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Structure and mechanism of ATP-dependent phospholipid transporters ☆

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Background: ATP-binding cassette (ABC) transporters and P4-ATPases are two large and seemingly unrelated families of primary active pumps involved in moving phospholipids from one leaflet of a biological membrane to the other.
Scope of review: This review aims to identify common mechanistic features in the way phospholipid flipping is carried out by two evolutionarily unrelated families of transporters.
Major conclusions: Both protein families hydrolyze ATP, although they employ different mechanisms to use it, and have a comparable size with twelve transmembrane segments in the functional unit. Further, despite differences in overall architecture, both appear to operate by an alternating access mechanism and during transport they might allow access of phospholipids to the internal part of the transmembrane domain. The latter feature is obvious for ABC transporters, but phospholipids and other hydrophobic molecules have also been found embedded in P-type ATPase crystal structures. Taken together, in two diverse groups of pumps, nature appears to have evolved quite similar ways of flipping phospholipids.
General significance: Our understanding of the structural basis for phospholipid flipping is still limited but it seems plausible that a general mechanism for phospholipid flipping exists in nature. This article is part of a Special Issue entitled Structural biochemistry and biophysics of membrane proteins.

1. Background

Biological membranes are the basis for highly defined and separated functional units. The cell membrane, or plasma membrane, defines the external boundary of every cell separating the cytoplasm from the surrounding environment. Eukaryotic cells contain in addition numerous subcellular membranes that divide the cytoplasm into multiple organelles, thereby allowing different functions to occur efficiently and simultaneously in different parts of the cell. Almost all biological membranes are organized as bilayers consisting of two leaflets structurally formed by phospholipids. Depending on their (sub)cellular location, they might also contain other types of phospholipids, including glycolipids and sterols. A remarkable feature of many biological membranes is that their phospholipids are asymmetrically distributed across the lipid bilayer, a phenomenon known as transbilayer lipid asymmetry. A prominent example is the plasma membrane of animal cells where the phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) are concentrated in the exoplasmic leaflet while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are restricted to the cytosolic leaflet [1]. Transbilayer lipid asymmetry is essential for several vital cellular functions, including regulation of membrane protein activity, signaling, and vesicle formation in the secretory and endocytic pathways [2–5]. In animals, loss of transbilayer lipid asymmetry has been related to processes like blood coagulation [6], macrophage recognition [7] and apoptosis [8]. Establishing and maintaining the asymmetry is thus crucial for the cells, and a number of proteins have evolved to fulfill a role as cross-bilayer phospholipid transporters.

Transbilayer lipid asymmetry is largely controlled by a diverse group of membrane proteins that catalyze the movement of phospholipids across membranes. Lipid translocators can be classified into two categories: (i) energy-independent transporters such as scramblases that randomize the distribution of lipids across the bilayer and (ii) ATP-driven, vectorial transporters that actively translocate specific lipids from one leaflet to the other. The latter class of transporters includes ATP-dependent flippases and floppases, which catalyze inward phospholipid movement to and outward phospholipid movement from the cytoplasmic leaflet of cellular membranes, respectively. Current genetic and biochemical evidence indicates that these proteins are primarily members of the ATP-binding cassette (ABC) and P-type family of transporters

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(Tables 1 and 2). However, measuring phospholipid translocation is not a trivial task, as the transporters are trapped in an environment (cellular membranes) formed by their own substrate (lipids). Therefore, in intact cells or organisms, assignment of phospholipid translocating activity is in most cases based on the use of fluorescent phospholipid analogs; only few studies have attempted to measure transport of natural phospholipids (Tables 1 and 2). Phospholipid transport observed in vivo need not be directly linked to the activity of ABC transporters or P4-ATPases, and might represent indirect effects. Only recently, advances in purification and reconstitution techniques have allowed demonstrating the capacity of some ABC transporters and P-type ATPases to directly translocate fluorescent phospholipid analogs, providing the best evidence so far that these transporters indeed have phospholipids as a substrate.

2. Scope of review

Several excellent reviews have surveyed the physiological relevance of phospholipid transporters recently [3,5,9]. Here, we will focus on recent advancements in the determination of the structure and mechanism of putative ATP-dependent phospholipid transporters.

3. ATP-dependent phospholipid transporters

Cellular, biochemical and recent reconstitution studies demonstrate that various ABC transporters and P-type ATPases couple ATP hydrolysis to translocation of specific phospholipids from one leaflet to the other and thereby help generate membrane lipid asymmetry (Tables 1 and 2). An important question arises when comparing the two classes of phospholipid flipping pumps: are their transport mechanisms the same or has nature evolved different ways of translocating phospholipids across the bilayer? In the next sections, we will review the main features of ABC transporters and P-type ATPases implicated in phospholipid transport and compare their putative transport mechanism(s).

3.1. ABC transporter family

The ABC transporter family is found in organisms throughout evolution and constitutes one of the largest superfamilies of integral membrane transporters. The family includes a wide range of proteins that share common structural features and transport a variety of organic and inorganic substrates, including phospholipids (Table 1). Several of these transporters are mutated in human disorders related to phospholipid transport and metabolism, including Tangier disease [10], Stargardt disease [11] and progressive familial intrahepatic cholestasis [12]. The functional transport unit comprises two nucleotide-binding domains (NBD), typical of ABC transporters, and two transmembrane domains (TMDs), each containing five to ten membrane-spanning regions (Fig. 1A). They can occur as one complete transporter, two half-transporters, or four polypeptides.

3.1.1. General structural features of ABC transporters

The NBDs of all ABC transporters contain highly conserved amino acid motifs. Each NBD contains two ATP-binding motifs: the Walker A motif (GXXGXGK(S/T)) and the Walker B motif (hhhhDE, where h is a

Table 1
Evidence for ABC transporter-catalyzed phosholipid transport.

Transporter	Lipid[1]	Movement, membrane[2]	Approach	Reference
Bacteria				
MsbA	**Lipid A**, PLs?	Out, PM	Intact cells; ATPase stimulation; Reconstitution	[72,137–139]
PglK	**Lipid-linked oligosaccharides**	Out, PM	Intact cells	[140]
LmrA	PE, **Lipid A**	Out, PM	Reconstitution; *E. coli* complementation; ATPase stimulation	[141,142]
Fungi				
Pdr5p, Yor1p	**PE,**	Out, PM	Intact cells	[143,144]
Ybt1p	PC	Out, vacuolar	Intact cells; Isolated vacuoles	[145]
Cdr1p	PE,PC,PS	Out, PM	Intact cells; Reconstitution	[63,146,147]
Cdr2p	PE,PC,PS	Out, PM	Intact cells	[63]
Cdr3p	PE,PC,PS	In, PM	Intact cells	[63]
Leishmania donovani				
LABCA1	PLs	Out, PM	Intact cells	[148]
LABCA2	PLs	Out, PM	Intact cells	[149]
LABCB4	PC	Out, PM	Intact cells	[150]
LABCG2	PS	Out, PM	Intact cells	[151]
LABCG4	PC	Out, PM	Intact cells; yeast vesicle assay	[152]
LABCG6	PLs	Out, PM	Intact cells	[153]
Mammals				
ABCA1	PC, PS, SM	Out, PM	Intact cells; reconstitution	[65,91]
ABCA3	PC, SM	Out, lysosomal vesicles	Expression in HEK-293 and A549	[154–156]
ABCA4	PE, N-retinylidene-PE	In, Disk	Reconstitution	[64,65,157]
ABCA7	PS, (PC, SM)	Out, PM	Intact cells; reconstitution	[65,158–160]
ABCB1 (MDR1)	PLs, SLs, **PAF**	Out, PM (apical)	Intact cells; yeast vesicle assay; reconstitution	[70,71,89,90,161–164]
ABCB4 (MDR3,Mdr2)	**PC**	Out, canalicular	Intact cells; yeast vesicle assay	[89,165–167]
ABCC1 (MRP1)	PLs	Out, PM (basolateral)	Intact cells; reconstitution	[168–171]

[1] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. PLs, phospholipids; SLs, sphingolipids; PAF, platelet-activating factor (1-Hexadecyl-2-acetyl-sn-glycero-3-phosphocholine); SM, sphingomyelin. Substrate specificities are mostly demonstrated by the use of fluorescent lipid probes. Evidences for translocation of natural lipids are indicated in bold.
[2] PM, plasma membrane; Disk, photoreceptor disk membranes.

Table 2
Evidence for P4-ATPase-catalyzed phosholipid transport.

Transporter	Lipid[1]	Movement, membrane[2]	Approach	Reference
Saccharomyces cerevisiae				
Dnf1p	PC, **PE**, (PS), **LPC**, **LPE**, **LPS**	In, PM	Intact cells	[126,143,172,173]
Dnf2p	PC, **PE**, (PS), **LPC**, **LPE**	In, PM	Intact cells	[143,172–174]
Dnf3p	PC, **PE**	In, Golgi	Purified vesicles	[175]
Drs2p	**PS**, **PE**	In, Golgi	Purified vesicles; Reconstitution	[174,176]
Leishmania donovani				
LdMT	PC, **PE**, (PS)	In, PM	Intact cells	[177,178]
Caenorhabditis elegans				
TAT-1	**PS**	In, PM	Intact cells	[179,180]
TAT-5	**PE**	In, PM	Intact cells	[181]
Arabidopsis thaliana				
ALA1	(PS)	In, PM	Yeast complementation	[104,182]
ALA2	**PS**	In, Prevacuolar compartment	Yeast complementation	[114]
ALA3	**PS**, **PE**, **PC**	In, Golgi	Yeast complementation	[113,114]
Mammals				
ATP8A1	PS, (PE)	In, Golgi	ATPase stimulation; Yeast vesicle assay	[183–186]
ATP8A2	PS, PE	Golgi, Disks	Reconstitution	[124,187,188]
ATP8B1	PS, (PE)	PM (apical)	Intact cells	[115,189,190]
ATP8B3	**PS**	PM	Intact cells	[191]
ATP11C	**PS**	PM	Intact cells	[192]

[1] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LPS, lyso-phosphatidylserine. PLs, phospholipids; SLs, sphingolipids; PAF, platelet-activating factor (1-Hexadecyl-2-acetyl-sn-glycero-3-phosphocholine). Substrate specificities are mostly demonstrated by the use of fluorescent lipid probes. Evidences for translocation of natural lipids are indicated in bold.
[2] PM, plasma membrane; AM, apical membrane; Disk, photoreceptor disk membranes.

hydrophobic amino acid) [13,14]. The Walker A motif establishes extensive interactions with the phosphate group of an ATP molecule [15,16]. The Walker B motif contains an aspartate involved in the coordination of water and Mg^{2+} at the catalytic site and a catalytic glutamate required for ATP hydrolysis [16–19]. A similar role in coordination has a conserved glutamine at the Q-loop located between the Walker sequences [16,19–21]. A conserved aromatic amino acid residue in the A-loop upstream of the Walker A motif is involved in interacting with the π-stack of the adenosine ring of ATP [22–24]. Downstream of Walker B are the D-loop and the H (or switch)-loop that contain a conserved aspartate and a conserved histidine, respectively, involved in the coordination of the γ-phosphate either through interaction with a water molecule (D-loop) [17,19,20,25,26] or through direct hydrogen bonding (H-loop) [19,27]. Finally, the ABC signature is a helical subdomain located in between the Walker sequences of each NBD containing the amino acid sequence LSGGQ, also involved in ATP binding [28–33].

When the transporter binds ATP, the two NBDs arrange in a head-to-tail manner, generating the so-called "closed-dimer" conformation, in which two nucleotide molecules are sandwiched in the interface between the two NBDs and interact with residues from the Walker A, Walker B, H- and Q-loops of one NBD and the D-loop and ABC signature of the other [15,17,34]. Due to this arrangement some ABC transporters show an allosteric behavior for hydrolysis of the two ATP molecules, presumably controlled by the D-loop [26,33,35].

ABC transporters present three different types of structural folding, depending on the organization of their transmembrane segments [36,37]. Thus, it is possible to distinguish between an "exporter" fold adopted by proteins involved in the transport of substrates away from the cytoplasm, and two distinct "importer" folds, typical of proteins that transport substrates toward the cytoplasm.

The typical ABC "exporter" fold contains a total of 12 transmembrane spanning α-helices that form the substrate binding site and provide the pathway for the movement of substances across the membrane [36] (Fig. 1A). TMDs from different transporters present low levels of homology, probably reflecting the wide variety of substrates transported by this protein family. The 12 membrane-spanning segments arrange into two bundles of six with a large cavity in between that can either be open-inwards (to the cytosol) or open-outwards (to the extracellular/luminal side) (Fig. 1B). In the open-inwards conformation, each bundle is commonly formed by interaction between transmembrane segment 4 (TM4) and TM5 of one half transporter or half monomer and TM3 and TM6 of the other half. In this conformation, a 2-fold pseudo-symmetry can be observed for TM1-3 with respect to TM4-6 for each individual TMD [38–40]. During catalysis, the TMDs suffer extensive structural rearrangement establishing a new interaction surface that changes the orientation of the exposed cavity in the membrane [38,39,41–43]. This reorganization results in the "open-outwards" conformation in which TM1 and TM2 in one monomer now interact with TM3 and TM6 of the other [44]. In the process, a high affinity binding site for the substrate in the "open-inwards" conformation changes into a low affinity binding site in the "open-outwards" conformation [45,46]. To date, only the bacterial ABC transporter MsbA has been crystalized in both the inward and outward facing conformations presumably adopted by the "exporters" during the catalytic cycle (Table 3) [39]. However, the similarities between the different structures suggest that the alternating-opening conformations also exist for other transporters.

In ABC "exporters", those TMDs that are not directly connected to NBDs are extended by α-helices that stretch out into the cytoplasm between 25 and 40 Å away from the surface of the lipid bilayer [40,47,48] (Fig. 1E and F). Such extended α-helices are not found in the "importer" fold, suggesting that they might be directly related to the exporter type mechanism and, in some ABC exporters, they have been linked to recognition of a soluble transported substrate [49–51]. All together, the cytosolic protrusions of TMD helices generate four intracellular loops (IL1–IL4) that contain the so-called coupling helices. These run parallel to the plane of the membrane and interact with the NBDs [36,52,53]. Due to the twisted arrangement of the transmembrane spanning segments, IL2 and IL4, each located in a different half transporter or half monomer, interact exclusively with the trans-NBD (in the opposite half), while IL1 and IL3 only interact with the cis-NBD. Besides, TM6 finishes in a cytoplasmic α-helix extension located just upstream of

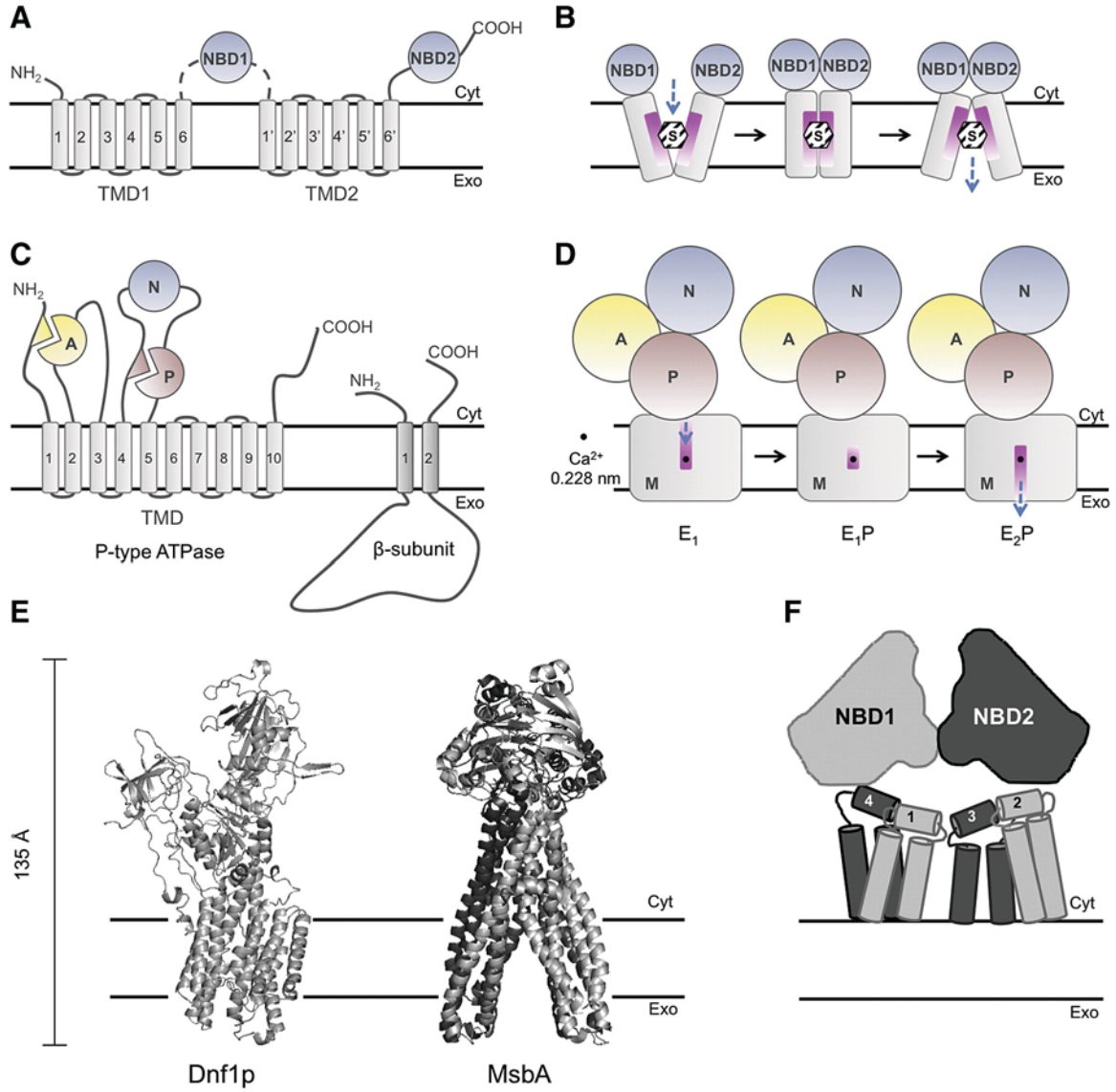

Fig. 1. General structure of ABC transporters and P-type ATPases. (A) Secondary structure of ABC transporters. The ABC transporter exists both as a monomer full transporter and as a dimer consisting of two half transporters. In either case, this type of transporter contains two transmembrane domains (TMD1 and 2), each harboring 6 transmembrane helices named 1 to 6 in TMD1 and 1′ to 6′ in TMD2. Furthermore, two nucleotide binding domains (NBD1 and NBD2) are present on the cytosolic side of the membrane. NBD1 is either situated at the C-terminal end of one half transporter or is connecting TMD1 and TMD2 in the full transporter; alternatively the domain architecture can have a reverse topology, i.e. NBD1-TMD1-NBD2-TMD2. (B) Model of substrate translocation carried out by ABC exporters. First, the substrate enters the cavity between the transmembrane domains, which are facing the cytosol. At this stage the transporter has high affinity for the substrate. Second, the substrate is occluded in the ABC transporter. Third, through conformational changes a cavity is now opened toward the exocytic side of the membrane the substrate specificity decreases and the substrate is being released. (C) Secondary structure of P-type ATPases. These transporters consist of one transmembrane domain (TMD) with eight to twelve transmembrane helices labeled 1 to 12. Both the N- and C-terminal ends are facing the cytosolic side of the membrane. The cytosolic domain of the transporter is divided into three major domains; The Actuator domain (A), the Nucleotide binding domain (N) and the phosphorylation domain (P). The P_4-ATPases in addition to the α-subunit consist of a β-subunit with two transmembrane spans and a large exoplasmic loop. (D) Model of ion transport through P-type ATPases. During the catalytical cycle the P-type ATPases undergoes large conformational changes during which a half channel is firstly opened toward the cytosolic side of the membrane. In this conformation the transporter has high affinity for the transported substrate. The enzyme becomes phosphorylated and undergoes a conformational change leading to the occlusion of the substrate followed by a closure of the half channel. An addition change in the conformation of the enzyme leads to the generation of an open half channel toward the exoplasmic side of the membrane in this conformation the substrate specificity decreases and the substrate is released. (E) Structural comparison between ABC transporters and P-type ATPases. Left, model of the yeast P4-ATPase Dnf1p [118]; Right, *Salmonella typhimurium* ABC transporter MsbA (PDB code 3B60). (F) Schematic model of the position of intracellular loops (ILs) in an ABC exporter. Light and dark gray colors represent the two independent halves of the transporter. ILs are designated by numbers (1–4). IL1 and IL2, corresponding to the first half transporter, interact with the *cis*-NBD and the *trans*-NBD, respectively. The same is the case for IL3 and IL4, corresponding to the second half transporter.

the NBD domain of each half monomer [39,40,52]. These interactions may allow for intramolecular communication between the NBDs and the TMD within the ABC transporter.

ABC "importers" present two distinct types of fold, Type I and Type II. Type I transporters mediate uptake of relatively small substrates (sugars, ions and amino acids) and are structurally represented by the bacterial molybdate transporter ModBC [54,55]. They typically contain 12 transmembrane segments, arranged into two core bundles of five helices in each half transporter or half monomer, with an N-terminal extra helix that wraps around the TMD of the other half transporter or half monomer [36]. In some cases, this extra helix is lacking, such as for the *E. coli* methionine importer MetNI [56], or additional regulatory helices might be present, as is the case for the bacterial maltose transporter $MalFGK_2$ [33,57]. Further biochemical and structural analyses are required to understand the consequences of this phenomenon for substrate movement.

Type II ABC "importers", involved in the transport of much bigger molecules, such as heme, metal chelates or vitamin B12, generally

Table 3
Available crystal structures for ABC transporters of the exporter fold.

Protein	Conformation	PDB entry*	Reference
Sav1866	Outward facing	2ONJ (3.40)	[45]
	Inward facing	2HYD (3.00)	[41]
MsbA	Outward facing	3B60 (3.70), 3B5Y (4.50), 3B5Z (5.20)	[39]
	Inward facing	3B5W (5.30), 3B5X (5.50)	[39]
TM287/288	Inward facing	3QF4 (3.90)	[48]
ABCB10	Inward facing	3ZDQ (2.85), 4AYW (3.30), 4AYX (2.90), 4AYT (2.85)	[40]
P-glycoprotein	Inward facing	3G61 (4.35), 3G60 (4.40), 3G5U (3.80), 4F4C (3.40), 4KSC (4.00), 4KSB (3.80), 4LSG (3.80), 4M1M (3.80), 4M2S (4.40), 4M2T (4.35), 4KSD (4.10)	[47,76,193,194]
CmABCB1	Inward facing	3WME, 3WMF, 3WMG (2.40/2.60)	[93]

* Numbers in brackets indicate structure resolution in Å.

contain 10 transmembrane helices in each half transporter, for a total of 20 hydrophobic helices in a full transporter [36]. Both Type I and Type II importers present shorter α-helices than the known ABC exporters and, at least the bacterial ones, require the presence of extracytosolic accessory proteins involved in substrate loading [58–60].

In a similar manner as the ABC "exporters", ABC "importers" have been crystallized both in "open-inwards" and "open-outwards" conformations, related to each order by rotation of the transmembrane domains as a consequence of the formation of a closed dimer between the NBDs [37]. Dimerization may be the result of ATP binding and its disruption a consequence of ATP hydrolysis. Coordination of the TMDs and the NBDs is achieved by the presence of two coupling helices extending from the TMDs of each half transporter. The coupling helices contain the so-called "EAA motif", a sequence of 15–20 amino acid residues considered to be a signature motif for ABC "importers" [61]. The EAA motif interacts with several residues in the NBDs, which are specific for Type I or Type II importers [62].

At least two ABC "importers", *Candida albicans* Cdr3p and mammalian ABCA4, appear to function as pumps catalyzing inward phospholipid transport [63–65]. Interestingly, in contrast to Cdr3p, two other members of the *C. albicans* Cdr family, Cdr1p and Cdr2p, have been characterized as outward phospholipid transporters [63]. Cdr3p possess 56 and 55% sequence identity to Cdr1p and Cdr2p, respectively, and based on secondary structure predictions, would be expected to fold similarly to these and other Cdr proteins [66]. Likewise, human ABCA4 and its closest homologue, ABCA1, have a similar predicted architecture, but transport phospholipids in opposite directions [65]. The transport activity of both ABCA1 and ABCA4 was demonstrated after reconstitution of purified proteins in proteoliposomes in the absence of accessory substrate binding proteins, which provides strong evidence that these proteins are directly catalyzing phospholipid flipping in opposite directions [64,65]. Does this mean that Cdr3p and ABCB4 present an "importer" fold in contrast to their homologues, Cdr1p and ABCA4, respectively, which would be predicted to present "exporter" folds? Membrane span predictions and whole sequence homology show that the architecture for these importers is not compliant with the bacterial importer type but, notably, ABCA4 contains a conserved EAA amino acid motif that is a signature for bacterial ABC importers [61,67]. It is possible that eukaryotic ABC "importers" present the same fold as "exporters", but have evolved an inverse affinity switch that allow them to transport the substrate inwards. Indeed, it has been demonstrated that some plant ABC transporters are capable of switching between the importer and exporter mode depending on substrate concentrations [68,69].

Of the five ABC "exporters" crystalized as full proteins to date, two have been shown to transport fluorescent phospholipid analogs in reconstituted systems (Tables 1 and 3) [70–72]. Due to the scarce information available about eukaryotic phospholipid "importers", in the following we will only discuss the putative transport mechanism of ABC "exporters". For a more concise review on the structural folding of ABC importers and its mechanistic consequences the reader is referred to [36,73].

3.1.2. Putative catalytic mechanism of ABC transporters of the "exporter" fold

The precise mechanism of coupling ATP binding and hydrolysis to transport is still not completely understood. Two general models try to explain this process: (i) the "ATP switch" model, and (ii) the "constant contact" model. Common for both is that they are "alternating access" models, according to which the pump alternates between two major conformational states. In one state, the binding site for the transported substrate faces one side of the membrane. In the other state, the site faces the other side of the membrane. A number of other proposals have been made that provide intermediate solutions for the mechanism of transport of some ABC exporters. The reader is referred to [53] for an extensive discussion on the subject.

According to the first "*ATP switch*" model (Fig. 1B) [25,74], the NBDs fully disassociate from each other during the catalytic cycle. Each catalytic cycle starts with the TMDs in an open-inward conformation and the NBDs separated from each other and none of them with bound nucleotide. The high affinity of the binding site will favor binding of the substrate inside the cavity. Substrate binding results in a rearrangement of the TMDs and repositioning of the NBDs, which allows for ATP binding at both sites and formation of a closed-dimer intermediate. According to this model, binding of ATP and subsequent movement of the NBDs into the closed-dimer configuration provides the power stroke that forces the transmembrane segments of the TMDs to close the cavity facing the cytoplasm and assume an open-outwards conformation. Rearrangement of amino acid residues with respect to the substrate during movement of the TMDs eliminates the high affinity binding site, which has the consequence that the substrate gets released to the extracellular side of the membrane. Finally, hydrolysis of either one or both molecules of ATP disrupts the closed NBD dimer and restores the ABC transporter in its original configuration. The driving force for this reaction comes from an increase in negative charge at the NBD due to the formation of ADP and Pi with subsequent electrostatic repulsion [75].

Although the ATP switch model can explain the action of many ABC transporters, it is still highly debated. Recent crystallographic characterization of human ABCB10 and the heterodimeric transporter TM287/288 from *Thermotoga maritima* suggests that the two NBDs are never far apart from each other during the catalytic cycle [40,48], in contrast with what was indicated by the apo-structures of MsbA and P-glycoprotein [39,47,76]. Moreover, it is still unclear whether it is ATP binding or ATP hydrolysis that actually drives conformational changes accompanying substrate translocation. The crystal structures of bacterial Sav1866 and MsbA in the presence of the non-hydrolysable ATP analog AMP-PNP present an open-outwards conformation [39,45]. This would suggest that ATP binding is sufficient to stabilize the open outwards conformation implying that the power stroke for conformational change happens before ATP hydrolysis. In contrast, crystallization of human ABCB10 and the heterodimeric *Thermotoga* transporter TM227/228 in the presence of the same ATP analog results in proteins locked in the open-inwards conformation [40,48], which in analogy with the

assumption above would indicate that ATP hydrolysis is required for the large conformational change. Whether these differences are due to mechanistic reasons or just an artifact of crystallization remains to be elucidated. Indeed, molecular dynamics simulations with human ABCB1 have led to the proposal that the very wide open-inwards conformations may represent non-physiological states that are stabilized by crystal lattice contacts [77].

According to the second "*constant contact model*", the two NBDs do not dissociate from each other during catalysis, and each ATP binding site hydrolyze ATP and open alternately [78,79]. This implies that a single catalytic cycle does not imply binding, hydrolysis and release of the same ATP molecule. Instead, in each transport cycle, one of the NBDs will hydrolyze a bound ATP, while the other NBD will unload ADP and Pi produced at this site during the previous catalytic cycle. In order to promote ADP and Pi release, the NBD containing hydrolyzed ATP rotates outwards which in turn forces the pump to acquire an inward-open conformation, as shown in the crystal structures of MsbA. The proposal that the NBDs never become distantly separated from each other is supported by the ABCB10 structures [40]. The biochemical and biophysical evidence supporting the "*constant contact model*" has been reviewed recently [80].

ABC "exporters" seem to have a stoichiometry of one to two molecules of ATP hydrolyzed per molecule of transported substrate. However, it has been shown that some of these transporters can be partially uncoupled in reconstituted systems [81,82]. A quest to understand the uncoupling process has led to extensive evidence that the two NBDs bind and hydrolyze ATP in a cooperative manner [83–86].

3.1.3. Putative phospholipid flipping mechanism of ABC transporters of the "exporter" fold

Although many details are known about the mechanism by which ABC exporters carry out ATP hydrolysis and transduce the rearrangement of the NBDs to the TMDs, very little is known about transport of the substrate. Of the five ABC transporters of the exporter fold crystallized to date only bacterial MsbA and P-glycoprotein (ABCB1) have been shown to transport lipidic substrates. Depletion of cellular MsbA or the presence of a conditionally inactivating mutation in this protein result in loss of lipid A and phospholipid transport from the cytoplasmic to the outer membrane in bacterial cells, suggesting a general flippase function for MsbA [87,88]. This evidence is confirmed by reconstitution experiments, in which MsbA has been shown to translocate a wide variety of fluorescent-labeled phospholipids, albeit with low activity [72]. In the case of P-glycoprotein, studies in cell lines and reconstituted systems have suggested the involvement of this protein in transport of a variety of phospho- and glycolipids [65,70,89–91]. However, this protein was first identified as the multidrug resistance transporter responsible for the inefficiency of long-term pharmacological treatments [92]. The flexibility in the recognition of substrates complicates greatly the task of defining a putative binding site for phospholipids within one of these crystal structures. Furthermore, most crystal structures for ABC "exporters" have a relatively low resolution (Table 3), which do not allow for confident modeling of the position of the amino acid chains. It has not been until very recently that the barrier of 3 Å resolution was broken and a putative entry pathway for an amphipathic substrate was proposed for the mitochondrial putative di-peptide transporter ABCB10 [40]. Recently, the crystal structure of an inward open half-size multidrug transporter, CmABCB1, was solved at 2.6 Å resolution, which, supported by mutant and transport analyses, revealed the structural basis for drug substrate gating [93]. Interestingly, a cavity lies exactly within the hydrophilic heads of the lipids and corresponds to the MsbA stretch where Cys mutants showed decreased basal activity [94].

Two speculative models on ABC-type flippases have been proposed, the "tilting model" and the "rotating helix model", that are however not exclusive [93,216]. In the tilting model, a variation of the alternating access model, the lipid enters the chamber of MsbA from the inner leaflet of the bilayer. ATP binding causes conformational changes of the TMDs, leading to the release of the substrate into the outer space (see Fig. 1B). In the rotating helix model, conformational changes of ABCB1 lead to the rotation of the TM helices reorienting the substrate-binding site from the inner leaflet of the lipid bilayer to the outer, from where the substrate is released.

Data from the ABCB1 structures revealed substantial TM helices repacking upon nucleotide binding, which is consistent with the rotating helix model [95]. However, in respect to the significant size of most lipids but especially the lipid-A core transported by MsbA, it seems likely that the flippase ABC does not completely enclose the lipid substrates, which is better covered by the alternating access model. This has been widely accepted for other flippases and is further supported by structural data on MsbA employing spin labeling and EPR [94]. However, the finding that both crystal structure and EPR studies indicate large conformational changes in MsbA that correlate with the huge size of the MsbA lipid-A substrate, might simply indicate that MsbA and ABCBs employ distinct flippase mechanisms.

Mutations in phospholipid binding domains should have a striking impact on the activity and substrate specificity of flippases. Site-directed mutagenesis has been carried out extensively in MsbA and substitution of a number of residues affected catalytic turnover by this transporter [94]. However, as only basal ATP hydrolytic activity was determined, it remains to be shown that in this system ATP hydrolysis is strictly coupled to transport. Interestingly, the suggested lipid–drug duality of flippases has been explained by a coupling of phospholipid transportation and drug transport [96]. According to this assumption ABCB1 would act as a phospholipid flippase caused as a side effect of its main drug transport function, while the opposite would hold true for MsbA [97].

In all ABCB10 structures, TM1-3 and TM6 of one monomer associate with TM4 and TM5 of the other monomer [40]. In the structure of the ABCB10 homodimer in the absence of bound nucleotide, TM1 and TM2 are separated from each other by a maximal distance of almost 14 Å, enough for an amphipathic substrate to be bound between the transmembrane loops. This distance is kept in a structure in which the NBDs are bound to the non-hydrolysable ATP analog AMP-PCP (Fig. 2A). Interestingly, the latter structure shows some electron density in the region between TM1 and TM2 that can be modeled to the aliphatic chain of a cardiolipin molecule (Fig. 2A). Although ABCB10 has never been implicated in the transport of cardiolipin, the structure demonstrates the possibility for the aliphatic chain of a lipid-like amphipathic substrate to penetrate sideways from the membrane into the TMD of the ABC exporter. Sequence homology alignments between different members of the ABCB family of ABC transporters have identified the presence of highly conserved residues in TM1-3 that have been suggested to form part of the substrate binding region (Fig. 2A) [40]. These residues are located toward the cytosolic side of the large central cavity of the ABC dimer where they have been proposed to coordinate the hydrophilic part of an amphipathic substrate, whereas its hydrophobic region could be accommodated in the gap formed by TM1 and TM2 (Fig. 2A). In a third structure, crystallized in the presence of the ATP analog AMP-PNP, the distance between TM1 and TM2 is reduced to about 9 Å, which suggests a conformation wherein the entry pathway is closed to prevent the substrate from moving back into the membrane before transport to the other leaflet takes place [40].

The proposed transport mechanism of ABCB10 is in agreement with the rotating helix model, in which rotation of the TMDs during the catalytic cycle allows for alternating opening not only of a central cavity, but also of lateral entry and exit pathways for the transported phospholipid substrate [95]. Unfortunately, all structures obtained for ABCB10 are in an open-inwards or closed-inwards conformation with the TMDs forming a central cavity opened to the cytosolic side, and the absence of a structure in the proposed open-outwards conformation limits our understanding of the catalytic cycle and how the transported substrate is delivered back to the membrane. However, although the AMP-PNP bound form of ABCB10 presents a closed-inwards conformation, the AMP-PNP-bound forms of the bacterial "exporters" MsbA and

Sav21866 show an open-outwards conformation, where the TMDs rearrange to create a substantial central cavity opened to the exoplasmic side of the membrane (Fig. 2A). TM1 and TM2 for one monomer, now associate with TM3–TM6 of the other monomer. TM3 and TM4 are now forming a lateral opening analogous to the one formed by TM1 and TM2 in the open-inwards conformation of ABCB10, which would allow for lateral exit to the membrane environment of a transported amphipathic substrate. Further biochemical and structural characterization is required to understand whether these gaps between transmembrane domains actually form pathways for entry and exit of the substrate.

3.2. Phospholipid flippases of the P-type ATPase family

P4-ATPases constitute a major group of phospholipid flippases that are not related to ABC transporters. Rather, they belong to the P-type ATPase superfamily, a large family of ATP driven biological pumps, which transport a number of different substrates but share the common feature that they form a phosphorylated intermediate during their catalytic cycle [98]. P-type ATPases are divided into five subfamilies (P1 to P5) characterized by their transported substrate [98]. P1 to P3-type ATPases are cation transporters, such as the heavy metal transporters involved in cytoplasm detoxification and loading of metals inside specific tissues and cellular compartments (P1-ATPases), the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA; a P2-ATPase) involved in maintaining calcium homeostasis during signaling, the plasma membrane Na^{+}/K^{+}-ATPase (a P2-ATPase) that generates the membrane potential in mammalian cells, and the plasma membrane H^{+}-ATPase energizing plant and fungal cell membranes (a P3-ATPase). Recently, structured logistic regression has been used as a machine approach to identify all P-type ATPases in the UniProtKB database and classify them in P-type ATPase subfamilies [99]. Using this automated method less than one hundred prokaryotic sequences can with low statistical significance be classified as P4- and P5-ATPases [95], but individual inspection of the sequences identified so far reveals that they all show higher overall homology to P2-ATPases than to P4-ATPases. We can thus conclude that while cation transporters of the P-type family are found in all three branches of life, members of the P4- and P5-ATPase subfamilies are only present in eukaryotic organisms. Whereas the transported substrate of P5-ATPases remains to be determined, P4-ATPases act as inward phospholipid transporters (Table 2).

3.2.1. General structural features of P-type ATPases

In contrast to ABC transporters, the catalytic subunit of P-type ATPases is always encoded by a single polypeptide (Fig. 1C). Several crystal structures are available that allow for visualization of various pump proteins at different steps during the catalytic cycle (Table 4). Even though the published P-type ATPase crystal structures show characteristic individual peculiarities, they all exhibit a common fold. Thus, four distinct structural regions, first defined and named by Toyoshima et al. (2000) [100], are present in all P-type ATPases (Fig. 1C): a membrane domain (M), that forms the pore through which the substrate is transported; a variable nucleotide-binding domain (N), that functions as a built-in protein kinase that phosphorylates the pump and where ATP docks mainly through hydrophobic stacking of the adenosine ring to a conserved phenylalanine; a phosphorylation domain (P) that contains the conserved amino acid sequence DKTG including the aspartate residue that gets phosphorylated during catalysis (hence P-type) and two other conserved motifs TGDN and GDGXND involved in the coordination of a Mg^{2+} ion required for ATP binding at the phosphorylation site; and a built-in protein phosphatase, named the actuator domain (A), comprising the amino acid sequence TGE, in which the glutamic acid generates a nucleophilic attack on the phosphorylated aspartate and causes dephosphorylation of the P-type pump. These four regions are connected to each other with amino acid linkers and suffer dramatic movements during the catalytic cycle, which involves two main protein conformations, *Enzyme 1* (E1) and *Enzyme 2* (E2), in which the substrate binding site presents different substrate affinities and is open to opposite sides of the membrane (Fig. 1D).

The conformational transitions between E1 and E2 are large and require very flexible pump molecules. For some P-type ATPases, the N- and/or C-terminal domains are autoinhibitory, preventing the conformational changes required for substrate transport by establishing intramolecular interactions with other parts of the P-type ATPase [101,102]. These autoinhibitory domains contain regulatory sequences that interact with accessory proteins, such as calmodulin or 14-3-3 proteins in order to trigger activation of the pump [103].

Like other P-type ATPases, P4-ATPases are predicted to have 10 transmembrane spanning segments and both N- and C-termini facing the cytosol [104]. The A-domain is formed by amino acid residues located at the N-terminal domain and the intracellular loop between TM2 and TM3, while the P- and N-domains are located to the intracellular loop between TM4 and TM5 [10399].

3.2.2. General catalytic cycle of P-type ATPases

The SERCA1 Ca^{2+} pump from rabbit skeletal muscle is by far the best structurally characterized P-type ATPase. Not only E1 and E2 forms of the protein have been crystallized, but also a large number of intermediate conformations representing almost all possible protein movements and interactions with the transported substrate, ATP or its hydrolysis products (Table 4). Taken together, this wealth of structural information makes it possible to outline a detailed mechanism for the action of this pump [105].

The catalytic cycle of cation transporting P-type ATPases starts when the protein in the E1 conformation binds the substrate at the M-domain in a high affinity site opened to the cytosolic side of the membrane [106,107]. This event initiates a structural rearrangement that is transmitted across the pump molecule and makes possible the phosphorylation of the P-domain by an ATP molecule already bound at the N-domain, generating the E1P conformation. Transition into the E2P state occurs when the A-domain rotates almost 120° and forces a reorganization of the M-domain, which brings the active site of the built-in protein phosphatase of the A-domain in close proximity to the phosphorylated aspartyl group. This reorganization results in an initial occluded state in which the ion is blocked inside the protein and, subsequently, the opening of a low affinity binding site to the exoplasmic side of the membrane, which causes release of the bound cytosolic substrate and, in some cases, binding of a counter-ion. Nucleophilic attack of the A-domain onto the phosphorylated aspartate leaves the protein in the E2 conformation that is unstable and transitions to the E1 conformation forcing the A-domain to rotate back to its original position. Movement of the A-domain results in reorganization of the M-domain, with the consequent release of the exoplasmic substrate (the counter-ion) to the cytosolic side of the membrane, see [108] for further detail.

Although no crystal structure is available for a P4-ATPase, these proteins are probably not very different in folding from other members of the P-type ATPase family, as suggested by the fact that (i) all P-type ATPases including P4-ATPases have conserved core sequence segments that are essential for catalysis [98,109], and (ii) all crystallized P-type ATPases show a very similar tertiary structure despite their varying TMD numbers and diverse substrates ranging from protons to heavy metals [108,110]. Thus, assuming an analogous transport mechanism for P4-ATPases as for the SERCA pump, the phospholipid would occupy the place of the counter-ion and therefore be bound in the E2P conformation at the exoplamic side of the membrane and released in the transition step to E2 on the cytosolic side. Whether an additional substrate is transported in the opposite direction is yet unknown.

3.2.3. Putative phospholipid flipping mechanism of P4-ATPases

In contrast to the different cations transported by members of the P1 to P3 subfamilies of P-type ATPases, phospholipids are voluminous substrates that contain both a substantial hydrophilic head group and two

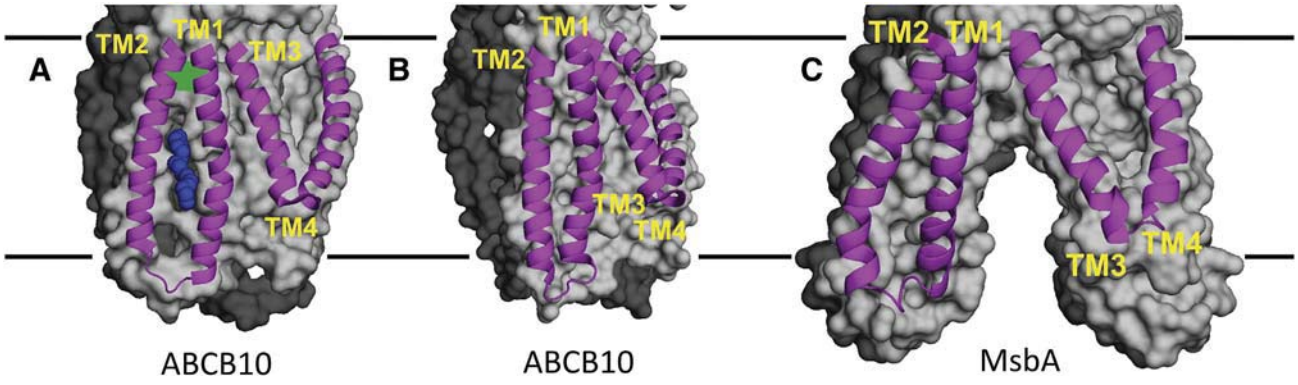

Fig. 2. Putative lipid translocating pathways for an ABC exporter. (A) In the Rod A crystal structure of ABAB10 (PDB code 4AYT), a lateral opening between TM1 and TM2 shows electron density that can be assigned to a cardiolipin molecule (dark blue). A putative lipid would be loaded by inserting its fatty acid tail in an analogous manner, while the head group interacts with hydrophilic residues (green). (B) A closing of the gap between TM1 and TM2 results in occlusion of the lipid as suggested by the plate form crystal structure of ABCB10 (PDB code 4AYW). (C) Based on the crystal structure of MsbA in the presence of AMP-PNP (PDB code 3B60), rotation of the alpha helices in the TMD generates a lateral opening to the membrane between TM3 and TM4 that can be used to unload the lipid.

long fatty acid chains of a hydrophobic nature (Fig. 3C). It is difficult to envisage how such a substrate can be introduced inside a protein pore opened to one side of the membrane, be entirely occluded during the structural transition of the protein through the catalytic cycle, and then exposed to the other side of the membrane for its release. So how do P4-ATPases manage their substrate?

A characteristic feature of P4-ATPases is the presence of a β-subunit required for the catalytic cycle. This protein belongs to the family of yeast Cdc50 proteins and contains two transmembrane domains and a large luminal loop that is heavily glycosylated [111] (Fig.1C). Topologically, this protein resembles a fusion of the β- and γ-subunits of mammalian Na^+/K^+-ATPases [112]. As in the case of the Na^+/K^+-ATPase β-subunit, the Cdc50 protein is required for proper folding and exit of the P-type ATPase from the ER [111,103–116]. For the Na^+/K^+-ATPase, the γ-subunit is involved in the modification of the kinetic parameters of cation transport depending on tissue and environmental conditions [117–119]. For the yeast P4-ATPases, the Cdc50 protein is strictly required for the generation of a phosphorylated intermediate and consequently for phospholipid transport, although it is not clear how this protein contributes to P4-ATPase activity [120,121]. It has been suggested that the Cdc50 protein is interacting with the transported phospholipid and responsible together with the P-type ATPase of generating a pathway for movement of the phospholipid through the membrane [121]. However, phospholipid specificity determinants for the P4-ATPase/β-subunit complex reside solely in the P-type protein [114,122], which makes it improbable for the β-subunit to directly participate in substrate binding during transport. Most likely, the β-subunit is required for stabilizing the P4-ATPase in some of the intermediate conformations during catalysis [123].

Recent mutational studies suggest two pathways by which P4-ATPases could transport their substrate (Fig. 3): (i) the canonical pathway with the phospholipid transported through the interior of P4-ATPases in analogy with the cation transport mechanism of well characterized P2-ATPases and (ii) a non-canonical pathway at the protein–membrane interface. An increasing amount of biochemical evidence seems to suggest that these two extreme models might be partial descriptions of the same mechanism.

In the thoroughly studied Ca^{2+}- and Na^+/K^+ transporting P2-ATPases, 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 [105]. Conserved acidic group(s) positioned in the middle of TM4 and TM6 secure charge neutralization of the transported cation, while two conserved asparagine residues in TM5 and TM6 provide oxygen groups for ion coordination. Further coordination is provided by backbone carbonyl oxygen atoms in TM4, that contains one or more highly conserved proline residues that function as helix-breakers [103]. Likewise, all P4-ATPases contain a conserved proline in the middle of TM4 (Pro-507 in Drs2p) suggesting the presence in these pumps of a central cavity with carbonyl oxygens contributing to the coordination of a hydrophilic group.

In contrast, P4-ATPases do not have preserved acidic residues in their predicted transmembrane segments [109]. However, a basic lysine residue (corresponding to Lys-1018 in the yeast P4-ATPase Drs2p; 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 [124]. Mutation of this residue has a dramatic negative impact on the apparent affinity of ATP8A2 for its substrate PS as judged by the ability of the substrate to induce dephosphorylation, or stimulate ATPase activity [124]. The binding energy of the substrate has to be transduced to the cytosolic domains in these assays, and either the binding or transduction could be perturbed by the mutation [124]. Given that several P4-ATPases tolerate drastic modifications of the acyl chains of the phospholipid substrate, this model would imply considerable flexibility of P4-ATPases to accommodate their substrate and especially the phospholipid tail when it traverses the membrane.

In contrast to this, substitution in the yeast P4-ATPase Dnf1p of the conserved lysine in TM5 (Lys-1194) results in a reduction of protein activity, but does not affect substrate specificity, suggesting that this residue is not directly binding the substrate [125]. Further mutational studies of 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, but not within the membrane region [122,125]. Two main residues determine the specificity of the Dnf1p protein: a tyrosine close to the cytosolic side of TM4 (Tyr-618 in Dnf1p) and a phenylalanine in the loop that connects TM3 and TM4 (Phe-587 in Dnf1p). Mutation of Tyr-618 to phenylalanine (the corresponding residue in the PS-transporting P4-ATPase Drs2p) confers Dnf1 the ability to transport PS, without affecting PC and PE translocation, while mutation of Phe-587 reduces PC recognition without affecting PE. This feature has led to the proposal of a two-gate system that would recognize the phospholipid on one side of the membrane, allow it to slide to the other side with the hydrophobic tail always protruding through a cleft toward the phospholipid bilayer and then release it on the other side of the membrane [125].

Indeed, a combination of biochemical analysis of mutants and protein modeling has been recently used to suggest a mechanistic model in which a conserved isoleucine in TM4 (corresponding to Ile-364 in the mammalian photoreceptor P4-ATPase ATP8A2) and its adjacent residues form a hydrophobic gate that allows the formation of alternating water-filled cavities on both sides of the groove formed by TM1, TM2, TM4, and TM6, similarly to the cation-gating role ascribed to the

Table 4
Available crystal structures for P-type ATPases.

Protein	Subclass	Conformation*	PDB entry**	Reference
Na^+,K^+-ATPase	P2	E2P	3N2F (4.10), 3 N23 (4.60), 3B8E (3.50), 3KDP (3.50), 3A3Y (2.80), 2ZXE (2.40)	[195–197]
		E1P	4HQJ (4.30), 3WGU(2.80), 3WGV (2.80)	[198–200]
H^+-ATPase (AHA2)	P3	E1	3B8C (3.60)	[110]
Cu^+-ATPase (CopA)	P1	E2P	3RFU (3.20), 4BBJ (2.80)	[201,202]
Ca^{2+}-ATPase (SERCA)	P2	E1	4H1W (3.10), 3W5A (3.01), 3W5B (3.20), 3TLM (2.95), 2C9M (3.00)	[203–206]
		E1P	3BA6 (2.80), 3N8G (2.59), 2DQS (2.50), 1VFP (2.90), 1T5S (2.60), 1T5T (2.90), 1SU4 (2.40)	[100,128,207–211]
		E2P	4J2T (3.20), 3N5K (2.20), 3AR2 (2.50), 3AR3 (2.30), 3AR4 (2.15), 3AR5 (2.20), 3AR6 (2.20), 3AR7 (2.15), 3AR8 (2.60), 3AR9 (2.60), 3B9B (2.65), 3FGO (2.50), 3FPB (2.55), 3FPS (3.20), 3N23(4.60), 3W5D (2.45), 2ZBD (2.40), 2ZBE (3.80), 2ZBF (2.40), 2ZBG (2.55), 2O9J (2.65), 1XP5 (3.00), 1WPG (2.30)	[129,133,196,204,207, 209–214]
		E2	4KYT (2.83), 2YFY (3.10), 3NAL (2.65), 3NAM (3.10), 3NAN (3.10), 3B9R (3.00), 3W5C (2.50), 2OA0 (3.40), 2EAR (3.10), 2EAS (3.40), 2EAT (2.90), 2EAU (2.80), 2C88 (3.10), 2C8K (2.80), 2C8L (3.10), 2BY4 (3.30), 2AGV (2.40), 1IWO (3.10)	[128,129,204,206,214–220]

* E1 and E2 denote totally dephosphorylated pumps, while E1P and E2P indicate the phosphorylated states and the transitions states to/from dephosphorylated conformations.
** Numbers in brackets indicate structure resolution in Å.

conserved glutamate residue present at the same position in other P-type ATPases [126]. These water-filled cavities would protect the hydrophilic phospholipid head group from the hydrophobic environment during its passage through the membrane, while the phospholipid tails protrude freely into the hydrophobic environment.

Further studies on the cooperation of Tyr-618 with other residues of TM1–TM3 has demonstrated that a number of amino acid residues located on the cytosolic side close to the M-domain contribute to a proposed exit gate for the phospholipid [127]. These residues also allow Dnf1p (and its homologue Dnf2p) to discriminate between mono- and di-acylphospholipids, which lead to the proposal that these yeast P4-ATPases function primarily as lyso-lipid transporters [127]. Based on homology modeling using the Na^+/K^+-ATPase as a template, these residues were mapped to a region of the SERCA Ca^{2+}-ATPase that forms a binding pocket for a PE molecule in the E2 conformation [127, 128]. This PE molecule has been proposed to assist Ca^{2+} entry into a deep, funnel-shaped and negatively charged path that leads to the Ca^{2+} coordination sites located halfway through the membrane bilayer [128,129]. The binding pocket is not accessible in the E1 conformation, implying that the PE molecule has to dynamically enter and exit the pocket as the SERCA Ca^{2+}-ATPase proceeds through the catalytic cycle. It is possible that this original phospholipid head group binding pocket and cation-loading funnel have evolved in P4-ATPases to generate a cleft for phospholipid translocation. Indeed, the homology model for Dnf1p in the E2 conformation shows a triangular shaped groove flanked by TM1 and TM3 on the sides and TM4 on the back [122]. Although the Dnf1p structural model has proven useful to identify residues for biochemical studies, conclusions based on it need to be carefully assessed, as the protein shows only 17% identity to the Na^+/K^+-ATPase used as a template. Further, the model cannot explain some of the most recent mutagenesis results. Two Dnf1p residues located inside the membrane plane in TM2 (Thr-254 and Asp-258) are involved in discrimination of lysophospholipids [127]. However, in the model, TM2 is located behind TM1 and has no access to the groove formed by TM1, TM3 and TM4, where the phospholipid is suggested to bind.

Interestingly, SERCA has been crystalized in the E2 conformation with bound thapsigargin, a potent inhibitor of the SERCA pump with phospholipid like features (Fig. 4, in blue) [128]. This crystal structure also shows electron density that can be assigned to a PE molecule (Fig. 4, in green). While PE is localized in a groove formed by TM2 and TM4 with TM1 covering the backside, thapsigargin binds in a similar groove on the opposite side of the M-domain, with TM3 and TM6 forming the sides and TM5 at the back. Notably, the acyl chains of thapsigargin sit deeply between the transmembrane sections of the SERCA Ca^{2+}-pump, which would be consistent with high structural flexibility [128]. Whether the position of the transmembrane segments in P4-ATPases would allow for binding of a phospholipid (and its subsequent transport) in a position analogous to that of PE or tharpsigargin in SERCA still needs to be demonstrated and would require the solution of a crystal structure for one of these proteins. However, it seems possible that P4-ATPases have evolved to generate a pathway through the membrane that would connect both binding sites, allowing for phospholipid movement across them.

3.3. Comparison of ABC transporters of the "exporter" fold and P4-ATPases

From a distant point of view, ABC transporters of the "exporter" fold and P4-ATPases might look as if they were similar machines: both exist in at least two conformers with different substrate affinities at each side of the membrane and ATP provides the driving force for a conformational interchange. Generally speaking the mechanism by which both proteins hydrolyze ATP is analogous, as can be inferred by the fact that both types of proteins are sensitive to vanadate [130,131], which mimics the transition state of ATP during hydrolysis [132].

A closer look reveals major differences between the transporters. Most notably, the fate of the γ-phosphate released from the hydrolysed ATP molecule, is very different in the two pump classes: P4-ATPases form a phosphorylated intermediate in which Pi is covalently bound to an aspartate residue located in a conserved amino acid motif within the nucleotide binding site. Release of this Pi requires active nucleophilic attack of a glutamic acid in the actuator domain [133]. In contrast, covalent protein phosphorylation has not been described for ABC transporters [74]. Further, P4-ATPases have a single location for ATP binding, while ABC transporters feature a closed NBD dimer that is stabilized by ATP as described in detail above. Up to now there are no structural reports of NBDs of P-type ATPases functioning as dimers. Moreover, in ABC exporters, ADP and Pi are suggested to be released by electrostatic repulsions after hydrolysis [74] and therefore ATP hydrolysis is most probably not used for the translocation step but for resetting the ABC transporter to its original conformation.

Despite these differences in energization, based on protein mass and structural models, the size of ABC exporters and P4-ATPases would be expected to be similar in the membrane (Fig. 1E) and the models proposed for explaining phospholipid transport by these two types of transporters present some remarkable similarities. Are ABC transporters and P4-ATPases using the same mechanism to transport phospholipids in opposite directions?

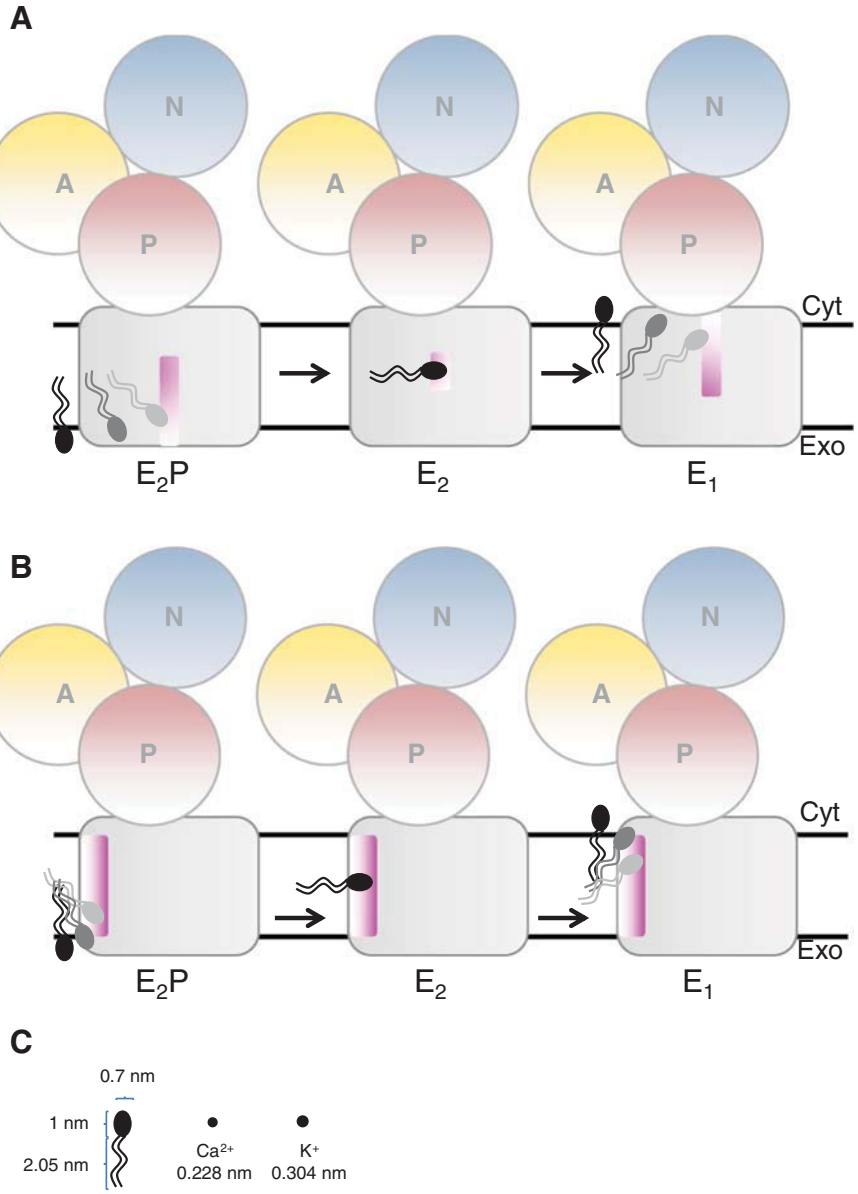

Fig. 3. The canonical versus the non-canonical model of lipid translocation by P4-ATPases. (A) The canonical pathway, in which the lipid enters the protein in a way analogous to the entry of cations in other P-type ATPases. After occlusion in the binding site, the rearrangement of the TM-domain results in opening of an exit pathway on the opposite side of the membrane. (B) The **non-canonical pathway**, in which the lipid head group is recognized by interaction with residues located in the boundary of the membrane, slides through the protein surface and is released on the other side of the membrane after rearrangement of the TM-domain. (C) Relative sizes of various substrates transported by P-type ATPases.

In both the "*alternating access model*" for ABC transporters and in the classical transport model for P4-ATPases, the phospholipid is proposed to gain access from one side of the membrane to a central cavity inside the transporter that is then closed and opened to the opposite side (Fig. 1B and 1D). The cavities supposedly used to allocate the substrate during transport would, nevertheless, be very different in size. In ABC transporters, the wide central cavity leaves plenty of space to accommodate a complete phospholipid molecule. Such an opening is unlikely to be present in P4-ATPases, yet lipids and lipid-like molecules like thapsigargin have been found deeply embedded in the crystal structure, suggesting a possible access pathway of the substrate to a central binding pocket, in analogy to other cation-transporting P-type ATPases. However, a substantial amount of evidence is still required to understand whether the big central cavity in ABC transporters is used for phospholipid translocation, and whether P4-ATPases and/or ABC transporters achieve an occluded state during phospholipid transport in which the phospholipid is buried within the pump molecule.

Likewise, the substrate binding mechanism proposed for the ABC transporter ABCB10 is similar to the non-canonical model for P4-ATPase transport. In both, it is suggested that a lateral groove in the transmembrane region will provide access of an amphipathic substrate directly from the membrane. For P4-ATPases, this groove would open for substrate access from the exoplasmic leaflet of the membrane in the E2P phospholipid-loading conformation and to the cytosolic leaflet in the E1 conformation after phospholipid release [122]. For ABC exporters, the groove would open to the cytosolic leaflet in the open-inwards, substrate-loading conformation and to the exoplasmic leaflet in the open-outwards conformation [40].

Finally, although P4-ATPases only contain 10 transmembrane domains, phospholipid translocation has only been shown for members of the family that interact with a β-subunit containing two transmembrane helices. Potentially, a total of 12 transmembrane domains might be required for flippase activity. Notably, ABC transporters related to phospholipid transport also contain the same number of transmembrane

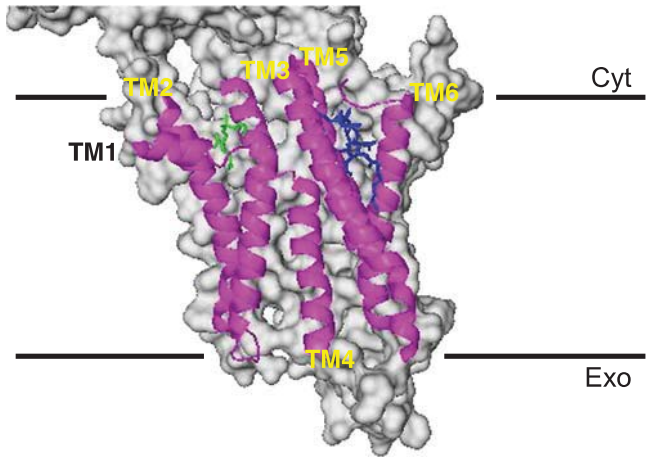

Fig. 4. Position of thapsigargin and phosphatidylethanolamine in the E2 conformation of Ca^{2+} pump sarcoendoplasmic reticulum calcium ATPase (SERCA1a). Thapsigargin and phosphatidylethanolamine (PE) bind in opposite sides of the SERCA E2 structure (PDB code 2AGV). PE (green) binds in a pocket formed by TM1, TM2 and TM4, while thapsigargin (blue), a molecule presenting lipid-like features, binds in a similar pocket on the other side of the protein between TM 3, TM5 and TM6.

segments. Further structural and biochemical characterization will be required to determine whether the twelve-TMD arrangement is the minimal optimized structure that allows for phospholipid translocation.

4. Major conclusions and general significance

Although an increasing amount of evidence is shedding light on the way phospholipid transporters discriminate their substrates, we are still very far from discerning the mechanism by which these substrates are translocated across cellular membranes. Unraveling this mechanism will require studies on the energy-dependent transporters reconstituted into proteoliposomes, a challenging task given the difficulties of purifying membrane proteins, the hydrophobic nature of the phospholipid substrates and the potential requirement for subunits and/or accessory proteins.

Promising steps have been taken to improve expression and large scale purification of P4-ATPases in complex with their subunit, a prerequisite for systematic crystallization trails [134]. Interestingly, a recent publication has reported on the ability of a P4-ATPase to counteract the toxic effects of overexpression of an ABC transporter in human cells [135], suggesting that novel strategies might be required to further improve heterologous expression production for these transporters. On the other hand, technology developments in membrane protein crystallization and synchrotron microcrystallography combined with advancements in protein stabilization using lipidic cubic phases may help to obtain structural information with smaller amounts of starting material [136]. Recent progress in the functional reconstitution of ABC transporters and P4-ATPases into vesicles and nanodiscs might allow in the near future for systematic studies aiming at identifying the phospholipid binding sites and at testing the existence of an occlusion state for the substrate during transport.

Finally, most evidence on phospholipid transport is based on labeled lipid analogs. In the future, new approaches will be needed to measure transbilayer phospholipid movement using natural phospholipids, such as shape-change visualization experiments in giant unilamellar vesicles.

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