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Review

Lipid flippases and their biological functions

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Abstract. The typically distinct phospholipid composition of the two leaflets of a membrane bilayer is generated and maintained by bi-directional transport (flip-flop) of lipids between the leaflets. Specific membrane proteins, termed lipid flippases, play an essential role in this transport process. Energy-independent flippases allow common phospholipids to equilibrate rapidly between the two monolayers and also play a role in the biosynthesis of a variety of glycoconjugates such as glycosphingolipids, *N*-glycoproteins, and glycosylphosphatidylinositol (GPI)-anchored proteins. ATP-dependent flippases, including members of a conserved subfamily of P-type ATPases and ATP-binding cassette transporters, mediate the net transfer of specific phospholipids to one leaflet of a membrane and are involved in the creation and maintenance of transbilayer lipid asymmetry of membranes such as the plasma membrane of eukaryotes. Energy-dependent flippases also play a role in the biosynthesis of glycoconjugates such as bacterial lipopolysaccharide. This review summarizes recent progress on the identification and characterization of the various flippases and the demonstration of their biological functions.

Keywords. Membrane assembly, membrane asymmetry, lipid flip-flop, *N*-glycosylation, ABC transporter, P-type ATPase, scramblase, vesicle budding.

Introduction

Eukaryotic cells are compartmentalized into distinct organelles by lipid bilayers. Assembly and maintenance of the various organellar membranes requires translocation of lipids from one leaflet of the bilayer to the opposing leaflet. Most phospholipids are synthesized in the endoplasmic reticulum (ER) membrane: since synthesis occurs at the cytoplasmic leaflet of the ER, roughly half of the newly synthesized lipid molecules must flip to the other side of the membrane to enable uniform propagation of the bilayer. A similar situation occurs in bacteria where phospholipids are synthesized on the inner face of the bacterial cytoplasmic membrane (bCM), and must be flipped to the external face for bilayer propagation. Mitochondria derive most of their membrane lipids from the ER through a non-vesicular pathway that exploits regions of close membrane contact between the two organelles [1, 2]: ER-derived lipids arriving at the mitochondrion's surface must flip across the mitochondrial outer membrane to gain access to the organelle interior [3]. Lipids are unequally distributed across both leaflets of the plasma membrane (PM), with aminophospholipids concentrated in the cytoplasmic leaflet. This compositional asymmetry cannot be explained by sidedness of lipid synthesis or breakdown and is thought to rely on active transport of specific lipids across the bilayer. Current data support a role for specific membrane proteins, termed 'lipid flippases', in facilitating the energetically unfavorable movement of a lipid's polar head group through the hydrophobic membrane interior. An ATP-independent flippase operating at the ER plays a critical role in bilayer

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propagation; ATP-dependent transporters regulate the transbilayer distribution of lipids between the two leaflets of the PMs. This review summarizes new information on this topic, focusing mainly on eukaryotic cells, while providing an overview of what is known about flippases and their biological functions.

Lipid flippases and membrane assembly

Flippase-mediated glycerophospholipid flip-flop in biogenic membranes

Most of the enzymes of glycerophospholipid biosynthesis in eukaryotic cells are membrane proteins located in the ER, a biogenic (self-synthesizing) membrane. (We use the terms glycerophospholipid and phospholipid interchangeably in this review. Phospholipids such as glycosylphosphatidylinositols, sphingomyelin or intermediates in the dolichol cycle of protein *N*-glycosylation are referred to explicitly.) The enzymes have their active sites in the cytoplasm and use cytoplasmically synthesized components for lipid synthesis. For example, cholinephosphotransferase, a polytopic membrane protein, synthesizes the abundant phospholipid phosphatidylcholine (PC) from diacylglycerol and cytoplasmically synthesized CDP-choline on the cytoplasmic face of the ER (Fig. 1). An identical scenario exists in other biogenic membranes such as the bacterial cytoplasmic membrane where, for example, the major bacterial phospholipid phosphatidylethanolamine is synthesized in two steps by phosphatidylserine (PS) synthase and PS decarboxylase on the cytoplasmic face of the bCM. The membrane topology of phospholipid biosynthesis dictates that newly synthesized phospholipids are located in the cytoplasmic leaflet of biogenic membranes. At least some of these molecules must be translocated (flipped) across the bilayer to populate the exoplasmic leaflet for uniform membrane propagation (Fig. 1). Furthermore, flipping must occur rapidly, on a time-scale commensurate with cell growth. It is likely that lipid flipping is necessary even in bolalipid-rich archaebacterial membranes with a predominantly monolayer architecture: since tetraether bolalipids are synthesized by condensation of two diether 'conventional' lipids, at least one of these must be flipped to provide a partner for the condensation reaction [4].

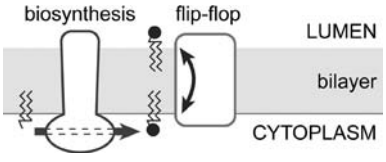

Figure 1. Lipid flippases in biogenic membranes. In biogenic membranes, such as the ER or bCM, active sites of phospholipid synthases are oriented towards the cytoplasm. Newly synthesized lipids are initially located within the cytoplasmic leaflet and rapidly equilibrated between both leaflets by membrane proteins acting independently of ATP. The flipping machinery translocates most, if not all, phospholipid classes, bi-directionally across the bilayer.

Transbilayer translocation (flipping) of phospholipids is energetically unfavorable because of the large energy barrier (20–50 kcal/mol) that has to be overcome to translocate the polar headgroup through the hydrophobic interior of the bilayer. Indeed, experiments with synthetic bilayers (*e.g.* [5–7]), or certain protein-containing non-biogenic biomembranes (*e.g.* animal cell PM, viral envelopes [8–10]), show that spontaneous phospholipid flip-flop occurs very slowly (half-times in the order of hours to days). However, phospholipid flip-flop in biogenic membranes is rapid (half-times about tens of seconds to minutes, [11–16]), suggesting that these membranes are endowed with a specific transport mechanism. There is an ongoing debate concerning what this mechanism might be. It has been proposed, for example, that non-bilayer arrangements of phospholipids [17], transient defects in bilayer structure, or non-protein biogenic membrane components such as isoprenoid lipids could be responsible for facilitating flip-flop [18]. It has also been noted that phospholipid flipping rates increase sharply at the gel-liquid crystalline phase transition in synthetic liposomes composed of a single molecular species of phospholipid [6, 19]. While these 'lipid-only' mechanisms are plausible, available data indicate that if they play any role in lipid flipping in biomembranes then it is only in concert with specific membrane proteins. There have been innovative proposals that peptides corresponding to transmembrane helices of membrane proteins can induce phospholipid flipping when incorporated into liposomes; however, experiments in support of this statement show that peptide-induced flipping is not especially fast (half-times about tens of minutes to hours) and that only certain phospholipids can be flipped [20, 21]. For example, the peptides tested could not facilitate flipping of the common phospholipid PC, indicating that the mere presence of membrane-embedded helices is not sufficient to promote generalized lipid flipping. However, it is possible that specific transmembrane peptide sequences may be required to flip particular phospholipids and that a wider range of peptides would need to be tested to identify those that promote PC flip-flop. In a variation of the idea of peptide-induced flipping, it has been proposed that any membrane proteins in the ER – or the mere presence of many membrane proteins – could promote lipid flip-flop, and that this effect is eliminated at the PM due to the high sterol content of that membrane [21, 22]. Although there are convincing data that argue against the possibility that flipping is promoted nonspecifically by membrane proteins, it remains a possibility that the sterol content of membranes such as the PM may play a role in reducing flip-flop. There are a few examples of chemically induced phospholipid flipping in protein-free liposomes where a

decrease in ambient pH [23–26], or the application of a small molecule capable of complexing specifically with phospholipid headgroups [27–29], reduces headgroup polarity and increases transbilayer translocation. Although the latter approach provides unique molecular tools for probing phospholipid flip-flop, it is unlikely that phospholipid flip-flop in a living cell proceeds by complexation of the lipid headgroup with a water-soluble molecule. Current data argue strongly for the idea that biogenic membranes possess a specific membrane protein, or flippase, that is capable of promoting glycerophospholipid flip-flop.

Phospholipid flip-flop in biogenic membranes is a rapid, ATP-independent, bi-directional process that is un-specific with regard to phospholipid headgroup: all the major phospholipids – PC, phosphatidylethanolamine (PE), PS, and phosphatidylinositol (PI) – are flipped [13, 12, 15, 30, 31]. Moreover, this process is also unspecific towards the glycerol backbone stereochemistry of the lipid: PI analogs with *sn*-1,2 diacylglycerol or *sn*-2,3-diacylglycerol backbones were recently shown to be flipped equally well across the ER membrane [16]. Attempts by many investigators to clearly establish the protein dependence of flipping by treating membrane preparations with proteases or protein modification reagents (such as the cysteine modifying reagent *N*-ethylmaleimide, NEM) yielded mixed results – typically no inhibition (*e.g.* [32, 33]) or only partial inhibition (*e.g.* [14, 30]) of flipping was seen. One explanation is that the flippase protein is largely buried in the membrane and that its functionally critical domains are inaccessible to proteases and other protein modification reagents. Another explanation for the non-effect or partial effect of protein modification reagents on flippase activity is that transmembrane elements of the flippase protein may be able to remain associated and functionally intact in the membrane even when exposed sequences of the protein are proteolytically cleaved or modified.

Biochemical reconstitution of phospholipid flippase activity and the search for a lipid flippases in biogenic membranes

Flippase activity in biogenic membranes was first described in the late 1970s [11, 34]. Although the activity has been extensively characterized during the past two decades, the flippase has yet to be identified. A first step towards flippase identification was taken in the late 1980s when Backer and Dawidowicz demonstrated that flippase activity, measured via an elegant density-shift method, could be reconstituted from detergent-solubilized rat liver ER; they also showed that protein-free vesicles or vesicles reconstituted with proteins from a non-biogenic membrane source were not active [33]. This approach was not pursued for over a decade until a series of papers published in the last few years established that flippase activity from rat liver and yeast ER, as well as the bCM of *Bacillus subtilis* and *E. coli*, could be readily reconstituted from Triton X-100 extracts of the membranes [35–38]. Furthermore, fractionation of the detergent extract prior to reconstitution yielded protein mixtures enriched in flippase activity and other fractions devoid of activity [33, 35, 37, 39, 40]. Velocity sedimentation analysis revealed that flippase sedimented slowly with an operational sedimentation coefficient of ~4S [35, 37]. Also, flippase activity could be destroyed with protein modification reagents (NEM and diethylpyrocarbonate) when the agents were applied to detergent-solubilized protein fractions rather than to intact vesicles [40]. The identification of fractions rich in membrane proteins but completely lacking flippase activity argues strongly that a specific protein is required to promote flip-flop and that the 'lipid-only' mechanisms described above are unlikely. Estimates from the reconstitution experiments indicate that flippase represents ~0.5% by weight of detergent-solubilized ER proteins [33, 35]. This suggests that a 200-fold enrichment of flippase activity should yield a pure protein. The ability to use standard chromatographic procedures to generate flippase-enriched fractions suggests that the identification of this enigmatic protein cannot be far off.

Possible mechanism for lipid flipping in biogenic membranes

Although a phospholipid flippase in biogenic membranes has yet to be identified, it is interesting to use available information speculate on how such a transporter might work to flip-flop phospholipids. Given its lack of stereospecificity and its ability to flip all major phospholipids, it is likely that the flippase does not specifically recognize the lipid it flips. An attractive mechanism [41–43] is that the flippase reorients phospholipids in its immediate vicinity to give rise to a hairpin-like, nonbilayer arrangement of phospholipids. This arrangement would effectively connect the two membrane leaflets allowing phospholipids to diffuse continuously between leaflets. An alternative view presented by Kol et al. [44] is to consider that transient defects generated by dynamic play in the transmembrane helices of the flippase would enable phospholipids to 'slip' into a mid-membrane transition state from which they could 'pop' into either leaflet of the membrane bilayer. We suggest a further variation on these ideas by proposing that the mechanism of action of a flippase may be likened to that of swiping a card through a card reader (Fig. 2). In this model the polar headgroup of the phospholipid (the magnetic strip on the card; the strip denotes all common phospholipid headgroups since the

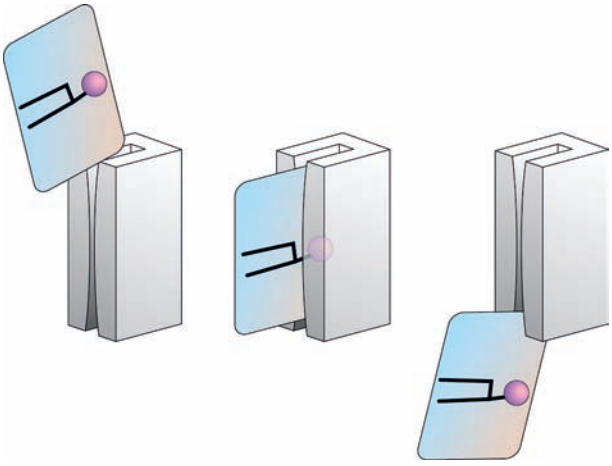

Figure 2. Model for substrate flipping by a lipid flippase in biogenic membranes. The translocation mechanism is imagined to resemble the swiping of a card through a card reader. The magnetic strip on the card (= polar headgroup of the phospholipid being transported) is protected from the lipid environment (by passage through the groove in the card reader) as it transits the hydrophobic interior of the membrane. See text for details and for a discussion of other possible flippase mechanisms.

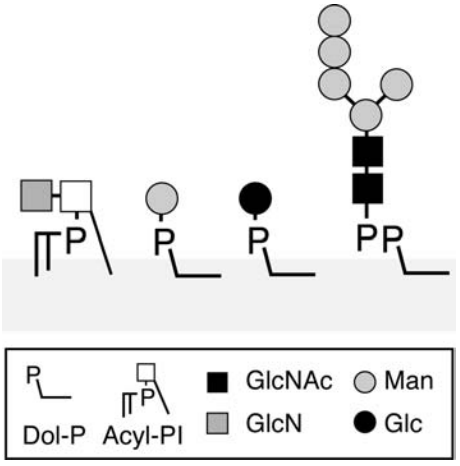

Figure 3. Glycolipids that are flipped across biogenic membranes. Protein and lipid glycosylation in eukaryotes and prokaryotes requires flipping of biosynthetic lipid intermediates such as glycosylphosphatidylinositol precursors (*e.g.* GlcN-acyl-PI), mannosyl-phosphodolichol, glucosyl-phosphoryldolichol, and an oligosaccharide-pyrophosphoryl-isoprenoid. Dol-P, dolichol phosphate; Acyl-PI, acyl-phosphatidylinositol; GlcNAc, N-acetylglucosamine; GlcN, glucosamine; Man, mannose; Glc, glucose.

flippase is unspecific) is protected during passage across the hydrophobic interior of the membrane until it emerges on the other side; the acyl chains of the lipid remain in the hydrophobic milieu of the membrane during this process. Thus the groove of the flippase/card-reader provides a low energy path for the lipid headgroup by sequestering it from the unfavorable hydrophobic environment of the membrane interior. Tests of these models awaits identification of the flippase.

Glycolipid flipping in the ER and bCM

Flippases are also required for the ER- and bCM-localized biosynthesis of glycolipid precursors of cell surface glycoconjugates such as *N*-glycosylated and glycosylphosphatidylinositol (GPI)-anchored proteins in eukaryotes and O-antigen-modified lipopolysaccharide (LPS) in Gram-negative organisms. In each case, biosynthesis of the precursor glycolipid occurs via a multi-step pathway that starts in the cytoplasmic leaflet of the ER or bCM and continues in the exoplasmic/periplasmic leaflet. This split topology necessitates transbilayer translocation of lipid intermediates, such as isoprenoid-P(P)-sugars, or GPIs (Fig. 3). Assays developed to monitor flipping of mannosyl-phosphoryldolichol, glucosyl-phosphoryldolichol and early GPI biosynthetic intermediates (*N*-acetylglucosamine-PI and glucosamine-PI; GlcNAc-PI and GlcN-PI) in ER membranes and reconstituted vesicles indicate that transbilayer translocation of these glycolipids is protein dependent and ATP independent [45–48]. In the case of the GPI intermediates, the flippase involved may be the same protein that facilitates glycerophospholipid flip-flop in the ER [48].

Genetic studies in yeast [49] and bacteria [50] have identified polytopic membrane proteins (Rft1p and Wzx, respectively) that appear to act as flippases for isoprenoid-PP-sugar lipids during the assembly of the glycolipid precursor of protein *N*-glycans and LPS O-antigen, respectively. Neither of these proteins has nucleotide binding domains, consistent with the reported ATP-independence of flipping. The Rft1 protein is essential in yeast and is found in all eukaryotes that synthesize mannosylated dolichol-PP-oligosaccharide precursors for consumption in protein *N*-glycosylation [51]. The flippase function of Rft1p remains to be biochemically established. Unlike Rft1p, Wzx is dispensable since it participates in the synthesis of a non-essential element (O-antigen) of the LPS structure. Using a radiolabeled, water-soluble analog of undecaprenol-PP-*N*-acetylglucosamine as reporter and membranes from Wzx-containing and Wzx-null bacterial cells, Rick and colleagues [52] provided evidence that the Wzx protein acts as an ATP-independent, bi-directional flippase in the assembly of the lipid precursor of O-antigen. Recent work on bacterial glycosylation suggests that the Wzx protein can be functionally replaced *in vivo* with the bacterial ATP-binding cassette (ABC) transporter PglK [53]. This is an unexpected result since lipid transport facilitated by the Wzx protein is bi-directional, whereas the PglK protein would be expected to function as a vectorial transporter. One way to reconcile these data is to suggest that lipid transport facilitated by Wzx is operationally unidirectional since lipids flipped from the cytoplasmic to the periplasmic face of the membrane would

be consumed by biosynthetic enzymes oriented towards the periplasm.

Flipping of glycolipids is also required for the Golgi-localized synthesis of glycosphingolipids. Glucosylceramide, the precursor of higher glycosphingolipids, is synthesized on the cytoplasmic face of the Golgi and must be flipped to the luminal face to be converted to lactosylceramide, a lipid that is then further elaborated in the Golgi lumen to generate a spectrum of glycosphingolipid species. Little is known about this flipping event except that it is ATP independent, specific for glucosylceramide (lactosylceramide is not flipped), and distinct from the phospholipid flippase activity discussed above [54].

Lipid flippases and membrane lipid asymmetry

Phospholipid flip-flop in the PM of eukaryotic cells

Phospholipid flipping in the ER is unspecific and serves to randomize the transbilayer distribution of phospholipids. Although there are no clear data on transbilayer phospholipid asymmetry in the ER [55], it is likely that the bulk compositional lipid asymmetry in this membrane is weak. In contrast to the situation in the ER, flip-flop of phospholipids in the PM of eukaryotic cells is highly regulated so that cells are able to maintain a nonrandom distribution of phospholipids across the PM [56, 57]. In general, the aminophospholipids PS and PE are restricted to the cytoplasmic leaflet, whereas PC, sphingomyelin and glycosphingolipids are enriched in the exoplasmic leaflet. This asymmetric lipid arrangement is thought to come about as a result of the action of energy-dependent flippases (P-type ATPases and ABC transporters; see below) that use ATP hydrolysis to move specific lipids against a concentration gradient (Fig. 4). In contrast to these energy-dependent flippases, the PM of certain cells contains a phospholipid scramblase activity that, when activated (typically, although not always, by elevation of cytoplasmic calcium concentration [58]), facilitates bi-directional movement of phospholipids that disrupts the PM lipid asymmetry set up by the ATP-dependent flippases [59–61]. Scramblase activity displays some lipid selectivity [62, 63]; this feature, together with the requirement for activation distinguishes scramblase activity from the constitutive phospholipid flippase activity found in the ER and other biogenic membranes.

The regulation of the nonrandom transbilayer lipid distribution at the PM of eukaryotic cells by the concerted action of three classes of lipid flippases is important for a variety of cellular functions. First, lipid asymmetry and its rapid change, *e.g.* by activation of the scramblase, provide a system to modulate the biological activity of the exoplasmic membrane leaflet. For example, exposure of PS on the cell surface signifies senescence and apoptosis,

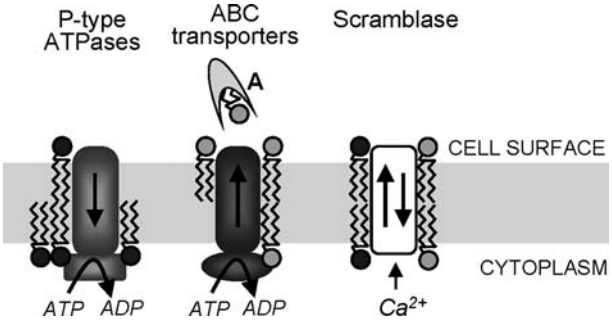

Figure 4. Lipid flippases in the PM of eukaryotic cells. In the PM of eukaryotic cells, flip-flop of phospholipids is constrained owing to the absence of constitutive bi-directional flippases. Thus, ATP-dependent flippases can maintain an asymmetric phospholipid distribution by moving specific lipids towards (P4-ATPase family members) or away from the cytosolic leaflet (ABC transporters). Alternatively, many ABC transporters might function in lipid exposure to an acceptor (A) rather than in the maintenance of membrane lipid asymmetry. Cellular activation triggered by cytosolic calcium can collapse the lipid asymmetry by the transient activity of an ATP-independent scramblase.

and results in engulfment of the cells by macrophages bearing PS receptors [64, 65]. There is also evidence that appearance of PS in the exoplasmic leaflet of erythrocytes invokes abnormal adherence to vascular endothelial cells [66]. In blood coagulation, rapid cell surface exposure of PS is an essential determinant in the assembly of coagulation factors on the activated platelet membrane [67]. Surface exposure of PS at the PM has also been observed during sperm capacitation [68] and myotube formation [69], and seems to be important for these fusion events. In the canalicular membrane of hepatocytes, lipid asymmetry is essential for preserving the specific phospholipid composition of bile [70]: although aminophospholipids and PC each represent some 35% of the canalicular membrane lipids, aminophospholipids are virtually absent from bile, whereas PC accounts for 95% of bile phospholipids.

Apart from these functions of an asymmetric lipid distribution in specific cells, the transfer of lipids from one leaflet to the other in cellular membranes appears to be of general significance for the functioning of individual cells. During cell division, PE is transiently exposed on the cell surface of the cleavage furrow. Immobilization of PE by a PE-binding peptide inhibits disassembly of the contractile ring, thereby preventing the final separation of daughter cells [71, 72]. These findings suggest that local redistribution of PE across the PM is essential for progression of cytokinesis. Furthermore, a dynamic regulation of the transbilayer lipid arrangement might act as a mechanism for signal transduction by modulating the activity of membrane proteins [73] and be crucial for membrane budding as discussed below.

P-type ATPases as potential energy-dependent inward lipid flippases

Work done in the mid-1980s demonstrated that human erythrocytes were capable of rapidly flipping exogenously added PS and PE to the cytoplasmic leaflet of the PM. PC was not transported, indicating that the activity was specific for aminophospholipids [10, 74]. Although first discovered in erythrocytes, aminophospholipid translocase activity is also found in the PM of many nucleated cells [75] as well as in membranes of chromaffin granules [76], synaptic vesicles [77], the *trans* Golgi network and post-Golgi secretory vesicles of budding yeast [78, 79]. Purification of aminophospholipid translocase activity from bovine chromaffin granules identified a protein known as ATPase II that displayed a striking similarity to Drs2p of *Saccharomyces cerevisiae* [80]. Drs2p is a member of the P4-ATPase subfamily of P-type ATPases [81]. Yeast cells in which the *DRS2* gene was deleted were shown to lack low temperature uptake of a fluorescently-tagged PS analog at the PM, further supporting a role for the ATPase II/Drs2p protein in aminophospholipid translocation [80, 82]. However, the function of Drs2p as an aminophospholipid translocase was subsequently questioned, since the uptake defect could not be confirmed in two other independent studies [83, 84]. The discrepancy in these observations was accounted for by the finding that Drs2p is primarily associated with the *trans* Golgi rather than with the PM [85]. This result indicates that, while Drs2p may play a role in lipid translocation at the PM, it is unlikely to be the only phospholipid translocase in the yeast PM. It is more likely that Drs2p has an indirect influence on lipid translocation at the PM since it appears to be involved in protein export from the *trans* Golgi [85–87], and may regulate the delivery of other lipid translocases to the PM. In fact, subsequent to the identification of Drs2p, two other proteins of the P4-ATPase subfamily were identified. These proteins, Dnf1p and Dnf2p, were found to be essential for the ATP-dependent inward transport of aminophospholipids at the yeast PM [88]. Unexpectedly, fluorescently tagged PC was also translocated, similar to the situation in some mammalian cells [89–91], indicating that members of the P4-ATPase family differ in their substrate specificity and that not all of them are specific for aminophospholipids. Notably, uptake of fluorescent lipid analogues in yeast has been reported to depend on the proton electrochemical gradient across the PM [92], which might be required for proper functioning of Dnf1p and Dnf2p. Moreover, recent data suggest additional protein-dependent flip mechanisms in the yeast PM [93].

Further evidence of a role for P4-ATPases as lipid transporters derives from the recent demonstration that Drs2p is required for translocation of aminophospholipids from the luminal to the cytosolic leaflet of late Golgi membranes and post-Golgi secretory vesicles [78, 79]. Importantly, loss of the Golgi-associated P4-ATPases Drs2p and Dnf3p, proved sufficient to abolish the asymmetric arrangement of endogenous PE in post-Golgi secretory vesicles [79]. These findings point to an essential role of P4-ATPases in generating and maintaining aminophospholipid asymmetry during membrane flow through the Golgi. Additional P4-ATPase family members have been identified and associated with lipid translocation in parasites [94], plants [82], mice spermatozoa [95] and mammalian cells [96]. Two members of the P4-ATPases (bovine ATP8A1 and murine Atp8a1) were purified and shown to be specifically stimulated by PS [97–99]. Collectively, these data support the proposed flippase activity of P4-ATPases, but do not rule out the alternative model in which they have an indirect role in lipid translocation by regulating either activity or localization of the proteins directly responsible for lipid translocation and membrane asymmetry. Unequivocal demonstration for a direct role in lipid transport will require reconstitution of purified P4-ATPases in model membranes.

In yeast, interestingly, mutations of an essential gene family (Lem3, Cdc50 and YNR048) that encodes integral membrane proteins, result in phenotypes that are similar to dnf1,2 and drs2 mutations. The disruption of the LEM3 gene causes a defect in the uptake of fluorescent analogues of PE and PC across the PM, while, surprisingly, translocation of the PS analogue is unaffected [100, 101]. Members of this protein family show no significant homology with P-type ATPases or other known transporters. They might have a regulatory function for the P4-ATPases and represent integral components of the translocation machinery, analogous to the β-subunit of Na^+, K^+-ATPases and H^+, K^+-ATPases (for a review see [102]). Indeed, Lem3p and Cdc50p were shown to interact with Dnf1p and Drs2p, respectively, and this association was demonstrated to be required for their correct localization [103]. Whether the proteins of the Lem3-Cdc50 family are also directly involved in the translocation process or in substrate recognition remains to be established.

ABC transporters: energy-dependent outward lipid flippases or lipid exporter?

A second class of ATP-dependent flippases, which may include members of the ABC transporter family, is apparently responsible for an active outward transport of lipids from the cytoplasmic leaflet to the exoplasmic leaflet of the PMs [104–107]. ABC transporters are members of a large family of evolutionarily conserved transmembrane proteins that transport a broad range of substrates, including ions, sugars, drugs and peptides across cellular membranes. First hints for a role of some family members in

the outward movement of lipids across the PM came from the finding that PC secretion into mouse bile required ABCB4 [108] and that this liver transporter enhanced transport of newly synthesized PC to the surface of transgenic fibroblasts [109]. In studies on short chain lipids, human ABCB4 was found to be specific for PC, whereas, unexpectedly, the closely related multidrug transporter ABCB1 translocated a wide variety of short chain lipids [110–113], including the short chain PC platelet-activating factor [114, 115]. The glutathione-dependent multidrug transporter ABCC1 transported short-chain PC, PS, sphingomyelin, and glucosylceramide analogs and has been suggested to maintain the outward orientation of natural choline phospholipids to the PM [116–119]. However, whether ABCB1 and -C1 translocate natural long chain lipids and whether this is physiologically relevant remains unclear. Likewise, members of these subfamilies in yeasts translocated various short-chain lipids to the outer face of the PM [120, 121], and the overexpression of these transporters caused endogenous aminophospholipids to accumulate at the cell surface [88, 122]. Many of these ABC transporters have also been implicated in the development of drug resistance. This suggests that the mechanism by which drugs are extruded from cells is closely related to the flippase mechanism by which lipids are translocated across membranes and that lipid translocation and drug transport take place through the same path in the transporters. However, evidence for this has only be provided so far with various short-chain fluorescent lipid derivatives [113].

Other ABC transporters are involved in sterol transport. This is unexpected since cholesterol with its small headgroup flips across membranes rapidly [123]. Mutations in ABCA1 cause impaired efflux of cholesterol and phospholipids across the PM to exogenous apolipoproteins [124–126]. Initially, a direct role of ABCA1 in sterol efflux was suggested but subsequent studies indicate that it might act as a PS transporter generating a microenvironment to facilitate binding of apolipoproteins [127–129]. In this context, it is also possible to speculate that the function of ABCA1 is not directly related to sterol flipping but rather to presentation of sterol to potential acceptors [130]. Alternatively, ABCA1 might mediate transport of PC as suggested by the recent demonstration of PC-stimulated ATPase activity of the purified protein [131].

Similarly, other members of the ABCA subfamily have been associated with the efflux of lipids. ABCA3 [132] is required for transporting lipid molecules, mostly saturated PC, to the lung surfactant membranes filling the lamellar bodies in lung epithelial type II cells [133, 134]. ABCA7 was shown to promote efflux of phospholipid and, to a lesser extent, cholesterol to apolipoproteins, when overexpressed in a fibroblast cell line [135, 136]. ABCA12 is needed for the transport of lipids, mainly glucosylceramide, to the membranes within the lamellar bodies in keratinocytes [137–139]. Two half-size ABC transporters ABCG5 [140] and ABCG8 [141], which are highly expressed in epithelial cells of the intestine and probably act as a heterodimer [142], have been linked to the efflux of plant sterols and cholesterol into bile [143], while ABCG1 and G4 are held responsible for transport of cholesterol onto high density lipoprotein particles [144–147]. Among these subfamilies of proteins candidate flippases have also been identified in many other eukaryotes, including an ABCG-like subfamily member in Arabidopsis involved in wax secretion on the stem surface [148], and ABCA-like members in the protozoan parasite *Leishmania* with a role in phospholipid trafficking [149–151].

Eukaryotes might not only express outward directed ABC transporters. In the yeast *Candida albicans*, a subfamily member (Cdr3p) has been identified that exhibits an inward-directed phospholipid translocase activity [121]. Two other ABC transporters (*S. cerevisiae* Aus1p and Pdr11p) facilitate exogenous sterol uptake by increasing the cycling of sterol between the PM and ER [152, 153]. In humans, a putative energy-dependent inward flippase is ABCA4. This photoreceptor cell-specific transporter has been associated with Stargardt macular dystrophy, a retinal degenerative disease that is accompanied by the defective transport of retinal PE derivatives from the luminal to the cytosolic leaflet of the outer-segment disc membrane [154–156]. The presence of CFTR/ABCC7 has been correlated with an increased uptake of the signaling lipids sphingosine-1-phosphate and lysophosphatidic acid [157, 158].

Finally, ABC transporters are also widely expressed in prokaryotes and some bacterial ABC transporters are attractive candidates for lipid flippases. One of these proteins, PglK, is implicated in the transport of isoprenoid-linked oligosaccharides as discussed above. Another ABC protein, MsbA [159], is an essential inner membrane transporter in Gram-negative *Escherichia coli* that is genetically linked to the export of the Lipid A core of lipopolysaccharides to the bacterial outer membrane [160–162]. Although MsbA has also been suggested to play a role in glycerophospholipid transport [161], available data suggest that this is not likely to be the case [22, 163].

A major unresolved question concerns the precise nature of lipid transport mediated by the various ABC transporter candidate flippases. Depending on the type of transporter essentially two different mechanisms can be envisaged [164, 165]. The flippase could bind the lipid substrate in the cytoplasmic leaflet and flip its polar head group across the membrane to deliver the molecule to the exoplasmic leaflet. This type of mechanism is supported by a few studies on natural long chain lipids: (i) ABCB1, Yor1p, Pdr5p and ABCA1 expressing cells exhibit an en-

hanced exposure of aminophospholipids on the outer PM leaflet, suggesting that these ABC transporter flip natural aminophospholipids towards the cell surface [88, 122, 127, 129]; (ii) in erythrocytes from ABCB1- or ABCB4-knockout mice, natural PC cell surface translocation was reduced [107].

Alternatively, the transporter could flip the molecule to present it for release to an acceptor. In this case, the transporter would be involved in lipid efflux rather than in the maintenance of membrane lipid asymmetry and even required for rapidly flipping lipids such as cholesterol. The primary function of ABCG5/8 may therefore very likely not be cholesterol transbilayer transport across the canalicular membrane, but rather facilitation of luminal cholesterol uptake (*e.g.* by mixed bile salt and PC micelles), possibly by pushing it partly into the aqueous phase. Whether such a mechanism indeed applies to ABCG5/8 and other transporters remains to be established. Interestingly, ABCA1 has been shown to directly interact with lipid-free apolipoproteins and this interaction is required for lipid efflux [128, 166].

Energy-dependent lipid flippases and vesicle formation

Several lines of evidence indicate that phospholipid translocation by ATP-dependent flippases in the PM, late Golgi and endosomal compartment is required for the formation of intracellular transport vesicles. For example, yeast lacking the two PM associated P4-ATPases, Dnf1p and Dnf2p, display a cold-sensitive defect in the biogenesis of endocytic vesicles [88] and inactivation of Drs2p results in a decrease in clathrin-coated vesicle budding from the trans-Golgi [86, 78]. Conversely, overexpression of ABC transporters with outward directed lipid translocase activity causes a defect in endocytosis [167, 120], and loss of ABCA1 function in Tangier fibroblasts is associated with enhanced endocytosis [168].

How would ATP-dependent transporters participate in vesicle biogenesis? One possibility is that a high concentration of specific phospholipids (PS, PE) in the cytosolic leaflet is required for the efficient recruitment of the vesicle-budding machinery. However, the requirement of an asymmetric PS distribution and the recruitment of PS-binding proteins to the *trans* Golgi network for vesicle formation can be ruled out since yeast strains that are unable to synthesize PS transport proteins normally via the secretory pathway and still require Drs2p to produce a specific class of secretory vesicles [78]. Moreover, removal of multiple P4-ATPases causes a marked decrease in the aminophospholipid content of cellular membranes [88]. This finding is hard to reconcile with the idea that maintenance of a high concentration of aminophospholipids in the cytosolic leaflet accounts for the requirement of P4-ATPases in vesicle formation, as down-regulation of aminophospholipid levels would have a counterproductive effect.

An alternative possibility is that ATP-dependent lipid flippases play a more direct and mechanical role in vesicle formation. The ATP-dependent transfer of lipids from one leaflet of the membrane to the other produces an area difference between the membrane leaflets (Fig. 5). According to the bilayer-couple mechanism [169], this area asymmetry will increase the spontaneous curvature of the bilayer, and may thus help deform the membrane during vesicle budding. Adopting a transbilayer lipid arrangement permissive for vesicle formation might not pose a problem to ER and *cis* Golgi membranes where phospholipids can rapidly cross the bilayer in both directions due to the presence of energy-independent, bi-directional flippases. Here, assembly of a protein coat and 'morphogenic' membrane proteins may exert a force sufficient to deform the bilayer into a bud [170]. In the PM, the late Golgi and endosomes, however, the free 'flip-flop' of phospholipids across the bilayer is constrained. In these organelles, it would be hard to accomplish the transbilayer lipid imbalance required for vesicle budding without assistance of ATP-dependent lipid flippases. This ATPase-dependent lipid transport might be particular important at low tem-

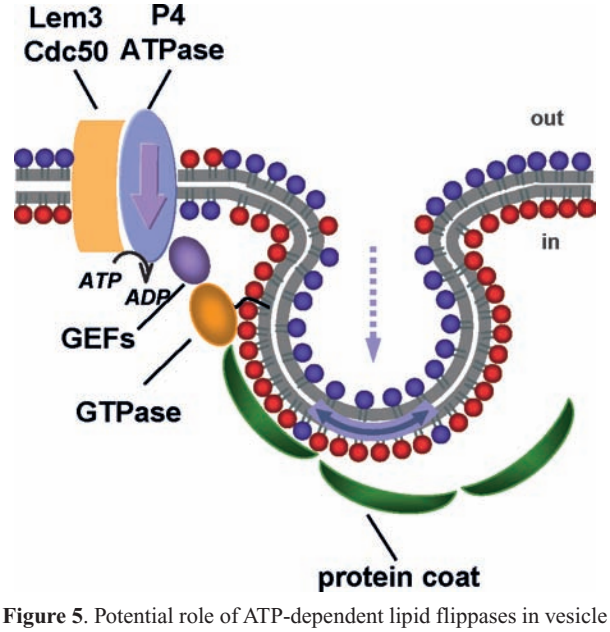

Figure 5. Potential role of ATP-dependent lipid flippases in vesicle formation. ATP-dependent lipid translocation might help deform the membrane by moving lipid mass towards the cytoplasmic leaflet. For P4-ATPases, proteins of the Lem3-Cdc50 family presumably represent a subunit regulating their localization and activity. Interaction of P4-ATPases with peripheral guanine nucleotide-exchange factors (GEFs) might cause activation of small GTPases, which subsequently bind to the membrane and facilitate the assembly of coat proteins such as clathrin. This would concentrate the vesicle budding machinery at sites of ATPase-dependent phospholipid translocation.

perature when a decreased fluidity of the membrane may prevent coat assembly from driving this process alone [79, 82, 88]. Direct participation of ATP-dependent lipid flippases in vesicle budding is supported by the observation that stimulation of the aminophospholipid translocase activity in red blood cells provokes the formation of endocytic vesicles [171, 172] and accelerates endocytosis in human erythroleukemia cells [173, 174]. In this model, coat assembly would determine the site on the membrane where budding occurs. Strikingly, family members of the P4-ATPases were found to interact with cytosolic proteins such as guanine nucleotide exchange factors and small GTPases that are crucial for the recruitment of coat proteins during membrane budding [85, 175, 176]. This may help concentrate the vesicle budding machinery at sites of ATPase-dependent phospholipid translocation.

Concluding remarks

Lipid flip-flop in cellular membranes seems to rely on two classes of lipid flippases. In early secretory organelles, as well as in the cytoplasmic membrane of bacteria, metabolic energy-independent flippases facilitate rapid flip-flop of lipids and allow them to equilibrate between the two membrane leaflets. These proteins appear to be absent from the PM, as well as the late Golgi and endosomal compartments. In these 'late' secretory organelles, ATP-dependent flippases are responsible for the net transfer of specific lipids to one side of the membrane, thus creating/maintaining transbilayer lipid asymmetry. Flippases involved in the rapid collapse of lipid asymmetry have not been identified, but a candidate phospholipid scramblase (PLSCR1) has been cloned from human erythrocytes [177]. Clearly, identification of the various lipid flippases remains a major challenge in current cell biology. The energy-dependent flippases belong to large protein families, making it necessary to map the subcellular localization of each family member, identify its substrates and determine how it is regulated. In many cases, final proof for their direct role in lipid transport remains to be obtained: this will require transport measurements of natural lipids by energy-dependent flippases reconstituted into proteoliposomes, a challenging task given the difficulties of purifying membrane proteins, the water insolubility of the substrates and the potential requirement for subunits and/or accessory proteins. Nevertheless, such studies are key to our understanding of the molecular mechanism of flippase action. The recent functional reconstitution of biogenic membrane flippases offers hope for similar studies with the ATP-dependent transporters. It will also be necessary to develop new approaches to measure transbilayer lipid movement such as assays based on shape changes of giant unilamellar vesicles that do not require labeled lipid analogues [178].

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