Transbilayer Movement of Phospholipids in Biogenic Membranes

4 AUTHORS, INCLUDING:

Matthijs A Kol
Universität Osnabrück
13 PUBLICATIONS   469 CITATIONS

SEE PROFILE

Josephine Antoinette Killian
Utrecht University
166 PUBLICATIONS   8,624 CITATIONS

SEE PROFILE

Current Topics

Transbilayer Movement of Phospholipids in Biogenic Membranes

Matthijs A. Kol, Anton I. P. M. de Kroon, J. Antoinette Killian, and Ben de Kruijff*

*Department of Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology,
Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Received December 8, 2003; Revised Manuscript Received January 20, 2004

ABSTRACT: Biogenic membranes contain the enzymes that synthesize the cell's membrane lipids, of which the phospholipids are the most widespread throughout nature. Being synthesized at and inserted into the cytoplasmic leaflet of biogenic membranes, the phospholipids must migrate to the opposite leaflet to ensure balanced growth of the membrane. In this review, the current knowledge of transbilayer movement of phospholipids in biogenic membranes is summarized and the available data are compared to what is known about lipid translocation in other membranes. On the basis of this, a mechanism is proposed, in which phospholipid translocation in biogenic membranes is mediated via membrane-spanning segments of a subset of proteins, characterized by a small number of transmembrane helices. We speculate that proteins of this subset facilitate lipid translocation via the protein−lipid interface, because they display more dynamic behavior and engage in less stable protein−lipid interactions than larger membrane proteins.

Structure, Function, and Assembly of Biomembranes

Biomembranes consist of a lipid double layer in which membrane proteins are embedded. Synthesis of the membrane building blocks occurs at distinct sites within the cell, making the assembly and growth of a complex structure such as a biomembrane a fascinating combination of cellular transport and membrane insertion processes of proteins and lipids. In the past two decades, much information about how the membrane proteins are synthesized, targeted, and inserted into the right membrane has been gathered (see, for example, ref *1* for a recent review). However, much remains to be learned about membrane assembly, in particular, about the transport of membrane lipids, which are synthesized in *biogenic* membranes, i.e., membranes that are capable of synthesizing a new membrane. The main lipid components of biomembranes are phospholipids, which are found in all species from bacteria to mammals. The active sites of the enzymes involved in phospholipid biosynthesis are usually confined to the cytoplasmic leaflet (*2*). Consequently, newly synthesized phospholipids are incorporated into this membrane leaflet only. To allow for balanced growth of the membrane, approximately half of the phospholipids must cross the bilayer to occupy the opposite leaflet, making this process a fundamental transport step in membrane biogenesis. This paper aims to review the available experimental data and their interpretation. While the data reflect the consensus characteristics of this process, their interpretation is still a matter of debate.

Problems and Solutions in Lipid Transport

Because of their amphiphilicity, phospholipids are faced with traveling problems. Moving through the aqueous phase would expose their hydrophobic tails to the water, whereas moving from one side of the membrane to the opposite side would expose their hydrophilic headgroup to the hydrophobic interior of the membrane. First, three possible solutions for moving through the aqueous phase will be briefly mentioned.

---

* To whom correspondence should be addressed. E-mail: b.dekruijff@chem.uu.nl.

Vesicular traffic of lipids mediates lipid transport without exposing the hydrophobic parts of the lipids during transport through the aqueous phase. During this transport from the main site of synthesis, the membrane of the endoplasmic reticulum (ER),[1] lipid (and protein) sorting occurs, maintaining the specific lipid (and protein) compositions of the membranes that are connected through vesicular traffic. The sorting mechanism remains to be elucidated. Possibly, lipids and proteins are cosorted (see, for example, ref *3*).

Alternatively, transport of lipids between membranes may be mediated via membrane contact sites, the paradigm being the sites of contact between the ER and the mitochondrial membrane (*4−7*). This mechanism also possibly provides an alternative route of transport from the ER to plasma membrane (*8*). It is proposed that two closely apposed membranes can exchange phospholipids, e.g., via collisional contact. However, the structure of these putative contact sites is unknown. Moreover, their constituting components, which may regulate lipid transport via contact sites, remain to be identified.

Finally, lipids could be transported by dedicated proteins that shield the hydrophobic parts of the lipid, after it is extracted from the membrane. The protein could then be targeted to the destination membrane and deliver its cargo there. Although some proteins have been shown to fulfill such a phospholipid transport function *in vitro*, there is no evidence of their involvement in membrane biogenesis. Rather, they seem to be important in various other cellular processes discussed elsewhere (*9, 10*).

Intramembrane or transbilayer transport presents another traveling problem for phospholipids, about which much insight has been derived from model membrane studies. The barrier for translocation consists of the polar phospholipid headgroup (*11*), having to cross the hydrophobic membrane interior. In the 1970s, Kornberg and McConnell (*12*) measured the translocation rate of phosphatidylcholine (PC) in small unilamellar vesicles composed of egg PC. The reporter PC, modified with a headgroup spin-label, was found to translocate with a half-time of 6.5 h, and an activation energy of 81 kJ/mol was reported for this process. Wimley and Thompson (*13*) reported similar activation energies for translocation of radiolabeled DMPC in pure DMPC or DMPC/DMPE vesicles. De Kruijff *et al.* (*14*) used ^{13}C-labeled PC to measure its translocation rate in vesicles composed of DOPC, and reported no changes in distribution of the label during incubation for several hours. Bai and Pagano (*15*) measured translocation of fluorescently labeled PC in vesicles composed of POPC and found a half-time of translocation of ∼7.5 h. Thus, the barrier for translocation seems to be rather high.

However, various studies have shown increased rates of phospholipid translocation in lipid model membranes under special circumstances. At the lipid main phase transition temperature, an increased rate of transbilayer movement can be observed (*16, 17*). Furthermore, a high degree of unsaturation of the acyl chains correlates with an increased level

of transbilayer movement (*18*). These results suggest that the acyl chain interactions are important in modulating translocation rates. Additionally, inducing asymmetric perturbances by enzymatically changing the phospholipid composition of only the outer membrane leaflet (*19, 20*) of the bilayer, or applying a large pH gradient across the bilayer (*21*), can elevate the rate of phospholipid translocation in model membranes. The examples given above notwithstanding, the conclusion from model membane studies is that phospholipid transbilayer movement occurs with half-times of several hours or even longer in unperturbed model membranes composed of only phospholipids that are resuspended in a regular buffer or salt solution. Generally, this is too slow to keep up with membrane growth *in vivo*. Thus, mechanisms for translocating phospholipids at an appreciable rate must exist in biological membranes.

Solutions to facilitating transbilayer transport of phospholipids have been characterized best in plasma membranes of eukaryotic cells, where inward and outward transbilayer movement occurs in a coordinated fashion. Plasma membranes from several sources have been shown to be endowed with an aminophospholipid translocation activity. This activity specifically mediates unidirectional transport of phosphatidylserine (PS) and phosphatidylethanolamine (PE). Transbilayer transport from the exoleaflet to the endoleaflet of the membrane is termed flip, whereas flop is transport in the opposite direction. The protein associated with transbilayer inward movement of PS and PE is therefore called the aminophospholipid flippase. It is ATP-dependent (*22*) and has a well-established substrate specificity. The membranes containing this enzyme have an asymmetric phospholipid distribution over the two membrane leaflets, with the aminophospholipids occupying the cytoplasmic leaflet, and the choline-containing phospholipids, which can be translocated by a floppase activity (*23* and references therein), predominantly residing at the exoplasmic side. The asymmetric distribution of phospholipids can be dissipated by the action of phospholipid scramblase activity, which is silent under normal circumstances but can be activated by a Ca^{2+} signal. This is important for cellular function, since exposure of aminophospholipids on the exoleaflet has been shown to trigger processes such as blood coagulation and phagocytosis. The reader is referred to refs *24−26* for reviews.

Members of the ATP binding cassette (ABC) transporter superfamily have been implicated in the outward movement of phospholipids. As the name implies, this directional transport depends on ATP just like inward transport by the aminophospholipid flippase. Typically, ABC transporters have been shown to extrude a variety of lipophilic compounds from the plasma membrane with a low level of or no structural similarity. As a consequence, upregulation of their activity results in multiple drug resistant phenotypes in cancer cells and bacteria. Because of their broad substrate specificity, and the frequent use of nonendogenous phospholipid analogues in translocation studies, their function as bona fide lipid translocators is still a matter of debate. Cell lines transfected with Mdr1 pgp, an extensively studied member, display enhanced secretion of the lipid metabolite platelet activating factor (*27*) and an increased level of exposure of fluorescently labeled PE and PC (*28*), suggesting a possible involvement in phospholipid translocation. Knockouts in the Mdr2 gene in mice fail to excrete phosphatidyl-

---

[1] Abbreviations: ABC, ATP binding cassette; BSA, bovine serum albumin; di-C_4, dibutyryl; DM, dimyristoyl; DO, dioleoyl; DP, dipalmitoyl; ER, endoplasmic reticulum; Lep, leader peptidase; NBD, nitrobenzoxadiazole; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PO, 1-palmitoyl-2-oleoyl; PS, phosphatidylserine.

choline into the bile (*29*), indicating that this protein could function as a phospholipid floppase. Further evaluation of the evidence is beyond the scope of the review; see, however, refs *30*−*33* for recent reviews. Also, putative lipid translocators such as Rft1 (*34*) and WzxE (*35*) that lack an ATP-binding domain have been identified. These are polytopic membrane proteins, like ABC transporters, and are thought to translocate lipid-linked sugar moieties across membranes without an apparent need for energy input. To our knowledge, their involvement in glycerophospholipid translocation has not been tested or hypothesized.

Against the background of protein-mediated transmembrane transport of lipids across plasma membranes, the knowledge of phospholipid transbilayer movement in biogenic membranes will be reviewed and discussed.

Characterization of Phospholipid Transbilayer Movement in Biogenic Membranes

The paradigms for prokaryotic and eukaryotic biogenic membranes are the bacterial cytoplasmic membrane and the ER membrane, respectively. The ER membrane can be regarded as a highly dynamic membrane, a factory where lipid and membrane protein synthesis and insertion take place. Because of the asymmetric lipid synthesis, and because in protein-free lipid bilayers phospholipid transbilayer movement is typically slow, the existence of a phospholipid translocase, i.e., an intrinsic proteinaceous component of biogenic membranes, was postulated (*36*). To eventually identify this elusive translocase, several groups set out to biochemically characterize phospholipid translocation in biogenic membranes. A number of these studies are summarized below, first for the ER membrane and then for the bacterial cytoplasmic membrane.

Phospholipid Transbilayer Movement in the ER. The occurrence of fast translocation of phospholipids in the ER membrane was first inferred from studies on intermembrane transport of phospholipids in in vitro systems (*37*−*39*). In these studies, transport of a radiolabeled phospholipid analogue from a donor membrane to an acceptor membrane is mediated by phospholipid transfer proteins. After separation of the donor and acceptor membranes, the extent of transfer of the radioactive label is used as a measure for intermembrane transport. It was found that PC as well as other important phospholipid classes in the ER membrane vesicles acts as a single pool available for intermembrane transport. This means that the lipids on either side of the microsomal membrane are not discernible within the time frame of the assay, and thus that transbilayer movement is not the rate-limiting step in the transport process. Under these circumstances, only an upper limit of the translocation half-time can be obtained. Thus, the half-times of equilibration of phospholipids over the two membrane leaflets were estimated to be 1 h or (much) less.

Bishop and Bell (*40*) reported another approach to the study of phospholipid translocation in microsomes. Using the water-soluble, radiolabeled phospholipid analogue dibutyrylphosphatidylcholine (di-C_4-PC), translocation was assessed as the redistribution of radioactivity over the intravesicular and extravesicular space. It was demonstrated that the probe entered the microsomal compartment, a process which was completed within 10 min. Translocation activity

was reported to be sensitive to protease treatment, and to alkylating agents, and it was shown that transport of di-C_4-PC was inhibited by addition of other short-chain PCs. Taken together, this led the authors to conclude that a dedicated protein mediates this process. Unfortunately, in this assay only the end point of the translocation process was measured, making the interpretation of the partial sensitivity toward protease ($\sim$30% inhibition) and NEM ($\sim$60% inhibition) difficult.

Subsequently, Herrmann *et al.* (*41*) reported on the outward−inward transbilayer movement of short-chain, spin-labeled phospholipids in microsomes. The partial water solubility of this probe allows it to be extracted from the outer leaflet of the membrane using bovine serum albumin (BSA). The amount of spin-labeled phospholipid associated with the microsomes after incubation with BSA is taken to be the amount of probe occupying the inner membrane leaflet. Several phospholipid analogues displayed a half-time of translocation of $\sim$20 min, showing low phospholipid headgroup specificity. The final distribution of the probe was approximately equal over the two membrane leaflets, indicating no preferential phospholipid distribution in this membrane. The spin-labeled analogues of lyso-PS and PC competed with each other in the translocation assay. Interestingly, translocation of spin-labeled analogues was not inhibited by addition of the dibutyryl derivative employed by Bishop and Bell, indicating different routes of translocation for these species. Preincubation of the microsomes with the sulfhydryl reagent *N*-ethylmaleimide (NEM) resulted in a reduction of the initial translocation rate.

In some recent studies, more insight into the transbilayer movement of phospholipids in the ER membrane was obtained by using assays with an improved time resolution. Buton *et al.* (*42*) assayed transport of short-chain, radiolabeled and spin-labeled analogues of major phospholipid classes by using a BSA back-exchange assay with a rapid membrane filtering step to reduce the dead time of the method. It was shown that phospholipid translocation in the rat liver ER proceeds bidirectionally, with a half-time of <25 s for all glycerophospholipids that have been tested. The equilibrium distribution of the phospholipids was found to be symmetrical, and competition between spin-labeled and radiolabeled analogues was demonstrated.

Marx *et al.* (*43*) circumvented the separation step and used a stopped-flow BSA extraction assay to study translocation of spin-labeled and NBD-labeled phospholipid analogues of PE and PC, using the differential spectral properties of the probes when residing in a membrane or when bound to BSA to discriminate between these populations. Both spin-labeled phospholipid classes were shown to translocate with a half-time of 8−16 s, whereas NBD-labeled phospholipids translocated at an 8-fold lower rate.

Phospholipid Flip-Flop in the Bacterial Cytoplasmic Membrane. The first insight into flip-flop in bacterial membranes was obtained from studies on the Gram-positive *Bacillus megaterium*. Its simple, single-membrane structure was shown to exhibit translocation of newly synthesized phospholipids *in vivo* with a half-time of $\sim$3 min (*44*), independent of metabolic energy, protein, and lipid synthesis (*45*).

An *in vitro* assay, using isolated membrane vesicles of *B. megaterium*, also demonstrated rapid translocation of phos-

pholipids. Short-chain, NBD-labeled analogues of phosphatidylglycerol (PG), PE, and PC were added from donor vesicles, in which the NBD fluorescence was quenched with rhodamine-PE. Upon addition of *B. megaterium* acceptor vesicles, a biphasic increase in fluorescence was observed, the fast phase representing incorporation in the outer leaflet of the acceptor membrane and the slow phase transbilayer movement. All three probes translocated with a half-time of ∼30 s at 37 °C, via a mechanism that was shown to be partially sensitive to protease treatment, but not to treatment with NEM (*46*).

Huijbregts *et al.* (*47*) studied transbilayer movement in *Escherichia coli* inner membrane vesicles (IMV) of endogenously synthesized PE, using the reaction of its amine function with fluorescamine as a method for detection. These experiments were performed using membranes from a PE-deficient strain [AD93 (*48*)], in which synthesis of (labeled) PE can be restored by adding labeled serine, CTP, and wild-type cell lysate containing the enzyme phosphatidylserine synthase. It was found that newly synthesized radiolabeled PE translocates with a half-time of <1 min in *E. coli* IMV. The translocation half-times remained at <1 min after treatment of right-side-out vesicles with protease, and treatment with NEM had no observable effect on rates of translocation measured in inside-out or right-side-out vesicles.

In inside-out inner membrane vesicles (IIMV) of wild-type *E. coli* W3899, transbilayer movement of several C_6-NBD phospholipids was assessed by determining the accessibility of the probe to dithionite, essentially as described by McIntyre and Sleight (*49*). Bidirectional transbilayer movement occurred with a half-time of ∼7 min, irrespective of the phospholipid headgroup, or pretreatment of the vesicles with NEM and proteinase K. Moreover, the process was found to be independent of ATP or the presence of a proton motive force across the membrane (*50*). However, some care should be taken with the interpretation of these results since the lack of kinetic readout of the assay may have masked subtle differences after the indicated treatments.

In agreement with the previous study, Kubelt *et al.* found bidirectional transport of C_6NBD phospholipids in IIMV of *E. coli* with translocation half-times of 0.5−3 min (*51*) in a different, wild-type MG1655 strain. Translocation was assessed using an on-line fluorescence BSA back-exchange assay as described previously (*43*), and the analogues of PE and PC behaved similarly in this assay.

It has been hypothesized that translocation of the polar phospholipid headgroup would require its accommodation in a proteinaceous pore in the bacterial membrane. Watkins *et al.* (*52*) tested an interesting putative candidate, the protein translocase from *E. coli*, for its ability to translocate exogenously added NBD-PS. It was shown that depletion of the protein translocase in a bacterial inner membrane system had no effect on *in vitro* PL translocation rates. This rules out the possibility that the protein translocase is the only phospholipid translocator in the *E. coli* IM.

Summary of Phospholipid Translocation in Biogenic Membranes. Several undisputed general characteristics of transbilayer movement in biogenic membranes are emerging, despite the limited number of studies, the variety of membrane systems that have been investigated, and the different methods that have been employed.

First, in contrast to translocation in phospholipid model membranes and in the plasma membrane of eukaryotes, transbilayer movement of phospholipids in biogenic membranes is fast, with translocation half-times on the order of minutes or less. Especially for the cytoplasmic membrane of rapidly growing bacteria should this be expected, since redistribution of newly synthesized phospholipids is a necessity in keeping up with growth.

Second, the ability to translocate phospholipids is an intrinsic property of the biogenic membrane since it occurs in isolated membranes, i.e., in the absence of other cellular components. Translocation does not occur in any lipid extract derived from a biogenic membrane, which taken together implies that transbilayer movement of phospholipids is a process mediated by a membrane protein(s).

Third, in diametric contrast, e.g., the aminophospholipid translocation activity in the plasma membrane of eukaryotes, translocation in biogenic membranes is bidirectional, energy-independent, and not specific for the phospholipid headgroup.

Though there is consensus on transbilayer movement being mediated by a membrane protein(s), there is controversy about the question of whether biogenic membranes contain one specific flippase or a group of flippases and/or have a membrane protein-borne intrinsic capacity to translocate phospholipids. To address this question, translocation assays have been performed on *in vitro*-reconstituted proteoliposomes, composed of lipids and detergent extracts (i.e., membrane proteins and membrane lipids) of ER membranes and bacterial membranes. A number of these studies are summarized in the next section.

In Vitro Reconstitution of Flip-Flop

Reconstitution of Flip-Flop Activity from Biogenic Membranes. Backer and Dawidowicz (*53*) reconstituted a protein extract of rat liver microsomes in vesicles, and obtained flippase activity in part of the vesicle population. The activity was measured as a shift in vesicle density in vesicles prepared with brominated PC. In donor vesicles with flippase activity, brominated PC was completely available for exchange with PCTP, resulting in a low-density vesicle fraction. This activity was reported to be insensitive to treatment with trypsin or NEM.

Hrafnsdottir and Menon (*54*) used the di-C_4-phosphatidylcholine-based assay as described above to show that membrane proteins from *Bacillus subtilis* could be reconstituted to yield transport-active proteoliposomes. The reconstituted activity was partially protease-sensitive, and the specific flip-flop activity could be enriched by fractionation, indicating the existence of a group of flippases in the *B. subtilis* membrane. With the same assay, Menon *et al.* (*55*) studied translocation in proteoliposomes, prepared of a detergent extract of rat liver ER membrane proteins. The probe was redistributed with a half-time of ∼2 min, and also here protein pools with higher specific activities of translocation were obtained by fractionation. The partial sensitivity to protease treatment is explained by postulating the existence of different pools of flippases. When present at a high concentration, all vesicles might contain a protease-resistant type of flippase. This line of reasoning reconciles the apparent discrepancies between the lack of protease sensitivity reported by Backer and Dawidowicz and the studies just

mentioned. Recently, a follow-up on this study was published (*56*), in which radiolabeled DPPC was used as a marker molecule instead of its water-soluble counterpart. The susceptibility of the probe to PLA_2 treatment was taken as a measure of the amount of probe in the outer leaflet. This study confirmed the characteristics of ER-derived proteoliposomes just described.

Using a lipid modification reaction and subsequent lipid analysis on thin-layer chromatography, Nicolson and Mayinger (*57*) demonstrated translocation of radiolabeled phosphatidylethanolamine in proteoliposomes prepared from a detergent extract from *Saccharomyces cerevisiae* microsomes, an activity that was also shown to be partially sensitive to protease treatment.

Exploiting the benefits of the on-line BSA back-extraction assay (*43*), Kubelt *et al.* (*51*) have also shown recently that a protein extract from the *E. coli* inner membrane is endowed with phospholipid translocating activity when reconstituted in proteoliposomes. A partial sensitivity to protease treatment was reported, which is in apparent contrast with the lack of protease sensitivity shown by Huijbregts *et al.* (*50*) in inner membrane vesicles from this organism. The studies cited above have revealed that the phospholipid translocating activity from biogenic membranes can be reconstituted *in vitro*. Combined with protein fractionation, this paves the way for isolation of the flippase(s).

Despite many efforts, as yet no protein responsible for phospholipid translocation in the ER or bacterial cytoplasmic membrane has been identified. Taking a different point of view, one can alternatively postulate that *the mere presence of membrane proteins in general* confers the ability to translocate phospholipids on biogenic membranes. Both hypotheses are *a priori* compatible with the general characteristics of transbilayer movement in biogenic membranes.

Membrane proteins interact with the lipid bilayer principally in two ways. They can either span the hydrophobic interior completely or be inserted into one leaflet of the bilayer. The latter type of interaction can be mediated by parts of the protein which interact with membrane−water interface, e.g., via amphipathic helices. To our knowledge, phospholipid translocation induced by membrane-associated proteins has not been described. There is a large body of evidence that amphipathic peptides can induce phospholipid translocation, but this is thought to occur only after these peptides have adopted a transmembrane orientation (see refs *58* and *59* for recent reviews), resulting in pore formation. Such a mechanism is unlikely to be involved in phospholipid translocation in membrane biogenesis.

Integral membrane proteins commonly span the membrane with one or more hydrophobic α-helical stretches. The hypothesis that the presence of membrane-spanning proteins is sufficient to induce phospholipid flip-flop was formulated and has been tested *in vitro* using a model membrane assay (*60*) using synthetic α-helical peptides.

In Vitro Flip-Flop Activity Mediated by Hydrophobic Membrane-Spanning Peptides. A series of well-characterized (*61−64*) membrane-spanning hydrophobic peptides have been employed as models for the membrane-spanning helices of proteins. These peptides, termed XALP*N*, consist of a hydrophobic core composed of alternating Ala and Leu (AL) residues, flanked at the N- and C-termini by two anchoring residues (X). The number *N* denotes the total number of residues. Charged or aromatic amino acids are typical anchoring residues of naturally occurring membrane-spanning segments (*65*) which have a characteristic length of around 20−25 residues depending on the type of membrane in which the protein resides (*66*). Therefore, the lysine-flanked peptide KALP23 [GKKL(AL)$_8$KKA] and the tryptophan-flanked peptide WALP23 [GWWL(AL)$_8$WWA] were used as mimics of naturally occurring α-helices of membrane proteins to study helix-induced translocation. Transbilayer movement was assessed by determining the accessibility of C6NBD-labeled phospholipids to reduction with dithionite. It was found that transmembrane peptides, incorporated in model membranes composed of *E. coli* phospholipids, induce phospholipid translocation. This process could be readily assessed at a peptide:lipid ratio as low as 1:2000. The rate of translocation increased linearly with the peptide:lipid molar ratio, up to rates corresponding to translocation half-times on the order of minutes at a peptide:lipid ratio of 1:250. Subtle differences in the rate of translocation between peptides with different anchoring residues were reported. From this study, it was concluded that the presence of transmembrane helices in the *E. coli* inner membrane might be sufficient to allow phospholipid translocation (*60*). This effect was not restricted to the XALP peptides, as it was shown that a naturally occurring transmembrane helix [H1, N-terminal transmembrane helix of the *E. coli* inner membrane protein leader peptidase (Lep)] induced phospholipid translocation with a similar efficiency (*67*).

Another study revealed that phospholipid translocation occurred not only in vesicles composed of *E. coli* phospholipids but also in XALP-containing vesicles composed of synthetic phospholipids such as dioleoylphosphatidylcholine (DOPC), DOPC/DOPE, DOPC/DOPG, and DOPC/cholesterol vesicles. The lipid composition of the vesicle was shown to modulate translocation rates. In DOPC/DOPE mixtures, a higher PE content of the membrane was found to moderately reduce the rate of translocation. The presence of 40% cholesterol was shown to be strongly inhibitory, reducing translocation rates 6-fold. It was suggested that a higher content of PE or cholesterol reduces the rate of phospholipid translocation by increasing the acyl chain order of the phospholipids. Various lipid probes were tested, revealing a strong dependence of the rate of transbilayer movement on the type of lipid headgroup. Apparently, a higher number of charges on the phospholipid headgroup correlated with a lower translocation rate, in accordance with the fact that the barrier for translocation is the polar lipid headgroup. It should be noted that a strong headgroup dependence is generally not observed in biogenic membranes. Possibly, the great variety of membrane-spanning segments found in biomembranes that may contain more polar or charged side chains in their hydrophobic core allows for a variety of phospholipid classes to be translocated.

In conclusion, these data indicate that transmembrane helix-induced translocation of phospholipids occurs in various lipid mixtures, and that the composition of the lipid mixture can modulate translocation efficiency. Thus, helix-induced translocation could in principle apply to biomembranes other than the *E. coli* inner membrane, and the lipid composition of some biomembranes may provide a means of regulating the rate of helix-induced translocation.
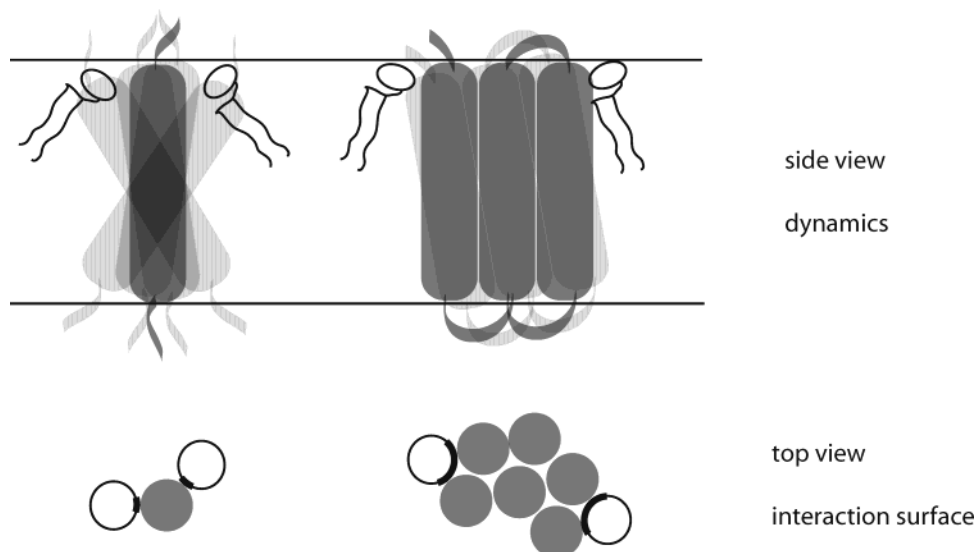
FIGURE 1: Slip-pop mechanism for phospholipid translocation in biogenic membranes. Schematic representation of a membrane, viewed from the side (top panel) and from the top (bottom panel), with either a single-membrane-spanning protein (in black, left-hand side) or a multi-membrane-spanning protein (in black, right-hand side) incorporated. The dynamic behavior (top panel, gray silhouettes suggesting motion) of single-membrane-spanning proteins is postulated to cause transient defects in the lipid−helix interface with more efficiency than a less dynamic multi-membrane-spanning protein. The bottom panel shows that incorporation of proteins with a large cross-sectional area may offer more "interaction surface" for phospholipids (dark thickened line in the empty circles) than a single helix, resulting in more stable lipid−protein interactions. As a result of these two phenomena, small, membrane-spanning proteins may be much more efficient in inducing phospholipid translocation than large membrane proteins.

In Vitro Flip-Flop Activity Mediated by a Subset of Bacterial Membrane Proteins. To validate the conclusions from the studies using synthetic peptides, a number of well-characterized membrane proteins from bacterial biogenic membranes have been tested for their ability to induce translocation (67). Purified membrane proteins were reconstituted into proteoliposomes with the typical phospholipid composition of *E. coli*, and the translocation of short-chain labeled NBD-PG was assayed essentially as described above.

The protein leader peptidase (Lep) from *E. coli* is an inner membrane protein with two membrane-spanning segments, and a catalytic periplasmic domain (P2). Lep was shown to induce translocation of NBD-PG, in agreement with the observation that the first TM segment of this protein also induces translocation. In contrast, the membrane surface-associated P2 domain did not induce appreciable phospholipid redistribution, indicating that the membrane-spanning segments of Lep are responsible for inducing the transbilayer movement. The potassium channel KcsA is a stable tetrameric protein (68), consisting of monomers that like Lep have two membrane-spanning segments. Tetrameric KcsA, although larger than Lep, was shown to mediate phospholipid transbilayer movement, with an efficiency similar to that of (monomeric) Lep. This might indicate that membrane-spanning helices are less effective in inducing translocation when they are components of a larger proteinaceous structure. MsbA from *E. coli*, a member of the ABC superfamily which forms a dimer with 12 transmembrane helices in total, has been shown to be involved in outer membrane biogenesis and has been postulated to be involved in (phospho)lipid translocation (69, 70). Reconstitution of this large protein in model membranes did not result in detectable phospholipid translocation activity in the proteoliposomes that were obtained.

On the basis of the model peptides and membrane proteins that have been tested, it could be concluded that the single-membrane-spanning proteins are more effective in inducing flop than polytopic membrane proteins. It seems that the cross-sectional size of the membrane-spanning part (i.e., of the helix or helix bundle) correlates with its efficiency in inducing translocation, which will be discussed shortly. The main conclusion from this study is that a subset of bacterial inner membrane proteins induces phospholipid redistribution via their α-helical membrane-spanning segments, in agreement with the results obtained with the model peptides described above.

How do transmembrane helices cause lipid redistribution? We propose that their presence causes transient disturbances in the bilayer, localized at the interface between the membrane-spanning parts and the lipids. As a result of this, a phospholipid adjacent to the peptide may "slip" into the bilayer's interior with its headgroup. This could be regarded as the transition state of the translocation process. From there, it may surface, or "pop up", with its headgroup on the other side (or the same side) of the membrane, after which translocation is accomplished. Thus, we propose the name "slip-pop" for the putative mechanism of transmembrane helix-induced flip-flop.

What is the nature of the disturbances that allow phospholipids to translocate? We speculate that they are created by dynamic processes, caused by, for example, the rotational motion, wobbling, tilting, or bending of the helix (see Figure 1, top panel). Increased helix dynamics may result in a less stable interaction between the helix and the adjacent phospholipid(s). Therefore, the phospholipid may have a greater probability of slipping into the transition state orientation, resulting in increased translocation rates for phospholipids in the presence of transmembrane helices in membranes. As was noted previously, single helices displayed more slip-

pop than multi-membrane-spanning proteins. This fits the model, since the former can be expected to have more motional freedom than the latter (see the top panel of Figure 1). We speculate that the tendency of the lipid itself to slip into the transition state correlates strongly and negatively with the number of charges on its headgroup.

In addition to the dynamic properties of the membrane-spanning protein, we speculate that its size *per se* may also influence the effect on the surrounding lipids. This is because proteins with a high cross-sectional area expose more surface to the lipids at which stable lipid−protein interactions can occur, as schematically shown in the bottom panel of Figure 1. The part of the lipid in contact with the protein is indicated for a single (left)- or multi-membrane (right)-spanning protein. More interaction surface would result in more stable lipid−protein interactions and consequently in a decreased probability of the lipid changing its orientation.

Concluding Remarks

The presence of membrane-spanning helices induces translocation of some phospholipid classes under conditions where bilayer integrity is preserved. This argues in favor of a general mechanism of constitutive flip-flop, or slip-pop in biogenic membranes. Although not a prerequisite, the asymmetric synthesis of phospholipids in these membranes may constitute a driving force for this process, giving rise to net phospholipid translocation in biogenic membranes, without the need for additional energy input.

We note that there must be restrictions to helix-induced translocation, as α-helical membrane-spanning segments are ubiquitous, also in membranes that typically display slow phospholipid translocation such as the erythrocyte membrane (*71*). Our model studies indicate that increasing the cholesterol content of a membrane inhibits slip-pop. It is tempting to speculate that the enrichment in cholesterol along the vesicular traffic pathway (i.e., from the ER to the plasma membrane) results in a gradual reduction of helix-induced translocation (*72*), which would make the maintenance of phospholipid asymmetry less costly. Cholesterol is known to influence the properties of biomembranes profoundly (see ref *73*); thus, an additional function as a modulator of phospholipid translocation is conceivable.

ACKNOWLEDGMENT

REFERENCES

1. Schnell, D. J., and Hebert, D. N. (2003) Protein translocons: multifunctional mediators of protein translocation across membranes, *Cell 112*, 491−505.
2. Bell, R. M., Ballas, L. M., and Coleman, R. A. (1981) Lipid topogenesis, *J. Lipid Res. 22*, 391−403.
3. Sprong, H., van der Sluijs, P., and van Meer, G. (2001) How proteins move lipids and lipids move proteins, *Nat. Rev. Mol. Cell Biol. 2*, 504−513.
4. Achleitner, G., Gaigg, B., Krasser, A., Kainersdorfer, E., Kohlwein, S. D., Perktold, A., Zellnig, G., and Daum, G. (1999) Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact, *Eur. J. Biochem. 264*, 545−553.
5. Shiao, Y. J., Lupo, G., and Vance, J. E. (1995) Evidence that phosphatidylserine is imported into mitochondria via a mitochondria-associated membrane and that the majority of mitochondrial

6. De Kroon, A. I., Koorengevel, M. C., Vromans, T. A., and De Kruijff, B. (2003) Continuous Equilibration of Phosphatidylcholine and Its Precursors between Endoplasmic Reticulum and Mitochondria in Yeast, *Mol. Biol. Cell 14*, 2142−2150.
7. Voelker, D. R. (2003) New perspectives on the regulation of intermembrane glycerophospholipid traffic, *J. Lipid Res. 44*, 441−449.
8. Pichler, H., Gaigg, B., Hrastnik, C., Achleitner, G., Kohlwein, S. D., Zellnig, G., Perktold, A., and Daum, G. (2001) A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids, *Eur. J. Biochem. 268*, 2351−2361.
9. Wirtz, K. W. (1997) Phospholipid transfer proteins revisited, *Biochem. J. 324*, 353−360.
10. Allen-Baume, V., Segui, B., and Cockcroft, S. (2002) Current thoughts on the phosphatidylinositol transfer protein family, *FEBS Lett. 531*, 74−80.
11. Ganong, B. R., and Bell, R. M. (1984) Transmembrane movement of phosphatidylglycerol and diacylglycerol sulfhydryl analogues, *Biochemistry 23*, 4977−4983.
12. Kornberg, R. D., and McConnell, H. M. (1971) Inside−outside transitions of phospholipids in vesicle membranes, *Biochemistry 10*, 1111−1120.
13. Wimley, W. C., and Thompson, T. E. (1991) Transbilayer and interbilayer phospholipid exchange in dimyristoylphosphatidylcholine/dimyristoylphosphatidylethanolamine large unilamellar vesicles, *Biochemistry 30*, 1702−1709.
14. de Kruijff, B., van Zoelen, E. J., and van Deenen, L. L. (1978) Glycophorin facilitates the transbilayer movement of phosphatidylcholine in vesicles, *Biochim. Biophys. Acta 509*, 537−542.
15. Bai, J., and Pagano, R. E. (1997) Measurement of spontaneous transfer and transbilayer movement of BODIPY-labeled lipids in lipid vesicles, *Biochemistry 36*, 8840−8848.
16. De Kruijff, B., and Van Zoelen, E. J. (1978) Effect of the phase transition on the transbilayer movement of dimyristoylphosphatidylcholine in unilamellar vesicles, *Biochim. Biophys. Acta 511*, 105−115.
17. John, K., Schreiber, S., Kubelt, J., Herrmann, A., and Muller, P. (2002) Transbilayer Movement of Phospholipids at the Main Phase Transition of Lipid Membranes: Implications for Rapid Flip-Flop in Biological Membranes, *Biophys. J. 83*, 3315−3323.
18. Armstrong, V. T., Brzustowicz, M. R., Wassall, S. R., Jenski, L. J., and Stillwell, W. (2003) Rapid flip-flop in polyunsaturated (docosahexaenoate) phospholipid membranes, *Arch. Biochem. Biophys. 414*, 74−82.
19. De Kruijff, B., and Baken, P. (1978) Rapid transbilayer movement of phospholipids induced by an asymmetrical perturbation of the bilayer, *Biochim. Biophys. Acta 507*, 38−47.
20. Contreras, F. X., Villar, A. V., Alonso, A., Kolesnick, R. N., and Goni, F. M. (2003) Sphingomyelinase activity causes transbilayer lipid translocation in model and cell membranes, *J. Biol. Chem. 278*, 37169−37174.
21. Hope, M. J., Redelmeier, T. E., Wong, K. F., Rodrigueza, W., and Cullis, P. R. (1989) Phospholipid asymmetry in large unilamellar vesicles induced by transmembrane pH gradients, *Biochemistry 28*, 4181−4187.
22. Seigneuret, M., and Devaux, P. F. (1984) ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes, *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751−3755.
23. Dekkers, D. W., Comfurius, P., Schroit, A. J., Bevers, E. M., and Zwaal, R. F. (1998) Transbilayer movement of NBD-labeled phospholipids in red blood cell membranes: outward-directed transport by the multidrug resistance protein 1 (MRP1), *Biochemistry 37*, 14833−14837.
24. Dolis, D., Moreau, C., Zachowski, A., and Devaux, P. F. (1997) Aminophospholipid translocase and proteins involved in transmembrane phospholipid traffic, *Biophys. Chem. 68*, 221−231.
25. Bevers, E. M., Comfurius, P., Dekkers, D. W., and Zwaal, R. F. (1999) Lipid translocation across the plasma membrane of mammalian cells, *Biochim. Biophys. Acta 1439*, 317−330.
26. Daleke, D. L., and Lyles, J. V. (2000) Identification and purification of aminophospholipid flippases, *Biochim. Biophys. Acta 1486*, 108−127.
27. Raggers, R. J., Vogels, I., and van Meer, G. (2001) Multidrug-resistance P-glycoprotein (MDR1) secretes platelet-activating factor, *Biochem. J. 357*, 859−865.

The continuation reference phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine, *J. Biol. Chem. 270*, 11190−11198.

28. Pohl, A., Lage, H., Muller, P., Pomorski, T., and Herrmann, A. (2002) Transport of phosphatidylserine via MDR1 (multidrug resistance 1) P-glycoprotein in a human gastric carcinoma cell line, *Biochem. J. 365*, 259−268.

29. Smit, J. J., Schinkel, A. H., Oude Elferink, R. P., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A., Ottenhoff, R., van der Lugt, N. M., van Roon, M. A., *et al.* (1993) Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease, *Cell 75*, 451−462.

30. Borst, P., Zelcer, N., and van Helvoort, A. (2000) ABC transporters in lipid transport, *Biochim. Biophys. Acta 1486*, 128−144.

31. Raggers, R. J., Pomorski, T., Holthuis, J. C., Kalin, N., and van Meer, G. (2000) Lipid traffic: the ABC of transbilayer movement, *Traffic 1*, 226−234.

32. Tannert, A., Pohl, A., Pomorski, T., and Herrmann, A. (2003) Protein-mediated transbilayer movement of lipids in eukaryotes and prokaryotes: the relevance of ABC transporters, *Int. J. Antimicrob. Agents 22*, 177−187.

33. Pomorski, T., Hrafnsdottir, S., Devaux, P. F., and van Meer, G. (2001) Lipid distribution and transport across cellular membranes, *Semin. Cell Dev. Biol. 12*, 139−148.

34. Helenius, J., Ng, D. T., Marolda, C. L., Walter, P., Valvano, M. A., and Aebi, M. (2002) Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein, *Nature 415*, 447−450.

35. Rick, P. D., Barr, K., Sankaran, K., Kajimura, J., Rush, J. S., and Waechter, C. J. (2003) Evidence that the wzxE gene of *Escherichia coli* K-12 encodes a protein involved in the transbilayer movement of a trisaccharide-lipid intermediate in the assembly of enterobacterial common antigen, *J. Biol. Chem. 278*, 16534−16542.

36. Bretscher, M. (1973) in *The cell surface in development*, pp 17−27, Wiley, New York.

37. Zilversmit, D. B., and Hughes, M. E. (1977) Extensive exchange of rat liver microsomal phospholipids, *Biochim. Biophys. Acta 469*, 99−110.

38. van den Besselaar, A. M., de Druijff, B., van den Bosch, H., and van Deenen, L. L. (1978) Phosphatidylcholine mobility in liver microsomal membranes, *Biochim. Biophys. Acta 510*, 242−255.

39. Jackson, R. L., Westerman, J., and Wirtz, K. W. (1978) Complete exchange of phospholipids between microsomes and plasma lipoproteins mediated by liver phospholipid-exchange proteins, *FEBS Lett. 94*, 38−42.

40. Bishop, W. R., and Bell, R. M. (1985) Assembly of the endoplasmic reticulum phospholipid bilayer: the phosphatidylcholine transporter, *Cell 42*, 51−60.

41. Herrmann, A., Zachowski, A., and Devaux, P. F. (1990) Protein-mediated phospholipid translocation in the endoplasmic reticulum with a low lipid specificity, *Biochemistry 29*, 2023−2027.

42. Buton, X., Morrot, G., Fellmann, P., and Seigneuret, M. (1996) Ultrafast glycerophospholipid-selective transbilayer motion mediated by a protein in the endoplasmic reticulum membrane, *J. Biol. Chem. 271*, 6651−6657.

43. Marx, U., Lassmann, G., Holzhutter, H. G., Wustner, D., Muller, P., Hohlig, A., Kubelt, J., and Herrmann, A. (2000) Rapid flip-flop of phospholipids in endoplasmic reticulum membranes studied by a stopped-flow approach, *Biophys. J. 78*, 2628−2640.

44. Rothman, J. E., and Kennedy, E. P. (1977) Rapid transmembrane movement of newly synthesized phospholipids during membrane assembly, *Proc. Natl. Acad. Sci. U.S.A. 74*, 1821−1825.

45. Langley, K. E., and Kennedy, E. P. (1979) Energetics of rapid transmembrane movement and of compositional asymmetry of phosphatidylethanolamine in membranes of *Bacillus megaterium*, *Proc. Natl. Acad. Sci. U.S.A. 76*, 6245−6249.

46. Hrafnsdottir, S., Nichols, J. W., and Menon, A. K. (1997) Transbilayer movement of fluorescent phospholipids in *Bacillus megaterium* membrane vesicles, *Biochemistry 36*, 4969−4978.

47. Huijbregts, R. P., de Kroon, A. I., and de Kruijff, B. (1998) Rapid transmembrane movement of newly synthesized phosphatidylethanolamine across the inner membrane of *Escherichia coli*, *J. Biol. Chem. 273*, 18936−18942.

48. DeChavigny, A., Heacock, P. N., and Dowhan, W. (1991) Sequence and inactivation of the *pss* gene of *Escherichia coli*, *J. Biol. Chem. 266*, 5323−5332.

49. McIntyre, J. C., and Sleight, R. G. (1991) Fluorescence assay for phospholipid membrane asymmetry, *Biochemistry 30*, 11819−11827.

50. Huijbregts, R. P., de Kroon, A. I., and de Kruijff, B. (1996) Rapid transmembrane movement of C6-NBD-labeled phospholipids across the inner membrane of *Escherichia coli*, *Biochim. Biophys. Acta 1280*, 41−50.

51. Kubelt, J., Menon, A., Muller, P., and Herrmann, A. (2002) Transbilayer movement of fluorescent phospholipid analogues in the cytoplasmic membrane of *Escherichia coli*, *Biochemistry 41*, 5605−5612.

52. Watkins, W. E., and Menon, A. K. (2002) Reconstitution of phospholipid flippase activity from *E. coli* inner membrane: a test of the protein translocon as a candidate flippase, *Biol. Chem. 383*, 1435−1440.

53. Backer, J. M., and Dawidowicz, E. A. (1987) Reconstitution of a phospholipid flippase from rat liver microsomes, *Nature 327*, 341−343.

54. Hrafnsdottir, S., and Menon, A. K. (2000) Reconstitution and partial characterization of phospholipid flippase activity from detergent extracts of the *Bacillus subtilis* cell membrane, *J. Bacteriol. 182*, 4198−4206.

55. Menon, A. K., Watkins, W. E. I., and Hrafnsdottir, S. (2000) Specific proteins are required to translocate phosphatidylcholine bidirectionally across the endoplasmic reticulum, *Curr. Biol. 10*, 241−252.

56. Gummadi, S. N., and Menon, A. K. (2002) Transbilayer Movement of Dipalmitoylphosphatidylcholine in Proteoliposomes Reconstituted from Detergent Extracts of Endoplasmic Reticulum, *J. Biol. Chem. 277* (28), 25337−25343.

57. Nicolson, T., and Mayinger, P. (2000) Reconstitution of yeast microsomal lipid flip-flop using endogenous aminophospholipids, *FEBS Lett. 476*, 277−281.

58. Hechard, Y., and Sahl, H. G. (2002) Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria, *Biochimie 84*, 545−557.

59. Shai, Y. (2002) Mode of action of membrane active antimicrobial peptides, *Biopolymers 66*, 236−248.

60. Kol, M. A., de Kroon, A. I., Rijkers, D. T., Killian, J. A., and de Kruijff, B. (2001) Membrane-spanning peptides induce phospholipid flop: a model for phospholipid translocation across the inner membrane of *E. coli*, *Biochemistry 40*, 10500−10506.

61. Demmers, J. A., van Duijn, E., Haverkamp, J., Greathouse, D. V., Koeppe, R. E., Jr., Heck, A. J., and Killian, J. A. (2001) Interfacial positioning and stability of transmembrane peptides in lipid bilayers studied by combining hydrogen/deuterium exchange and mass spectrometry, *J. Biol. Chem. 276*, 34501−34508.

62. de Planque, M. R., Boots, J. W., Rijkers, D. T., Liskamp, R. M., Greathouse, D. V., and Killian, J. A. (2002) The effects of hydrophobic mismatch between phosphatidylcholine bilayers and transmembrane α-helical peptides depend on the nature of interfacially exposed aromatic and charged residues, *Biochemistry 41*, 8396−8404.

63. Rinia, H. A., Boots, J. W., Rijkers, D. T., Kik, R. A., Snel, M. M., Demel, R. A., Killian, J. A., van der Eerden, J. P., and de Kruijff, B. (2002) Domain formation in phosphatidylcholine bilayers containing transmembrane peptides: specific effects of flanking residues, *Biochemistry 41*, 2814−2824.

64. Weiss, T. M., Van Der Wel, P. C., Killian, J. A., Koeppe, R. E., Jr., and Huang, H. W. (2003) Hydrophobic Mismatch between Helices and Lipid Bilayers, *Biophys. J. 84*, 379−385.

65. De Planque, M. R., and Killian, J. A. (2003) Protein−lipid interactions studied with designed transmembrane peptides: role of hydrophobic matching and interfacial anchoring, *Mol. Membr. Biol. 20*, 271−284.

66. Bretscher, M. S., and Munro, S. (1993) Cholesterol and the Golgi apparatus, *Science 261*, 1280−1281.

67. Kol, M. A., van Dalen, A., de Kroon, A. I., and de Kruijff, B. (2003) Translocation of phospholipids is facilitated by a subset of membrane-spanning proteins of the bacterial cytoplasmic membrane, *J. Biol. Chem. 278*, 24586−24593.

68. Heginbotham, L., Odessey, E., and Miller, C. (1997) Tetrameric stoichiometry of a prokaryotic K+ channel, *Biochemistry 36*, 10335−10342.

69. Doerrler, W. T., Reedy, M. C., and Raetz, C. R. (2001) An *Escherichia coli* mutant defective in lipid export, *J. Biol. Chem. 276*, 11461−11464.

70. Chang, G., and Roth, C. B. (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters, *Science 293*, 1793−1800.

71. van Meer, G., Poorthuis, B. J., Wirtz, K. W., Op den Kamp, J. A., and van Deenen, L. L. (1980) Transbilayer distribution and mobility of phosphatidylcholine in intact erythrocyte membranes.

A study with phosphatidylcholine exchange protein, *Eur. J. Biochem. 103*, 283−288.

72. Kol, M. A., de Kruijff, B., and de Kroon, A. I. (2002) Phospholipid flip-flop in biogenic membranes: what is needed to connect opposite sides, *Semin. Cell Dev. Biol. 13*, 163−170.

73. Brown, D. A., and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts, *J. Biol. Chem. 275*, 17221−17224.