Functional roles of lipids in membranes

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1. Introduction and overview

Lipids as a class of molecules display a wide diversity in both structure and biological function. A primary role of lipids in cellular function is in the formation of the permeability barrier of cells and subcellular organelles in the form of a lipid bilayer (Fig. 1). Although the major lipid type defining this bilayer in almost all membranes is glycerol-based phospholipid, other lipids are important components and vary in their

![Diagram of membrane structure](image)

**Fig. 1.** Model for membrane structure. This model of the plasma membrane of a eukaryotic cell is an adaptation of the origin model proposed by Singer and Nicholson [1]. 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 headgroups) are enriched in cholesterol and contain a glycosylphosphatidylinositol-linked (GPI) protein. The light gray headgroups depict lipids in close association with protein. The irregular surface and wavy acyl chains denote the fluid nature of the bilayer.
presence and amounts across the spectrum of organisms. Sterols are present in all
eukaryotic cytoplasmic membranes and in a few bacterial membranes. The ceramide-
based sphingolipids are also present in the membranes of all eukaryotes. Neutral
glycerol-based glycolipids are major membrane-forming components in many Gram-
positive bacteria and in the membranes of plants while Gram-negative bacteria utilize
a glucosamine-based phospholipid (Lipid A) as a major structural component of the
outer membrane. Additional diversity results in the variety of the hydrophobic domains
of lipids. In eukaryotes and eubacteria these domains are usually long chain fatty acids
or alkyl alcohols with varying numbers and positions of double bonds. In the case of
archaebacteria, the phospholipids have long chain reduced polyisoprene moieties, rather
than fatty acids, in ether linkage to glycerol. 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 the hundreds. In more complex eukaryotic organisms
with greater diversity in both the phospholipids and fatty acids, the number of individual
species is in the thousands.

If one or two phospholipids are sufficient to form a stable bilayer structure, why is the
above diversity in lipid structures present in biological membranes [2]? The adaptability
and flexibility in membrane structure necessitated by environment is possible only with
a broad spectrum of lipid mixtures. The membrane is also the supporting matrix for a
wide spectrum of proteins involved in many cellular processes. Approximately 20–35%
of all proteins are integral membrane proteins, and probably half of the remaining
proteins function at or near a membrane surface. Therefore, the physical and chemical
properties of the membrane directly affect most cellular processes making the role of
lipids dynamic with respect to cell function rather than simply defining a static barrier.

In this chapter, the diversity in structure, chemical properties, and physical properties
of lipids will be outlined. Next, 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.

2. **Diversity in lipid structure**

Lipids are defined as those biological molecules readily soluble in organic solvents such
as chloroform, ether, or toluene. However, some very hydrophobic proteins such as the
F₀ subunits of ATP synthase are soluble in chloroform, and lipids with large hydrophilic
domains such as lipopolysaccharide 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.

2.1. **Glycerol-based lipids**

The primary building blocks of most membranes are glycerol phosphate-containing
lipids generally referred to as phospholipids (Fig. 2). The diacylglycerol backbone in
Fig. 2. Structure of glycerol phosphate-based lipids. The complete lipid structure shown is 1,2-distearoyl-
$\text{sn}$-glycerol-3-phosphocholine or phosphatidylcholine (PC). Substitution of choline in the box with the
headgroups listed below results in the other phospholipid structures. CDP-diacylglycerol has a CMP and
phosphatidic acid has a hydroxyl group in place of choline (not shown). Diphosphatidylglycerol, which
contains two phosphatidic acids joined by glycerol, is commonly referred to as cardiolipin (CL).
eubacteria and eukaryotes is $\text{sn}$-3-glycerol esterified at the 1- and 2-position with long
chain fatty acids. In archaeabacteria (Fig. 3), $\text{sn}$-1-glycerol forms the lipid backbone and
the hydrophobic domain is composed of phytanyl (a saturated isoprenyl) groups in ether linkage at the 2- and 3-position (an archaeol). In addition two $\text{sn}$-1-glycerol groups
are found connected in ether linkage by two biphytanyl groups (dibiphytanyldiglycerophosphatetetraether) [3] to form a covalently linked bilayer. Some eubacteria (mainly
hyperthermophiles) have dialkyl (long chain alcohols in ether linkage) glycerophosphate lipids and similar ether linkages are found in the plasmalogens of eukaryotes. The
headgroups of the phospholipids (boxed area of Fig. 2) extend the diversity of lipids
defining phosphatidic acid (PA, with OH), phosphatidylcholine (PC), phosphatidylserine
Fig. 3. Structure of dialkylglycerols in archaeabacteria. Archaeabacteria have phytanyl chains in ether linkage to the 2- and 3-positions of sn-1-glycerol (archaeol). The 1-position 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 glycolipid with either a mono or disaccharide (glucose or galactose) at the 1-position of sn-1-glycerol. The R groups are ether-linked phytanyl chains. Similar glycolipids are found in eubacteria and plants with a sn-3-glycerol backbone and ester-linked fatty acid chains at the 1- and 2-positions.

(PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin (CL). Archaeabacteria analogues exist with headgroups of glycerol and glycerolmethylphosphate as well as all of the above except PC and CL (Chapter 3). Archaeabacteria also have neutral glycolipid 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 glycolipids with mono- or disaccharides linked to the 3-carbon of sn-3-diacylglycerol (Chapter 4). Therefore, the diversity of glycerol-based lipids in a single organism is significant, but the diversity throughout nature is enormous. The lipid composition of various biological membranes is shown in Table 1.
Table I

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Erythrocyte</th>
<th>Myelin</th>
<th>Mitochondria</th>
<th>Endoplasmic reticulum</th>
<th>E. coli</th>
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<tr>
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<td>22</td>
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<td>6</td>
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<tr>
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<td>17</td>
<td>10</td>
<td>39</td>
<td>40</td>
<td>–</td>
</tr>
<tr>
<td>Sphingomyelin</td>
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<td>8</td>
<td>–</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
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<td>7</td>
<td>9</td>
<td>2</td>
<td>5</td>
<td>–</td>
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<tr>
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<td>–</td>
<td>–</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10</td>
</tr>
<tr>
<td>Glycolipid*</td>
<td>3</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Others</td>
<td>13</td>
<td>8</td>
<td>–</td>
<td>27</td>
<td>–</td>
</tr>
</tbody>
</table>

The data are expressed as weight % of total lipid.

* Ceramide based.

* Human sources.

* Rat liver. Inner and outer mitochondrial membrane.

* Inner and outer membrane excluding Lipid A.

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 archaeaebacteria. 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 archaeaebacteria. Many of these organisms exist in harsh environments that call for more chemically stable lipid bilayers which is afforded by the above lipids. How the physical properties of the more commonly studied lipids change with environment will be discussed later.

2.2. Diglucoseamine phosphate-based lipids

The outer membrane of Gram-negative bacteria (Fig. 4) contains a lipid made up of a headgroup derived from glucosamine phosphate (Chapter 3). The core lipid (Lipid A, see Fig. 5 and Chapter 3) in E. coli is a phospholipid containing two glucoseamime 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 α(1–3) linkage) results in KDO2-Lipid A that is further modified by an inner core, an outer core, and the O-antigen. Laboratory strains of Salmonella typhimurium and E. coli such as K-12 lack the O-antigen found in the wild-type and clinically important strains.

The complete structure either with or without O-antigen is referred to as lipopolysaccharide or LPS. The core Lipid A forms the outer monolayer of the outer membrane bilayer of Gram-negative bacteria; the inner monolayer of the outer membrane (Fig. 4) is made up of glycerophosphate-based lipids. The whole lipopolysaccharide structure defines the outer surface of Gram-negative bacteria, but only the KDO2-Lipid A struc-
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 KDO₂-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 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 crosslinked peptidoglycan gives structural rigidity to the cell envelope. One-third of the lipoproteins (*lpp* gene product) is covalently linked via its carboxyl terminus to the peptidoglycan and in complex with the remaining lipoproteins as trimers that associate 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.

Structure is essential for viability of laboratory strains. However, the remainder of the lipopolysaccharide structure is important to survival of Gram-negative bacteria in their natural environment. This structure is modified post-assembly in response to environment including host fluids, temperature, ionic properties, and antimicrobial agents [4]. In addition, both enteric and non-enteric Gram-negative bacteria show a great diversity in all component parts of the LPS structure. Studies of Lipid A biosynthesis is of clinical importance because it is the primary antigen responsible for toxic shock syndrome caused by Gram-negative bacterial infection.
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 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 effect’ as developed by Charles Tanford [5]. The ‘fluid mosaic’ model for membrane structure further popularized these concepts [1]. This model, since extensively refined, envisioned membrane proteins as undefined globular structures freely moving in a homogeneous sea of lipids.

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 that readily interact with water. The basic premise of the hydrophobic effect is that the hydrocarbon domains of polar lipids disrupt the stable hydrogen bonded structure of water and therefore are at an energy minimum when such domains self associate to minimize the total surface area in contact with water. The polar domains of lipids interact either through hydrogen bonding or
ionic interaction with water 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 versus steric and ionic repulsive forces of the polar domains in 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 favorable interaction of the polar domain with water is outweighed by the unfavorable interaction of the hydrophobic domain with water. 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]. The larger the hydrophobic domain, the lower the critical micelle concentration due to the increased hydrophobic effect. However, the larger the polar domain, either because of the size of neutral domains or charge repulsion for ionic domains, the higher the critical micelle concentration due to the unfavorable steric hindrance in bringing these domains into close proximity. The critical micelle concentration of amphipathic molecules with a net charge is influenced by ionic strength of the medium due to dampening of the charge repulsion effect. Therefore, the critical micelle concentration of the detergent sodium dodecyl sulfate is reduced ten-fold when the NaCl concentration is raised from 0 to 0.5 M.

These physical properties and the shape of amphipathic molecules define three supramolecular 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 packing of amphipathic molecules at an energy minimum by balancing the attractive force of the hydrophobic effect and the repulsive force of close headgroup 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 14 and above self associate at a concentration around 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
Fig. 6. Polymorphic phases and molecular shapes exhibited by lipids. The space filling model for the micellar phase is of β-D-octyl glucoside micelle (50 monomers). The polar portions of the detergent molecules (oxygen atoms are black) do not cover completely the micelle surface (hydrocarbons in gray) leaving substantial portions of the core exposed to bulk solvent. Inverted cone-shaped molecules form micelles. Model adapted with permission 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 (HII) structure depending on the geometry of molecule (cylindrical or cone-shaped, respectively) and environmental conditions. The Lp (order gel) and Lo (liquid crystalline) bilayer phases differ in the order within the hydrophobic domain and in mobility of the individual molecules.

a continuous single bilayer and an aqueous core much like the membrane surrounding cells. Liposomes can also be made by physical extrusion of lamellar structures through a small orifice or by dilution of a detergent–lipid mixture below the critical micelle concentration of the detergent.

Cylindrical 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 headgroups 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 (HII) and cubic phases (a more complex organization similar to the HII 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 bilayer or the HII phase, depending on solvent conditions, alkyl chain composition, and temperature.

Both cone-shaped and inverted cone-shaped lipids are considered as 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 have no tendency to bend away from or toward the aqueous phase due to their cylindrical symmetry. In such a system, the hydrophobic domain orients toward the air. Monolayers of the asymmetric cone-shaped lipids (HII-forming) tend to bend toward from 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 mis-match in lipid mixtures will be covered later.

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 headgroups, 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 31P-NMR and microcalorimetry. The difference between the ordered gel (Lβ) and liquid crystalline (Lα) 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 also space occupied by the hydrophobic domain of lipids also increases. 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 Lβ or Lα phase mainly dependent on the alkyl chain composition and the temperature. Non-bilayer-forming lipids such as PE exist at low temperatures in the Lβ phase, at intermediate temperatures in the Lα phase, and at elevated temperatures in the HII or cubic phase (Fig. 7). The last transition is temperature dependent but also depends on the shape of the lipid. The shape of lipids with relatively small head groups can change from cylindrical to conical (HII phase) with increasing unsaturation or length of the alkyl chains or with increasing temperature. As can be seen from Fig. 7, the midpoint temperature (Tm) of the transition from the Lβ to Lα phase increases with an increase in the length of the fatty acids, but the midpoint of the transition temperature (TmHII) between the Lα and HII phases decreases with increasing chain length (or increasing unsaturation, not shown).
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 \( L_\alpha \) to \( L_\beta \) transition and the smaller peaks at higher temperatures are due to the \( L_\alpha \) to \( H_\beta \) 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 \( H_\beta \). Figure adapted with permission from Seddon et al. [7]. Copyright 1983 American Chemical Society.

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 \( L_\alpha \) 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 \( L_\alpha \) 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 (see the end of 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 to the aqueous phase. Mixtures of lipids with dissimilar phase properties can also generate phase separations with local domain formation. 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 Le 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 Le phase and favor a less fluid but more ordered structure than the Le 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 headgroup 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 headgroups of CL and PA allowing organization into the H0 phase. Low pH has a similar effect on the headgroup of PS. Since Ca\(^{2+}\) is an important signaling molecule that elicits many cellular responses, it is possible that part of its effects may be 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 cardiolipin

CL has the unique property of being both a bilayer and non-bilayer lipid depending on the absence or presence of divalent cations. CL is found almost exclusively in eukaryotic mitochondria and in bacteria that utilize oxidative phosphorylation for proton pumping across the membrane. A property of CL that has gone largely unrecognized is the ionization constants of its two phosphate diesters. Rather than displaying two pK values in the range of 2–4, pK2 of CL is \(>8.5\) [8] indicating that CL is protonated at physiological pH (Fig. 8). This property may make CL 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 results in a reduction in cell growth dependent on oxidative processes. Therefore, CL is not absolutely essential, but it appears to be required for optimal cell function.

3.4. What does the membrane bilayer look like?

The functional properties of natural fluid bilayers not only include the hydrophobic core and the hydrophilic surface but the interfacial region containing bound water and ions. Fig. 9A shows the distribution of the component parts of dioleoylphosphatidylcholine across the bilayer [9] and illustrates the dynamic rather than static nature of the membrane. The bilayer thickness of 30 Å is defined by the length of the fatty acid chains. However, the thickness is not a static number as indicated by the probability of finding CH2 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. What is generally not appreciated is the width (15 Å on either side of the bilayer) of the interface region between the hydrocarbon core and the free water phase of the cytosol. This region contains a complex mixture of chemical species defined by the ester linked glycerophosphate moiety, the variable headgroups, and bound water and ions. Many biological processes occur within this interface region and are dependent on its unique
Fig. 8. Ionization state of CL and deoxy-CL at physiological pH. CL is only partially ionized under these conditions (pK2 > 8.5) and therefore can trap a proton by hydrogen bonding with the sn-2 hydroxyl of glycerol that joins the two phosphatidic acids in the CL structure. Deoxy-CL lacking the sn-2 hydroxyl is fully ionized and cannot trap a proton.

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 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 never learn the molecular bases for other requirements. Over the past 20 years, genetic approaches have been successfully used to establish the biosynthetic pathways of most of the common lipids. The challenge is to use this genetic information to manipulate the lipid composition of cells without
severely compromising cell viability. In those cases where this has been possible, the combination of the genetic approach to uncover phenotypes of cells with altered lipid composition and the dissection in vitro of the molecular basis for the phenotype has proven to be a powerful approach to defining lipid function. The more complex the organelle content and accompanying membrane structure of a cell the more difficult is the application of the genetic approach. Therefore, 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 [2]. Unlike many other mutations affecting the metabolic pathways in E. coli, mutants in phospholipid biosynthesis cannot be bypassed by supplementation of the growth media with phospholipids due to the barrier function of the outer membrane. Therefore, the isolation and study of E. coli phospholipid auxotrophs has not been possible.

With the exception of the synthesis of CL, mutants in all steps of phospholipid biosynthesis were thought to be lethal even under laboratory conditions. To date, no growth conditions have been established for cells unable to synthesize CDP-diacylglycerol. Null mutants in the pgsA gene (encodes phosphatidylglycerophosphate synthase) that cannot synthesize PG and CL are lethal, but a suppressor of this mutation has been identified [10]. In such mutants, the major outer membrane lipoprotein precursor (see Fig. 4), that 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 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 5.4 and 5.5.

The amine-containing lipids, PS and PE, were also thought to be essential based on initial mutants carrying temperature sensitive alleles of the genes (pssA and psd) encoding their respective biosynthetic enzymes. However, the growth phenotype of these mutants (as well as pssA null strains) with reduced amine-containing lipids could be suppressed by adding Ca\(^{2+}\), Mg\(^{2+}\), and Sr\(^{2+}\) in millimolar concentrations to the growth medium. These mutants, although viable, have a complex mixture of defects in cell division, reduced growth rate, loss of outer membrane barrier function, defects in energy metabolism, mis-assembly of membrane proteins, and defects in sugar and amino acid transport.

The key to defining new functions for the anionic and zwitterionic phospholipids of E. coli was the design of strains in which the content of PG/CL and PE could be
regulated in a systematic manner in viable cells. The *pgsA* gene (encoding the phosphatidylglycerophosphate synthase) was placed under the control of the exogenously regulated promoter *lacOP* (promoter of the *lac* operon) that is controlled by isopropyl-β-thiogalactoside levels in the growth media. Variation in PG plus CL levels were correlated in a dose–response manner with the functioning of specific cellular processes both in vivo and in vitro to determine lipid function. Similarly, the involvement of PE in function was uncovered by comparing phenotypes of cells with and without PE or by placing the *pssA* gene (encoding PS synthase) under exogenous regulation. Therefore, these genetically altered strains have been used as reagents to define potential lipid involvement in cellular process in vivo that can be verified by biochemical studies in vitro.

4.2. Alteration of lipid composition in yeast

The pathways of phospholipid synthesis and the genetics of lipid metabolism in yeast *Saccharomyces cerevisiae* [11] are as well understood as in *E. coli*. Yeast have pathways (see Chapters 3 and 8) similar to those in the *E. coli* for PE and PG synthesis. 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, yeast utilize the mammalian pathways for synthesis of PI, PE, and PC including the methylation of PE to form PC (Chapter 8).

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 since *pssl* (encodes PS synthase) mutants also lacking a sphingolipid degradative enzyme that generates ethanolamine internally, require ethanolamine supplementation [12].

No gene products involved in lipid metabolism are encoded by the mtDNA which in *Saccharomyces cerevisiae* encodes eight proteins (subunits I, II, and III of cytochrome *c* oxidase, cytochrome *b*, the 3 subunits that make up the Fₐ component of ATP synthase, and the *VAR1* gene product which is part of the mitochondrial ribosome). The enzymes necessary for synthesis of PE from PS, and for PG and CL, are all encoded by nuclear genes and imported into the mitochondria. Null mutants of *crdl* (encodes CL synthase) 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 phosphatidylglycerophosphate synthase) results in the inability to utilize non-fermentable carbon sources for growth. Mitochondrial membrane potential is reduced to near undetectable levels although remains sufficient to support the import of all nuclear encoded proteins thus far investigated. Similar effects are seen in mammalian cells with a mutation in the homologous *PGS1* gene. The surprising consequence of 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 would indicate that
either 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 or the lack of these lipids indirectly affects both mitochondrial and cytoplasmic mRNA translation.

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 are dependent on the chemical and structural anatomy of lipids (headgroup, 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, thickness, shape, packing properties) of the lipids organized into membrane structures.

5.1. The bilayer as a supramolecular lipid matrix

Biophysical studies on membrane lipids coupled with biochemical and genetic manipulation of membrane lipid composition have established that the Lα 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 make up dramatically. The complex membrane lipid composition including cholesterol stabilizes mammalian cell membranes in the Lα 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 exist in the Lα 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 of unsaturated fatty acids from the growth medium and incorporate these fatty acids to adjust membrane fluidity in response to growth temperature. Mutants grown at low temperature with high unsaturated fatty acid content lyse when raised rapidly to high temperature probably due to the increased membrane permeability of fluid membranes and a transition from the Lα to HII phase of the lipid bilayer. Conversely, growth at high temperatures with high saturated fatty acid content results in growth arrest after a shift to low temperature due to the reduced fluidity of the membrane. Wild-type cells, that 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 a favorable membrane fluidity.

Bacterial cells also regulate the ratio of bilayer to non-bilayer-forming lipids in response to growth conditions [2]. Bacterial non-bilayer-forming lipids are PE with unsaturated alkyl chains, CL in the presence of divalent cations, and monoglucoyl diacylglycerol (MGDG). Extensive studies of lipid polymorphism have been carried out on *Acholeplasma laidlawii* because this organism alters its ratio of MGDG (capable of assuming the H\(_{II}\) phase) to DGDG (diglucoyl diacylglycerol, which only assumes the L\(_{α}\) or L\(_{β}\) phase) in response to growth conditions. High temperature and unsaturation in the fatty acids favor the H\(_{II}\) phase for MGDG. At a given growth temperature, the MGDG to DGDG ratio is inversely proportional to the unsaturated fatty acid content of MGDG. As growth temperature is lowered, *A. laidlawii* either increases the incorporation into MGDG of unsaturated fatty acids from the medium or increases the ratio of MGDG to DGDG 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.

Contrary 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. 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 to 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\(^{2+}\)) as these ions induce the formation of non-bilayer phases of CL. Neither growth of the mutant nor the H\(_{II}\) phase for CL is supported by Ba\(^{2+}\). 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? There are numerous biological processes that can be envisioned to require discontinuity in the membrane bilayer. Integration of proteins into the bilayer might require ‘annular lipids’ (those in close proximity to the protein) to interface with the more regular structure of the bilayer. 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.

Since cells homeostatically adjust their 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 [14]. Protein kinase C is a peripheral membrane protein that binds to the membrane 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 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 or not 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$a$ to H$\|b$ transition at a lower temperature than the D-serine isomer [15]. 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 may provide the unique allosteric switch regulating 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 well-known stabilizer of the bilayer phase, inhibits the enzyme. Thus phospholipase C appears to be regulated by the overall geometry and composition of the bilayer [16] 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 from initial studies which property of lipids, i.e. chemical or physical, is required for optimum function. 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 optimal reconstitution of function in vitro for more than 50 membrane proteins. If one considers specific lipid requirements for membrane association and activation of peripheral membrane proteins, the number is in the hundreds. Integral membrane proteins fold and exist in a very complex environment and have three modes of interaction with their environment. The extramembrane parts 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 (see Fig. 9). The remainder of the protein is buried within the approximately 30 Å thick hydrophobic interior of the membrane.
Peripheral membrane 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 has uncovered new roles for lipids. Most exciting have been recent results from X-ray crystallographic analysis of membrane proteins which have revealed lipids in specific and tight association with proteins [17]. 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 interfacial region (at the cytoplasmic side of the membrane) (Fig. 10A). Bonding interactions between CL and the protein involve either direct contacts of the phosphate oxygens of the lipid headgroup with basic amino acids and backbone amide groups exposed at the protein surface, or indirect contacts with amino acid side chains that are mediated by bound water molecules. 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.

Bacteriorhodopsin is a light-driven ion pump that is found in the purple membrane of the archaeabacterium *Halobacterium salinarum*. Bacteriorhodopsin monomers consist of a bundle of seven transmembrane α-helices that are connected by short inter-helical loops, and enclose a molecule of retinal that is buried in the protein interior, approximately half way across the membrane. 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 (a reduced isoprenoid) and the methyl ester of phosphatidylglycerophosphate is required to maintain normal photochemical cycle behavior. In a high-resolution (1.55 Å) structure of bacteriorhodopsin, 18 full or partial lipid acyl chains per monomer were resolved (Fig. 10C), four pairs of which were 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 probable hold the complex together.
Fig. 10. Atomic structure of protein bound lipids. (A) Model of CL (green) tightly bound to the surface of photosynthetic reaction center (blue) from *R. sphaeroides*. The space-fill model was derived from X-ray crystallographic data that resolved between 9 and 15 carbons of the acyl chains of CL. *R. sphaeroides* contains mainly 18:0 and 18:1 fatty acids. Figure adapted with permission from McAuley et al. [18]. Copyright 1999 National Academy of Sciences, USA. (B) 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. Figure adapted with permission from Ferguson et al. [19]. Copyright 1999 Elsevier Science Ltd. (C) 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-fill models) as viewed from the extracellular side. Three phytanyl chains of lipid (gray) lie in the crevices formed between the A and B domain of one monomer and the D and E domain of the adjacent monomer. The central core of the trimer is filled with a sulfated triglycoside attached to archaeol (glycolipid S-TGA-1). Red denotes the oxygen molecules of the sugars in white. (D) Cross section of the lipid bilayer showing phytanyl archaeol molecules (blue hydrophobic domain with red and white for the headgroups) extending from both sides of the bilayer and interacting with protein monomers (red and yellow helices) of bacteriorhodopsin. In the central core of the protein are two glycolipid S-TGA-1 molecules on the extracellular side of the membrane with a the gap on the cytosolic side of the membrane. The black lines indicate the boundary of the interface region. Figure adapted with permission from Essen et al. [20]. Copyright 1998 National Academy of Sciences, USA.
This organization explains the requirement for the natural archaeol lipids to maintain structure and function of the protein. The X-ray data demonstrate a good match between the hydrophobic face of membrane proteins and selective highly ordered surrounding lipids. The positioning of two glycolipid S-TGA-1 molecules to the extracellular side of the central hole in the bacteriorhodopsin trimer (Fig. 10D) results in a 5 Å ‘membrane thinning’ relative to the surrounding bilayer. This may cause a steeper electric field gradient across the central core than in the bulk lipid phase that might affect the proton pathway in bacteriorhodopsin.

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 aqueous pore on one side and an interface with the membrane bilayer on the other (Fig. 10B). X-ray crystal structure of E. coli outer membrane ferric hydroxamate uptake receptor (FhuA) contains a bound lipopolysaccharide in 1∶1 stoichiometric amounts. 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 headgroup of lipopolysaccharide makes extensive interactions with charged and polar residues of the protein near the outer surface of the membrane. Most of the favorable interactions are contributed by a cluster of 8 positively charged residues on the surface of the barrel, which interact by hydrogen bonding at distances around 3 Å or electrostatically at longer distances. 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 near the membrane interface region has been observed in other membrane proteins and may be involved in π-bonding interactions with the headgroups of lipids.

5.2.3. Organization of protein complexes
Rather than being associated with the exterior surface of membrane proteins, many phospholipids, particularly anionic ones like PG and CL, are found wedged between the subunits of monotropic and heterotropic 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 bc₁ complex, ATP synthase, cytochrome c oxidase, and the ATP/ADP translocase.

The crystal structure of bovine cytochrome c oxidase has been determined to a resolution of 2.3 Å. This integral membrane protein (a homodimer with 13 different subunits in each monomer) is responsible for the reduction of molecular oxygen to water during aerobic respiration, with concomitant proton pumping across the mitochondrial inner membrane. Several lipids were resolved in this structure: three PEs, seven PGs, one PC and two CLs. One of the CLs is located at the interface between the two monomers of the dimer and the remaining phospholipids are located between subunits
within each monomer. The resolution of CL in the structure of the bovine cytochrome c oxidase is particularly intriguing because it is well documented that CL cannot be removed from cytochrome c oxidase without a loss of enzyme activity.

In the above case, specific lipids mediate protein–protein contacts within a multimeric complex and may be 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. Using lipids as interface material reduces the need for highly complementary protein–protein interactions and provides for flexible interactions between subunits.

5.2.4. Binding sites for peripheral membrane proteins
A common mechanism of cellular regulation is to organize functional complexes on demand from existing components. In many cases, the components are initially distributed between membranes and the cytosol. Post-translational modification of a protein, or the appearance of a cytoplasmic or membrane component, signals the organization of a functional complex on the membrane surface. There are many examples of primarily anionic phospholipids being either the signals or the organization site for such protein complexes at the membrane surface. Three structure-specific domains have been recognized, mostly in eukaryotes, that serve as specific lipid binding domains.

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 the protein kinase C to specific membrane sites [21]. 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 phospholipase C, phospholipase A$_2$, PI-3-phosphate kinase and calcium-dependent protein kinase C [22]. The crystal structure of C2 domain of protein kinase C$_\alpha$ in complex with PS reveals that the recognition of PS involves a direct interaction with two Ca$^{2+}$ ions.

The PH, or pleckstrin homology, domain is shared by protein kinase C$_\beta$ and some phospholipases C. This domain is responsible for associating peripheral membrane proteins with the membrane via the phosphoinositide head group of polyphosphorylated PIs in an enantiomer-specific manner [23]. PH domains consist of 7-stranded β-sheets with positively charge pockets that attract the negatively charged PI head group.

In prokaryotic cells, the protein structural features defining lipid-binding domains is 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 protein and SecA protein (see Section 5.3) are peripheral membrane proteins in E. coli that carry out 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 anionic lipid content could be controlled [2]. DnaA protein is required for initiation of DNA replication and is active in its ATP- but not ADP-bound form. In vitro, the exchange of ATP for ADP in the complex is greatly stimulated by almost any anionic phospholipid including non-E.
coli lipids like PI. In vivo DnaA function is compromised in mutants with reduced PG/CL levels. This phenotype can be suppressed by mutations that bypass the need for DnaA. An anionic specific membrane binding domain has been identified 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.

What was once thought to be a specific interaction of SecA and DnaA 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 highly elevated levels of phosphatidic acid still initiate DNA replication (DnaA) and export protein (SecA) [10]. Both of 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 both of 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, an early precursor to 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. 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. 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 free fatty acids (micelle forming) has the opposite effect. Surface association of the amphipathic helices would be favored by the decrease in hydration of the interfacial region due to the induced negative curvature, and penetration into the surface of the bilayer would reduce the stress imposed by negative curvature [24].

5.3. Translocation of proteins across membranes

Movement of proteins across membranes is 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 role of lipids in this process has only recently received considerable attention. 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 [2]. The in vivo translocation of the outer membrane precursor proteins, prePhoE and proOmpA, was severely hampered in these cells. When the expression of the *pgsA* gene (encodes phosphatidylglycerophosphate synthase) placed under control of the *lac* promoter/operator was used to fine-tune the level of PG in the membrane, the translocation rate of the proteins was directly proportional to the amount of PG. The molecular basis for this anionic lipid requirement is for the function of SecA. SecA is a peripheral membrane protein that acts as a translocation ATP-driven motor 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 pore component SecY for high affinity binding to the membrane, for membrane penetration, and for high level ATPase-dependent function. Functional reconstitution of purified and delipidated SecYEG complex from E. coli and Bacillus subtilis into liposomes of defined lipid composition revealed an absolute requirement for PG [25]. 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.

The N-terminal signal peptides of E. coli secreted proteins possess at least one positively charged amino acid. Protein translocation efficiency is dependent on both the number of positive charges and the anionic phospholipid content of the membrane. Photocrosslinking of the secreted proteins with the fatty acid chains of membrane lipids demonstrated direct contact between the signal peptide and lipids during early stages of protein translocation.

Non-bilayer-forming lipids are also required for protein translocation of proteins across the membrane of E. coli. The only non-bilayer-forming lipid in E. coli mutants lacking PE is CL in the presence of Mg$^{2+}$ or Ca$^{2+}$. Protein translocation into inverted membrane vesicles prepared from PE-deficient cells is reduced with divalent cation-depletion but can be enhanced by inclusion of Mg$^{2+}$ or Ca$^{2+}$ [2]. Translocation in the absence of divalent cations is restored by incorporation of non-bilayer PE (18:1 acyl chains) but not by bilayer-prone PE (14:0 acyl chains). These results would indicate that lipids with a tendency to form non-bilayer structures provide a necessary environment for the translocase 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. Most such proteins are organized with several $\alpha$-helical transmembrane domains spanning the membrane bilayer. These helices are connected by extramembrane loops alternately exposed on either side of the membrane. In bacteria these proteins generally obey the 'positive inside' rule in which those extramembrane loops on the cytoplasmic side of the membrane have a net positive charge and the loops exposed to the exterior are either neutral or negatively charged. Variation in the positive charge density of these loops as well as the anionic phospholipid content of the membrane indicates that these positive loops are anchored to the inside of the cell by interaction with anionic phospholipids.

5.4.1. Lipid-assisted folding of membrane proteins

The membrane clearly serves as the solvent within which integral membrane proteins fold and function [9]. However, do lipids act in more specific ways to guide and determine final membrane protein structure and organization? Recent evidence supports a role for lipids analogous to that of protein molecular chaperones in the folding of membrane proteins. Molecular chaperones facilitate the folding of substrate proteins by interacting with non-native folding intermediates but do not interact with native or totally unfolded proteins, and are not required to maintain native conformation. Lipids
that fulfill these requirements in assisting the folding of specific membrane proteins have been termed ‘lipochaperones’ to distinguish their function from simply providing a solvent for the folding process.

The major evidence for the existence of lipochaperones comes from studies on the requirement for PE in the assembly and function of lactose permease (LacY) of *E. coli* [26]. LacY is a polytopic membrane protein with 12 transmembrane-spanning domains connected by alternating cytoplasmic and periplasmic loops (Fig. 11). LacY carries out transport of lactose either in an energy-independent mode to equilibrate lactose across the membrane (facilitated 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 (active transport). Uncovering a lipochaperone role for PE in the assembly of LacY came about by the fortuitous availability of reagents and techniques. A large number of biochemical and molecular genetic resources are available for studying LacY including antibodies and molecularly engineered derivatives of LacY. The availability of viable *E. coli* strains that either lack PE, or in which the level of PE can be regulated, provided reagents to study the requirement of PE in the assembly of LacY in vivo and in isolated membranes. The development of a blotting technique termed an ‘Eastern-Western’ made possible the screening for lipids affecting the refolding of LacY in vitro or the conformation of LacY made in vivo.

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 by direct assay. This combined blotting technique allows the detection of membrane protein conformational changes as influenced by individual lipids during refolding. Since many membrane proteins including LacY retain significant amounts of secondary structure even in sodium dodecyl sulfate, refolding from sodium dodecyl sulfate detects intermediate and late steps of folding.

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 mixtures containing PE, both active and facilitated transport was restored. In mixtures containing only PG and/or CL or even 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 had full transport function, but cells lacking PE only displayed facilitated transport even though bioenergetic parameters of the membrane were normal. 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
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 (NH₂) to the carboxyl (COOH) terminus. The even-numbered hydrophilic loops (‘C’ and open) connect the transmembrane domains on the cytoplasmic side (IN) of the membrane. The odd-numbered hydrophilic loops (‘P’ and filled) connect the transmembrane domains on the periplasmic side (OUT) of the membrane. (B) The topological organization of lactose permease when assembled in the absence of PE. Note the first six transmembrane domains along with their connected hydrophilic loops are inverted with respect to the plane of the membrane when compared to A. Transmembrane domain VII is very hydrophilic and is postulated to reside outside of the membrane in the altered structure.

the presence of specifically PE (or other primary amine-containing phospholipids such as PS). 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. Furthermore, LacY extensively denatured in urea–sodium dodecyl sulfate (which eliminates most secondary structure) cannot be renatured by simple exposure to PE. 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 molecular chaperone [26].

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 [27]. In the absence of PE the first six transmembrane domains and the loops that connect them assume an inverted topology with respect to the plane of the membrane bilayer (Fig. 11). Cytoplasmic loops become periplasmic, and periplasmic loops become cytoplasmic. If LacY is first assembled in the absence of PE and then post-assembly PE synthesis is initiated, there is correction of topology and regain of full transport function. These results dramatically illustrate the specific effects of membrane lipid composition on both structure and function 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.

5.4.2. Scope of lipochaperone function
Secondary transport proteins (those that couple active transport to proton uptake) in E. coli show high overall structural homology to LacY. Amino acid permeases specific for Phe, Lys, Pro, and aromatic amino acids are defective in active transport in cells lacking PE. The melibiose permease requires PE in order to reconstitute transport function in vitro. Therefore, the lipochaperone function of PE may be a general requirement for proper assembly of this family of secondary transport proteins. A lipochaperone requirement in E. coli has been reported for the proper folding of the \( \beta \)-barrel outer membrane protein PhoE. During passage of the protein through the inner membrane of E. coli, interaction with precursors to lipopolysaccharide is required to obtain assembly competent protein upon arrival at the outer membrane [28]. Therefore, a role for lipids to act in membrane protein folding in a more specific manner than providing the solvent has been established.

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 are all utilized to compartmentalize functions. The original fluid mosaic model envisioned the membrane bilayer as a homogenous sea of lipid into which proteins are dispersed (Fig. 1). The current view of biological membranes is that they contain microdomains of different lipid and protein composition than is reflected by the whole membrane and that these domains serve to further compartmentalize cellular processes.

Lipid mixtures made up of defined lipids undergo phase separations due to lipid polymorphism and differences in steric packing of the acyl chains. Mixtures of bilayer and non-bilayer lipids undergo multiple phase transitions supporting the existence
of segregated domains within the bilayer. In model systems amphipathic polar lipid analogues self associate into domains if their hydrophobic domains are the same even if their polar domains carry the same net charge. Therefore, headgroup repulsive forces can be overcome by orderly packing of the hydrophobic domains. There is considerable acyl chain mismatch between phospholipids and sphingolipids (see Chapter 14 for structures), i.e., phospholipids tend to have shorter acyl chains (16–18) with higher degrees of unsaturation as 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 and expanded domains of unsaturated acyl chains of phospholipids. Lipid segregation can also be facilitated by specific polar headgroup 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 \( pK_a \), as noted earlier, may be the basis for the formation of clusters of CL in natural and artificial membranes [29].

5.5.1. Lipid rafts

Lipid rafts are liquid ordered phases of cholesterol, glycosphingolipids (gangliosides), sphingomyelin, and proteins that exist as microdomains within the more dispersed liquid crystalline bilayer. Lipid rafts are operationally defined as the membrane fraction of eukaryotic cells that is resistant to solubilization in the cold by the detergent Triton X-100 (detergent-resistant membrane fraction). This fraction is greatly enriched in cholesterol, glycosphingolipids, sphingomyelin and a subset of membrane proteins. The proteins co-clustered in lipid rafts are soluble globular proteins tethered to the raft lipids via covalent linkage to fatty acids, cholesterol, or phosphatidylinositol (Chapter 2) [30]. The latter glycosylphosphatidylinositol-linked proteins are attached 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 glycosylphosphatidylinositol-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 glycosylphosphatidylinositol-linked proteins. The hydrogen-bonding properties of the glycosphingolipids with themselves and with the constituents of the glycosylphosphatidylinositol-linked proteins stabilizes 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 be why it is not dissipated by detergent extraction.

Lipid rafts appear to be a mechanism to compartmentalize various processes on the cell surface by bringing together various receptor-mediated and signal transduction processes. A general phenomenon is that when glycosylphosphatidylinositol-linked proteins aggregate on the surface, they also become enriched in the detergent-resistant
Fig. 12. Model of lipid raft. A glycosylphosphatidylinositol-linked protein is attached to the exterior monolayer of the membrane and a 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 glycosylphosphatidylinositol-linked protein is hypothetical. Clustered (dark gray) around the glycosylphosphatidylinositol are ordered (straight alkyl chains) glycosphingolipids, sphingomyelin, and PC with intercalated cholesterol. The phospholipids with kinked (unsaturated) chains indicate the more disordered liquid crystalline state of the surrounding bilayer. Reprinted (abstracted/excerpted) with permission from Simons and Ikonen [30]. Copyright 2000 American Association for the Advancement of Science.

membrane fraction as well as phosphorylated. For example, clustering of the IgE receptor, FcεRI, by binding its ligand on the cell surface results in its phosphorylation by a Src-family kinase that activates a signaling cascade. Only the receptor associated with the detergent-resistant membrane fraction is phosphorylated. The Src-family of
kinases is 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).

A class of lipid domains related to rafts are caveolae which are invaginations on the surface of the plasma membrane of eukaryotic cells (Chapter 20). They contain the protein caveolin which when complexed with cholesterol forms a hairpin structure believed to induce curvature in the bilayer to form the caveolae. Caveolin is an integral part of the intracellular cholesterol transport machinery and caveolae are involved in regulating influx and efflux of cellular cholesterol. Caveolae are also resistant to detergent solubilization and also contain several proteins responsible for signal transduction suggesting a regulatory role for these structures in response to cholesterol.

The existence of lipid rafts and their function is still an evolving area. 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 the native state, presumably due to their small size and possibly their loose association and dynamic properties. The raft components may normally have low affinity for each other, but increase their affinity when clustering is induced by other processes. It still is not clear how glycosylphosphatidylinositol-linked proteins on the exterior of the cell interact with acylated proteins that appear to be associated with rafts on the interior surface of the membrane.

5.5.2. Lipid domains in bacteria
One of the few examples of lipid domains being observed in living cells comes from the staining of CL in the membrane of *E. coli* by the fluorescent dye 10-N-nonyl acridine orange [31]. This reagent is highly specific for CL and has little or no interaction with other lipids or cell components. In wild-type cells, fluorescence is observed at the poles of the cell and at the division septum. In filamentous cells of *E. coli* with multiple genomes distributed along their length, the fluorescence is localized between the genomes. These fluorescence-enriched domains may be due either to regions enriched in CL (or possibly anionic lipids including CL) or areas enriched in lipid relative to protein. Initiation of DNA replication and the formation of the septal domain of *E. coli* have been postulated to be dependent on anionic phospholipids that would be consistent with the localization of CL domains.

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.

Phospholipids in biological membranes are distributed asymmetrically between the inner and outer leaflets of the lipid bilayer [32]. In the plasma membrane of eukaryotic cells PE and PS are localized to the inner leaflet and PC and sphingomyelin are enriched in the outer leaflet. Using a cyclic peptide 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. Immobilization of cell surface PE by the PE-binding peptide inhibited disassembly of the contractile ring resulting in formation of a long cytoplasmic bridge between the daughter cells. Removal of the peptide from the surface of arrested cells allowed cell division to proceed with disappearance of exposed PE. Furthermore, a mutant cell line defective in PE biosynthesis was isolated as a variant resistant to the cytotoxicity of the PE-binding peptide [33]. This cell line required either PE or ethanolamine for normal growth and cell division. In ethanolamine-deficient medium these mutant cells arrested with a cytoplasmic bridge between the two daughter cells. Addition of PE or ethanolamine restored normal cytokinesis. These findings provide the evidence that transbilayer movement of PE at the cleavage furrow contributes to regulation of cytokinesis.

In *E. coli*, cell division is initiated after genome duplication by organization of FtsZ protein monomers 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 filamentous cells. The FtsZ protein complex is recruited to the division site, but the FtsZ ring fails to constrict [34]. This phenotype is not observed in strains with specific defects in other steps of phospholipid biosynthesis. Although not firmly documented, prokaryotic membranes also appear to have an asymmetric enrichment of PE on the inner leaflet of the cytoplasmic membrane [35]. It is likely that PE is essential for cytoskeletal organization in the completion of cytokinesis in prokaryotic cells as well as mammalian cells.

6. Future directions

The roles lipids play in cellular processes is as diverse as the chemical structures of lipids found throughout nature. Although a single phospholipid can form a sealed bilayer vesicle in solution, a diversity of lipid structure and physical properties is necessary to fill the broad range of roles that lipids play in cells. Lipid structures vary greatly from the archaeabacteria, with their often-harsh environments, to the eubacteria that also must carry out a diversity of processes in one or two membrane structures, and to eukaryotic cells that have specialized organelles with different lipid compositions tailored to their function.

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. Biological membranes 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, their physical properties 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 subdomains such as lipid rafts, lipid domains, and organizations of membrane associated complexes with their own unique composition. These complexes
can be made up of specific lipids as the organization site for integral and peripheral membrane proteins and many times are transient responding to cellular signals that can themselves be lipids. Lipids are integral components of stable complexes and serve specific structural roles by affecting protein conformation, by serving as the ‘glue’ that holds complexes together, or by providing the flexible interface between protein subunits. Lipids provide the complex hydrophobic–hydrophilic solvent within which membrane proteins fold and function, but they can also act in a more specific manner as molecular chaperones directing the attainment of final membrane protein organization. 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 macromolecules. At the same time they can organize into the very stable but highly dynamic supramolecular structures we know as membranes.

The challenge for the future will be to determine the function at the molecular level for the many lipid species already discovered. 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

<table>
<thead>
<tr>
<th>Code</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>CL</td>
<td>cardiolipin</td>
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<tr>
<td>DGDG</td>
<td>diglucosyl or digalactosyl diacylglycerol</td>
</tr>
<tr>
<td>MGDG</td>
<td>monoglucosyl or monogalactosyl diacylglycerol</td>
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<tr>
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<td>phosphatidic acid</td>
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<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
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<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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References


