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INVITED REVIEW

P4-ATPases: lipid flippases in cell membranes

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Abstract Cellular membranes, notably eukaryotic plasma membranes, are equipped with special proteins that actively translocate lipids from one leaflet to the other and thereby help generate membrane lipid asymmetry. Among these ATP-driven transporters, the P4 subfamily of P-type ATPases (P4-ATPases) comprises lipid flippases that catalyze the translocation of phospholipids from the exoplasmic to the cytosolic leaflet of cell membranes. While initially characterized as aminophospholipid translocases, recent studies of individual P4-ATPase family members from fungi, plants, and animals show that P4-ATPases differ in their substrate specificities and mediate transport of a broader range of lipid substrates, including lysophospholipids and synthetic alkylphospholipids. At the same time, the cellular processes known to be directly or indirectly affected by this class of transporters have expanded to include the regulation of membrane traffic, cytoskeletal dynamics, cell division, lipid metabolism, and lipid signaling. In this review, we will summarize the basic features of P4-ATPases and the physiological implications of their lipid transport activity in the cell.

Keywords Flippase · Lipid asymmetry · P-type pump · CDC50 protein · Vesicle biogenesis · Importer

Introduction

Cellular membranes are organized as bilayers consisting of two leaflets, which are structurally formed by hundreds of different lipid species. In eukaryotic cells, the distribution of lipids is heterogeneous, and the membranes of different organelles have different lipid compositions. Furthermore, there are striking differences in the lipid distribution across the bilayer. Lipids in the late Golgi, endosome, and plasma membrane adopt an asymmetrical distribution with the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) concentrated in the cytosolic leaflet and phosphatidylcholine (PC) and the sphingolipids (i.e., sphingomyelin and glycosphingolipids) enriched in the exoplasmic leaflet [38, 96]. This lipid asymmetry has been implicated in numerous cellular processes. For example, asymmetric distribution of specific lipids may induce membrane curvature, a prerequisite for vesicle formation in the secretory pathway, and controlled disruption of lipid asymmetry is critical in physiological processes such as blood coagulation, apoptosis, cytokinesis, cell fusion, and host–virus interactions.

Growing evidence indicates that the asymmetric transbilayer distribution of lipids is largely determined by a diverse group of lipid translocators that use the energy of ATP hydrolysis to move specific lipids across the bilayer. These translocators include ATP-dependent flippases and floppases, which catalyze the inward movement of phospholipids from the extracellular/luminal leaflet to the cytoplasmic leaflet and the outward movement of other lipids, respectively [20, 25]. The specificity of each of these transporters defines the resulting asymmetry that is generated. Current biochemical evidence indicates that these proteins are primarily members of the P-type and ATP-binding cassette (ABC) family of transporters. In addition to these energy-dependent translocators, certain eukaryotic cells contain phospholipid scramblases; putative membrane proteins that upon activation facilitate a rapid bidirectional movement of phospholipids across the two plasma membrane leaflets, disrupting the lipid asymmetry created by the ATP-dependent translocators. The molecular identity of the scramblase activity has not been unequivocally established yet, but several candidates have been identified [79, 87, 88, 103, 112]. This review

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focuses on lipid flippases belonging to the P4 subfamily of P-type ATPases (P4-ATPases). Several excellent reviews have recently treated different aspects of P4-ATPases [18, 77, 86, 94]. In this review, we will first provide an overview on the functional implications of their activities for the cell and then highlight the current focus of some of the research efforts in understanding the transport mechanism of P4-ATPases.

P-type ATPase superfamily

The P-type ATPase family comprises a large number of evolutionarily related membrane-bound pumps with the common feature that they form a phosphorylated intermediate during their catalytic cycle, hence the designation P type (Fig. 1; [57]). Based on sequence similarity, the P-type ATPase family is divided into five subfamilies (P1–P5) with different transport specificities [58]. Among the members, heavy metal-transporting P1-ATPases are in charge of detoxifying the cytoplasm and loading of heavy metals in specific cell compartments. Prominent members of P2-ATPases include the Na^+/K^+-ATPase that maintains electrochemical gradients for Na^+ and K^+ across the plasma membrane of animal cells, the human H^+/K^+-ATPase which is primarily responsible for the acidification of the stomach contents, and the sarcoplasmic reticulum Ca^{2+}-ATPase which restores Ca^{2+} levels in the sarcoplasmic reticulum after muscle contraction. In both plants and fungi, plasma membrane-localized proton-pumping P3-ATPases extrude H^+ from the cell to generate a proton-motive force and a pH gradient across the plasma membrane. While P1-, P2-, and P3-ATPases are found in all three branches of life, members of the P4-ATPases are only present in eukaryotic organisms and represent by far the largest P-type ATPase subfamily. In humans, there are 14 genes that encode P4-ATPases, while in yeast, there are five: Drs2, Dnf1, Dnf2, Dnf3, and Neo1. Mutations in one of the human members of this subfamily, ATP8B1, give rise to the rare autosomal recessive diseases progressive familial intrahepatic cholestasis (PFIC12 or Byler disease) and the related but less severe benign recurrent intrahepatic cholestasis (BRIC1) [10]. These diseases result in defects in bile salt secretion in the liver canaliculi, leading to episodes of jaundice and severe pruritus, together with non-hepatic symptoms that can include growth defects, diarrhea, and hearing loss [45]. ATP8B1 is expressed in several epithelial tissues and, notably, in the canalicular membrane of the liver [28] and the stereocilia membrane in hair cells [83]. Studies in mouse models show that P4-ATPases fulfil multiple important physiological functions; deficiencies result in a wide variety of neurological phenotypes, liver disease, immunological problems, type 2 diabetes, and diet-induced obesity (for recent reviews, see [18, 94]). How all these distinct phenotypes relate to a defective flippase activity

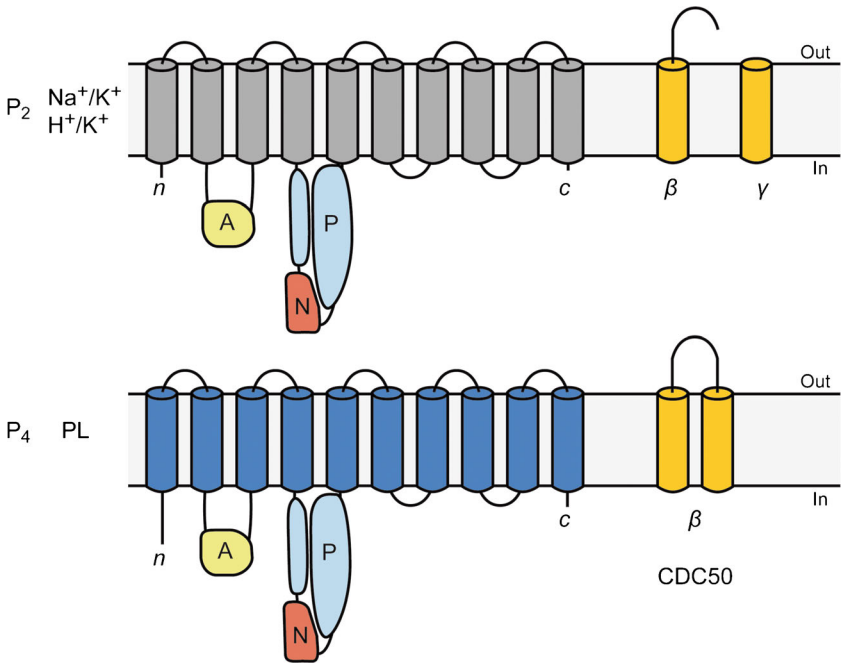

Fig. 1 Membrane topology of P2- and P4-ATPases and their subunits. P-type ATPases consist of an actuator (*A*), a phosphorylation (*P*), a nucleotide-binding domain (*N*), and 10 transmembrane spanning helices. The P domain contains the canonical aspartic acid phosphorylated during the reaction cycle. The beta subunits associated with P2-ATPases are type II membrane proteins with one transmembrane segment, a short cytoplasmic tail, and a large, heavily glycosylated ectodomain with three disulfide bridges. In some cases, a gamma subunit belonging to the FXYD protein family is associated to the P2-ATPases. The CDC50 subunits of P4-ATPases consist of two membrane-spanning domains with a large extracellular loop containing four possible N-linked glycosylation sites and two disulfide bridges. Both the membrane and extracellular domains of CDC50 are required for assembly with the P4-ATPase [17, 68]

remains to be elucidated. Accumulating evidence reveals that P4-ATPases operate as heterodimers in combination with protein subunits from the ligand-effect modulator (LEM)3 / cell division cycle (CDC50) family to flip phospholipids from the exofacial to cytosolic side of cell membranes. This active, unidirectional flip of specific phospholipid species against their concentration gradient has been implicated in generating lipid asymmetry, in scavenging exogenous lipids, and in inducing membrane curvature.

Physiological implications of P4-ATPase-catalyzed phospholipid transport

P4-ATPases and phospholipid asymmetry

Studies in yeast, parasites, and higher eukaryotes uncovered that P4-ATPases localize to the plasma membrane and are found in various intracellular compartments of the late secretory and endocytic pathways (Table 1). This implies that P4-ATPases act at multiple cellular sites to establish and maintain phospholipid asymmetry (Fig. 2(a)). Indeed, studies on the five members of this subfamily in the yeast *Saccharomyces cerevisiae* support this notion. For example, loss of the yeast plasma membrane-associated P4-ATPases Dnf1p and Dnf2p causes an aberrant exposure of endogenous aminophospholipids at the cell surface [15, 65]. Removal of the Golgi-associated P4-ATPases Drs2p and Dnf3p disrupts aminophospholipid transport and asymmetry in post-Golgi secretory vesicles [1, 56]. Thus, membrane aminophospholipid asymmetry appears to be established in late Golgi compartments and maintained at the plasma membrane by the help of P4-ATPases. How the PC translocase activity of several P4-ATPases relates to membrane asymmetry is still unknown, but certain cell types might restrict PC to the cytoplasmic leaflet as well. An asymmetric transbilayer lipid distribution provides the two membrane leaflets of organelles with different characteristics necessary for their respective physiological functions. Furthermore, membrane lipid asymmetry might be involved in the proper sorting of membrane proteins destined for the plasma membrane [34]. It is, therefore, not surprising that a system for sensing lipid asymmetry has been reported in yeast [41].

P4-ATPases as lipid scavengers

Some P4-ATPases are characterized as plasma membrane flippases with relatively broad phospholipid specificity. It is, therefore, tempting to speculate functions for these P4-ATPases beyond their role in maintaining membrane lipid asymmetry. In the yeast *S. cerevisiae*, for example, extracellular lysophospholipids can be taken up by cells and used as substrates for regeneration of phospholipids [73]. Lysophosphatidylethanolamine (lyso-PE) and lysophosphatidylcholine (lyso-PC) are transported into the cell and subsequently acylated to PE and PC, respectively, by the ALE1-encoded lysophospholipid acyltransferase, which also utilizes lysophosphatidic acid as a substrate [43, 90]. Loss of both plasma membrane P4-ATPases Dnf1p and Dnf2p or of their subunit Lem3p blocks the uptake of radiolabeled lyso-PC or lyso-PE and inhibits lyso-PC- or lyso-PE-dependent growth, respectively, supporting a nutrient scavenger role for these pumps [72, 73]. Notably, parasitic protozoa and helminths have developed unique metabolic pathways that allow them to survive and multiply by scavenging nutrients from the host but are unable to synthesize the majority of their own lipids de novo (reviewed in [6]). There is evidence suggesting that several parasites can take up host phospholipids in vivo and from the growth medium in vitro. Whether P4-ATPases are used by parasites for lipid acquisition remains to be demonstrated. Studies on mammalian cells uncovered a role of the human P4-ATPase beta subunit CDC50A (TMEM30a), in the uptake of the inflammatory lipid platelet-activating factor (PAF, a short-chain PC) and other exogenous short-chain choline phospholipids, implying that P4-ATPases in complex with their beta subunit can participate in inward translocation of lipid signaling molecules from outside of the cell (Fig. 2(b)) [14].

P4-ATPases as motors in vesicle formation

Genetic studies in yeast, worms, parasites, plants, and mammals have uncovered an unexpected role of P4-ATPases in vesicular trafficking. In yeast, removal of the plasma membrane-associated P4-ATPases Dnf1p and Dnf2p causes a cold-sensitive defect in the formation of endocytic vesicles [65]. Inactivation of the Golgi-resident P4-ATPase Drs2p rapidly blocks the formation of a clathrin-dependent class of post-Golgi secretory vesicles carrying exocytic cargo [13, 32, 39], while temperature-sensitive variants of the endosome-associated P4-ATPase Neo1p cause defects in receptor-mediated endocytosis, vacuolar biogenesis, and vacuolar protein sorting [40, 102]. Likewise, deletion of the P4-ATPase MgATP2 in the rice plant pathogenic fungus *Magnaporthe grisea* decreases the secretion of extracellular enzymes and results in abnormal Golgi-like cisternae [33].

Trafficking defects associated with aberrant P4-ATPase function are also observed in higher eukaryotes. The *Caenorhabditis elegans* P4-ATPase transbilayer amphipath transporter 1 (TAT-1) is required for yolk uptake in oocytes and for an early step of fluid-phase endocytosis in the intestine [22, 74]. TAT-1 forms a complex with the Cdc50 family protein CHAT-1, and both proteins are important in maintaining normal endocytic sorting/recycling by promoting membrane tubulation of the early endosome [12]. Further, TAT-5 has been linked to the regulation of ectosome shedding [100]. Loss of ALA3, a Golgi-resident P4-ATPase in *Arabidopsis thaliana*, causes a defect in the production of slime vesicles containing

Table 1 P4-ATPase/Cdc50 complexes and their substrate specificities

Organism	P4-ATPase	Sub class	Cdc50 subunit	Location	Substrates[a]	Biological role	References
Saccharomyces cerevisiae	Drs2p	1	Cdc50p	Golgi, SV	**PS**, **PE**	Vesicle formation, cell polarity	[1, 5, 34, 39, 56, 65, 72, 73, 84, 113]
	Neo1p	2	–	Endosome	–	Vesicular transport	
	Dnf1p	3	Lem3p	PM	PC, **PE**, (PS), **LPC**, **LPE**, **LPS**	Endocytosis, cell polarity, lysolipid uptake, protein sorting	
	Dnf2p	3	Lem3p	PM	PC, **PE**, (PS), **LPC**, **LPE**	Endocytosis, Lysolipid uptake, protein sorting	
	Dnf3p	4	Crf1p	Golgi, SV	PC, **PE**	Vesicular transport	
Leishmania donovani	LdMT	1	LdRos3	PM	PC, **PE**, (PS)	–	[64, 101]
Caenorhabditis elegans	TAT-1	1	CHAT-1	PM	**PS**	Endocytosis	[12, 22, 100]
	TAT-5	2	–		**PE**	Vesicular transport	
Arabidopsis thaliana	ALA3	1	ALIS1/3/5	Golgi	**PS**, **PE**, PC	Vesicular transport	[8, 16, 17, 27, 47, 59, 60, 82, 89, 93, 95]
	ALA2	(4)[b]	ALIS1/3/5	PVC	PS	Vesicular transport	
	ALA1	5	ALIS1/3/5	PM	(PS)	Chilling tolerance	
Homo sapiens	ATP8A1	1	CDC50A/B	Golgi, SV	PS, (PE)	Cell migration	[8, 16, 17, 27, 44, 47, 59, 60, 82, 89, 93, 95]
	ATP8A2	1	CDC50A	Golgi, disk	PS, PE	Neurite outgrowth	
	ATP8B1	1	CDC50A/B	PM, AM	PS, (PE)	Membrane integrity	
	ATP8B2	1	CDC50A/B	PM	–	–	
	ATP8B3	1	CDC50C	PM	**PS**	Sperm acrosome formation and capacitation	
	ATP8B4	1	CDC50A/B	PM	–	–	
	ATP9A	2	Not detected	TGN, EE	–	–	
	ATP9B	2	Not detected	TGN	–	–	
	ATP10A	5	CDC50A	PM	–	–	
	ATP10B	5	CDC50A	Vesicles	–	–	
	ATP10D	5	CDC50A	PM	–	–	
	ATP11A	6	CDC50A	PM, EE	–	–	
	ATP11B	6	CDC50A	PM, EE	–	–	
	ATP11C	6	CDC50A/B	PM	**PS**	–	

PM plasma membrane, *SV* secretory vesicle, *PVC* prevacuolar compartment, *AM* apical membrane, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PS* phosphatidylserine, *LPC* lyso-phosphatidylcholine, *LPE* lyso-phosphatidylethanolamine, *LPS* lyso-phosphatidylserine, *disk* photoreceptor disk membranes, *EE* early endosomes

[a] Substrate specificities are mostly demonstrated by the use of fluorescent lipid probes, typically NBD-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-lipid. Evidences for translocation of natural lipids are indicated in bold

[b] Closely related to class 4

polysaccharides and enzymes for secretion [66]. In mouse mastocytoma cells, knockdown of ATP8B5 profoundly perturbs the structural organization of the Golgi complex and causes loss of constitutive secretion at lower temperature [107]. Recently, ATP8A1 in complex with CDC50A was linked to the generation of membrane ruffles in cell motility [44], and studies in neuronal PC12 cells and rat hippocampal neurons indicate a role for ATP8A2 in promoting neurite outgrowth [108].

All these studies indicate that P4-ATPases are required to support vesicle formation in multiple parts of the intracellular trafficking pathways. At least two models, not mutually exclusive, have been proposed to explain how P4-ATPases contribute to vesicle formation (Fig. 2(c)). One model is that P4-ATPases directly catalyze an inward-directed phospholipid translocation across the lipid bilayer, which creates an imbalance in phospholipid numbers between the two leaflets. In turn, this causes an inward bending of the membrane leading to budding and vesicle formation, which is stabilized by recruitment of coat proteins (e.g., clathrin or COPII proteins). Consistent with this hypothesis, insertion of exogenous phospholipids in the exoplasmic leaflet of the plasma membrane and their subsequent translocation to the cytosolic leaflet by a lipid flippase causes dramatic shape changes of red blood cells [21, 78]. Similarly, lipid flipping can provoke the formation of endocytic-like vesicles [53] and accelerates endocytosis [30]. Direct participation of ATP-driven lipid transport in vesicle budding is further supported by the observation that giant

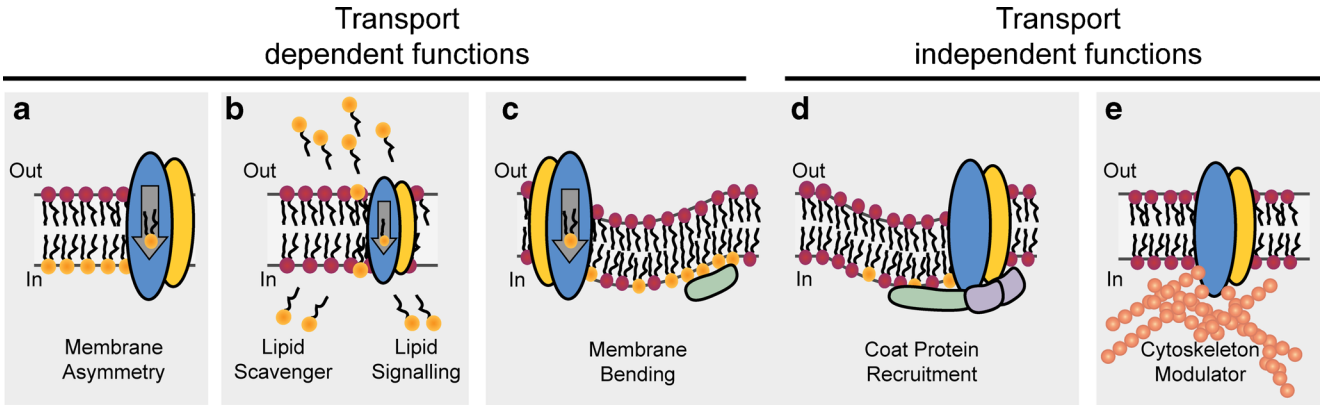

Fig. 2 Cellular functions involving P4-ATPases. P4-ATPases appear to exert their cellular functions by combining an enzymatic phospholipid translocation activity with an enzyme-independent action. These functions are not mutually exclusive. Active transport of lipids from the exoplasmic to the cytosolic membrane leaflet can maintain lipid asymmetry (*a*), scavenge lipids (*b*), and drive membrane budding by generating a lipid imbalance across the bilayer and/or a membrane environment permissive for vesicle budding (*c*). Enzyme-independent functions of P4-ATPases include recruitment of proteins involved in coat assembly (*d*), cellular signaling, and cytoskeleton regulation (*e*). See text for details

proteoliposomes formed from erythrocyte membrane fragments undergo budding in the presence of ATP [29].

A second model is that ATP-driven lipid translocation by P4-ATPases is needed to create a membrane environment permissive for vesicle budding. A high local concentration of aminophospholipids in the cytosolic leaflet may favor the recruitment or activity of peripherally associated proteins with a critical function in vesicle budding, such as small ADP ribosylation factor (Arf) GTP-binding proteins, clathrin, coat protein complex II, amphiphysin, and endophilins. Consistent with such a functional role is the observation that the yeast plasma membrane P4-ATPases Dnf1p and Dnf2p in complex with their Lem3p subunit regulate the fast membrane dissociation of cell division cycle protein 42, a small GTPase with a central role in establishing cell polarity in eukaryotic cells [23, 76]. As discussed in more detail below, various P4-ATPase family members in yeast differ in their substrate specificity. The biological implications of this are unknown, but it could be important for the recruitment or function of cytosolic proteins.

P4-ATPases as membrane scaffolds

In addition to their function as lipid flippases, P4-ATPases appear to serve also transport-independent functions (Fig. 2(d, e)). The yeast P4-ATPases Drs2p and Neo1p 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 [7, 11, 15, 31, 92, 102]. Furthermore, a systematic search for Drs2p-binding partners yielded proteins involved in phosphoinositide metabolism [70]. Phosphoinositides are important signaling molecules in membrane traffic that help establish organelle identity through recruitment of effector proteins. Further, blocking ATP8B1 expression in polarized Caco-2 cells was shown to result in a profound disorganization of the apical actin cytoskeleton and a substantial loss in microvilli without affecting aminophospholipid transport and asymmetry across the apical bilayer [97]. These results suggest that ATP8B1 may provide a molecular scaffold in the apical membrane to recruit structural components or modulators of the actin cytoskeleton involved in microvilli formation. Consistent with a putative scaffold function, ATP8B1 expression on the apical membrane of Caco-2 cells increases dramatically during differentiation, concomitantly with cell polarization.

Notably, also the catalytic alpha subunits of the Na^+/K^+-ATPase and the closely related H^+/K^+-ATPase interact with ankyrin, a cytoskeletal protein implicated in epithelial junction formation [106, 111]. In a similar manner in yeast, functionality of both Golgi-localized Drs2p and plasma membrane Dnf1p is dependent on their interaction with Sla1p [48], a protein that is part of an endocytic coat/adaptor complex with clathrin. Sla1p binds to proteins involved both in endocytosis and in regulation of actin dynamics, allowing for activation of proteins involved in actin polymerization [99].

Linking the cytoskeleton to specialized areas of the plasma membrane may thus be a feature shared among several members of the P-type ATPase superfamily. Thus, intracellular P4-ATPases that seem to act without a beta subunit, e.g., Neo1p in yeast or ATP9B in mammals [89], could serve a lipid transport-independent function as scaffold switches to recruit structural components or effectors.

P4-ATPases display different substrate specificities

Initial biochemical evidence indicated that P4-ATPases flip aminophospholipids only. For example, the first P4-ATPase to

be characterized was identified as the result of purification of an aminophospholipid translocase activity [91]. This protein, also known as ATPase II or, currently, ATP8A1, was isolated from bovine chromaffin granules and displays a striking similarity to the yeast protein Drs2p. The observation that removal of Drs2p caused a specific defect in the inward translocation of fluorescently labelled PS across the yeast plasma membrane provided further support for a role of P4-ATPases in translocation of di-acyl aminophospholipids. Although the function of Drs2p as an aminophospholipid translocase was subsequently questioned [52, 80], lipid transport assays with purified Golgi membranes containing a temperature-sensitive drs2ts allele indicated that Drs2p is directly coupled to flipping of PS acyl labelled with the fluorescent probe nitrobenzoxadiazole (NBD) [56]. Indeed, recent reconstitution of NBD-PS translocase activity with purified Drs2p demonstrates that this enzyme catalyzes PS translocation [113].

Further studies on individual P4-ATPase family members from fungi, plants, and animals have revealed that P4-ATPases differ in their substrate specificities and transport lipid substrates other than di-acyl aminophospholipids (Table 1). In the yeast *S. cerevisiae*, for example, removal of the P4-ATPases Dnf1p and Dnf2p abolishes inward translocation of fluorescently labelled PS, PE, and even PC across the plasma membrane [65], but see also [84]. Moreover, Dnf1p and Dnf2p are also capable of translocating the natural lipids lyso-PE and lyso-PC across the plasma membrane [72, 73]. In addition, the double *Δdnf1Δdnf2* mutant cells are defective for the uptake of alkylphosphocholine derivatives and are, therefore, resistant to the toxic effects of these drugs [65]. In fact, recent data suggest that Dnf1p prefers lysolipids rather than di-acyl phospholipids as its substrate [5]. Similarly, *Leishmania* parasites deficient for Dnf1p orthologs are defective in inward translocation of PE, PC, and alkylphosphocholine derivatives at the plasma membrane [63, 101].

Studies in the plant *A. thaliana* further substantiate the notion that members of the P4-ATPase family do not serve exclusively as aminophospholipid-specific translocators. The *Arabidopsis* genome encodes 12 P4-ATPases, designated ALA1 to ALA12 (for aminophospholipid ATPase) [2]. So far, only three ALA proteins have been partially characterized: ALA1, ALA2, and ALA3. ALA1 localizes to the plant plasma membrane, while ALA2 and ALA3 are located to the prevacuolar compartment and the Golgi apparatus, respectively. Complementation studies in yeast mutants lacking the P4-ATPases Dnf1p, Dnf2p, and Drs2p revealed that ALA2 specifically transports PS, while ALA3 has broader specificity and facilitates transport of PS, PE, and PC, but not of the lyso-PC derivative miltefosine [50, 66].

In mammals, at least 14 P4-ATPases [36], designated ATP8A1 through ATP11C, have been identified, but their substrate specificities are still poorly defined. Among the P4-ATPases expressed in mammalian cells, ATP8A1, ATP8A2, ATP8B1, ATP8B3, and ATP11C have, so far, been connected to PS translocation. ATP8A1 is dependent on PS and PE for ATPase activity [27, 59] and is able to translocate fluorescent PS upon expression in yeast [82]. Likewise, ATP8A2, a P4-ATPase highly expressed in the brain, testes, and retina, exhibits PS-dependent ATPase activity and the ability to translocate fluorescent PS, and to some extend PE, in proteoliposomes [16, 17]. Deficiency of ATP8B1, a P4-ATPase located in the canalicular membrane of liver cells, is accompanied by enhanced recovery of PS, but not PC or PE in bile [61], and heterologous expression of ATP8B1 restores the non-endocytic uptake of NBD-PS in PS transport-defective CHO mutant cells [60, 93]; see also [97]. In the spermatozoa of mice, ATP8B3 is necessary for PS asymmetry and fertilization [98]. ATP11C, a P4-ATPase essential for B cell development, was found to play a crucial role in PS translocation [109]. Notably, recent data show that also mammalian P4-ATPases do not only include aminophospholipid-specific translocators; ATP8B5 from mouse testes was found to transport PC and PE upon heterologous expression in yeast [107]. Recently, a role of ATP8B1 as a cardiolipin flippase [71] has been suggested but requires further investigations [62].

Based on sequence similarity, P4-ATPases have been further subdivided into six classes (designated classes 1–6) carrying conserved class-specific amino acid sequences [35, 37]. However, it seems unlikely that these sequence similarities are linked to functional similarities as the subdivision into classes does not correlate directly with the different substrate specificities so far reported for various P4-ATPase members (Table 1). For example, class 1 P4-ATPases comprise flippases that, in some organisms, are specific for PS (e.g., *C. elegans* TAT-1) and, in others, also transport PE (e.g. *S. cerevisiae* Drs2p) and, in others, PC as well (but barely PS, as *Leishmania donovani* LdMT). All eukaryotic organisms analyzed contain genes encoding class 1 and class 2 P4-ATPases (Table 1). *C. elegans* and *Arabidopsis* express class 5 P4-ATPases as well, while mammals and *Drosophila* express both class 5 (ATP10) and class 6 (ATP11) P4-ATPases. In contrast, class 3 and class 4 P4-ATPases are solely expressed in yeast (Table 1).

The requirement for a beta subunit for P4-ATPases

The catalytic subunit of P4-ATPases associates with a beta subunit from the Cdc50 family, which is required for functional maturation of the enzyme. Structurally, P4-ATPase beta subunits contain two transmembrane domains and a large exoplasmic loop that is heavily glycosylated (Fig. 1). This resembles a fusion of the two subunits for the Na^+/K^+-ATPase, the beta subunit with one transmembrane domain and a large glycosylated ectodomain and the gamma subunit with one transmembrane domain and very short cytosolic and luminal

extensions. P4-ATPase beta subunits have, therefore, been suggested to be analogous to the Na^+/K^+-ATPase beta and gamma subunits [67, 69]. The role of the P4-ATPase beta subunit also seems to be similar to that of the Na^+/K^+-ATPase accessory subunits. They act as chaperones required for the P4-ATPase to leave the endoplasmic reticulum (ER), and they seem to affect the catalytic properties of the complex, being required for lipid translocation.

The function as chaperone was demonstrated by the fact that the association of Cdc50 proteins with P4-ATPases is a prerequisite for stability and ER export of the transporter complex [8, 31, 50, 51, 60, 64, 66, 75]. Notably, yeast Neo1p as well as the related mammalian P4-ATPases ATPA9 and ATP9B are able to exit the ER independent of CDC50 proteins. In co-immunoprecipitation studies, none of these proteins formed a stable complex with CDC50 proteins, and experimental evidence that they catalyze phospholipid transport is lacking. This suggests that Neo1p, ATP9A, and ATP9B possess a biochemical activity that is different from the other P4-ATPases, and for which, they might not require a Cdc50-binding partner.

Based on genetic studies in yeast, it was suggested that the beta subunit is required not only for ER exit but also for proper localization of the P4-ATPase to the membrane. Thus, yeast Drs2p and its beta subunit, Cdc50p, are localized to the trans-Golgi network, while Dnf1p and Dnf2p, sharing a common beta subunit, Lem3p, localize both to the plasma membrane. However, this does not seem to be the case in multicellular organisms. Plant ALA1, ALA2, and ALA3 can use three different beta subunits for ER exit, and each P4-ATPase will reach a different final subcellular localization in the presence of each of these subunits [50, 51]. Likewise, several human P4-ATPases can interact with all three identified human Cdc50p homologues to exit the ER [8]. It is a possibility that CDC50 proteins from multicellular organisms have evolved to interact with P4-ATPases in a different way than their yeast counterparts. In a yeast cell, all P4-ATPases and beta subunits are expressed at the same time within the same cell, and it might be necessary for proper function that each beta subunit will only recognize one interacting partner. In multicellular organisms, however, expression of each protein can be controlled both temporally and spatially to avoid undesired interactions. Thus, it might be more effective for the cell to maintain a number of interchangeable beta subunits that will be ready at each given moment to assist ER exit of a number of P4-ATPases expressed under different conditions and fulfilling distinct physiological roles.

The P4-ATPase beta subunit appears to also serve a role in lipid translocation. It has been suggested that transmembrane flipping might occur at the interface between a P4-ATPase and its Cdc50-binding partner, an arrangement in which Cdc50 proteins would contribute directly to the transport specificity of the complex [16, 69, 113]. This idea is consistent with the observation that the yeast trans-Golgi P4-ATPases Drs2p and Dnf3p, which exhibit different translocation profiles [1], interact with different Cdc50 homologues. Drs2p interacts with Cdc50p [75] and Dnf3p with Crf1p [31], whereas the plasma membrane P4-ATPases Dnf1p and Dnf2p, which have the same substrate specificity [65], both interact with Lem3p [31, 75]. However, co-expression studies of different plant Cdc50 proteins and P4-ATPases show no influence of the beta subunit on lipid specificity, implying that the determinants for substrate specificity primarily [50]. Recent studies uncovered a series of residues involved in defining the substrate specificity of P4-ATPases within the catalytical subunit [4], consistent with this notion. Studies on yeast Drs2p suggest that Cdc50 proteins directly participate in the P4-ATPase reaction cycle [46] and perhaps help create a high-affinity phospholipid-binding site in the membrane domain of P4-ATPases [86]. Similar findings have been reported for the closely related Na^+/K^+-ATPase where the subunit affects the K^+ affinity of the pump. Whether all P4-ATPases require a CDC50-binding partner to accomplish lipid transport is unknown.

Regulation of P4-ATPase activity

In light of their physiological relevance, P4-ATPases are expected to be highly regulated. However, not much is known about this aspect. Studies in yeast have provided initial evidence that P4-ATPases are targets of kinase-dependent phosphorylation, a common mode of regulation of P-type ATPases. Yeast Dnf1p and Dnf2p can be phosphorylated in vitro by flippase kinase 1 (Fpk1p), and in vivo experiments have shown that Fpk1p and its homologue Fpk2p are required for normal levels of inward-directed phospholipid transport across the plasma membrane of yeast [54]. The specific amino acid position phosphorylated by Fpk1p and the direct impact of this phosphorylation on Dnf1/2p flippase activity remain to be established. Interestingly, Drs2p and Dnf3p also get phosphorylated by Fpk1p in vitro, but to a lesser extent. Neo1p, which has not yet been shown to transport phospholipids, is not phosphorylated by Fpk1p. The physiological significance of these results remains to be determined, and currently, it is unknown whether other kinases interact with these P4-ATPases.

The activity of Drs2p in the trans-Golgi was recently found to be regulated through interaction with cytosolic proteins and specific lipids that play a critical role in membrane budding (Table 2). For example, the guanine nucleotide exchange factor Gea2p and phosphatidylinositol 4-phosphate bind to regions in the C-terminal cytosolic tail of Drs2p, and this binding is required for Drs2p activity [11, 42, 55]. Furthermore, binding of the small GTPase Arl1p (ADP ribosylation factor-like protein 1) to the N-terminal tail of Drs2p stimulates its flippase activity [92]. Collectively, these findings imply that

Table 2 P4-ATPase interactors

P4-ATPase	Interactor	Method	Function	Reference
Neo1p	Ysl2p	Co-IP	Potential GDP/GTP exchange factor for the Arl1p small GTPase	[102]
Drs2p	AP-1	Co-IP (cross-linking)	Tetrameric clathrin adaptor	[49]
	Gea2p	Co-IP, two-hybrid membrane	GDP/GTP exchange factor for Arf1p	[11]
	Rcy1p	Co-IP	F box protein involved in endocytic recycling	[31]
	Sac1p	Pull-down (cross-linking), two-hybrid membrane	Phosphatidylinositol 4-phosphatase	[70]
	Itr1p	Pull-down (cross-linking), two-hybrid membrane	myo-Inositol transporter	[70]
	Ino1p	Pull-down (cross-linking), two-hybrid membrane	myo-Inositol 1-phosphate synthase	[70]
	Tcb3p	Pull-down (cross-linking), two-hybrid membrane	Synaptotagmin ortholog	[70]
	Arl1p	Co-IP, two-hybrid membrane	Small GTPase	[92]
	Sla1p	Two-hybrid membrane	Clathrin adaptor protein	[92]
Dnf1p, Dnf2p	Fpk1p, Fpk2p	Phosphorylation	Serine/threonine kinase	[54]

Co-IP co-immunoprecipitation

the C-terminal tail of Drs2p contains an autoinhibitory domain and that Drs2p flippase activity is tightly coupled to the vesicle budding machinery at the trans side of the Golgi. The challenge is now to dissect the order and regulation of the dynamic and multiple interactions between Drs2p and its partners that eventually result in membrane budding. Additionally, further studies will be necessary to unravel how the activity of other flippases such as Dnf1/2p is regulated through interaction with lipids and cytosolic proteins.

The mechanism of P4-ATPase-catalyzed phospholipid transport

Even though available data support a direct role of P4-ATPases in lipid translocation, it is unclear how these enzymes acquired the ability to translocate lipids instead of much smaller ions. Early studies on aminophospholipid translocases pointed at the glycerol backbone and the lipid head group as the key recognition elements for the flippase. More recent data indicate that several P4-ATPases are capable of transporting a broad range of lipid substrates, including synthetic alkylphospholipids lacking a glycerol backbone, and define the phosphoryl head group as the key element for substrate selection by these transporters (Table 1). Thus, P4-ATPases must at least provide a sizeable hydrophilic pathway for the polar head group to pass through the membrane.

Recent mutational studies suggest two pathways by which P4-ATPases could transport their substrate: (1) the classical pathway with the lipid transported through the interior of P4-ATPases in analogy with the cation transport mechanism of well-characterized P2-ATPases and (2) a nonclassical pathway at the protein–membrane interface (Fig. 3). In the thoroughly studied Ca^{2+}- and Na^{+}/K^{+}-transporting P2-ATPases, for which a wealth of crystal structures are available [9], the cation-binding sites are present in small central cavities primarily formed by charged or polar residues in the center parts of transmembrane segments TM4, TM5, and TM6 (Fig. 3). Contributing to cavity formation, all "classical" cation-translocating P-type ATPases possess one or more highly conserved proline residues in TM4 that function as helix breakers that allow backbone carbonyl oxygen atoms to be exposed and participate in cation coordination [57]. Notably, all P4-ATPases also contain a conserved proline in the middle of TM4 (Pro-507 in Drs2p) suggesting the presence of a central cavity in these pumps with carbonyl oxygen contributing to coordination of a hydrophilic group. Besides this feature, P4-ATPases have only a few conserved residues in their transmembrane segments that share properties with those in the membrane domain of P2-ATPases. Most notably is the presence of two conserved asparagine residues in TM5 and TM6 (Asn-1019 and Asn-1050 in Drs2p), which in P2-ATPases provide oxygen groups for ion coordination. In P2-ATPases, conserved acidic group(s) positioned in the middle of TM4 and TM6 secure charge neutralization of the transported cation. In contrast, P4-ATPases have not preserved acidic residues in their predicted transmembrane segments. However, a basic lysine residue (corresponding to Lys-1018 in Drs2p and Lys-873 in the mammalian photoreceptor P4-ATPase ATP8A2) is conserved in all P4-ATPases and situated in the middle of TM5 at the same position as cation-coordinating residues in P2-ATPases. As mutating this residue has a dramatic impact on the activity of ATP8A2, it has been proposed that P4-ATPases have evolved a canonical substrate-binding site centrally located in the membrane domain of the pump, which, with the lipid head group, is deeply embedded within the protein [19]. It has been hypothesized that the role of lysine is to neutralize an acidic group in the transported phospholipid molecule, which could be its phosphate group [19]. Given the large size of a phospholipid as compared to

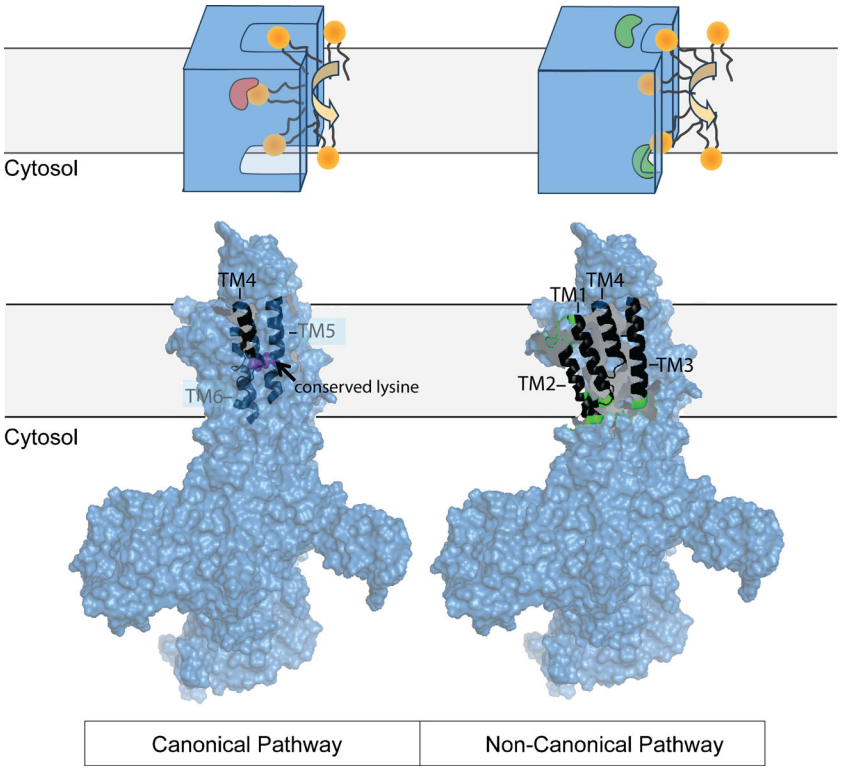

Fig. 3 Schematic overview of two proposed phospholipid transport pathways in P4-ATPases. In the classical model, the lipid is transported through a space in the transporter analogous to the cation transport mechanism of well-characterized P2-ATPases. Here, an occluded state is expected with the transported lipid deeply buried in a central cavity (*red*) within the P4-ATPase with the entrance and exit pathways closed. By contrast, in the external surface model, the lipid is transported at a cleft on the membrane-facing surface, and only the lipid head group is protected from the lipid environment. The presence of two substrate-selecting gates (*green*) acting sequentially on opposite sides of the membrane has been reported [4]. In both cases, the relative positioning of the transmembrane segments critical for phospholipid binding/transport is highlighted on a homology model of Dnf1p based on the crystallized Na^+/K^+-ATPase in the E2P conformation [3]. The rest of the structure is shown in surface representation

ions, this model implies a considerable flexibility of P4-ATPases to accommodate their substrate and, especially, the lipid tail when it traverses the membrane. Interestingly, long-chain thapsigargin analogs do adjust their fatty acid chain deeply between the transmembrane sections of the Ca^{2+} pump sarcoendoplasmic reticulum calcium ATPase (SERCA), consistent with high structural flexibility [24, 81, 105]. Furthermore, the E1–sarcolipin structure of the P2-SERCA displays a deep, funnel-shaped, and negatively charged path that leads to the Ca^{2+} sites located halfway through the membrane bilayer, and phospholipid head groups have been proposed to assist Ca^{2+} entry to these sites [104]. A similar funnel might have evolved into a phospholipid head group-loading pathway in P4-ATPases.

In the nonclassical transport pathway proposed for P4-ATPases (Fig. 3), interaction between the pump and the phospholipid involves only the hydrophilic head group and amino acid residues all along the membrane-facing surface of the protein [3]. In this "sliding card" model, only the head group of the phospholipid makes a direct contact with the P4-ATPase protein during transport, while the acyl chains remain within the surrounding lipid environment of the membrane at all stages [4]. In line with this model mutational studies of yeast Dnf1p identified amino acid residues involved in substrate selection at the lumenal side of TM1 and the cytosolic sides of TM1, TM2, TM3, and TM4 [3]. Mutation of the Drs2p residue corresponding to Lys-873 in ATP8A2 reduced Drs2p activity but did not change the specificity of the pump. Dnf1p residues shown to be involved in phospholipid recognition at the cytosolic side of the membrane can be mapped to a region of SERCA that, in the E2 conformation, forms a binding pocket for the head group of a PE molecule [4]. This pocket is not accessible in the E1 conformation, implying that in the SERCA Ca^{2+}-ATPase, the PE molecule has to dynamically enter and exit as it proceeds through the catalytic cycle. This original phospholipid head group-binding pocket might have evolved in P4-ATPases to generate a cleft for lipid translocation.

While the two models appear to oppose each other, it is useful to consider a mechanistic model that combines the two. According to such a model, P4-ATPases function mechanistically according to the same principles as P2-ATPases, but with some modifications. In both classes of pumps, the transported ligand enters from one side of the membrane

through a half-channel leading to a ligand-binding cavity in the middle of the plane of the membrane. After occlusion of the ligand, which involves closure of the entrance pathway, a major conformational change results in the opening of an exit half channel through which the ligand is able to leave the pump protein. What distinguishes P4-ATPases from P2-ATPases in this context is that the transported ligand is so large that the pump protein cannot accommodate all of its mass within its structure. Therefore, part of the ligand, in this case the lipid tail, has to protrude out from the flippase during passage through the membrane and, during this process, never makes contact with the pump protein. To enable this, both the entrance and exit half channels are open towards the lipid bilayer and take form as clefts. In any case, conclusive evidence for the nature of the phospholipid head group-binding site(s) and the transport pathway of P4-ATPases potentially requires successful crystallization and structural elucidation of a P4-ATPase, which has not been achieved to date.

Concluding remarks

As is clear from this overview, phospholipid translocation and asymmetry in eukaryotic cells require P4-ATPases, and reconstitution experiments with purified enzymes demonstrate that these enzymes participate directly in lipid translocation. Yet, many fundamental questions still remain to be investigated. First, the mechanism of phospholipid transport by P4-ATPases is still an enigma. A major unresolved question is whether lipids are transported through the interior of P4-ATPases in analogy with the cation transport mechanism of well-characterized P-type ATPases such as the Na^+/K^+- and Ca^{2+}-ATPases or other mechanisms should be considered. Further biochemical studies are required to unravel the enzyme kinetic parameters and the role of the CDC50 beta subunit in the catalytic cycle of these pumps. Notably, an improved, higher resolution assay for studying phospholipid transport has recently been developed [85] that can help to address these issues in more detail. Secondly, the substrate specificity of the individual P4-ATPases is still largely unknown. Its further elucidation will require transport measurements with natural lipids by P4-ATPases reconstituted into proteoliposomes, a challenging task given the difficulties of purifying membrane proteins, the water insolubility of the substrates, and the requirement for subunits and/or accessory proteins. The recent purification and functional reconstitution of two P4-ATPases [16, 113] offer hope for similar studies on other P4-ATPases. Third, we still lack a detailed understanding on how P4-ATPases participate in vesicle budding. Recent progress in membrane protein reconstitution into giant vesicles [26, 110] can help to test whether P4-ATPase-catalyzed phospholipid transport has a primary role in driving vesicle budding in the late secretory and endocytic pathway. Finally, while a number of interacting partners have been found for the yeast P4-ATPases, little or nothing is known about the interaction partners for P4-ATPases in higher eukaryotes. Several phenotypes have been associated to defects in P4-ATPase expression and activity in mammalians and plants, but the mechanisms underlying these phenotypes are largely unknown. At the same time, many P4-ATPases from higher eukaryotes do not have an assigned physiological role, most probably due to functional redundancy. Further characterization of mutant lines and screening for protein–protein interactions are required to shed some light onto these points and get a better understanding of P4-ATPase function and regulation.

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