The barrel is 270 Å long and 230 Å wide at the equator. (B) Location of the structural underpinnings of the molecule with the catalytic domains removed. (C) Organization of the alpha-subunit hexamer. (D) Organization of one β-subunit trimer (adapted from Lomakin et al. [13] with permission). (See page 163 in this volume.)

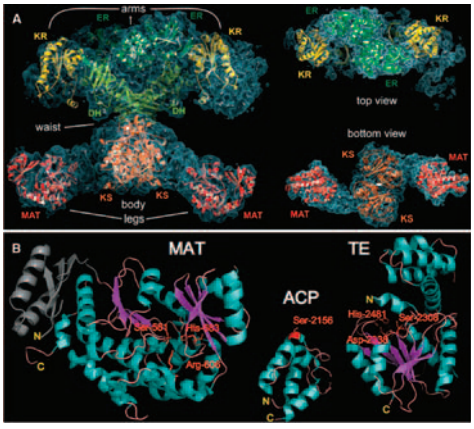

Plate 5. Structure of the animal FAS. (A) An overview of the entire complex (reproduced with permission of Maier et al. [9]). Fitted homologous domains are shown with a semi-transparent surface representation of the experimental 4.5-Å-resolution electron density. Two white stars indicate the pseudosymmetry-related suggested attachment regions for the ACP and thioesterase ACP and TE, where more density is visible on the right side. MAT, malonyl/acetyltransferase. (B) High-resolution structures of the ACP and MAT domains (Structural Genomics Consortium) and the TE domain (F.A. Quiocho, 2004) of the human FAS showing locations of active-site residues. The gray region of the MAT (residues 422–484) represents a structured linker region that probably interacts with the adjacent KS domain and is not part of the MAT catalytic domain. (See page 165 in this volume.)

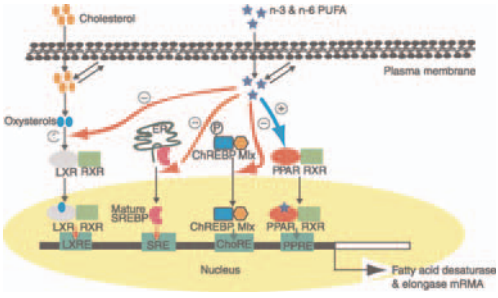

Plate 6. Coordinated transcriptional regulation of fatty acid desaturases and elongases in mammals. PUFA, polyunsaturated fatty acids; +, stimulation; –, inhibition; LXR, liver X receptor; RXR; retinoid X receptor; SREBP, sterol regulatory element binding protein; ChREBP, carbohydrate response element binding protein; Mlx, Max-like receptor; PPAR-α, peroxisome proliferator activated receptor alpha; LXRE, liver X receptor response element; SRE, sterol response element; ChoRE, carbohydrate response element; PPRE, peroxisome proliferator response element. (See page 206 in this volume.)

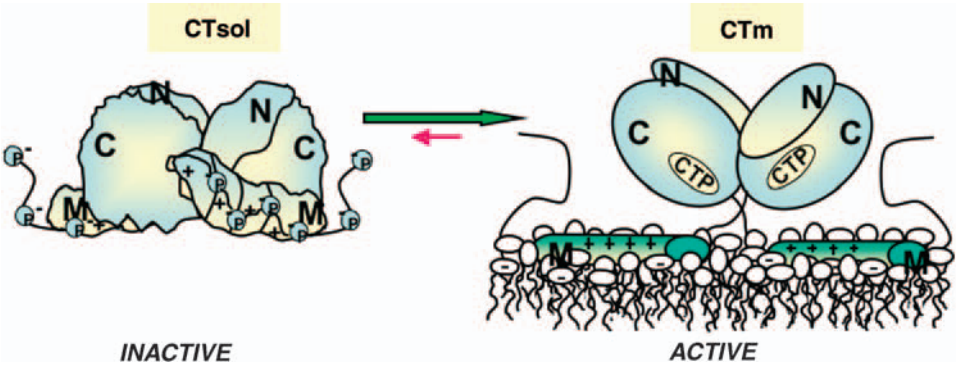

Plate 7. Translocation of CTP:phosphocholine cytidylyltransferase (CT) from an inactive soluble form (CTsol) to a membrane-associated activated form (CTm). The reversible interaction of CT with membranes involves the amphipathic helical region lying on the surface of the membrane so that the hydrophilic region interacts with the negatively charged lipid head groups and the hydrophobic side intercalates into the membrane core. N, amino terminal domain; C, carboxyl terminal domain; M, membrane-binding domain. Figure kindly supplied by Prof. R. Cornell, Simon Fraser University. (See page 226 in this volume.)

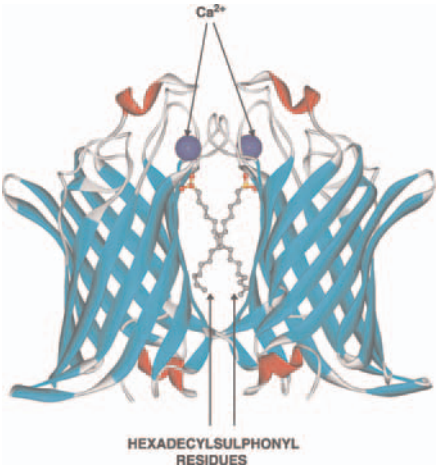

Plate 8. Crystal structure of the outer membrane phospholipase A dimer from *E. coli* shown in the plane of the membrane. The top half of the molecule is located in the lipopolysaccharide monolayer facing the exterior of the cell. The phospholipid monolayer of the outer cell membrane would be located around the bottom half of the protein. Two calcium ions are shown at the active sites while Ser-144 of each active site is covalently modified with a hexadecylsulfonyl moiety represented in a ball and stick format. Structure is adapted from Ref. [12]. (See page 312 in this volume.)

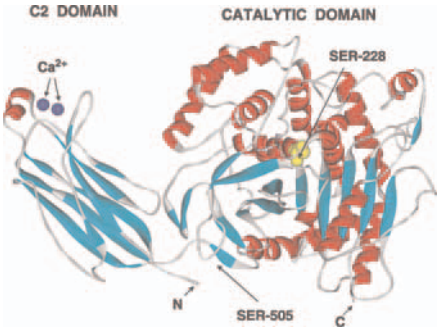

Plate 9. Crystal structure of group IV cytosolic PLA_2 showing the C2 and catalytic domain. The two calcium ions bound to the C2 domain and the active-site Ser-228 are highlighted. The phospholipid interface would be parallel to the top surface of the molecule as shown. A flexible region between residues 499 and 538 is not seen in the crystal structure but contains Ser-505, the approximate position of which is indicated by the arrow. The N-and C-termini of the protein are indicated. Adapted from A. Dessen (1999) and Ref. [24]. (See page 321 in this volume.)

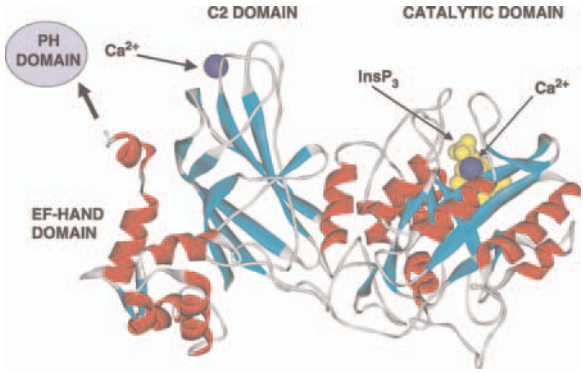

Plate 10. Crystal structure of phosphatidylinositol-PLCδ showing the C2 and catalytic domains. The position of the PH domain that would be attached to the EF-hand domain is indicated. The membrane surface would be parallel to the top surface of the molecule as shown. Calcium ions are shown bound to the active site and to the C2 domain. The position of inositol trisphosphate in space-filling format at the active site is indicated. Adapted from L.-O. Essen (1996) and Ref. [32]. (See page 325 in this volume.)

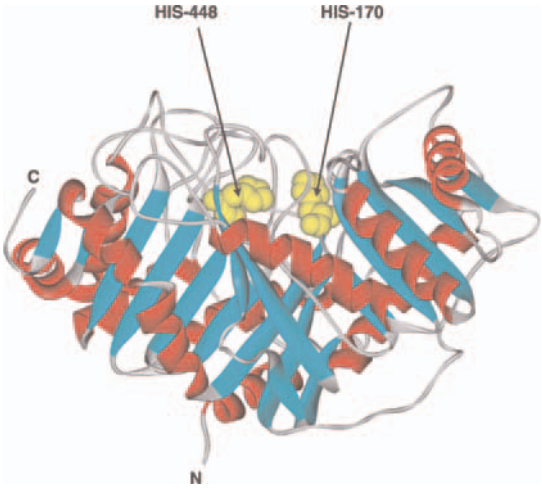

Plate 11. Crystal structure of the PLD from *Streptomyces* sp. showing two active-site histidines in space-filling format together with the N- and C-termini of the protein. Adapted from I. Leiros (2000). (See page 326 in this volume.)

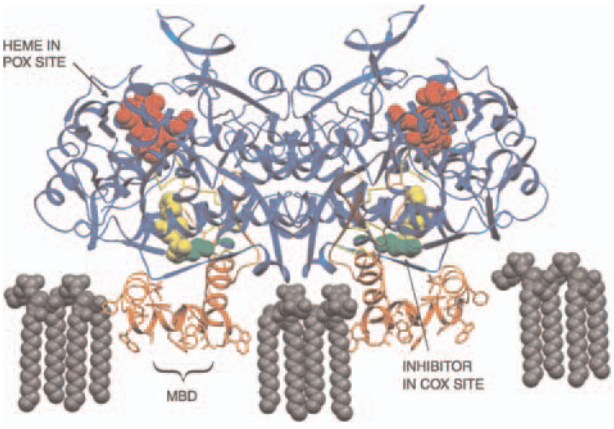

Plate 12. Ribbon diagram of the structure of ovine PGH synthase-1 homodimer interdigitated via its membrane-binding domain (MBD) into the lumenal surface of the endoplasmic reticulum. (See page 340 in this volume.)

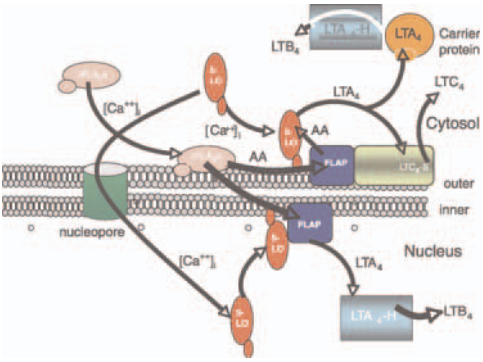

Plate 13. Proposed model for the location of biosynthetic events occurring during leukotriene (LT) biosynthesis at the nuclear membrane of cells. Arachidonic acid is released from membrane glycerophospholipids by nuclear membrane-associated cPLA$_2\alpha$ and is then presented to 5-lipoxygenase via 5-lipoxygenase activating protein (FLAP). LTA$_4$ is either converted by nuclear membrane associated LTC$_4$ synthase (LTC$_4$-S) into LTC$_4$ or carried to LTA$_4$ hydrolase (LTA$_4$-H). These events can occur from the cytosolic side or the nucleoplasmic side of the dual lipid bilayer of the perinuclear region. (See page 350 in this volume.)

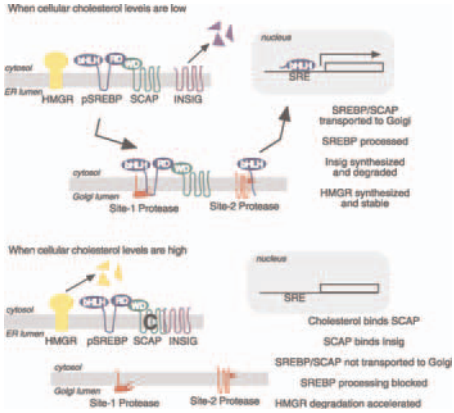

Plate 14. Current model of cholesterol regulation of SREBP proteolysis. The sterol regulatory element-binding protein (SREBP) precursor is inserted into the endoplasmic reticulum (ER) membrane. The SREBP regulatory domain (RD) interacts with the SREBP cleavage-activating protein (SCAP), likely through SCAP's WD repeats. When cholesterol levels are low, SCAP escorts SREBP to the Golgi where the transcription factor is released by site-1 protease cleavage of a lumenal loop followed by site-2 protease cleavage within a transmembrane span. The mature SREBP translocates into the nucleus and activates gene transcription. HMG-CoA reductase (HMGR) is synthesized and stable in the ER membrane, whereas Insig is synthesized and quickly degraded. In cholesterol-fed cells, cholesterol binds SCAP, which induces SCAP to bind Insig. SCAP and Insig form a stable ER complex, anchoring the SREBP precursor in the ER. Thus, the SREBP precursor is not proteolyzed to release the basic helix-loop-helix (bHLH) transcription factor. Degradation of HMG-CoA reductase is accelerated. (See page 410 in this volume.)

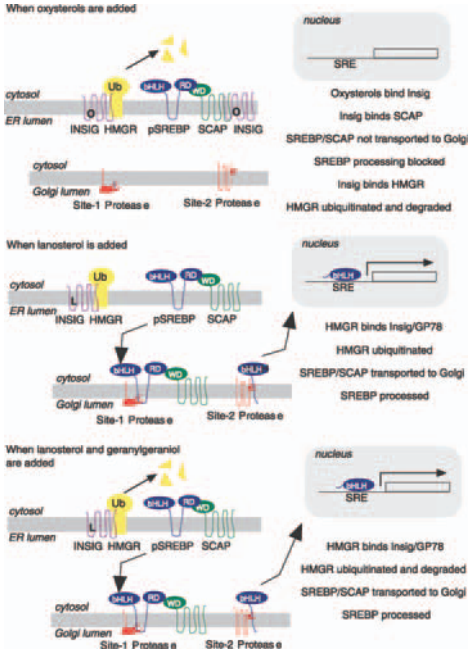

Plate 15. Sterol regulation of SREBP processing. Oxysterols bind to Insig and induce Insig to anchor SCAP/SREBP in the ER membrane. Oxysterols also lead to HMG-CoA reductase (HMGR) ubiquitination and degradation. Lanosterol causes Insig-Gp78 complexes to bind to HMG-CoA reductase, leading to HMGR ubiquitination. SREBP is transported to the nucleus and processed. If geranygeraniol is also present, the ubiquitinated HMG-CoA reductase is extracted from the membrane and degraded. SREBP transport and processing continues. (See page 414 in this volume.)

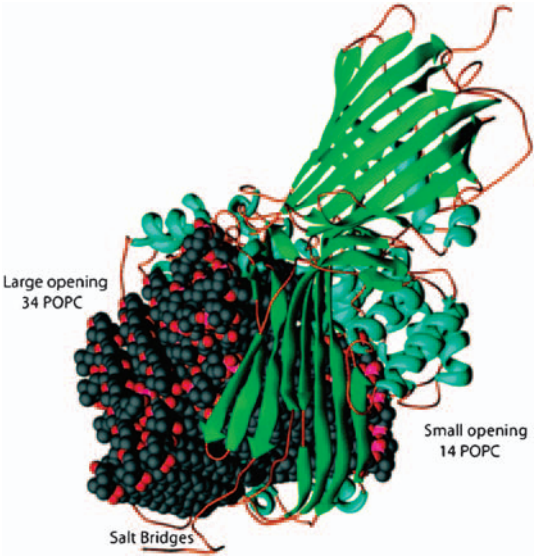

Plate 16. The lipid pocket of apo B. Apo B22.5 is represented with β-strands shown as green arrows and α-helices shown as turquoise tubes. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) is shown with carbon atoms in gray and oxygens in red. There are 34 POPC molecules in the large opening and 14 POPC molecules in the small opening. Thus, a total of 48 POPC molecules fit into the lipid pocket. From Ref. [24], with permission. (See page 511 in this volume.)

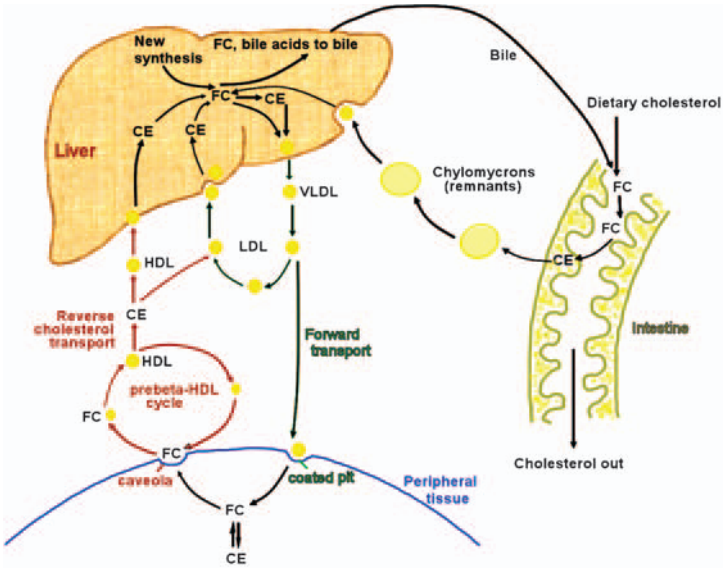

Plate 17. Summary of major ‘forward’ and ‘reverse’ lipid transport pathways through the extracellular compartment that link the liver and intestine with peripheral tissues. FC, unesterified cholesterol. For other abbreviations see list of abbreviations. (See page 536 in this volume.)