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Review

Diversity and versatility of lipid–protein interactions revealed by molecular genetic approaches☆

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Abstract

The diversity in structures and physical properties of lipids provides a wide variety of possible interactions with proteins that affect their assembly, organization, and function either at the surface of or within membranes. Because lipids have no catalytic activity, it has been challenging to define many of their precise functions in vivo in molecular terms. Those processes responsive to lipids are attuned to the native lipid environment for optimal function, but evidence that lipids with similar properties or even detergents can sometimes partially replace the natural lipid environment has led to uncertainty as to the requirement for specific lipids. The development of strains of microorganisms in which membrane lipid composition can be genetically manipulated in viable cells has provided a set of reagents to probe lipid functions. These mutants have uncovered previously unrecognized roles for lipids and provided in vivo verification for putative functions described in vitro. In this review, we summarize how these reagent strains have provided new insight into the function of lipids. The role of specific lipids in membrane protein folding and topological organization is reviewed. The evidence is summarized for the involvement of anionic lipid-enriched domains in the organization of amphitropic proteins on the membrane surface into molecular machines involved in DNA replication and cell division.

Keywords: Amphitropic protein; Protein topology; Phospholipid; Cell division; Lipid domain; DNA replication; Lipochaperone; Protein folding; Membrane

Contents

Abbreviations: CL, cardiolipin; DGlcDAG, diglucosyl diacylglycerol; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; LacY, lactose permease; Lep, leader peptidase; LPS, lipopolysaccharide; MGalDAG, monogalactosyl diacylglycerol; MGlcDAG, monoglucosyl diacylglycerol; NAO, 10-*N*-nonyl acridine orange; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PheP, phenylalanine permease; PI, phosphatidylinositol; PS, phosphatidylserine; TM, transmembrane domain

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1. Introduction

Lipids provide a complex solvent for integral membrane proteins and a varied surface for interaction of soluble amphitropic proteins with the membrane. The large diversity in lipid structures allows for a broad spectrum of chemical and physical properties for the membrane bilayer that affects protein function and organization. The ability of lipids to form subdomains of unique protein and lipid composition provides a mechanism to regulate and compartmentalize protein function within a membrane. However, defining the function of lipids in molecular terms in cellular processes has been a challenging undertaking. Although the physical and chemical properties of individual lipids and lipid mixtures have been extensively studied, it is not clear how to relate this information to the in vivo state. Lipids have no inherent catalytic activity. Therefore, biochemical studies have relied on the secondary effects of lipids on in vitro reconstituted processes. Careful consideration has not always been given to the physical and chemical properties of lipids, and it is difficult to sort out which properties of a given lipid or lipid mixture are important. Although in vitro approaches have provided insight into the role of lipids, in vivo verification has been limited. Determination at atomic resolution of the structure of a few membrane proteins has shown specific lipid species in association with proteins. However, the tight association of detergents in these structures indicates that other lipids may have been lost during isolation, resulting in alterations in structure and function. Furthermore, structural studies face limitations in revealing the dynamic nature of lipid–protein interactions.

The development of bacterial and yeast strains in which lipid composition can be manipulated has extended and complemented in vitro approaches. Molecular genetic manipulation of lipid composition in living cells has provided in vivo verification of lipid function and revealed new roles for lipids in cellular processes. Such strains have provided information on the role of lipids in such dynamic processes as membrane protein folding, DNA replication, cell division, and transient structural arrangements of proteins. This review will focus on lipid–protein interactions that have been revealed with the aid of microorganisms genetically altered in their lipid composition.

2. Diversity and similarity of lipids

Fig. 1 outlines the pathway and genes responsible for the synthesis of all phospholipids in *Escherichia coli* and CL in eukaryotic cells [1]. Initial genetic studies indicated that elimination of the major phospholipids, i.e., phosphatidylethanolamine (PE^3, 70%) or both phosphatidylglycerol (PG, 20%) and cardiolipin (CL, 5%), was lethal, supporting an essential role for these lipids. However, subsequent studies have shown remarkable plasticity of *E. coli* with respect to membrane lipid composition while revealing unexpected roles for the major lipid classes [2]. Conditions have been established for viability of *E. coli* lacking phosphatidylserine (PS) and PE, lacking PE with accumulated PS, lacking PG and CL, or lacking CL with accumulated PG. In all cases, the resulting strains have defects that have been used to probe the role of specific lipids in important cellular

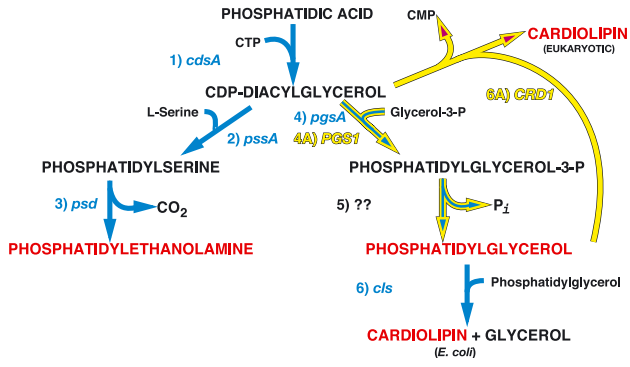

Fig. 1. Biosynthetic pathways for phospholipids in *E. coli* and CL in eukaryotic cells. The genes encoding the enzyme for each step (1–4, 6 in blue) in *E. coli* and eukaryotic cells (4A and 6A in yellow) are shown. No gene has been identified for step 5. Note the difference in substrates for CL synthesis shown in steps 6 and 6A for *E. coli* versus eukaryotic cells, respectively.

processes. Incorporation of foreign lipids into some of the above lipid mutants partially suppresses the phenotypes of these strains. These studies indicate specific roles for lipid–protein interactions in supporting optimal function, while also indicating considerable flexibility with regard to a specific lipid species requirement.

Such flexibility lies in the inherent nature of lipids as diverse in structure but sharing common physical and chemical properties. Lipids are not rigid but highly deformable molecules that easily fit between dynamic and mobile protein structures. Structural features, chemical properties, and the collective physical properties of lipids in association with each other must be considered when assessing lipid–protein interactions [1]. For instance, PE is a zwitterionic glycerophosphate-based diacyl lipid with no net charge that favors the formation of a typical membrane bilayer structure (lamellar) when both fatty acids are saturated, but favors nonbilayer structures (hexagonal and cubic phases) with increasing unsaturation of its fatty acids and with increases in temperature. The primary amine is capable of donating a proton and, along with the phosphate group, is capable of hydrogen bonding. Phosphatidylcholine (PC), on the other hand, is nearly always bilayer forming and like PE capable of charge dampening due to its lack of a net charge. PC and PE can interact through π bonding with aromatic residues via their respective charged amines, but the quaternary amine of PC can neither hydrogen bond nor donate a proton. PS, CL, phosphatidic acid (PA), PG, and phosphatidylinositol (PI) all have a net negative charge, and are anionic, but have different physical properties. PS, PA, and CL can form nonbilayer structures in the presence of divalent cations, whereas all the anionic lipids can hydrogen bond and provide a net negative membrane surface. Finally, monoglucosyl diacylglycerol (MGlcDAG) [3] contains no charge character, can both hydrogen bond and partially ionize [4] via its sugar hydroxyls, and can either be bilayer or nonbilayer forming depending on temperature and fatty acid content. Diglucosyl diacylglycerol (DGlcDAG) is similar to MGlcDAG but only forms bilayer structures.

A unique property of biological membranes is that they are a complex mixture of several lipids with respect to head group each with a spectrum of fatty acid compositions. The head groups determine many of the structural and chemical properties of the membrane surface while the combination of head groups with different fatty acids determines the collective physical property of the membrane [1]. Although an overall bilayer structure is essential to cell survival, all membranes contain some nonbilayer-forming lipids that affect the collective property of the membrane. Bilayer-forming lipids align the head groups of each monolayer parallel to each other. Nonbilayer lipids introduce concave curvature with respect to the surface of each monolayer because of the larger volume occupied by the hydrophobic domain in comparison to the head group. Introduction of nonbilayer-forming lipids into both monolayers introduces stress in the bilayer, as each monolayer tends to curve away from the other. Association of lipids with common fatty acid chain lengths results in phase separation into domains of varied bilayer thickness and mixtures of bilayer and nonbilayer-forming lipids form separate domains overcoming charge repulsion between identically charged head groups.

Identifying processes that involve lipids has been challenging, but numerous examples now exist for roles of lipids in specific cellular processes. A greater challenge is defining the precise property of a given lipid in molecular terms that supports a particular function. The major contributions of genetic approaches to the study of lipid–protein interactions have been in vivo verification of lipid function and the uncovering of unsuspected novel roles for lipids. Through genetic alteration of lipid composition, some insight is emerging as to which property of a given lipid is important for its function.

3. Lipid mutants as biological reagents to study lipid function

3.1. Cells lacking amine-containing phospholipids

Point mutants in either *pssA* or *psd* (step 2 or step 3 of Fig. 1, respectively) that significantly lower PE or raise PS at the expense of PE, respectively, were isolated as conditional lethal temperature-sensitive mutations and showed very similar growth phenotypes (see reviews in Refs. [1,2,5]). When PE levels were reduced from the normal level of about 70% to below 35% with or without accumulation of PS, cells filamented and stopped growing. However, supplementation of the growth medium with a subset of divalent cations (see below) allowed slow growth but did not suppress filamentation. A null mutation in the *pssA* gene (lacking both PS and PE) is lethal but can be suppressed by millimolar addition to the growth medium of Ca^{2+}>Mg^{2+}>Sr^{2+} (in the indicated order of effectiveness) but not Ba^{2+}. In the presence of Mg^{2+}, cells completely lacking PE and PS (*pssA* null [6]) or containing 76% PS and 5% PE (*psd* point mutant [7]) are still filamentous, suggesting the lack of a neutral zwitterionic lipid (PE) and/or the high negative charge density on the membrane surface affects the cell division machinery. However, in *pssA* mutants, several secondary solute transport systems are defective that are functional in *psd* mutants, indicating a specific requirement for PE and/or PS for the transporters. As discussed below, mutants lacking both PS and PE have been used to investigate the role of membrane lipid composition in proper functioning of the cell division machinery, in the folding and topological organization of integral membrane proteins, and the formation of lipid-enriched domains within the membrane.

The absolute requirement for amine-containing phospholipids is suppressed by addition of a subset of divalent

cations, as noted above, but not trivalent cations, polyamines, or molar concentrations of monovalent cations [8,9]. Therefore, phenotypes of these mutants are not due to the aberrant high negative charge density of the membrane surface. The level of CL present in *pssA* null cells is dependent on the divalent cation added to the growth medium and its effectiveness in promoting the nonbilayer phase for CL. Ca^{2+} is more effective than Mg^{2+} or Sr^{2+} in promoting the nonbilayer phase for CL and results in inducing the lowest CL content. Ba^{2+} does not induce the nonbilayer phase for CL, which presumably is why it does not support growth. The collective property of lipids extracted from these cells and analyzed in the presence of the divalent cation supplied during growth mimics the bilayer to nonbilayer transition properties of lipids extracted from wild-type cells and analyzed in the absence of divalent cation. To maintain the bilayer to nonbilayer phase transition temperature near but below the growth temperature, *E. coli* normally adjusts the collective properties of the membrane by changing the ratio of saturated to nonsaturated fatty acid in the PE pool. Interestingly, *E. coli* is viable with no PE and PS (*pssA* null) in the presence of a divalent cation, with 0.2% CL provided that PE is present (*cls* null), but not viable when PE, PS, and CL are eliminated in a *pssA cls* double mutant. These results taken together strongly suggest that *E. coli* balances the ratio of bilayer to nonbilayer lipids to achieve the optimal collective property of the membrane even when confronted with an unnatural lipid content. Nonbilayer lipids, CL plus divalent cations or PE, are also required for efficient translocation of proteins across the inner membrane [10]. The above studies emphasize the importance of nonbilayer-forming lipids in cell membrane structure and cell function. Still unresolved is a molecular understanding of how the collective properties of the membrane bilayer affect protein assembly and function.

3.2. Cells deficient in anionic phospholipids

Point mutants and deletion mutants of *cls* (step 6, CL synthase, Fig. 1) only show a mild phenotype of reduced viability after prolonged incubation in stationary phase [11]. However, cells either lacking or with less than 2% PG plus CL (step 4, *pgsA* null mutants) are not viable [12], but the phenotype can be suppressed by mutation of the *lpp* gene [13]. This gene encodes a high copy outer membrane protein that is posttranslationally modified by diacylglycerol derived from PG. Failure to modify this protein due to limiting PG leads to high accumulation of the apoprotein in the inner membrane and cell lysis [14]. Cells mutant in *lpp* and completely lacking PG and CL are viable, suggesting no specific role for these lipids. However, extensive studies described below strongly indicate that the accumulation of the anionic lipid precursors PA and CDP-diacylglycerol partially substitute for PG in the essential organization of amphitropic proteins on the membrane surface. However, this substitution is not complete because these mutants are not viable at 40 °C without additional mutations that have not been fully characterized [13]. Therefore, optimal growth and viability require PG and CL, but other anionic lipids can suboptimally substitute.

Eukaryotic cells appear to show more structural specificity in their requirement for PG and CL than bacterial cells. These lipids are primarily found in mitochondria in close structural and functional association with the energy-transducing systems of the inner membrane. In yeast, complete lack of PG and CL (step 4A, *pgs1* null mutants) results in severe mitochondrial dysfunction due to the lack of translation of one of the nuclear-encoded and all of the mitochondrial-encoded components of the electron transport chain [15]. The molecular basis for this loss in translation is not known. Similar morphological and growth defects are seen in Chinese hamster ovary cell conditional *pgs1* mutants with low PG and CL levels [16]. A much milder phenotype of poorer growth on a carbon source requiring mitochondrial respiratory function results from lack of CL (step 6A, *crd1* null mutant) with accumulation of PG [17]. The apparent substitution of PG (normally 10-fold lower than CL) for CL is not completely surprising because PG can substitute for CL in the reconstitution of delipidated energy-transducing complexes [18]. The molecular basis for the reduced growth rate of cells lacking CL is discussed in Section 7.

Molecular genetic manipulation of lipid metabolism in bacteria and yeast has provided viable strains with a broad range of membrane lipid compositions. These strains have proven valuable in dissecting the roles of lipids at the molecular level in a multitude of cellular processes.

4. Lipid-assisted protein folding

The phospholipid bilayer as a solvent influencing the structure and function of membrane proteins is well established. However, the role of individual phospholipid molecules as a part of the protein folding process has been understudied. Can lipids act as molecular chaperones in the folding of proteins? By definition, molecular chaperones facilitate the folding of proteins by interacting noncovalently with nonnative folding intermediates and not with either the native or totally unfolded protein. When folding is complete, molecular chaperones are not required to maintain proper conformation.

The most compelling evidence for a specific role of phospholipids in membrane protein folding is the requirement for PE in the folding of the integral membrane protein lactose permease (LacY) of *E. coli*. Normal assembly of LacY occurs into membranes containing an abundance of PE so that a separation between phospholipid-assisted and -unassisted folding pathways cannot be distinguished. However, LacY assembled in a PE-lacking mutant of *E.*

coli (*pssA* null mutant) only carried out facilitated, but not active, transport of substrate [19]. Moreover, the loss of this transport ability correlates with misfolding of periplasmic domain P7 (see Fig. 2) that is crucial for active transport of substrate [20]. Thus, LacY is "denatured" in vivo with respect to the P7 domain by assembly in a nonnative lipid environment.

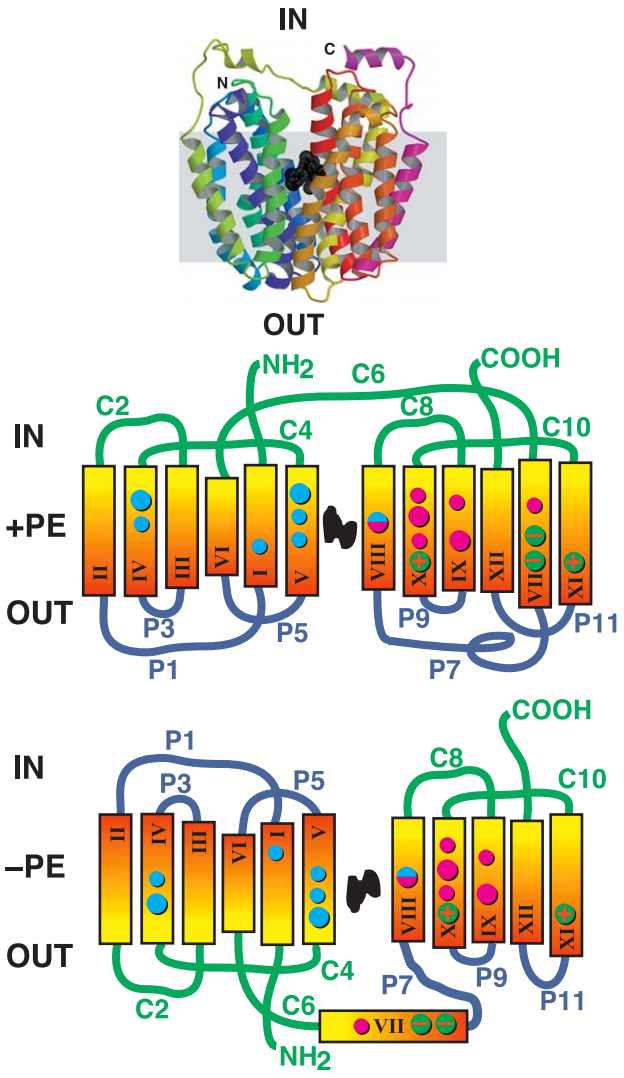

Fig. 2. Topological organization of LacY from *E. coli* in PE-containing and PE-lacking cells. The upper panel shows the packing of LacY into the two separate six TM N-terminal (N) and C-terminal (C) regions with respect to the plane of the membrane bilayer (IN, cytoplasmic side and OUT, periplasmic side). The position of bound substrate is between the two separate domains of the C154G mutant locked in the inwardly open conformation [31]. Reproduced with permission from *Science*. The center and lower panels are a planar depiction of the nearest neighbor relations and orientation of TMs (color gradient from light to dark in the outward direction for PE-containing cells) of LacY in PE-containing (+**PE**) and PE-lacking cells (−**PE**), respectively. The TMs are numbered in Roman numerals consecutively from the N-(NH_2) to C-(COOH) terminus. The cytoplasmic (C, green) and periplasmic (P, blue) extramembrane domains are similarly numbered consecutively as they are oriented in PE-containing cells. The light blue and pink circles indicate the position of residues most important for substrate binding and proton translocation, respectively. The + and − symbols refers to charged residues in the TMs. Note that C6 (light green helix, top of top panel) connects the two separate helical bundles and that P7 is shown with different conformations in PE-containing and PE-lacking cells.

SDS-polyacrylamide gel electrophoresis, which only partially denatures LacY, followed by Western blotting analysis of LacY from PE-containing cells, demonstrated that the protein could be separated from PE and still retain its native structure with respect to domain P7 (as determined by a conformation-specific monoclonal antibody), consistent with molecular chaperone action. The misfolding of LacY due to assembly in PE-lacking cells was corrected by employing the Eastern–Western blotting technique in which LacY was exposed to phospholipids during renaturation from SDS in the Western blotting procedure, followed by probing with a conformation-specific antibody [20]. LacY regained the native conformation of domain P7, which was absent during in vivo assembly, after renaturation specifically in the presence of the PE or PS. Anionic lipids PG and CL and "foreign" lipids such as PC did not support proper refolding. Proper refolding did not occur with protein extensively denatured by SDS–urea treatment. Taken together, these data strongly suggest that PE assists the folding of LacY via a transient noncovalent interaction with a folding intermediate and fulfills the minimum requirements of a molecular chaperone.

The interaction between lipids and partially folded LacY during refolding was found to be dependent on both the chemical properties of the individual lipid molecules and the collective properties of phospholipid mixtures [21]. There was a specific requirement for an ionizable amine-containing diacyl phospholipid (i.e., PE, PS, mono- and dimethyl-PE, but not PC) of natural chirality (both the backbone and the head group) and preference for bilayer organization to facilitate proper folding of LacY into its native conformation.

Although refolding of denatured full-length protein in vitro provides useful information, it does not reflect in vivo folding of a nascent peptide that begins during translation. To more closely mimic the in vivo situation, LacY translation and membrane insertion were coupled with co- or posttranslational synthesis of PE in membranes originally isolated from PE-lacking cells. The insertion of LacY into the membrane was independent of PE. However, post-assembly synthesis of PE corrected the improper folding observed in domain P7 [22]. Thus, PE appears to facilitate in vitro refolding and in situ folding into a fully native conformation by interacting with late nonnative folding intermediates like most protein molecular chaperones.

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 made up of different phospholipids [23]. Dimyristoyl-PE, a bilayer-forming PE, 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. PCs containing a wide range of fatty acids were either nonsupportive of refolding or inhibited refolding. Therefore, LacY and the peroxidase share common requirements for proper refolding dependent on lipids.

Do lipids influence the folding of other membrane proteins? Proper folding of OmpA, a monomeric protein of the *E. coli* outer membrane, may require lipopolysaccharides (LPS composed of lipid A plus Kdo plus a polysaccharide). Lipid A is a diglucosamine phosphate acylated with six fatty acids in amide and ester linkage [24], which forms the outer leaflet of the outer membrane, and is covalently linked to two 3-deoxy-D-manno-octulosonic acid (Kdo) monosaccharides comprising the minimal required LPS structure (characteristic of deep rough mutants); Kdo is normally decorated with ethanolamine phosphate, derived from PE, in pyrophosphate linkage. Wild-type laboratory strains have a polysaccharide extension added to Kdo that together with Kdo makes up the inner and outer core of LPS (wild-type LPS). Rough mutants contain various truncated versions of this polysaccharide extension. Addition of wild-type LPS to denatured OmpA in vitro resulted in quantitative conversion to native OmpA [25]. Similar results were obtained using only the lipid A portion of LPS but not with a range of phospholipids or the oligosaccharide portion of LPS. Moreover, only the immature processed form of OmpA and not the precursor pro-OmpA or mature OmpA underwent a conformational change upon addition of LPS, suggesting that LPS assists with folding of OmpA by interacting only with a folding intermediate [26].

Interaction of LPS might also be crucial in the assembly of the outer membrane porin PhoE of *E. coli* [27,28]. The functional unit of this protein is a trimer. LPS is required for the correct and efficient folding of PhoE protein into a form that is competent for stable trimer formation. *E. coli* phospholipids did not substitute for LPS and truncation of the polysaccharide of LPS reduced folding efficiency. A negative charge in the inner core of LPS and the number and nature of the six fatty acids of lipid A were critical to its function. The collective properties of LPS molecules were also important. Nonbilayer organization of LPS favored proper assembly while LPS organized in a bilayer inhibited proper folding. Thus, extensive hydrophilic and hydrophobic interactions between LPS and protein, involving the negative charges in the inner core and lipid A, respectively, seem to be required for efficient folding of the PhoE monomer in the inner membrane.

Based on all these results, a broad range of lipids can function as molecular chaperones (tentatively named lipochaperones) that specifically mediate the folding of proteins, thereby extending the definition of chaperones to other biomolecules in addition to proteins [29].

5. Lipid-dependent membrane protein topogenesis

The number of transmembrane segments, their orientation in the membrane, and the length of extramembrane domains that connect successive transmembrane domains (TMs) define the topology of a membrane protein. Major emphasis has focused on determining the role that the protein insertion machinery and amino acid sequence of membrane proteins play in determining topology, but many questions still remain unresolved. Only a limited number of studies have investigated the role of phospholipids in determining membrane protein topology. Do phospholipids act as specific topological determinants? Are protein topogenic signals within a membrane protein sequence "written" for a given membrane environment?

5.1. Amine-containing phospholipids as topological determinants

Combining the biochemical and genetic information on LacY with its expression in cells either with or without PE and PS (*pssA* null mutant) revealed a surprising dependence of protein topology on membrane phospholipid composition and explained many of the properties described in Section 4 for LacY assembled in PE-lacking cells [30]. Single amino acid substitutions by cysteine in the extramembrane domains of an otherwise cysteineless LacY were expressed in PE-containing or PE-lacking cells, and the accessibility of these cysteines to a membrane-impermeable sulfhydryl reagent was used to determine topology. Examination of whole cells, permeabilized cells, and inverted membrane vesicles showed that, when expressed in PE-lacking cells, the N-terminal six TMs (Fig. 2, TM I–VI) with their associated extramembrane domains were topologically inverted relative to the orientation in PE-containing cells. Cysteine replacements in TM VII (Bogdanov and Dowhan, unpublished results) were partially accessible from outside of the PE-lacking cell, indicating that TM VII was localized to the periplasmic space.

Recent crystallographic structural information for LacY [31] coupled with the topological, structural, and functional effects resulting from lack of PE during assembly can be summarized in a the model shown in Fig. 2. The N-terminal and C-terminal half of LacY fold into two independent six TM-bundles connected by a long cytoplasmic linker (C6). The interface of these two large domains forms the channel through which substrate passes. The detailed structure was determined on a mutant (C154G in TM V) of LacY locked in the inward open conformation [32]. This mutant can bind substrate but due to reduced flexibility cannot complete the transport process by opening the channel to the periplasm. Interestingly, most of the residues involved in substrate binding lie within the N-terminal domain, which as a group is inverted, in PE-lacking membranes. The ability of LacY in PE-lacking cells to perform facilitate transport demonstrates that the N-terminus has retained most of the structure

required to bind substrate. All of the residues involved in symport of a proton required for active uptake of substrate lie within the C-terminal domain that retains normal topology except for the displacement of TM VII to the periplasm. Such a displacement, which is facilitated by the low hydrophobicity of this domain, would disrupt the established salt bridges between TM VII and TMs X/XI, disrupt the conformation of domain P7 (required for active transport), and presumably disrupt the proton wire necessary for active transport. The ability of LacY to adopt alternative conformations in different lipid environments is consistent with the existence of two separately folded subdomains that can be coexpressed independently within the cell and associate into a functional protein [33]. Therefore, each domain can respond independently to the lipid environment. The substrate-binding pocket at the interface between these domains displays considerable flexibility [34] to open the channel alternatively to each side of the membrane during transport. The C154G mutation reduces flexibility of LacY and prevents transport. Immobilizing domain P7 by binding with antibody also blocks active transport [35].

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 lipid–protein interactions or depends on the lipid requirements of the components of the assembly machinery. To address this question, misassembled LacY or properly assembled LacY was purified from PE-lacking or PE-containing cells, respectively, and then reconstituted into liposomes of various lipid compositions [36]. Irrespective of the source of LacY, the final lipid composition of proteoliposomes determined both topology and function. LacY assembled in total *E. coli* phospholipids had the C6 and P7 domains on opposite sides of the liposome and supported both active and facilitated transport of substrate. Assembly in the presence of PG and CL alone resulted in C6 and P7 on the same side of the liposomes and only facilitated transport of substrate. Surprisingly, inclusion of PC into the latter liposomes resulted in wild-type topological organization of the C6 and P7 domain, but active transport was not restored. This result suggests that topological organization may be sensitive to the net charge on the membrane surface, i.e., both PE and PC would dilute out the high negative charge density of a bilayer consisting of only PG and CL. PE plays a dual role in establishing proper topology and supporting active transport. Therefore, both the topological organization and transport function of LacY are determined solely by the phospholipid composition independent of the cellular protein assembly.

Is lipid-assisted membrane protein topogenesis a general phenomenon of secondary transport systems and possibly other membrane proteins? The high-affinity phenylalanine permease (PheP) shares many similarities with LacY. PheP assembled in a mutant of *E. coli* lacking PE exhibited significantly reduced active transport function and a complete inversion in topological orientation for the N-terminus and adjoining transmembrane hairpin (NT-TM I-P1-TM II-C2), compared with PheP in a PE-containing strain [37]. An identical inversion of the N-terminal hairpin was observed for the *E. coli* γ-aminobutyric acid permease (GabP) expressed in PE-lacking cells (Zhang, King, Dowhan, unpublished result). In both amino acid transporters, the common N-terminal hairpin precedes an extended TM III (over 30 amino acids). This region of PheP has been associated with substrate binding and transport function [38] and may possess a high degree of flexibility. The extended length of TM III, which is rich in glycine, may provide the flexible interface between two independently folded domains. The flexible hinge region of LacY that allows a topological inversion in the absence of PE occurs in the center of the protein, which is associated with substrate transport and is also a very flexible region of the protein. Therefore, atypical TMs, either abnormally hydrophilic (LacY) or extended (PheP and GabP), may serve as discontinuities in the orderly packing of TM helices providing hinge points between separately folded domains that can respond differently to the lipid environment. Proteins without such a hinge region either cannot fold and are degraded or cannot assume different topologies in response to changes in lipid environment. Several other secondary transporters (lysine, aromatic amino acid, the high affinity Na^+/proline, and melibiose permeases) are compromised for active transport in PE-lacking cells (Bogdanov and Dowhan, unpublished), suggesting that lipid-determined topogenesis might be a general property of such transporters.

The evidence that membrane protein sequence is written for a specific lipid environment is most convincing for secondary transporters, but there are several other examples that support this concept. The P-glycoprotein is localized to mammalian cytoplasmic membranes and is an ATP-binding cassette transporter responsible for multidrug resistance. Like LacY, it is a highly flexible protein that undergoes large conformational changes during its catalytic cycle [39] and during membrane insertion [40]. In its native host, the protein exhibits 12 TMs with both the N- and C-terminus exposed to the cytoplasm. When expressed in *E. coli*, the N-terminal half of the protein assumes the same topology as in the native host. However, TM VII no longer spans the membrane, TMs VIII–XII assume an inverted orientation, and the C-terminus is exposed to the periplasmic side of the membrane [41]. Similarly, a citrate carrier of *Klebsiella pneumoniae* displays 11 TMs when inserted into dog pancreas endoplasmic reticulum membranes but only 9 TMs when expressed in *E. coli* [42]. Ductin exists as a subunit of the vacuolar H^+-ATPase and in an opposite orientation in endoplasmic reticular membranes as a component of the connexon channel of gap junctions [43]. An endoplasmic reticular epoxide hydroxylase is found with a different topology in the sinusoidal plasma membrane where it

mediates bile acid transport [44]. These results have important implications for the expression of foreign membrane proteins in heterologous systems.

How might proteins interact with phospholipids to determine topology? An attractive explanation is the occurrence of direct interactions between membrane proteins and phospholipids during assembly. Ordinarily, the orientation of TMs is consistent with the "positive–inside rule," whereby the positively charged extramembrane domains of membrane proteins tend to be localized to the cytoplasm [45]. The topology of the C-terminus of LacY is completely consistent with the positive–inside rule. However, the N-terminal cytoplasmic domains of LacY (C2, C4 and C6) and PheP (C2) contain a negatively charged amino acid near the membrane–cytoplasm interface and possess a low positive charge density in the adjacent cytoplasmic domains. Placing a single negatively charged amino acid in a cytoplasmic domain within six residues of the end of a TM can result in mislocalization of the cytoplasmic domain to the periplasm with inversion of TM topology [46]. These negatively charged residues of LacY and PheP might make these domains more prone to translocation, but in PE-containing cells remain anchored by interaction with zwitterionic PE. Lack of PE increases the negative charge density at the membrane surface perhaps favoring the translocation of cytoplasmic domains of low positive charge or containing acidic amino acids. Indeed, increasing the anionic lipid content in a liposome system favored the translocation of weakly positively charged domains in opposition to the positive inside rule [47]. Therefore, interaction between charged amino acids and phospholipid head groups at the membrane surface seems to be a critical determinant of protein topology.

5.2. Anionic phospholipids as topological determinants

In an *E. coli* strain in which expression from the *pgsA* gene (see Fig. 1) is under control of the *lac* promoter, the levels of PG and CL (normally13% and 3%, respectively) drop to 1% each without induction, but the anionic lipid precursors (PA and CDP-diacylglycerol) accumulate so that the total anionic phospholipid pool drops from about 18% to 7% [12]; the remainder of the phospholipid is PE. This strain was employed to test whether anionic phospholipids influence the transmembrane orientation of leader peptidase (Lep). Lep has two TM helices (N-terminus periplasmic) separated by a large cytoplasmic domain. Lep derivatives with varying amounts of positive charge within the cytoplasmic extramembrane domain were expressed in *E. coli* cells growing with or without *lac* inducer [45]. At low anionic phospholipid content, a higher positive charge was required to prevent translocation of the cytoplasmic domain. Increasing anionic phospholipid content resulted in increased retention of cytoplasmic domains with a lower positive charge. This result not only supports the "positive–inside" rule for orientation of cytoplasmic domains but also extends the mechanism to an interaction of positive amino acid side chains with the anionic head groups of phospholipids to provide a cytoplasmic anchor. Because many membrane proteins in *E. coli* follow the positive–inside rule for all of their cytoplasmic domains, elimination of PE would not be expected to affect their topology consistent with the viability of PE-lacking cells. However, the above conclusion that anionic lipids play a direct role as topological determinants in vivo does not rule out the possibility of the influence of lipids on known or not yet identified components of the membrane protein assembly machinery.

Can anionic lipids alone determine the transmembrane orientation of integral membrane proteins? OEP7 is an outer envelope protein of spinach chloroplasts with a single TM, N-terminus in and C-terminus out, flanked by soluble domains with net charge of +1 and +2, respectively. The content of PC in the outer leaflet of the outer envelope is 50% compared with 6% in the inner leaflet; in contrast, PG appears to be exclusively present in the inner leaflet. The topology of OEP7 was inverted when reconstituted in liposomes composed of the average lipid content of the chloroplast outer envelope [48]. However, native orientation was achieved by reducing the PG content, i.e., by mimicking the composition of the outer leaflet of the outer envelope of chloroplasts suggesting that the topology of OEP7 in vivo is sensitive to the lipid asymmetry of PC and PG between the two leaflets of the outer envelope. A mutant OEP7 protein with equal positive charge distribution on both sites of the TM inserted into PC-enriched liposomes in a more random fashion but still with preference for the normal orientation.

5.3. Reversible lipid-triggered protein conformational switches

Once inserted, TM segments should not oscillate readily back and forth across the membrane due to the large free energy barrier to transfer polar sequences back through the bilayer. Most membrane protein topology studies are consistent with a static and permanent location of extramembrane domains facing either one side or the other of the membrane. However, the actual structure of a membrane is very dynamic. Therefore, is the lipid bilayer really a nonflipping zone for integral membrane proteins?

Further studies with LacY misassembled in PE-lacking cells (see Fig. 2) indicate that TM organization of membrane proteins is responsive to changes in lipid environment and potentially more dynamic than previously assumed [30]. Placing the *pssA* gene under control of the regulatable *araB* promoter provided a cell in which repressed gene expression (growth in glucose) resulted in less than 2% PE. Expression of LacY under these conditions showed the expected misorientation, lack of active transport function, and lack

of properly folded P7 domain. Induction of *pssA* gene expression (growth in arabinose), under conditions of no new LacY synthesis, resulted in a return of PE to 70% and regain of active transport function, the native topology of domain C6, and the native conformation of domain P7. A similar restoration of transport function and topological organization for the misassembled N-terminal hairpin of PheP was observed upon incorporation of PE into PE-lacking cells [37]. For both transporters, topology and function were dependent on the membrane phospholipid composition and displayed dynamic topological organization in response to lipid environment after stable membrane insertion.

The above results suggest that the orientation of individual TMs may not be "fixed" during assembly, after native structure has been attained, or during catalytic transport cycles. A flexible region or hinge point between more stable domains appears to be a common feature of LacY and PheP enabling a switching of topological organization within a folded compact structure in response to changes in phospholipid composition. For PheP, this hinge point is the extended hydrophobic TM III rich in glycine residues, which might disrupt the orderly packing between the first two and last ten TMs of the protein. Such mismatch in the length of TMs may induce conformational instability and flexibility to polytopic membrane proteins by tilting the TM at an angle in the bilayer. For LacY, the hinge point is the relatively hydrophilic TM VII, which contains two acidic amino acids and can exist stably out of the membrane. Uncompensated charges within the bilayer strongly interfere with TM insertion and stability [49]. In LacY, salt bridges to TMs X/XI compensate for these charges, but during catalytic cycles these contacts may be temporarily broken, contributing to the required mobility of P7 and TM VII during active transport [35]. Topological rearrangement due to the absence of PE may also disrupt these salt bridges favoring displacement of TM VII from the bilayer.

Highly mobile TMs, either uncharacteristically hydrophilic or with unique organization in the bilayer, have been reported for several other membrane proteins. These domains are usually directly involved in the function of these proteins. These properties may be shared by other secondary transporters that are also dysfunctional in PE-lacking *E. coli* cells. A potential role has been proposed for the movement of TM 3 of plasma membrane Chinese hamster P-glycoprotein into and out of the bilayer during the transport cycle [50]. TM 10 of band 3 protein, which is of low hydrophobicity, is transiently exposed to the lumen during assembly and then inserted into the membrane [51]. The region surrounding TM 8 of the cystic fibrosis transmembrane conductance regulator consists of a relatively long hydrophobic sequence (34 residues) and contains two charged residues. During the early stages of its membrane insertion, TM 8 extends significantly farther into the endoplasmic reticulum lumen than would be expected based on its final organization in the membrane [52]. The extended hydrophobic TM III of PheP has been proposed to form a U-shaped loop in PE-lacking membranes with both ends of the TM exposed to the same side of the membrane [37]. Recent crystallographic studies demonstrate that glycerol facilitator *glpF* [53] and the water channel aquaporin AQP1 [54] contain two oppositely oriented U-shaped loops that together make a TM. Protease mapping studies demonstrate that one of these loops is not fixed but undergoes posttranslational topological reorientation late in maturation [55], demonstrating topology reorganization during folding.

The lipid-induced conformational and topological changes observed for LacY and PheP do not occur in wild-type cells, but may be a consequence of the functional properties or reflect intermediates present during folding of these proteins. However, there are several examples where large lipid-induced changes in protein structure are of physiological significance. Reversible binding of CTP:phosphocholine cytidylyltransferase to lipids is accompanied by a lipid-triggered transition of the membrane binding domain from a mixture of conformations to an ordered continuous α-helix [56], resulting in activation of the enzyme in response to changes in membrane lipid composition. PG triggers a conformational switch from disordered to amphipathic helix in the membrane-binding domain of the enzyme IIA component of the *E. coli* phosphotransferase system [57]. This conformation change is essential to the activity of the enzyme. In the presence of lipids, apolipoprotein A1 structure changes from random coils and β-strands to a single continuous amphipathic α-helix that may form a belt around the high-density lipoprotein particle [58]. Binding of β-amyloid peptide to membranes containing specifically ganglioside GM1 results in a rapid conformational transition from random coil to an ordered conformation rich in β-sheet structure [59,60]. A reversible switch of secondary structure from a fusion-permissive α-helix to a nonfusogenic β-sheet regulates a peptide involved in vesicle fusion [61]. The "on/off" activation of this switch is governed by minor amounts of lyso-phospholipids in target membranes that cause a drop in α-helix and a dramatic increase in β-sheet content. Apolipophorin III undergoes a rearrangement of its five tightly bundled amphipathic α-helices upon association with dimyristoyl-PC [62]. The resulting structure consists of two separate helical bundles of two and three helices connected by a hydrophobic hinge structure.

Lipids play a central role in defining both the static and dynamic state of membrane protein topological organization. Lipids play specific roles during de novo folding and after proteins are fully assembled in the membrane. Large changes in protein structural organization should be investigated more fully in response to dynamic changes in membrane lipid composition and as proteins move along the protein trafficking pathway of eukaryotic cells.

6. Lipid interactions with amphitropic proteins

Amphitropic proteins [63] are localized to both the cytosol and the membrane. Membrane association, through either a membrane protein or lipid, is reversible and binding affinity is subject to regulation. Association can be solely with the hydrophilic surface of the membrane or involve penetration into the hydrophobic membrane core. Regulation occurs through protein conformational switches resulting from reversible phosphorylation, ligand binding, or transient appearance of a membrane-binding site. Amphitropic proteins can be in a globular hydrophilic state, or they can expose hydrophobic and positively charged domains important for binding directly to membrane lipids. They are classified into three major categories: (1) proteins containing a binding motif for specific lipid monomers, such as PH domain proteins; (2) proteins with covalent lipid anchors, such as Ras; and (3) proteins with amphipathic helices, as discussed below. Proteins use amphipathic helices to sense membrane lipid composition, particularly the general anionic lipid content rather than a specific anionic lipid. Amphipathic helices also sense changes in electrostatic potential, lipid packing density or membrane curvature. A number of reviews describe in detail a wide range of amphitropic proteins such as CTP:phosphocholine cytidylyltransferase, protein kinase C, phospholipase C, vinculin (see Refs. [64,65]). Here we will focus on amphitropic proteins related to the cell cycle and cell division in *E. coli* that undergo dynamic polymerization on the membrane and participate in the formation of lipid–protein membrane domains.

6.1. Lipid domains in bacterial membranes

The fluid mosaic model of Singer and Nicolson [66] assumed membrane lipid homogeneity. However, cells require a network of membrane domains that produce the specific environment for the action of membrane proteins [67,68] and the membrane association of amphitropic proteins. A number of results demonstrate that bacterial membranes share with eukaryotic cells the presence of defined lipid domains. A heterogeneous distribution of phospholipids was shown in *Micrococcus luteus* [69] with spatial distribution of phospholipids and glycolipids in the membrane varying during the cell cycle [70]. Gel and fluid lipid domains were directly visualized in mycobacteria by the use of fluorescent lipophilic probes [71]. Lateral heterogeneity in the membrane of *Acholeplasma laidlawii* was suggested based on experiments with endogenously produced pyrene–lipid probes and liposomes composed from major lipids of this bacterium [72]. Coexistence of two types of lipid domains of different order and polarity was demonstrated in *E. coli* membranes [73] consistent with one being more enriched in lipids and the other more enriched in protein. Introduction into the *E. coli* membrane, either separately or concomitantly, of pyrene-labeled analogs of PE and PG indicated that the two labeled phospholipids are sequestered into separate preexisting domains in the membrane [74]. Uneven distribution of the fluorescent hydrophobic dye FM 4–64 [75] may be a reflection of lateral heterogeneity of phospholipid distribution in *E. coli* membranes. Specific lipid domains, formed by DNA–membrane interactions, were proposed to be involved in regulating DNA replication, segregation, and cell division [76–78].

The CL-specific fluorescent dye 10-*N*-nonyl acridine orange (NAO) allows direct visualization of domains enriched in CL in living wild-type or filamentous PE-lacking *E. coli* cells. Domains were located at cell poles and in the center of dividing wild-type cells after nucleoid segregation or between segregated nucleoids in filamentous cells, suggesting their possible role in the cell division process [79]. CL localization at the poles, from which minicells (large cytoplasm-containing vesicles lacking DNA) bud off, is consistent with the fourfold increase in content of CL of minicells compared with normal *E. coli* cells [80].

A model explaining the specificity and spectral shift of NAO interaction with CL domains was developed [81]. CL has been proposed [82] to specifically form a 1:2 ratio complex with NAO resulting in a spectral shift in emission from green to red due to the stacking of the conjugated rings of NAO. The specificity and spectral shift were postulated to be due to the binding of positively charged NAO to the two ionized phosphate groups of CL in contrast to the single phosphate of other anionic phospholipids. However, due to the central hydroxyl of the glycerol connecting the two domains of CL, the phosphate groups of CL display one acid pK_a and one basic pK_a > 8.5 [83]. Therefore, at physiological pH, CL is not fully ionized. Spectral shift upon NAO binding to CL is due to ππ bond stacking, analogous to acridine orange binding to DNA, but the interaction is neither ionic in nature nor pH dependent, thus making it unlikely that it is due to the interaction of two NAOs with one CL. Instead, it was proposed that the binding is hydrophobic through the insertion of the nonyl group into the hydrophobic domain made up of the four fatty acid chains of CL rather than through the phosphate residues [81]. The stacking would then arise from the close proximity of CL molecules packing in a domain or separate lipid phase within membranes.

Staining with NAO was successfully used for detection of CL-rich domains in *Bacillus subtilis* Marburg membranes [84]. As is the case for *E. coli* [79], the domains were localized in septal regions and on the cell poles. These domains were only scarcely detectable in exponentially growing cells in which the gene for CL synthase was disrupted, which is consistent with only trace amounts of CL in the mutant. Visualization of CL domains in *B. subtilis* cells in different phases of sporulation revealed specific targeting of CL into the engulfment and forespore membranes. The low amount of

CL found in CL synthase mutants was also concentrated in the forespores. These mutants exhibited a delay in the sporulation process. Both findings are significant because a specific role of CL in vivo is often clouded by its apparent dispensability.

Specific NAO detection of CL does not distinguish between whether these domains are lipid domains enriched in CL or simply lipid-enriched domains of average lipid composition but reduced in protein content relative to the surroundings. In either case, these domains would contain a higher negative charge density than neighboring domains. In PE-lacking cells, the negative charge of these domains would be further increased over that in PE-containing cells. These domains would provide a site of negative charge density for the binding of amphitropic proteins, whether they are CL-enriched or simply lipid-enriched.

6.2. Cell division machinery

Polymerization of cytoskeletal protein FtsZ, the prokaryotic ancestor of tubulin [85,86], in a ring structure (Z-ring) on the cell membrane at the division site is the first known step in the assembly of the cell division machinery. Dual localization of FtsZ in a cytosolic and membrane-bound state defines FtsZ as an amphitropic protein. The crystal structure of FtsZ does not provide any evidence for existence of a domain for direct interaction with a lipid phase [86]. Either ZipA protein or FtsA protein is required for the stable localization of the Z-ring on the membrane [87]. FtsZ proteins across many bacteria contain a highly conserved N-terminus required for polymerization [88,89] and a highly conserved C-terminus that is important for interactions with other proteins such as FtsA and ZipA [90]. GTPase activity of FtsZ is coupled to its polymerization [91]. FtsZ can form a variety of polymer structures in vitro depending on conditions. The status most approximating its in vivo organization is formation of mini-rings and/or thin sheets of protofilaments in the presence of GDP or GTP when FtsZ is adsorbed to a zwitterionic phospholipid monolayer [92] supporting an association of FtsZ with lipid.

ZipA contains an N-terminal membrane anchor, a flexible linker, and a cytoplasmic C-terminus that binds FtsZ [93]. FtsA is a member of the actin ATPase superfamily [94] and can be classified as an amphitropic protein because it is present both in the cytoplasm and associated with the cytoplasmic membrane [95]. FtsA interacts with the extreme C-terminal domain of FtsZ [90,96] in a FtsA/FtsZ ratio of 1:100 [97]. The ability of FtsA to bind ATP (utilize metabolic energy) and to undergo a phosphorylation–dephosphorylation cycle (a potential regulatory switch) suggests its important role in cytokinesis. The localization of GFP-tagged early cell division proteins, FtsZ, FtsA, and ZipA, into a Z-ring structure on the membrane in *pssA* null mutants of *E. coli* that lack PE [98] demonstrated that this mutation causes inhibition of cell division resulting in long multinucleated filamentous cells. Importantly, FtsZ, FtsA, and ZipA still localize to division sites between segregated nucleoids, but they are often assembled into aberrant spiral rather than ring structures. Therefore, although FtsZ ring formation in vitro appears to require zwitterionic phospholipid, localization of FtsZ to the membrane in vivo still takes place in the absence of zwitterionic PE. However, the ability of FtsZ to undergo constriction is strongly inhibited.

Discontinuity between nucleoid segregation and cell division in the absence of PE may result from aberrant organization of FtsZ polymers but 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. In a related observation, movement of PE between the inner and outer leaflet of the cytoplasmic membrane of animal cells in the vicinity of the division septum is required for cell division. This movement plays a critical role in disassembly of the actin contractile ring [99]. A similar process might be involved in disassembly of FtsZ/FtsA polymers in bacteria. Defects in cell division in the mutant lacking PE suggest that PE either provides a nonbilayer forming lipid with no net charge or dilution of the high negative membrane surface charge necessary for either proper organization of the amphitropic components of the cell division machinery or their dissociation from the membrane surface. Introduction of the neutral nonbilayer forming glycolipid MGlcDAG into the PE-lacking mutant partially suppresses both the filamentous growth and the defective division phenotypes [100].

Localization of CL-rich or lipid-enriched domains at mid-cell is consistent with their functional role as the binding sites for the polymerization of FtsZ into the Z-ring [76] at a proposed, but undefined, nucleation site [101]. Experiments in *E. coli* strongly suggest that initiation of DNA replication at *oriC* occurs at mid-cell [102], which is consistent with the initial localization of the replication machinery (replisome) at this site. DnaA, the initiator of DNA replication, requires anionic phospholipids for membrane association [103,104], and anionic phospholipids (see Section 6.4) are required for DnaA function in vivo [105–107]. In *B. subtilis,* a mid-cell structure defines a nucleation site for Z-ring formation that is masked by the replisome complex once it is assembled at *oriC* [101,108]. It is subsequently unmasked after a round of replication, when the two replisomes separate and move away from mid-cell exposing the mid-cell to allow Z-ring formation possibly at a lipid-enriched domain.

The role of lipid domains might not be limited only to providing specific binding sites for division proteins, but such domains might play an active role in membrane invagination leading to cell constriction. Fluid membranes can exhibit two-dimensional bending and form spherical segments. The spontaneous curvature of the domain

depends on the molecular structure of the bilayer and its interaction with the environment, which can be controlled by concentration of anchored proteins [109]. A model has been proposed suggesting that such perturbation of the membrane might favor binding of FtsZ polymers, possibly in combination with linker proteins such as ZipA and/or FtsA, converting these domains into the invaginating fold responsible for division [110].

6.3. Min proteins

The Min system owes its name to the aberrant division near the poles in *min* mutants of *E. coli*, resulting in minicell formation and short filaments [111,112]. It includes the MinCDE proteins, products of the *minB* operon [113]. Studies with GFP fusions to these proteins have shown that MinD, along with MinC, rapidly oscillates from one pole of the cell to the other. This oscillation depends upon MinE which itself oscillates in the cell, lagging slightly behind the MinCD complex [114–117]. These oscillations have been modeled using differential diffusion rates to show that the concentration of MinC, the inhibitor of FtsZ assembly, would be lower in the cell center, thus allowing formation of the Z-ring there [118–121]. MinC is a cytosolic protein recruited to the membrane through binding to MinD and passively follows the distribution of MinD [122]. MinD protein is an amphitropic ATPase. The ATP-bound form of MinD is localized to the membrane (Fig. 3A). The binding of MinE to MinD induces hydrolysis of ATP and release of MinD into the cytoplasm [123] (right side of Fig. 3B). The ATP-binding cycle induced by MinE results in the rapid movement of MinD from one cell pole to the opposite cell pole, alternately forming membrane associated zones extending from each pole [114]. The crystal structure of MinD indicates that residues around the ATP-binding site are required for the direct interaction with MinC [124], and that ATP binding and hydrolysis function as a molecular switch to control MinCDE oscillation [125]. The C-terminal region of MinD contains a highly conserved motif [126,127] that is essential for membrane localization. This motif is not structured in crystals of MinD [124,128] 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 positive charged amino acids [126,127]. 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 the phospholipid bilayer and α-helix formation (see Fig. 3A). Such amphipathic helices usually align parallel to the membrane surface so that the hydrophobic residues directly interact with lipid acyl chains, whereas the cationic residues on the opposite face of the helix interact with the head groups of anionic phospholipids (for review, see Ref. [65]).

Direct interaction of the C-terminal α-helix of MinD with phospholipids was confirmed in vitro [126,129]. Studies with purified MinD and liposomes demonstrated that

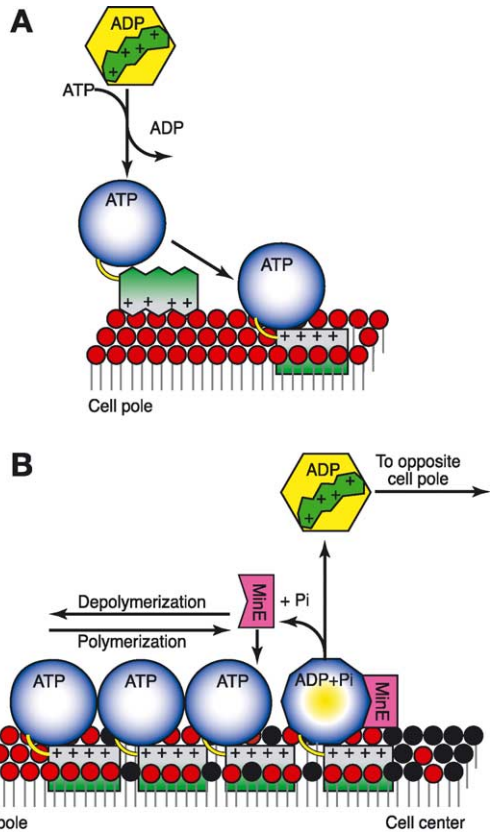

Fig. 3. Scheme for membrane insertion and polymerization–depolymerization of MinD. (A) At the cell pole, cytosolic MinD-ADP (yellow) with its unstructured C-terminal domain (green) exchanges ADP for ATP with an accompanying conformational change in MinD-ATP (blue), resulting in exposure of the C-terminal amphipathic motif (green–gray) followed by electrostatic attraction to the anionic lipid-enriched domain (red phospholipid head groups) at the cell pole. Penetration into the phospholipid bilayer is accompanied by amphipathic α-helix formation with alignment parallel to the membrane surface and complementary hydrophobic and ionic interactions. (B) Consecutive binding of MinD-ATP monomers to the membrane as shown in A results in formation of MinD-ATP polymer growing from cell pole to cell center. Near the cell center, binding of MinE (pink) to MinD-ATP induces ATP hydrolysis coupled to a return to the MinD-ADP conformation accompanied by detachment of MinDE. Consecutive detachment of MinD results in depolymerization from the cell center to the cell pole. Cytosolic MinD-ADP diffuses to the opposite cell pole where the cycle is repeated.

affinity of MinD to the membrane depends on its phospholipid composition, and MinD has a preference for anionic over zwitterionic phospholipids [130]. A comparison of affinity of the *E. coli* and *B. subtilis* C-terminal membrane targeting sequence of MinD to different anionic phospholipids suggests that the polar surface of each α-helix has been evolutionarily "tuned" for interaction with the specific phospholipid composition of each bacterial membrane [131]. In many (including *E. coli*), but not, all MinD orthologs, the C terminal amphipathic domain is flanked at its N-terminus with three glutamates. As was shown recently for CTP:phosphocholine cytidylyltransferase, acidic amino acids of an amphipathic α-helix also influence selectivity for anionic phospholipids [132]. These phospho-

lipids create a low-pH environment at the membrane surface that favors protonation of acidic amino acids and consequently decrease the repulsion of the helix. Affinity of MinD to phospholipids depends not only on negative charge of head groups but also on the level of unsaturation of the acyl chains [130]. This is consistent with a notion that the higher degree of disorder in unsaturated phospholipid bilayers might create more favorable conditions for insertion of a short amphipathic α-helix oriented parallel to the membrane surface [133].

Orientation of amphipathic α-helices in the membrane can be predicted by an hydrophobic moment plot analysis [134]. Calculation of the sequence hydrophobic moment according to Ref. [134] for the C-terminal domain of *E. coli* MinD places this domain on a conventional plot of hydrophobic moment versus hydrophobicity [135] within the area, indicating parallel orientation to the plane of the membrane, but very near the border between parallel and oblique orientation. This is consistent with the localization of the C-terminal α-helix parallel to the membrane surface; however, oblique orientation in the membrane cannot be excluded [130]. Therefore, the MinD C-terminal domain may be finely tuned in its affinity for the wild type phospholipid composition of *E. coli* to allow reversible interaction with the membrane surface. If the membrane-binding affinity of MinD depends on lipid composition, changing the protein retention time on the membrane and/or its orientation at the surface by changes in the surface distribution of phospholipid types could modulate both spatial and temporal oscillatory characteristics of the protein. An increase in the anionic lipid content of the membrane in PE-lacking cells would be expected to disrupt normal oscillation as was shown [130] and discussed below.

Another property necessary for the functioning of MinD is its reversible polymerization on the membrane surface (see Fig. 3B). Resonance energy transfer between two differentially fluorescent-labeled MinD molecules was induced upon binding MinD to liposomes indicating direct interaction between MinD monomers [130]. These results, together with self-enhanced binding of MinD to liposomes [130,136], formation of MinD filament bundles in solution [137], cryo-electron microscopy images of MinD forming tubes upon binding to liposomes [123], and finally fluorescent images of MinD coiled structures in *E. coli* cells [138] strongly suggest that MinD binding to the membrane surface is accompanied by protein polymerization. A higher concentration of CL found at the cell poles in CL- or lipid-enriched domains described earlier may be important for self-assembly of MinD providing nucleation sites for growing polymers.

In vivo approaches also demonstrated that MinD has a higher affinity for anionic phospholipids over zwitterionic phospholipids [130]. The localization dynamics of a GFP-tagged derivative of MinD expressed in a mutant of *E. coli* that lacks PE showed that GFP-MinD assembled into dynamic focal clusters that often appeared to follow a helical "zigzag" path (right upper panel of Fig. 4A). This helical pattern of movement is supported by recent results showing that the broad zones of MinD localization in wild-type cells actually consist of helical filaments (top left panel of Fig. 4A). Organization of MinD takes place within coiled structures (bottom left panel of Fig. 4A) that wind around the cell beginning at the cell pole and depolymerize at the center proximal end due to interaction with MinE [138]. Organization of MinD in PE-lacking cells, in which negative charge density on the membrane surface is very high, into compact clusters (top right upper and lower panels of Fig. 4A) may be caused by increased local concentrations of MinD due to high concentration of anionic phospholipids. The pattern of localization of GFP-MinD in compact zones in PE-lacking cells is similar to the localization of CL-domains in this mutant [79]. Other amphitropic proteins also support a co-localization with CL-enriched domains. MGlcDAG synthase of *A. laidlawii*, when expressed in PE-lacking *E. coli* cells, exhibited a focal point association to the membrane (right panel of Fig. 4B) similar to the distribution of CL-enriched domains and MinD in this mutant [100]; in wild-type cells, MGlcDAG synthase is localized to the membrane without formation of focal clusters and reaches its highest concentration at the poles (left panel of Fig. 4B). The MGlcDAG synthase has a similar (but longer) anionic-lipid-binding amphipathic α-helix [100]. *E. coli* MurG contains an N-terminal hydro-

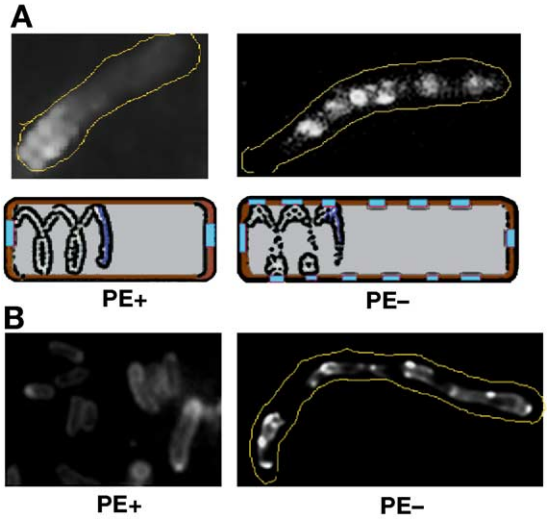

Fig. 4. Distribution of MinD and MGlcDAG synthase in wild-type and PE-lacking *E. coli*. (A) Fluorescent image of YFP-MinD (yellow fluorescent protein) in a living cell (yellow line encircles cell of normal length) with wild-type phospholipid composition (top left) [138]. Schematic representation (bottom left) of coiled MinD polymer structure originating at the cell pole enriched in anionic phospholipids (blue). Fluorescent image of GFP-MinD (top right) in PE-lacking cell (yellow line encircles multinucleated filamentous cell) displayed as distinct focal points [130]. Schematic representation (bottom right) of coiled MinD polymer interacting as concentrated domains in association with multiple anionic phospholipid domains (blue). (B) GFP-MGlcDAG synthase localization at the poles of wild-type cells (left) and as focal points in filamentous PE-lacking cells (right) [100].

phobic patch surrounded by cationic residues putatively involved in association of the protein with the membrane. MurG activity is stimulated by CL, and its overexpression results in formation at the cell poles of membrane vesicles enriched in CL and MurG [139].

6.4. DnaA protein

DnaA protein initiates DNA replication in *E. coli* and shows homology across many phylogenetic groups, suggesting that the mechanism for initiation of chromosomal replication has been largely conserved. Multiple DnaA molecules bind to *oriC*, the unique origin of chromosomal replication, which then becomes the nucleation site for recruitment of the remaining proteins of the replisome machinery [140–142]. DnaA protein has high affinity for ATP and ADP. The ATP-bound form initiates DNA replication in vitro while the ADP-bound form, which still binds to DNA, cannot [143]. DnaA protein has intrinsic ATPase activity that slowly converts the active to the inactive form, which can be reactivated by exchange of bound ADP for ATP. The slow rate of release of bound nucleotide can be greatly accelerated by addition of anionic phospholipids in vitro, but rejuvenation (see Fig. 5) of a functional form of DnaA-ATP only occurs in the presence of both anionic lipid and *oriC* DNA [144]. The in vivo nucleotide concentrations would favor the ATP-bound active form [105]. Cell fractionation studies [145] and immunogold labeling of whole cells [146] support the amphitropic character of DnaA protein. Introduction of acidic amino acids into a basic amphipathic α-helix of DnaA protein blocked CL-dependent exchange of nucleotides [147]. Binding of DnaA to liposomes is selective for anionic phospholipids but also involves intercalation of a protein domain into the hydrophobic core [148]. The requirements for cluster formation of anionic lipids for the activation of DnaA protein was demonstrated using lipid mixtures in which anionic lipids were either evenly dispersed or clustered into domains [103]. These observations suggest that in mixed lipid bilayers, a domain structure of anionic lipids seems to be an important parameter to active DnaA protein.

In vivo evidence for a requirement of anionic phospholipids in DnaA protein function was obtained using regulated expression of the *pgsA* gene under the control of the *lac* promoter. This strain also carried a wild type copy of the *lpp* gene (Section 3.2) so that systematic reduction in anionic phospholipid content from 18% and 7% correlated with a decline in cell growth rate to zero. However, growth arrest was bypassed by introducing mutations that allowed DNA replication to proceed by a DnaA-independent mechanism [106]. Also, growth arrest was suppressed by mutations in the DnaA anionic lipid-binding domain [149]. Growth arrest was suggested to result from the high demand of the *lpp* gene product on the limited PG pool, thereby maintaining the pool below a critical level required for DnaA nucleotide exchange. Elimination of the *lpp* gene product removed this drain resulting in a higher steady state level of the total anionic phospholipid pool for support of DnaA protein function [14]. Physiological changes in lipid composition for the Gram-positive bacterium *Staphylococcus aureus* occur upon transition of cells from the exponential phase to the stationary phase of growth. These changes have been correlated with inhibition of DnaA activity [150]. The basic phospholipid lysyl-phosphatidylglycerol accumulates as cells enter stationary phase and was shown to strongly inhibit PG- and CL-facilitated release of ATP from DnaA in vitro. Mutants unable to synthesize lysyl-phosphatidylglycerol had an increased number of replication origins supporting an in vivo role for regulation of initiation of DNA replication by lipids.

Heterogeneous distribution of lipids in the membrane of bacteria has been clearly demonstrated and supported by direct observation using fluorescent probes. These domains play a central role in organizing membrane and amphitropic proteins into molecular machines on the membrane surface.

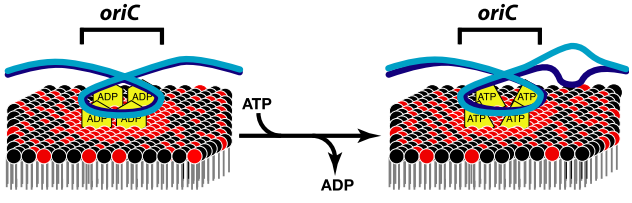

Fig. 5. Rejuvenation of DnaA activity by anionic phospholipids and *oriC*. Multiple DnaA molecules (yellow), bound with either ATP or ADP, bind to *oriC* near the initiation site on the DNA duplex (blue). Only the DnaA-ATP form (right diagram) is active and capable of opening the DNA duplex as shown to allow assembly of the replisome and initiation of replication. The inactive DnaA-ADP form can be rejuvenated by exchange of ATP for ADP, but this only occurs when DnaA is bound to both *oriC* and anionic phospholipids shown as red phospholipid head groups organized into an anionic lipid domain on the membrane surface.

7. Lipids as components of protein complexes

Lipids have been resolved as integral components within the crystal structures of several membrane proteins. The distribution of these lipids falls into two classes. In one group, the lipids lie at the surface of these proteins, with the head groups interacting with amino acid side chains at the membrane–aqueous interface and the fatty acids aligned within hydrophobic grooves on the protein surfaces that lie within the bilayer. Examples are the lipid A portion of LPS associated with the outer membrane protein FhuA of *E. coli* [151], PC associated with the cytochrome *c* oxidase from *Paracoccus denitrificans* [152], PE associated with surface of subunit VII and CL within a groove of yeast cytochrome bc_1 (Complex III) [153], which has been suggested as the point of interaction [154] with cytochrome *c* oxidase (Complex IV). Alternatively, lipids lie between subunits of multisubunit complexes. Examples are squalene, the methyl

ester of PG-phosphate, and a sulfoglycolipid intercalated between the subunits of bacteriorhodopsin from *Halobacterium salinarum* [155], PC, PI, PG, and CL within the bovine multisubunit cytochrome *c* oxidase [156], PE occupying a deep groove between the protein surfaces formed by the TM α-helices of the subunits of the photosynthetic reaction center from *Thermochromatium tepidum* [157], and CL occupying a depression formed by α-helices of the TMs of photosynthetic reaction center from *Rhodobacter sphaeroides* [158]. Both head groups and fatty acid chains resolved within integral membrane proteins are very often highly disordered and display considerable conformational heterogeneity, which may provide a deformable flexible surface between subunits in contrast to more rigid protein–protein interfaces. The resolution of detergent molecules in the crystal structures of many membrane proteins suggests that there may be additional specific but weaker lipid association sites.

There also appears to be a higher order lipid-dependent organization of membrane proteins into lipid domains best characterized by the formation of lipid rafts in mammalian cells [159] and CL-rich lipid-domains in bacteria. The role of CL particularly in mitochondria has received considerable attention because of its ubiquitous presence in membranes that carry out oxidative energy transduction. Therefore, it was quite surprising that yeast completely lacking CL (Fig. 1, step 6A, *crd1* null strain) were only mildly compromised for growth under conditions requiring a functional mitochondrial electron transport chain [160,161]. Such mutants grow slower than wild-type cells, and the growth difference is accentuated at higher temperatures. However, lack of CL does not result in loss of viability or destabilization of mitochondrial DNA [17]. The mild phenotype of these mutants is most likely due to an accumulation of PG, the immediate precursor of CL in eukaryotic cells (Fig. 1). In vitro reconstitution studies of delipidated bovine Complex III (cytochrome bc_1) demonstrated that PG can substitute for the required CL in this enzyme [18]. CL has also been implicated in the function of the ADP/ATP translocator [162], the carnitine:acylcarnitine translocase [163], and, as noted above, Complex IV (cytochrome *c* oxidase). The phenotype of the CL-lacking yeast strains strongly suggests that PG also partially substitutes in the above examples. Mutants of yeast [164] lacking both PG and CL (step 4A, *pgs1* null strains) and mammalian cell mutants with reduced PG and CL levels [16] display severe mitochondrial dysfunction. However, the yeast phenotypes are due to the lack of translation of many of the components of the energy-transducing systems of the mitochondria, so conclusions concerning the absolute requirement in vivo for these anionic lipids for function cannot be made [15].

CL is clearly an important structural component of several membrane protein complexes, but it may also play a role in higher-order organization of supermolecular complexes among the individual energy-transducing complexes of the mitochondria. Solubilization of yeast mitochondrial proteins by nondenaturing detergents followed by gel electrophoresis in the absence of detergent and in the presence of a charged dye displayed a heterodimeric supermolecular complex composed of the individual homodimers of Complex III and Complex IV [165]. A similar experiment using mitochondria from the mutant lacking CL showed most of Complexes III and IV as individual homodimers with greatly reduced level of the heterodimeric supermolecular complex [166]. Using a mutant of yeast in which the level of CL was altered by regulation of expression of the *CRD1* gene showed a CL-dose-dependent relationship between cell growth rate, final maximum cell density, and level of supermolecular complex [166]. Although these results support a direct role of CL in catalytic efficiency and interaction between Complexes III and IV in vivo, only the reduced stability of a supermolecular complex in vitro in the absence of a CL has been shown and not the state of such a complex in cells lacking CL. Similar results were reported on the reduced stability of the supermolecular complex in vitro from CL-lacking cells. However, electrophoretic separation in the absence of detergent and charged dye showed mostly the heterodimeric supermolecular complex and less of the individual homodimers of Complex III and Complex IV [154]. A more direct measure of the interaction between Complexes III and IV in vivo is required to resolve this question. However, substitution of a highly conserved arginine that is responsible for the positioning CL between TM helices of the reaction center from *R. sphaeroides* results in the absence of the CL in the crystal structure of the mutant protein [167]. Increased thermal sensitivity of the mutant protein is accompanied by the loss of packing interactions between TM helices supporting a role for CL is stabilizing protein–protein interactions. Also, clustering of CL into domains has been proposed as an organization site and proton sink for electron transport complexes [81,168].

8. Plasticity of lipid–protein interactions

Lipids are highly deformable, flexible molecules that contribute to membrane structure and protein function through their specific chemical properties and their collective physical properties as a membrane bilayer. Although each lipid molecule has unique physical and chemical properties, these are shared to different degrees over the spectrum of lipid molecules. Therefore, it is not entirely surprising that mutants lacking individual lipids survive, although not optimally, by utilizing remaining lipids to support function or that defects can be partially corrected by foreign lipids not normally present. The proposed substitution of CL in the presence of divalent cation as a nonbilayer-forming lipid for PE in mutants lacking PE and PS was discussed earlier (see Section 3.1). PC supports proper

topological organization of LacY in vitro but not active transport function (see Section 5.1).

A general requirement for anionic phospholipids by *E. coli* runs throughout this review, but the lack of head group structural specificity is illustrated by the viability of mutants lacking PG and CL but containing their anionic precursors PA and CDP-diacylglycerol [13]. In this mutant, lipid-enriched domains might still contain significant clusters of PA or CDP-diacylglycerol to attract amphitropic proteins that clearly favor negatively charged lipid domains. DnaA protein of *E. coli* requires anionic phospholipids for activation. However, in vitro activation occurs with all the structurally diverse *E. coli* anionic lipids (CL, PG, PA, and PS), as well as with PI [169], the anionic ganglioside GM1 from brain [170], and even with negatively charged hydrophobic compounds that cluster into anionic domains [103]. Similarly, translocation of outer membrane protein proPhoE and proOmpA and periplasmic prePhoA protein across the inner membrane of *E. coli* was shown to be dependent on anionic phospholipids in a dose-dependent manner in a strain of *E. coli* in which *lac* promoter regulated *pgsA* expression was used to vary PG plus CL content [171,172]. Protein translocation could be reconstituted in vitro in PG-depleted membranes using PG but also using structurally diverse anionic phospholipids such as CL, PA, PS, phosphatidylethanol, or PI [173].

Functional reconstitution of purified and the delipidated inner membrane protein translocation complex SecYEG from *E. coli* and *B. subtilis* into liposomes of defined lipid composition required PG in a dose-dependent manner [174]. However, optimal translocation activity of *B. subtilis* translocase required the presence of 70% PG and 30% PE while the *E. coli* complex required 30% PG and 70% PE, which in both cases reflects the phospholipid composition of their respective host cells. Therefore, translocase activity not only required anionic phospholipids, but the optimal functioning of each complex is tuned to its native membrane phospholipid composition.

The introduction of foreign lipids into mutants lacking normal lipids can provide useful information about which property of a lipid is important to a cellular process. The *E. coli* mutant (*pssA* null) lacking PE is impaired in several membrane-related processes, such as active transport of sugars and amino acids, topological organization of transport proteins, cell division, and a dependence on divalent cations in the growth medium to prevent growth arrest and cell lysis. Expression of the MGlcDAG synthase from the mycoplasma *A. laidlawii* in *E. coli* cells lacking PE resulted in MGlcDAG levels of 30–50% of total lipid and partial correction of the above phenotypes [100].

PE and MGlcDAG have several physical and chemical properties in common. Both lipids form bilayer and nonbilayer liquid-crystalline phases under similar conditions and have similar radii of spontaneous curvature. These physical properties may be responsible for reducing the dependence on Mg^{2+} for both growth and maintenance of cell membrane integrity by providing the necessary nonbilayer forming lipids. The hydrophilic component of both lipids can ionize and engage in hydrogen bonding and thereby in contrast to PC may support active transport by LacY. Both would dampen the negative charge density of a bilayer composed of only anionic lipids although the chemical differences between the zwitterionic PE head group, and the uncharged, stiff, glucose ring of MGlcDAG are substantial. These properties may suppress topological misorganization of membrane proteins (Xie, Bogdanov, and Dowhan, unpublished data for MGlcDAG) and aberrant behavior of amphitropic cell division and cell cycle proteins [100]. Thus interchangeability of PE and MGlcDAG can be interpreted in terms of their physicochemical similarities and differences.

The substitution of neutral glycolipids for PE in support of LacY active transport function may be a general physiologically important property of secondary transport proteins and their requirement for lipids. The branched chain amino acid transporter of *Streptococcus cremoris* functions as an active transporter when reconstituted into proteoliposomes containing PE, MGalDAG (Gal refers to galactose), or MGlcDAG, but was inactive with combinations of only PG, CL, and PC [175]. Similarly, the latter phospholipids do not support active transport of reconstituted LacY. *E. coli* LacY expressed in *Corynebacterium glutamicum*, which has no amine-containing lipids but does contain MGlcDAG, carried out active transport of substrate in vivo [176]. The osmosensing ABC transporter OpuA from *Lactococcus lactis*, a bacterium similar in lipid composition to *A. laidlawii*, is functional in *E. coli* and in proteoliposomes containing PE but not PG alone [177]. Therefore, either the glycolipids or PE but unlike the ineffective phospholipids, may provide hydrogen bonding capability, ionization potential, and dilution of high negative charge density of the bilayer surface.

The Ca^{2+}-ATPase of sarcoplasmic reticulum reconstituted with either dioleoyl-PE or MGalDAG exhibited both high initial rates of ATP-dependent Ca^{2+} uptake and coupling between ATP hydrolysis and Ca^{2+} uptake. Reconstitution with lipids of increasing degrees of methylation (PE, monomethyl PE, dimethyl PE, and PC) or increasing degrees of glycosylation (MGalDAG vs. DGalDAG) revealed a progressive decrease in both ATP-dependent Ca^{2+} uptake and the coupling ratio. In both cases, the progressive loss of function correlates with a shift from nonbilayer to bilayer potential for the lipid [178].

Lipids are the solvent for membrane proteins and their apparent interchangeability, particularly in in vitro reconstitution studies, has masked the importance of specific lipids in supporting optimal functioning of cellular processes. Molecular genetic manipulation of lipid metabolism and the introduction of foreign lipids into living cells have defined more precisely the roles particular lipid properties play in supporting optimal function.

9. Summary

The overall intent of this review is to focus attention on lipids as important components in a wide range of biological processes and to stimulate greater in-depth analysis of the precise molecular involvement of specific lipids in cell function. Because lipid structure and properties are interrelated, it is difficult to distinguish a specific role for lipid structure from the collective properties of lipid mixtures. Development of cells in which lipid composition can be varied has demonstrated considerable flexibility with respect to lipids in supporting protein function but has also uncovered new roles of lipids, provided in vivo verification of function, begun to define the specific properties of lipids required for function, and defined in molecular terms the multitude of processes that require specific lipids for optimal function. General principles of membrane protein folding, dynamic response of proteins to changes in lipid environment, organization of cytoplasmic proteins on the membrane surface, and the formation of structures on the macro level emerging from studies in microorganisms can now be applied to the study of more complex biological systems.

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