Cell. Mol. Life Sci. 65 (2008) 3119 – 3125
1420-682X/08/203119-7
DOI 10.1007/s00018-008-8341-6

Cellular and Molecular Life Sciences

Visions & Reflections (Minireview)

Flippases: still more questions than answers

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Received 19 June 2008; received after revision 31 July 2008; accepted 15 August 2008
Online First 15 September 2008

Abstract. Our understanding of flippase-mediated lipid translocation and membrane vesiculation, and the involvement of P-type ATPases in these processes is just beginning to emerge. The results obtained so far demonstrate significant complexity within this field and point to major tasks for future research. Most importantly, biochemical characterization of P_4-ATPases is required in order to clarify whether these transporters indeed are capable of catalyzing trans-membrane phospholipid flipping. The β-subunit of P_4-ATPases shows unexpected similarities between the β- and γ-subunits of the Na^+/K^+-ATPase. It is likely that these proteins provide a similar solution to similar problems, and might have adopted similar structures to accomplish these tasks. No P_4-ATPases have been identified in the endoplasmic reticulum and it remains an intriguing possibility that, in this compartment, P_{5A}-ATPases are functional homologues of P_4-ATPases.

Keywords. Flippases, vesicle formation, phospholipid flipping, P_4-ATPases, P_{5A}-ATPases.

Trans-bilayer lipid flipping: a prerequisite for membrane trafficking in eukaryotes?

The secretory and endocytotic pathways are specific features of eukaryotic organisms. Exocytosis and endocytosis are prime events in these processes and both require formation of membrane vesicles from other membrane structures. Membrane vesicles are stabilized by coat proteins, such as clathrin and the coat protein complexes COPI and COPII. Although coat proteins can deform membranes by themselves, their action might not be sufficient to initiate vesicle budding [1].

The local accumulation of phospholipids on one side of biological membranes and a corresponding decrease on the other side (surface area asymmetry) has been suggested to be the prime event in vesicle budding [1–4]. Indeed, mathematical models show that translocation of a few lipids from one monolayer to the other in a lipid bilayer triggers membrane deformations, which resemble vesicle budding [5]. Membrane lipid asymmetry can be generated *in vitro* by addition of exogenous lipids to giant liposomes, which results in shape deformations and in some cases formation of bud-like structures [6, 7]. The remaining question is whether the generation of surface area asymmetry is a prerequisite for formation of membrane buds *in vivo*. In order for this question to be answered, we need first to identify the proteins that catalyze membrane lipid flipping.

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Do P_4-ATPases translocate phospholipids?

Flippases are hypothetical membrane proteins that sustain the inward-directed translocation of phospholipids across biological membranes. P_4-ATPases, which form a subfamily of the large P-type ATPase superfamily, are so far the main flippase candidates [8–12]. A large body of genetic evidence primarily obtained from the yeast *Saccharomyces cerevisiae* but recently also in the plant *Arabidopsis thaliana* [10], shows that P_4-ATPases are involved in vesicle formation and membrane trafficking in the secretory and endocytotic pathways [10, 13–17].

The possible physiological functions of the *S. cerevisiae* P_4-ATPase isoforms, Drs2p, Dnf1p, Dnf2p, Dnf3p and Neo1p, in vesicle-mediated protein traffic have been reviewed recently [3]. For example, Drs2p is linked genetically to the formation of clathrin-coated vesicles [14, 18, 19] and is capable of translocating fluorescent phosphatidylserine (PS) analogues and to a lesser extent phosphatidylethanolamine (PE) analogues [20, 21]. Surprisingly, PS is not essential for Drs2p function *in vivo*, as exocytic vesicle production that requires an active Drs2p is still on-going in a PS-deficient yeast strain [21]. This suggests that Drs2p transports some other substrate across the Golgi membrane, which plays an important role in vesicle formation.

P_4-ATPases may contribute to lipid flipping either, i) indirectly by supporting function of a hitherto unidentified flippase protein or, ii) directly by being the actual lipid translocators. In the first scenario, P_4-ATPases transport a substrate required as a co-factor for the function of another unidentified protein, which may be the actual flippase, or in another indirect way contribute to the function of a flippase system. Such a possibility has to be considered given the pleiotropic nature of P_4-ATPase mutants. For example, a defect in phospholipid internalization is just one of the phenotypes associated with a lesion in the yeast *DRS2* P_4-ATPase gene. Others include a defect in ribosome assembly [22], and increased sensitivity towards cold [22], zinc, cobalt and amiodarone [23, 24]. The latter is also one of many phenotypes of cells carrying disruptions in two other Golgi localized pumps: the *PMR1* P-type Ca^{2+}-ATPase and *VMA7* and *VMA13*, subunits of the V-type ATPase [23]. Thus, it appears that a defect in any transport system contributing to the function of elements of the secretory pathway, *e.g.* the Golgi, gives rise to pleiotropic phenotypes not directly associated with the nature of the transported ligand [23]. This does not rule out a direct function of P_4-ATPases in phospholipid flipping, but calls for a note of caution. Whatever the transport specificity, it remains out of question that P_4-ATPases are essential for proper functioning of the secretory pathway, which includes flippases as central components.

In the second scenario, the P_4-ATPases would bind and flip a lipid molecule across the membrane. One genetic argument for direct flipping of fluorescently labelled phospholipids is that an *Arabidopsis* P_4-ATPase, *ALA3*, complements a disruption of yeast *DRS2* that is compromised in lipid internalization, but presents a translocation profile of fluorescently labelled phospholipids, which is dissimilar to the Drs2p-generated translocation profile [10]. Thus, in yeast, Drs2p is primarily involved in translocation of fluorescent analogues of PS [20, 21], while ALA3 primarily contributes to transport of analogues of PE [10]. This would not have been expected if the ALA3 protein and Drs2p contribute to the function of the same flippase.

The case, however, would be much stronger if it could be shown that a purified P_4-ATPase flips natural phospholipids when reconstituted in liposomes. Biochemical characterization of a partially purified mammalian P_4-ATPase, Atp8a1, has shown that ATPase activity, which is lost during detergent solubilization, can be recovered specifically by addition of PS [25]. Furthermore, analysis of the phosphorylated intermediate of Atp8a1 (and related isoforms) shows a clear dependence on PS for dephosphorylation during the catalytic cycle [26]. Is this evidence that PS is transported by Atp8a1? It is a common observation that delipidation during detergent mediated solubilization of P-type ATPases causes pump inactivation, and that activity can be restored by readdition of phospholipids [27–29]. For example, it was shown already in 1970 that PS effectively reactivates delipidated Na^+/K^+-ATPase [30].

It should be noted that the Atp8a1 isoform is specifically reactivated by PS and not by any other type of anionic phospholipids [25], in contrast to the Na^+/K^+-ATPase [31] and the Ca^{2+}-ATPase [32]. Furthermore, Atp8a1 activation by PS is totally dependent on the stereochemistry of the glycerol derived backbone, suggesting a very specific interaction between the lipid and the protein. In contrast, the specificities for lipid reactivation and lipid flipping by Atp8a1 do not appear to match, as N-methyl-PS, which is transported by the flippase in intact systems, cannot reactivate detergent solubilized Atp8a1 [25]. Taken together, the reactivation pattern of Atp8a1 indicates some kind of specific interaction between PS and this P_4-ATPase, but no biochemical evidence proves the capacity of the pump to transport this lipid.

During the catalytic cycle of P-type ATPases and following pump phosphorylation, specific cations are first pumped out of the cytoplasm [33] and subsequently, as the pump gets dephosphorylated, counter-

ions are transported in the opposite direction [34]. Phospholipid transport by P_4-ATPases would correspond to counterion transport as the direction of lipid flipping is from an extra-cytoplasmic to the cytoplasmic leaflet (the side from which ATP is bound). According to this model, phospholipid binding would trigger dephosphorylation of the pump, as for Atp8a1 [26], whereas binding from the cytoplasmic side of another ligand should initiate the phosphorylation process. What could the nature of this theoretical ligand be? Dissipation of the membrane potential with the proton ionophore CCCP inhibits internalization of fluorescently labelled phosphatidylcholine (PC) and PE at the plasma membrane of yeast cells [35–37]. Could this be taken as evidence that P_4-ATPases also transport protons as counterions? Probably not. If transport of phospholipids is dependent on an already established proton gradient, the direction of proton transport is expected to be downhill and from the outside to the inside (i.e. in the same direction as lipid flipping). Furthermore, the dependence of such a gradient would characterize the flippase system as a coupled H^+-symporter, not an active primary pump. We conclude that biochemical evidence for a direct role of P_4-ATPases in lipid flipping or in the transport of any other ligand is still lacking.

What is the role of the Cdc50p homologues?

In 2004, a family of P_4-ATPase putative β-subunits was identified in yeast. In this organism, the subunit family contains three members: Cdc50p, Lem3p and Crf1p [38]. Cdc50p homologues have also been found in humans [39], *Leishmania* [12], and in plants [10]. Cdc50p-like proteins seem to be involved in trafficking of the interacting P_4-ATPase [11, 12, 38]. In plants, although an additional role of the subunit in localization cannot be ruled out, Cdc50p homologues (here named ALIS proteins) seem to be required for the contribution of the ALA3 protein to flipping of phospholipid analogues [10]. Furthermore, Cdc50p homologues may be involved in determination of substrate specificity as Drs2p and Dnf3p, which exhibit distinct translocation profiles [20], interact with different Cdc50p homologues (Cdc50p and Crf1p, respectively) [38, 40], while Dnf1p and Dnf2p, having the same substrate specificity [17], both interact with Lem3p [38, 40]. However, direct proof for these suggestions is not available yet.

Most P-type ATPases only need a single α-subunit polypeptide in order to carry out ATP hydrolysis and ion pumping. A prominent exception is the Na^+/K^+-ATPase, which requires two subunits for full functionality. The β-subunit serves as chaperone for the newly synthesized α-subunit, is essential for correct folding and proper membrane insertion, plays an important role in trafficking of the pump, and, finally, is involved in controlling transport related properties of the α-subunit [41]. The γ-subunit, which is very small, has a role in fine tuning the activity of the Na^+/K^+-ATPase [42].

Functional similarities between Cdc50p homologues and Na^+/K^+-ATPase β- and γ-subunits thus appear to be well documented. Both groups of proteins share important roles in proper folding and trafficking of their associated P-type ATPase [11, 12, 38, 41]. Furthermore, both proteins appear to be involved in modifying the activity of their respective ATPase [10, 41, 42].

Interestingly, Cdc50p related proteins structurally resemble a fusion between the β- and γ-subunits of the Na^+/K^+-ATPase in terms of polypeptide lengths and membrane segment topology. Specifically, transmembrane segment one (TM1) and the large cytosolic loop of Cdc50p homologues structurally resemble the Na^+/K^+-ATPase β-subunit, while TM2 and the C terminus of Cdc50 proteins are comparable to the γ-subunit (Fig. 1A-B). Furthermore, like the β-subunit of the Na^+/K^+-ATPase, Cdc50p homologues interact directly with the P_4-ATPase [10] and are heavily N-glycosylated [43, 44]. This points to a hitherto not suggested structural link between Cdc50p-like proteins and Na^+/K^+-ATPase β- and γ-subunits.

A sequence alignment shows that Cdc50p homologues and Na^+/K^+-ATPase β- and γ-subunits have very low sequence similarity (Fig. 1C; around 13 % similarity of the Cdc50 proteins with the β-subunit and 12 % with the γ-peptide). Dissimilar protein sequences often fold into similar tertiary structures [45]. P-type ATPases, such as the *Arabidopsis* plasma membrane H^+-ATPase AHA2 and the rabbit Ca^{2+}-ATPase SERCA1a, share low sequence identity (20 %), but a structural comparison shows the overall fold to be remarkably similar, also in transmembrane regions with far lower sequence similarity [46]. The messenger RNA export factors Mtr2 from yeast and p15 from human, which have neglible sequence similarity, also present an analogous structural conformation [47]. Despite low sequence identity it is therefore an intriguing possibility that Cdc50p-like proteins and Na^+/K^+-ATPase subunits have structural features in common. Further understanding of the structure and physiological function of Cdc50p homologues will help clarify their putative resemblance to the subunits of Na^+/K^+-ATPases and related pumps.

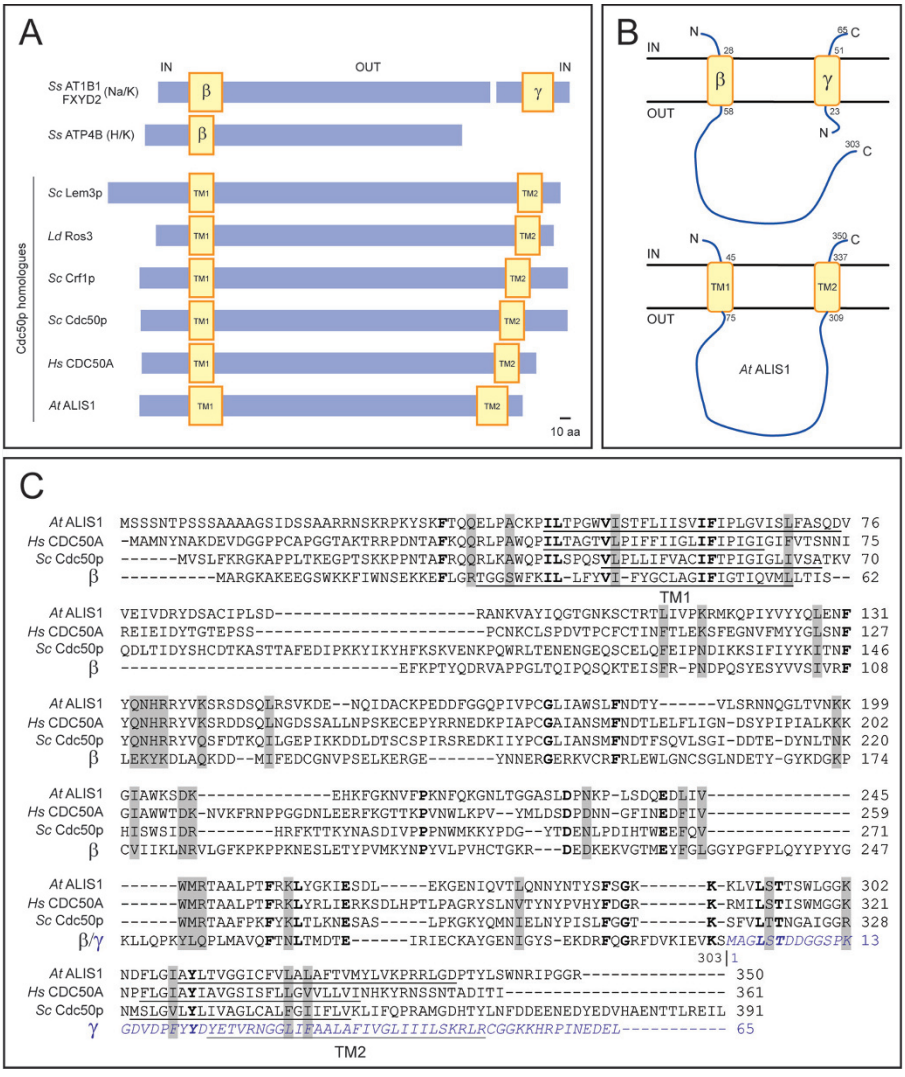

Figure 1. (*A*) Size and structural comparison of the β- and γ-subunits of the Na^+/K^+- and H^+/K^+-ATPase from *Sus scrofa* (*Ss*) and Cdc50p homologues from *Saccharomyces cerevisiae* (*Sc*), *Leishmania donovani* (*Ld*) *Homo sapiens* (*Hs*) and *Arabidopsis thaliana* (*At*) for which interaction with at least one P_4-ATPase has been proven (**Ex**pert **P**rotein **A**nalysis **Sy**stem identification: P05027, Q58K79, P18434, P42838, Q0P0L8, P53740, P25656, Q9NV96 and Q9LTW0, respectively). Transmembrane regions are predicted using TMHMM server version 2.0 [73]. The transmembrane domains of ALIS1 are predicted according to structural alignment between this protein and the β- and γ-subunits of the Na^+/K^+-ATPase from *Sus scrofa*. (*B*) Schematic representation of the structural resemblance between the plant Cdc50p homologue ALIS1 and the β- and γ-subunits of the Na^+/K^+-ATPase. Top panel, topology for the β- and γ-subunits. Lower panel, predicted topology for ALIS1. Size of fragments is indicated by the amino acid residue number. (*C*) Sequence alignment of Cdc50p homologues from *Arabidopsis*, human, yeast and the β- and γ-subunits of the Na^+/K^+-ATPase from *Sus scrofa*. The sequence alignment was produced online at http://www.tcoffee.org/ using the T-coffee Advanced mode with default settings. Small gaps and alignment segments have manually been fused in the final alignment. The γ-subunit is marked in grayish blue and italic. The transition between the β- and γ-subunit sequences is marked with (|). Transmembrane domains are underlined and marked TM1 and TM2. *Bold*, conserved amino acid residues; *Grey shade*, similar amino acids. Abbreviations: TM, transmembrane domain; aa, amino acid.

Do all P_4-ATPases require a Cdc50 protein?

So far, a higher number of P_4-ATPase isoforms compared to Cdc50p homologues have been identified in each organism. This imbalanced ratio is particularly obvious in multicellular organisms, humans having 14 P_4-ATPases [48] and only three CDC50 proteins [39], while in *Arabidopsis* 12 P_4-ATPase isoforms [49] and five subunits are present [10].

So what does this mean? Do some P_4-ATPases act alone or does one Cdc50p homologue interact with several P_4-ATPases? No interaction partner among the Cdc50p homologues has thus far been found for Neo1p, indicating that some P_4-ATPases may act without a Cdc50p homologue. Lem3p interacts and sustains functionality of Dnf1p [38] and Dnf2p [40], and in multicellular organisms several Cdc50p homologues have been shown capable of activating the

same P_4-ATPase [10, 11], which supports the notion that one Cdc50p isoform can interact with several P_4-ATPases.

What are the flippases of the endoplasmic reticulum?

In the endoplasmic reticulum (ER) a fast and unspecific bidirectional protein-mediated translocation of phospholipids [50, 51] acts against the formation of an asymmetric phospholipid distribution in this membrane. Still, it is known that vesicles are formed and released from the ER and that both coat proteins and ATP are required for vesicle formation [52]. If phospholipid translocation by P_4-ATPases is indeed one of the major driving forces in vesicle initiation at the *trans*-Golgi, the endosomes, and the plasma membrane, it is a fair assumption that a local ATP-dependent protein-mediated translocation of phospholipids may be required for a vesicle to form at the ER membrane in the early steps of the secretory pathway. Intriguingly, no P_4-ATPases have been localized to the endoplasmic reticulum (ER). What would then be the flippase responsible for vesiculation in the anterograde pathway from ER to Golgi? We propose that P_{5A}-ATPases, which are related to P_4-ATPases, could be prime candidates for mediating an ATP-dependent lipid translocation in the ER required for vesiculation in this compartment.

Like P_4-ATPases, P_5-ATPases have been identified in every eukaryotic genome sequenced so far, but are absent from all prokaryotic genomes. This family of proteins can be divided in two subgroups, P_{5A} and P_{5B} [53]. All the members of the P_{5A}-ATPase family investigated to date have been localized to the ER: one in *Arabidopsis* [54, 55], one in *S. cerevisiae* [56, 57], which has also been observed in *cis*-Golgi membranes [58], and a third homologue in *Schizosaccharomyces pombe* [59]. The two homologues from *Arabidopsis* and *S. cerevisiae* have both been linked to processes in the secretory pathway, in particular the anterograde traffic between the ER and *cis*-Golgi [55, 57, 58, 60, 61]. P_{5A}-ATPases have been suggested to be involved in calcium homeostasis, but no evidence for Ca^{2+}-translocation by these proteins has been found [56], and the substrate of these pumps remains to be identified.

A P_{5B}-ATPase (human ATP13A2) has been localized within the lysosome [62] indicating a distinct function for the second group of P_5-transporters. Support for this notion is that the closest homologue to Spf1p, yor291w, is a P_{5B}-ATPase which seems to have no physiological function in common with Spf1p [56, 63, 64]. So far the physiological function of P_{5B}-ATPases is unknown; however human ATP13A2 has been linked to Parkinson and dementia [62] and ATP13A4 to language development [65].

An unexpected similarity between P_4- and P_{5A}-ATPases is that they appear to have homologous interaction partners. Liu and co-workers [1] have recently suggested that a guanine nucleotide exchange factor (GEF) and a small GTPase have to act in concert with P_4-ATPases in order to induce vesiculation from a lipid bilayer. For example, the yeast Golgi P_4-ATPase Drs2p interacts with the GEF Gea2p involved in activation of ADP-ribosylation factors (Arfs), which are small GTPases required for vesicle formation [19]. Similarly, the yeast P_4-ATPase Neo1p interacts with the GEF Ysl2p/Mon2p [66] and the Arf Arl1p [67], and cooperation between these three proteins is important for recruitment of clathrin adaptors [68]. Interestingly, a yeast mutant lacking the single P_{5A}-ATPase, Spf1p, presents an altered localization of the GEF Sec12p [58], a membrane protein localized in the ER [69]. Sec12p interacts with the small GTPase Sar1p, which has been suggested to be an early regulator of vesiculation and COPII assembly in the ER [70–72]. Taken together, the genetic evidence points to similarities between interaction partners of Drs2p, Neo1p and Spf1p, and suggests the possibility that these three proteins play similar roles in the *trans*-Golgi network, the endosomes and the ER, respectively. Whether or not P_{5A}-ATPases translocate phospholipids is currently unknown; nevertheless, increasing evidence points to their involvement in the vesiculation machinery at the ER, suggesting them to be good candidates for the functional homologues of P_4-ATPases in this cellular compartment.

Acknowledgements. This work was supported by the Danish National Research Foundation.

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