Intracellular Targeting Signals and Lipid Specificity Determinants of the ALA/ALIS P_4-ATPase Complex Reside in the Catalytic ALA α-Subunit

Rosa L. López-Marqués,*† Lisbeth R. Poulsen,*,‡ Susanne Hanisch,* Katharina Meffert,† Morten J. Buch-Pedersen,* Mia K. Jakobsen,*,§ Thomas Günther Pomorski,*,‡ and Michael G. Palmgren*

*Center for Membrane Pumps in Cells and Disease, PUMPKIN, Danish National Research Foundation, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, DK-1871, Frederiksberg C, Denmark; and Faculty of Mathematics and Natural Science I, Institute of Biology, ‡Humboldt-University Berlin, 10115 Berlin, Germany

Submitted August 4, 2009; Revised November 25, 2009; Accepted December 24, 2009
Monitoring Editor: Reid Gilmore

Members of the P_4 subfamily of P-type ATPases are believed to catalyze flipping of phospholipids across cellular membranes, in this way contributing to vesicle biogenesis in the secretory and endocytic pathways. P_4-ATPases form heteromeric complexes with Cdc50-like proteins, and it has been suggested that these act as β-subs in the P_4-ATPase transport machinery. In this work, we investigated the role of Cdc50-like β-subs in the P_4-ATPase for targeting and function of P_4-ATPase catalytic α-subs. We show that the Arabidopsis P_4-ATPases ALA2 and ALA3 gain functionality when coexpressed with any of three different ALIS Cdc50-like β-subs. However, the final cellular destination of P_4-ATPases as well as their lipid substrate specificity are independent of the nature of the ALIS β-subs they were allowed to interact with.

INTRODUCTION

A fundamental cellular process that requires a dynamic regulation of transbilayer lipid arrangements is the biogenesis of endocytic and secretory vesicles. Recently, accumulating evidence has pointed to an important role in this process for P_4-ATPases, which form one of the five subfamilies of P-type ATPases (Tang et al., 1996; Axelsen and Palmgren, 1998; Palmgren and Harper, 1998). P-type ATPases constitute a large family of membrane pumps that are transiently auto-phosphorylated at a conserved aspartate residue, hence the designation P-type. Evidence for an important role of P_4-ATPases in membrane vesiculation has mainly come from genetic studies (Graham, 2004). For example, 1) inactivation of a Saccharomyces cerevisiae P_4-ATPase, DRS2, rapidly blocks formation of a clathrin-dependent class of post-Golgi secretory vesicles carrying exocytotic cargo (Gall et al., 2002); 2) S. cerevisiae Δdnf1Δdnf2 mutant cells show a cold-sensitive defect in endocytosis (Pomorski et al., 2003); 3) conditional alleles of the essential yeast P_4-ATPase Neo1p perturb ADP-ribosylation factor–dependent vesicle formation from endosomes (Hua and Graham, 2003; Wicky et al., 2004); 4) loss of ALA3, a Golgi-resident P_4-ATPase in Arabidopsis thaliana, causes a defect in the production of slime vesicles containing polysaccharides and enzymes for secretion, and impairs growth of roots and shoots (Poulsen et al., 2008a); and finally, 5) the Caenorhabditis elegans P_4-ATPase TAT-1 is required for yolk uptake in oocytes and for an early step of fluid-phase endocytosis in the intestine (Darland-Ransom et al., 2008; Ruaud et al., 2009).

At least three models can be proposed to explain how P_4-ATPases contribute to vesicle formation. One model is that P_4-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 (PM), and their subsequent translocation to the cytosolic leaflet by a lipid flippase causes dramatic shape changes of red blood cells (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985). Similarly, lipid flipping can provoke the formation of endocytic-like vesicles (Muller et al., 1994) and accelerates endocytosis (Farge et al., 1999). An unresolved question with this model is the “giant substrate” problem. All other P-type ATPases characterized so far, e.g., Na^+ /K^+- and Ca^{2+}-ATPases (Ebashi and Ebashi, 1962; Jor-
gensen et al., 2003), pump small cations and three-dimensional (3D) structures of such pumps show that the transported ions are occluded in the center of the transmembrane part of the protein, from where they have alternating access to either side of the membrane (Toyoshima et al., 2000; Morth et al., 2007; Pedersen et al., 2007; Shinoda et al., 2009). Consequently, it is unclear how this mechanism can be utilized to transport a phospholipid across the membrane bilayer. A second model is that Pγ-ATPases contribute to vesicle budding by serving as scaffold proteins that recruit coat proteins (e.g., clathrin or COPII) to the membrane through protein–protein interactions and/or by increasing the concentration of specific lipids in the cytosolic leaflet, which in turn could be required for binding of coat proteins. This model is supported by the observation that S. cerevisiae Drs2p directly interacts with the ADP-ribosylation factor (ARF) activator, Geo2p (Chantalat et al., 2004). The latter protein is a GTP-exchange factor that regulates recruitment of ARF, adapter protein-1 (AP-1) and clathrin coat proteins to membranes of the trans-Golgi. However, a recent study shows that membrane association of ARF, AP-1, and clathrin does not require Drs2p (Liu et al., 2008).

In a third model, it is hypothesized that Pγ-ATPases, in view of their overall similarity to cation-transporting P-type ATPases, are cation pumps that only contribute indirectly to lipid flipping (Axelsen and Palmgren, 2001; Kuhlbrandt, 2004). According to the model, Pγ-ATPases pump cations to generate a transmembrane electrochemical gradient in secretory and endocytic pathway membrane structures. In turn, a second transport protein operating by a symport mechanism energized by this gradient drives a fundamental process in vesicle budding, which could be lipid translocation. This protein could in principle be a subunit of the flipase complex (Axelsen and Palmgren, 2001; Kuhlbrandt, 2004). Prime candidates for such accessory proteins would be the recently identified Cdc50-like β-subunits of Pγ-ATPases (Katoh and Katoh, 2004; Saito et al., 2004; Paulusma et al., 2008; Poulsen et al., 2008a). Because Cdc50 proteins lack any similarity to known transporters, it has recently been suggested that transmembrane flipping might require cooperation between both subunits and occur at the interface between a Pγ-ATPase and its Cdc50 binding partner, an arrangement in which Cdc50 proteins would contribute directly to the transport specificity of the complex (Coleman et al., 2009; Puts and Holthus, 2009; Zhou and Graham, 2009). Alternatively, creation of a high-affinity phospholipid binding site may require Cdc50-induced conformational changes in the membrane domain of Pγ-ATPases, analogous to the role of the β-subunit in the oligomeric Na+/K+-ATPase (Geering, 2001; Puts and Holthus, 2009). In this work, we aimed at investigating closer the role of Cdc50-like β-subunits of Pγ-ATPases for targeting and function of Pγ-ATPases in higher eukaryotes using the plant Arabidopsis as a model organism. In this plant, Pγ-ATPases are encoded for by ALA genes and Cdc50p-like β-subunits by ALIS genes (Gomés et al., 2000; Poulsen et al., 2008a). First, we identified a novel Pγ-ATPase, ALA2, in Arabidopsis. We found that it only gained functionality while coexpressed with an ALIS protein, and in its absence ALA2 never exited the endoplasmic reticulum (ER) in planta. However, the final cellular destination of ALA2 as well as its lipid substrate specificity was not affected by the ALIS protein it was allowed to interact with. Further, when compared with ALA2, the related Pγ-ATPase ALA3, that also requires an ALIS protein for ER export, shows a different subcellular location and lipid specificity, which similarly was unaffected by the accompanying ALIS protein. We conclude that targeting signals and lipid specificity determinants of Pγ-ATPases reside in the ALA catalytic α-subunit. This would suggest that plant Cdc50p-like proteins mainly play a role in functional maturation and ER export of Pγ-ATPases, but do not affect the nature of the transported phospholipid and the final localization of the putative flipase complex.

MATERIALS AND METHODS

DNA Cloning and Sequence Analysis

The ALA2 (A5g44240) cDNA was isolated by PCR amplification using a cDNA library of size-fractionated (3–6 kb) cDNAs (Kieber et al., 1993) as template. Primers were designed on the basis of information contained in the P-type ATPase database (http://biobase.dk/axe/Pathbase.html). All fragments were amplified using Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) and cloned into the pJet1 blunt-end cloning vector (Fermentas, Hanover, MD) for sequencing. Full sequencing of the cDNA clone revealed four point mutations causing amino acid changes in the protein sequence, which were subsequently corrected by site-directed mutagenesis giving rise to four silent mutations. The corrected clone was used as a template in new PCRs in which the full-length cDNA sequence was amplified (via sequencing or when sequence corresponding to a hemagglutinin (HA) epitope (YPYDVPDYA) was included at the N-terminal end. The PCR products were cloned into the Gateway compatible vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA) using the pENTR/D-TOPO cloning kit. Prior to fusion to the five ALIS proteins (Arabidopsis, we successfully cloned ALIS1, ALIS3, and ALIS5 (Poulsen et al., 2008a). HA:ALA2 was cloned into a modified version of yeast plasmid pRS425GAL1-10 and its derivatives containing RGS12/ALIS gene fusions (Poulsen et al., 2008a) using the Gateway technology. Employing an overlapping PCR strategy an HA-tagged version of ALA2, ala2D381A, was generated and cloned in a similar manner into pENTR/D-TOPO and the Gateway-compatible yeast plasmids.

To obtain a green fluorescent protein (GFP)-tagged version of ALA2 for tobacco transformation, untagged ALA2 was transferred into plasmid pMD1C3 (Curtis and Grossniklaus, 2003) using the Gateway technology. Likewise, for generating C-terminal fusions of ALIS1, ALIS3, and ALIS5 to yellow fluorescent protein (YFP), the corresponding genes were transferred from pENTR/D-TOPO clones (Poulsen et al., 2008a) to plant binary plasmid pEarleyGate 104 (Earley et al., 2006) using the Gateway technology.

Topology predictions for ALA2 were carried out using the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) servers. Sequence identity/homology scores were calculated using CrustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Yeast Strains and Media

Functional complementation and lipid translocation assays were carried out employing S. cerevisiae mutant strain ZHY709 (MATa his3 leu2 ura3 met15 dystA duruA drs2::LEU2; Hua et al., 2002), and strain BY4741 (MATa his3 leu2 ura3 met15 EURO5CARF) as wild type. Cells were grown at 30°C in standard rich medium with glucose (YPD) or galactose (YPG), or rich synthetic SD or SG media (Rose and Broach, 1990) containing yeast synthetic dropout medium with glucose (YPD) or galactose (YPG), or rich synthetic SD or SG media (Rose and Broach, 1990) containing yeast synthetic dropout medium without histidine (Sigma-Aldrich; see also Supplemental Figure 1). Heavy metal-containing media were generated by incorporation of 200 μM CoCl2 or 2 mM ZnCl2. Solid media were added 2% agar (Villalba et al., 1992). Papuamide B (Flintbox, Lynsey Huxham; www.flintbox.com/), duramycin (Sigma-Aldrich, St. Louis, MO), and miltefosine (Calbiochem, La Jolla, CA) were added to rich synthetic SD or SG media to the indicated concentrations.

Yeast Transformation and Growth

Yeast cells were transformed by the lithium acetate method (Gietz and Woods, 2002). Transformants were incubated in liquid SG medium for 4 h and then diluted with water to 0.1, 0.01, and 0.001 OD600. Drops (5 μl) were spotted on plates and incubated at 20°C for 6–8 d or at 30°C for 2–3 d as indicated. All experiments were repeated independently at least three times.

Yeast Membrane Preparation and Protein Immunodetection

Total cellular membranes for protein expression analysis were prepared as previously described (Villalba et al., 1992). Quantification of proteins content and Western blot analysis were carried out as described (Poulsen et al., 2008a). For sucrose-density fractionation, fresh yeast transformants were inoculated in 25 ml selective SD media and grown for 24 h at 28°C under 160 rpm shaking. Cells were harvested (1,500 × g, 5 min, 24°C), washed twice with water, and inoculated into 500 ml YPG media. Cultures were grown to 1–1.2 OD600 at 28°C under 160 rpm shaking. Cells (400 ml) were harvested...
by centrifugation (1500 × g, 4°C, 5 min), and washed in 25 ml ice-cold lysis buffer (10 mM Tris-HCL, pH 7.5, 1 mM EDTA, 0.6 M sorbitol, 1 µg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). After lysis, the cells were resuspended in 4 ml of lysis buffer, and 2-ml aliquots were vortexed with glass beads (1.2 × g; 200 µm; Sigma-Aldrich). The cell lysate was clarified by centrifugation (1000 × g, 10 min, 4°C), and PM-enriched membranes, devoid of mitochondria and most of the endomembranes, were subsequently collected by centrifugation (8000 × g, 30 min, 4°C). Pelleted membranes were resuspended in 500 µl lysis buffer and centrifuged for 10 min, 4°C before loading onto two-step sucrose gradients: 7.6 ml 43% (wt/wt) sucrose on top of 3.8 ml 53% (wt/wt) sucrose prepared in lysis buffer. After centrifugation (120,000 × g, 17 h, 4°C), 1.2-ml fractions were collected from the top. Equal volumes per fraction were used for Western blot analysis. Sucrose was quantified in each fraction using a PAL refractometer (Atago, Kirkland, WA).

**Lipid Translocation Assays and Flow Cytometry**

1-Palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-phosphocholine (NBD-PC), phosphoethanolamine (NBD-PE), and phospho-serine (NBD-P5) were from Avanti Polar Lipids (Birmingham, AL). All NBD-lipid stocks (10 mM) were prepared in DMSO. Uptake experiments were performed essentially as previously described (Poulsen et al., 2008a) except that cells were grown in selective rich synthetic medium to midlogarithmic phase (0.5 1.0 OD600/ml). Cells (10 OD600/ml) were incubated in the same medium with 60 g/ml kanamycin or 50 µg/ml puromycin for 2 h, washed twice in ice-cold medium containing 4% (wt/vol) bovine serum albumin, and resuspended to 3 × 106/ml in NBD-lipid from the NBD liposome mix (Poulsen et al., 2008a). Flow cytometry of NBD-labeled cells was performed on a Becton Dickinson FACSCalibur (San Jose, CA) equipped with an argon laser using Cell Quest software. One microliter of 1 mg/ml propidium iodide in water was added to 1 × 107 cells in 1 ml buffer (137 mM NaCl, 10 mM Na2HPO4, 2 mM NaH2PO4, pH 7.4) just before analysis. Thirty thousand cells were analyzed without gating during the acquisition. Live cells were selected based on forward/side-scatter gating and propidium iodide exclusion. A histogram of the green fluorescence (NBD) of living cells was used to calculate the mean fluorescence intensity of total cells (see Figure 2; Supplemental Figure 1).

**Transient Expression in Tobacco Epidermal Leaf Cells**

Agrobacterium tumefaciens strain CS81 (Koncz and Schell, 1986) was transformed by electroporation, and transformants were selected on YEP plates (1% yeast extract, 2% peptone, 1.5% agar) containing 25 µg/ml gentamicin and 50 µg/ml kanamycin or 50 µg/ml spectinomycin as required. Transformants were either directly used for infiltration or resuspended in a 15% glycerol solution, frozen in liquid nitrogen, and kept at −80°C until needed. Transient expression in tobacco epidermal cells was carried out as described (Sparkes et al., 2006) using 3-wk old Nicotiana benthamiana plants. To facilitate high expression of recombinant proteins Agrobacterium strains carrying the different constructs were coinfilitrated with a strain carrying the p19 gene encoding the viral p19 protein that specifically inhibits plant posttranscriptional gene silencing (Voinnet et al., 2002) except that cells were grown under the restrictive temperature (Figure 1A). Among these, ALA1, ALA2, and ALA3 are the most divergent phylogenetically, whereas ALA4 to ALA12 group closely together. ALA1 has been partially characterized and shown to be involved in chilling tolerance in Arabidopsis (Voinnet et al., 2002), whereas ALA3 seems to be required for vesicle secretion in actively secreting cells at the plant root tip (Poulsen et al., 2008a) and for normal development of plant trichomes (Zhang and Oppenheimer, 2009). When expressed in the presence of different ALIS proteins, ALA3 is capable of complementing yeast mutants carrying deletions in P4-ATPases (Poulsen et al., 2008a). None of the other 10 members of the P4-ATPase family in Arabidopsis has been investigated so far. In this work, we aimed at studying ALA2 for the first time.

An Arabidopsis cDNA fragment containing the full-length ALA2 gene (At5g44240) was amplified from a cDNA library from 3-d-old hypocotyls (Kieber et al., 1993). This cDNA corresponds to the gene contained in the genomic clone AB005239 (accession no. AF419611). ALA2 encodes a protein with 1107 amino acid residues and a molecular weight of ~124 kDa, predicted to have 10 transmembrane spanning segments and containing the conserved domains of the P-type ATPase superfamily and the characteristic motifs of the P2b-ATPase subfamily (Axelsen and Falmgren, 1998). ALA2 shares 27 and 29% sequence identity with ALA1 and ALA3, respectively, and 27% identity with Drs2p.

**ALA2 in Combination with an ALIS Functionally Complements an S. cerevisiae P2c-ATPase Mutant**

To investigate if ALA2 encodes a functional P2c-ATPase, the gene was expressed in a S. cerevisiae mutant strain lacking three endogenous P2c-ATPases (Δdrs2Δdnf1Δdnf2), which displays a cold-sensitive phenotype (Hua et al., 2002). In our system, expression of heterologous genes is carried out under the control of a bidirectional galactose-inducible promoter. Thus, glucose-grown yeast cells containing the desired plasmids will show no expression of the heterologous proteins, whereas culturing the yeast cells with galactose as a carbon source will result in heterologous protein overexpression. The bidirectionality of the promoter also allows for overexpression of two heterologous proteins at the same time. When transformed with a plasmid containing ALA2 alone, the yeast was unable to grow on galactose plates at 20°C (Figure 1A). Recently, we showed that functional complementation of this yeast strain by ALA3, is dependent on the presence of an ALIS gene (Poulsen et al., 2008a). When ALA2 was expressed in combination with ALIS1, ALIS3, or ALIS5, the yeast was able to grow to different extents below the restrictive temperature (Figure 1A). Among these, ALIS1 and ALIS5 were the most efficient at complementing the yeast cold-sensitive phenotype. A mutant version of ALA2 in which the aspartate residue being phosphorylated as part of the catalytic reaction is replaced by an alanine residue (ala2D381A) was not capable of rescuing the yeast cold-sensitive phenotype (Figure 1A), supporting that a catalytically active pump is required for functionality.

Next, we carried out Western blot analysis on total membranes isolated from yeast overexpressing epitope-tagged ALA2 alone or in combination with different ALIS proteins (Figure 1C). The epitope-tagged ALA2 was in all cases immunodecorated with an anti-HA antibody, indicating that the inability of ALA2 to complement the yeast cold-sensitive phenotype in the absence of an ALIS is not derived from lack of expression. ALA2 expression levels were found to be comparable in the presence of the different ALIS proteins, whereas ALIS3 was expressed at levels lower than ALIS1 and higher than ALIS5. This suggests that the differences in complementation of the cold-sensitive phenotype are not related to protein expression levels. Expression of the catalytically inactive mutant form ala2D381A seems to be lower than ALA2 expression in all cases, suggesting that lack of complementation by the mutant protein might be due to low expression. However, a catalytically inactive ALA3, analogous to the ala2D381A mutant used in this work, is unable to complement the cold-sensitive phenotype of the Δdrs2Δdnf1Δdnf2 mutant strain even when expressed at similar levels to active ALA3 (Poulsen et al., 2008a). This
ALA2 and negative controls, respectively. ALA2 hemagglutinin (HA) epitope and ALIS genes under the control of a bidirectional galactose-inducible/H9004 a ZnCl2. (C) Western blot analysis of membranes from yeast overexpressing ALA2 in combination with ALIS, ALA2-ALIS1, ALA2-ALIS3, ALA2-ALIS5, ala2-ALIS1, and ala2-ALIS5.

Figure 1. ALA2 in combination with ALIS genes functionally complement the cold- and heavy metal–sensitive phenotype of a Δdrs2Δdnf1Δdnf2 yeast mutant. The triple yeast mutant Δdrs2Δdnf1Δdnf2 expressed ALA2 alone or in combination with ALIS genes under the control of a bidirectional galactose-inducible promoter. Yeast DRS2 and an empty vector were used as positive and negative controls, respectively. ALA2 was tagged with the hemagglutinin (HA) epitope and ALIS with the RGS3 epitope. ALA2 expressed in concert with ALIS allows growth of the cold-sensitive yeast strain at 20°C, whereas the catalytically inactive mutant, ala2D381A, in combination with ALIS, did not support growth at 20°C. (A) Yeast cells were dropped on rich media at 30°C and at the restrictive temperature 20°C. (B) Yeast cells dropped on minimal media without metals or containing 200 μM CoCl2 or 2 mM ZnCl2. (C) Western blot analysis of membranes from yeast overexpressing HA:ALA2 or HA:ala2D381A in concert with RGS3:ALIS proteins. Control, cells grown on glucose plates to repress expression; Induced, cells grown on galactose plates to induce overexpression; Empty, yeast cells transformed with an empty plasmid control; ala2, ala2D381A.

supports the hypothesis that a catalytically active ALA2 would be required for functional complementation of the yeast mutant phenotype.

Single Δdrs2 and double Δdnf1Δdnf2 mutant strains are unable to grow on elevated concentrations of Zn2+ and Co2+ ions (Siegmund et al., 1998; Pomorski et al., 2003), and, although the nature of this defect is unknown, it has been taken as evidence for overlapping physiological functions between Drs2p and members of the Dnf subfamily. ALA2 alone was unable to complement the heavy-metal–sensitive phenotype of Δdrs2Δdnf1Δdnf2 (Figure 1B). However, coexpression of ALA2 with any ALIS allowed growth on 200 μM CoCl2. Interestingly, in contrast to results obtained when testing complementation of the cold-sensitive phenotype, the combination of ALA2 and ALIS1 presented a slower growth on CoCl2-containing plates and was unable to restore growth on plates containing 2 mM ZnCl2 (Figure 1B).

ALIS1, Dnf1p, and Dnf2p have recently been used in the characterization of the lipid flipping activity of the ALA3/ALIS complex with different NBD lipids (Poulsen et al., 2008a). Herein, we used palmitoyl-NBD lipids that only differ in their headgroup to characterize the lipid transport activity of ALA2. We chose ALIS1 as an example of a β-subunit, because this protein promotes lipid transport in the presence of ALA3 (Poulsen et al., 2008a). Coexpression of ALA2 with ALIS1 in the yeast triple mutant background resulted in a population of cells with increased internalization of NBD-PS, but not -PC or -PE (Figure 2, A and B). ALA2 alone was unable to promote internalization of NBD-lipids (Figure 2B). Likewise, the mutant protein ala2D381A lacking the ATPase activity in combination with ALIS1 was unable to support NBD-lipid translocation (Figure 2C), which confirms that P1-ATPase activity is a requirement for lipid internalization at the PM.

All ALA2/ALIS Combinations Result in the Same Lipid Transport Specificity

It has been suggested that P1-ATPase β-subunits could have a role in determining the transport specificity of the protein complex (Lenoir et al., 2007; Puts and Holthuis, 2009). The fact that ALA2 was capable of complementing the yeast mutant phenotype in the presence of three different members of the ALIS family allowed us to test this hypothesis. Coexpression of ALA2 in yeast with ALIS3 and ALIS5 resulted in internalization of NBD-PS to levels similar to those observed before for ALIS1 (Figure 2, B and C). No internalization was observed for NBD-PE or -PC. These results suggest that the specificity of lipid transport by ALA2 is not dependent on the nature of the coexpressed β-subunit or alternatively, that all β-subunits used in this work generate the same lipid specificity.

Because the lipid transport assay requires a flippase activity at the PM, we tested whether ALA2 was present in this membrane. PM-enriched yeast membranes expressing ALA2 alone or in the presence of an ALIS protein were subjected to discontinuous sucrose gradient fractionation followed by Western blot analysis. The ER marker protein dolichyl-phosphate-mannose synthase 1 (Dpm1p) was restricted to the lower sucrose concentrations, whereas the PM proton ATPase Pma1p was enriched at fractions containing 48% sucrose (Figure 3A). Although ALA2 and the ALIS proteins were heavily represented in ER fractions, as expected for overexpressed heterologous proteins, a significant amount reached the PM in all cases (Figure 3, A and B).

ALA2/ALIS Combinations Regulate Plasma Membrane Distribution of PS in Yeast Cells

Even when fluorescently labeled lipids are internalized, this needs not be the case for naturally occurring lipids. We therefore investigated the capacity of ALA2 and ALA2/ALIS combinations to promote the transport of natural lipids in the Δdrs2Δdnf1Δdnf2 triple mutant yeast strain exhibiting an aberrant exposure of PE and PS at the cell surface.
(Pomorski et al., 2003; Chen et al., 2006). Such abnormal lipid distribution at the PM can be detected using duramycin and papuamide B, which are cytolytic peptides that in order to exert their cytotoxicity require binding to cell surface–exposed PE and PS, respectively (Márki et al., 1991; Parsons et al., 2006). Thus, wild-type yeast that confines PS and PE in the cytoplasmic leaflet of the PM is less sensitive to peptide-induced cytolysis compared with the triple yeast mutant (Figure 4A). Yeast Δdrs2Δdnf1Δdnf2 mutant cells expressing ALA2 alone or in combination with an ALIS protein were spotted on selective plates containing different concentrations of cytotoxic peptides (Figure 4B). A wild-type strain transformed with an empty plasmid or overexpressing ALA2 alone were highly sensitive to both the PS- and the PE-binding peptide. Expression of ALA2 in combination with ALIS proteins promoted inward translocation of natural PS, but not PE, across cellular membranes. ALA2/ALIS3 and ALA2/ALIS5 combinations promoted a better growth on papuamide B than ALA2/ALIS1 (Figure 4B). Interestingly, ALA2 in combination with ALIS3 or ALIS5 was also more potent in supporting yeast growth on elevated Cobalt and Zinc concentrations than when expressed with ALIS1 (Figure 1B). However, coexpression of ALA2 with ALIS1 or ALIS5 was more effective at rescuing the cold-sensitive phenotype of the Δdrs2Δdnf1Δdnf2 mutant strain (Figure 1A), and this could not be directly correlated to protein expression levels (Figure 1C) or cellular distribution (Figure 3, A and B). Whether this differences reflect physiologically related properties of ALA2/ALIS complexes remains to be determined.
Yeast wild-type strains can take up miltefosine, a toxic lyso-PC analog, and are unable to grow in the presence of this lysolipid, whereas any strain lacking Dnf1p and Dnf2p (like the \( \Delta \)drs2\( \Delta \)dnf1\( \Delta \)dnf2 mutant) is capable of growing on moderate amounts of this drug (Pe´rez-Victoria et al., 2006; Riekhof and Voelker, 2009). Under our experimental conditions, the triple mutant strain was resistant to 2.5 \( \mu \)g/ml miltefosine, whereas no growth could be detected for the wild-type strain under these conditions (Supplemental Figure 2). All strains expressing ALA2/ALIS combinations or Drs2p were able to grow in the presence of miltefosine, suggesting that neither of them is capable of transporting lysolipid across the PM of yeast cells.

**ALA2 Is Retained in the Plant ER in the Absence of a \( \beta \)-Subunit**

To study the physiological importance of ALA2, we decided to investigate its subcellular localization in planta. For this purpose, a GFP N-terminally tagged version of the protein was generated and transiently expressed in tobacco leaf epidermal cells alone or in the presence of ALIS. GFP:ALA2 was expressed in tobacco leaf epidermal cells alone or in the presence of ALIS. (A) When expressed alone, GFP:ALA2 co-localized with an ER-retained YFP: HDEL: left, YFP fluorescence; middle, YFP fluorescence; right, fluorescent signal overlay on the bright-field image. (B–D) On coexpression with an untagged ALIS, GFP:ALA2 was localized to vesicular structures; (B) coexpression with ALIS1; (C) coexpression with ALIS3; and (D) coexpression with ALIS5. In each case, two different magnifications are shown; (E) Prevacuolar compartment visualized by expression of the fusion protein BP-80:YFP containing the targeting signals for a plant homologue of the yeast receptor protein Vsp10p: left, YFP fluorescence; middle, YFP fluorescence; right, overlay of YFP signal on a bright-field image. (F) GFP:ALA2 expressed in the presence of ALIS colocalized with BP-80:YFP in aberrant prevacuolar compartment structures. Coexpression with ALIS3 is shown: left, GFP fluorescence; middle, YFP fluorescence; right, overlay of the fluorescent signals on the bright-field image. Arrows indicate the position of nuclei when visible. Bar, 25 \( \mu \)m.
and GFP fluorescent signals colocalized (Figure 5A), indicating that GFP:ALA2 is indeed retained in the ER.

**ALA2 Coexpressed with an ALIS Exits the ER to the Prevacuolar Compartment**

To study the effect of the β-subunits on trafficking of ALA2, the GFP:ALA2 gene fusion was expressed in leaves of *N. benthamiana* in the presence of an untagged ALIS protein. In addition to the ER, the GFP fluorescent signal could now be localized in highly mobile vesicular structures of 1–4-μm diameter (Figure 5, B–D), and, in some cases, in structures that resemble the vacuolar membrane (cf. with Supplemental Figure 3D). The number of vesicular structures varied from cell to cell and between experiments, but these bodies were always associated with expression of ALA2/ALIS combinations, indicating that all ALIS proteins tested cause ALA2 to exit the ER and accumulate in similar organelles.

Vesicular structures similar to those apparent in Figure 5 were not observed in the absence of ALA2 (Figure 5E), but have been described in tobacco cells as aberrant prevacuolar compartment (PVC), that results from defects in the cell vesicular machinery (Kotzer et al., 2004; Tse et al., 2004; Wang et al., 2009). To test this hypothesis, we repeated the leaf infiltration with the combination of GFP:ALA2 and untagged ALIS3, this time in the presence of the PVC marker YFP:BP80 construct (Tse et al., 2004). Fluorescent signals for GFP:ALA2 and YFP:BP80 colocalized, indicating a PVC localization of GFP:ALA2 (Figure 5F).

**ALA3 in Combination with an ALIS Generates Transport of NBD-PE, -PS, and -PC**

To compare the properties of ALA2 with those of ALA3 (Poulsen et al., 2008a), we tested lipid internalization by ALA3 under the same conditions used before for ALA2. Overexpression of ALA3 with any of the ALIS proteins promoted the internalization of not only NBD-PS, but also -PE and -PC (Figure 6, A and B). These results suggest that the specificity of lipid transport of plant P₄-ATPase/β-subunit complexes is determined by the nature of the P₄-ATPase catalytic α-subunit.

**ALA3/ALIS Combinations Regulate PM Asymmetry in Yeast Cells**

All ALA3/ALIS combinations reduced the sensitivity of cells to both papuamide B and duramycin, whereas yeast strains transformed with a mutant form of ALA3, ala3D413A, displayed comparable sensitivity to the empty vector control (Figure 4B). On the basis of these results, we conclude that ALA3 with any ALIS indeed promotes transport of natural PS and PE. Notably, coexpression of ALA3 with any ALIS reduced the sensitivity of cells to papuamide B to a lesser extent compared with the results obtained for ALA2, in agreement with the results obtained for the translocation assays with fluorescent lipids.

Similarly to ALA2, ALA3/ALIS combinations did not increase miltefosine sensitivity (Supplemental Figure 2), suggesting that ALA3 is also not capable of transporting the lysolipid.

**ALA3 Requires a β-Subunit for Exit from the ER**

When expressed alone in *N. tabacum*, ALA3 can apparently exit the ER (Poulsen et al., 2008a). However, expression of a GFP:ALA3 fusion in *N. benthamiana* together with the ER-retained YFP:HDEL construct, resulted in colocalization of the fluorescent signals (Figure 7A), indicating that GFP:ALA3 is retained in the ER in this tobacco species. This result could be explained assuming that *N. tabacum* is equipped with a Cdc50-like protein that allows for ER exit of the heterologous *Arabidopsis* ALA3. In contrast, when the GFP:ALA3 construct was expressed in the presence of untagged ALIS1, ALIS3, or ALIS5, fluorescence could be detected in punctuated structures resembling Golgi bodies (Figure 7, B–D). Based on the expression pattern on a YFP-tagged version of the Golgi marker sialyl transferase (ST:YFP; Saint-Jore et al., 2002), these punctuate structures most likely represent the Golgi apparatus (Figure 7E; Poulsen et al., 2008a).

**Localization of ALIS Proteins Is Determined by the Coexpressed P₄-ATPase**

At this point, we wondered whether ALIS proteins are able to exit the ER in the absence of a coexpressed P₄-ATPase. To

---

**Figure 6.** Coexpression of ALA3 and ALIS proteins complements the lipid uptake defect of the ΔΔrs2Δdnf1Δdnf2 yeast mutant. Yeast mutant cells expressing different protein combinations and wild-type cells were labeled with 1-palmitoyl-NBD lipids and then washed and analyzed by flow cytometry. Accumulation of NBD lipids was expressed as percentage of fluorescence intensity relative to control ΔΔrs2Δdnf1Δdnf2 yeast mutant. (A) Coexpression of ALA3 and ALIS resulted in cells displaying a broad distribution of NBD-PS uptake. Representative histograms are shown. For quantitative analysis of lipid uptake the fluorescence intensity of the total population was determined. (B) ALA3 facilitated the internalization of NBD-PS, -PE, and -PC in the presence of ALIS proteins; (C) The catalytically inactive mutant ala3D413A failed to promote NBD-lipid internalization. Results in B and C are averages ± SE from five independent experiments. One hundred percent corresponds to 51 ± 5 arbitrary units (NBD-PS), 40 ± 5 arbitrary units (NBD-PE), and 42 ± 4 arbitrary units (NBD-PC). WT, wild type; ala3, ala3D413A.
test this, YFP-tagged versions of the different ALIS genes were transiently expressed in *N. benthamiana* epidermal cells, together with GFP:HADEL encoding an ER-retained fusion protein (Hawes *et al.*, 2001). Both fluorescent signals were found to colocalize in all cases, indicating that the β-subunits expressed alone are retained in the ER (Figure 8A; Supplemental Figures 4A and 5A).

To study the effect of an ALA protein on ALIS localization in the cell, we coexpressed ALIS:YFP constructs with either GFP:ALA2 or GFP:ALA3. In combination with GFP:ALA2, YFP-tagged ALIS1, ALIS3, and ALIS5 all localized to large vesicular structures (Figure 8B; Supplemental Figures 4B and 5B). In the case of ALA3, coexpression of tagged ALIS3 and ALIS proteins appeared to be toxic for the cells. Therefore, we coexpressed ALIS:YFP fusions together with an untagged version of ALA3 (Figure 8C; Supplemental Figures 4C and 5C). The fluorescent signals for YFP could then be detected in punctate structures resembling Golgi bodies as shown for ALA3. The different localization for ALIS proteins in the presence of ALA2 or ALA3 suggests that the P₄-ATPase determines the final intracellular localization of the protein complex after exit from the ER.

**DISCUSSION**

Defining the minimal composition of the P₄-ATPase dependent flippase machinery is a major challenge in cell biology. In this work we tested whether Cdc50 related β-subunits of P₄-ATPases add novel features to the P₄-ATPase catalytic subunit. We show that *Arabidopsis* P₄-ATPases ALA2 and ALA3, which are putative aminophospholipid flippases, are retained in the ER when expressed in planta in the absence of an ALIS Cdc50-like β-subunit. After coexpression with any of three different ALIS β-subunits they leave the ER from where ALA2 travels to the endosomal system and ALA3 to the Golgi apparatus. Both ALA proteins are catalytically inactive in the absence of an ALIS protein but, in the presence of any ALIS β-subunit, ALA2 and ALA3 both promote transmembrane flipping of PS whereas ALA3 shows a broader transport specificity. This holds true both for labeled lipid probes as well as for natural lipids. We conclude that intracellular targeting signals and lipid specificity determinants reside in the catalytic ALA subunit. P₄-ATPases have been suggested to contribute indirectly to lipid flipping, e.g., by generating an electrochemical gradient in various membrane systems that drives specific lipid flippases. However, this is difficult to reconcile with our results showing that different ALA proteins when expressed in a heterologous host can generate flipping activities with different lipid specificities. Therefore, our results are in line with a direct role of P₄-ATPases in lipid translocation as recently suggested (Coleman *et al.*, 2009; Zhou *et al.*, 2009).

It has previously been proposed that Cdc50-like proteins may help determine the substrate specificity of the flippase complex. It has been argued that the yeast *trans-Golgi* P₄-ATPases Drs2p and Dnf3p, which exhibit different translocation profiles (Alder-Baerens *et al.*, 2006), interact with different Cdc50 homologues (Cdc50p and Crf1p, respectively; Saito *et al.*, 2004; Furuta *et al.*, 2007). Furthermore, the yeast plasma membrane P₄-ATPases Dnf1p and Dnf2p, which have the same substrate specificity (Pomorski *et al.*, 2003), both interact with Lem3p (Saito *et al.*, 2004; Furuta *et al.*, 2007). However, these are only circumstantial pieces of evidence and do not produce a causative link between the nature of the subunits and the lipid specificity of the protein complex.

A higher number of P₄-ATPase isoforms compared with Cdc50p homologues has been identified in most organisms, which argues against the notion that Cdc50-like proteins add specific features to P₄-ATPase catalytic subunits. This imbalanced ratio is striking in multicellular organisms, because humans have 14 P₄-ATPases and only three Cdc50 proteins (Paulusma and Oude Elferink, 2005), whereas in *Arabidopsis* 12 P₄-ATPase isoforms (Gomès *et al.*, 2000) and five subunits (Poulsen *et al.*, 2008a) are present. No interaction partner among the Cdc50p homologues has thus far been
found for yeast Neo1p, indicating that some P₄-ATPases may act without a Cdc50p homologue. Lem3p interacts and sustains functionality of Dnf1p and Dnf2p (Saito et al., 2004; Furuta et al., 2007). In multicellular organisms several Cdc50p homologues have been shown capable of activating the same P₄-ATPase (Poulsen et al., 2008a; Paulusma et al., 2008), which supports the notion that one Cdc50p isoform can interact with several P₄-ATPases.

In light of the results presented in this study, it seems unlikely that Cdc50-like proteins directly participate in lipid binding and flipping. However, we cannot exclude the possibility that they support the transport function of the ALA subunit or play other role(s) in catalysis of the putative flipase complex. Indeed, three lines of evidence indicate that Cdc50 subunits directly participate in the P₄-ATPase catalytic mechanism. First, separation of Drs2p from its binding partner Cdc50p affects its ability to form a phosphoenzyme intermediate (Lenoir et al., 2009). Second, association of the Cdc50 subunit with the Drs2p ATPase fluctuates during the reaction cycle, with the strongest interaction occurring at or near a point where the enzyme would be loaded with phospholipid ligand (Lenoir et al., 2009). Third, if Cdc50-like proteins are indispensable for catalytic function of P₄-ATPases, mutation of Cdc50-like genes should also block phospholipid transport and phenocopy mutations in P₄-ATPase genes. Indeed, mutations in members of the Cdc50 protein family produce such phenotypes. In yeast, this family consists of three members: Cdc50p, Crf1p, and Lem3p. CDC50 was identified in a screen for cold-sensitive, cell-division-cycle mutants, but how cdc50 causes a cold-sensitive block in the G1-to-S transition is unknown (Saito et al., 2004). At low temperatures, Δcdc50 mutant cells exhibit depolarization of cortical actin patches and mislocalization of polarity regulators, such as Bni1p and Gic1p, in a manner similar to the Δdrs2 mutant (Saito et al., 2004). LEM3 (previously ROS3) was recovered in two unbiased, genetic screens for yeast mutants that either aberrantly expose endogenous phosphatidylethanolamine on the cell surface (Kato et al., 2002) or are resistant to a toxic phosphatidylcholine analogue (Hanson et al., 2003). Deletion of LEM3 causes a defect in the translocation of NBD-PC and -PE across the plasma membrane and in this way phenocopies deletion mutants in the P₄-ATPases dnf1 dnf2 (Pomorski et al., 2003).

Besides P₄-ATPases, another subfamily of P-type ATPases is known to have additional subunits, namely the P₂C subfamily of Na⁺/H⁺ and H⁺/K⁺-ATPases. With only 20–30% overall sequence identity, the three Na⁺/H⁺-ATPase β-subunit isoforms and one H⁺/K⁺-ATPase β-subunit are much less conserved than the α-subunits. Yet all share the same basic overall structure: a short cytosolic N-terminal tail, followed by a single membrane span and a large C-terminal ectodomain with multiple glycosylation sites and disulfide bonds. As pointed out previously (Poulsen et al., 2008b), Cdc50 proteins mimic a fusion between the β- and γ-subunits of the Na⁺/H⁺-ATPase in terms of polypeptide chain lengths and membrane topology. Both Cdc50 proteins and β-subunits are heavily N-glycosylated and contain highly conserved, disulfide bridge-forming cysteine residues (Geering, 2001; Kato et al., 2002; Lenoir et al., 2009; Shinoda et al., 2009).

**Figure 8.** ALIS1 leaves the ER and localizes to different compartments in the presence of ALA2 and ALA3, respectively. A C-terminal fusion of ALIS1 to YFP was expressed in tobacco leaf epidermal cells alone or in combination with ALA2 and ALA3. (A) When expressed alone, ALIS1:YFP colocalized with an ER-retained GFP:HDEL: left, YFP fluorescence; middle, GFP fluorescence; right, overlay image of YFP and GFP fluorescent signals on the bright-field image. (B) When expressed with GFP:ALA2, ALIS1:YFP colocalized to vesicular structures: left, YFP fluorescence; middle, GFP fluorescence; right, overlay of YFP and GFP fluorescent signals. (C) On coexpression with an untagged ALA3, ALIS1:YFP was detected in Golgi-resembling structures: left, YFP fluorescence; right, overlay of YFP signal on the bright-field image. Arrows indicate the position of nuclei when visible. Bar, 25 μm.
The β-subunit of P2C-ATPases is required for the correct membrane insertion, functional maturation, and ER export of the α-subunit (Gottardi and Caplan, 1993; Geering, 2001). The γ-subunit is not required for function, but has a role in fine-tuning Na\(^+\)/K\(^+\)-ATPase activity in a tissue-specific manner (Geering, 2008). Similarly, Cdc50-like proteins required for P\(_2\)-ATPase stability and export of the P\(_2\)-ATPase from the ER (Paulusma et al., 2008; Saito et al., 2004; Chen et al., 2006; Furuta et al., 2007; this work). In this respect, Cdc50 proteins and P2C-ATPase β-subunits appear to be functionally similar. Hence, it seems that Cdc50 proteins function as chaperones responsible for the proper maturation of P\(_2\)-ATPases, rather than serving as translocases or sympotyers themselves. P2C-ATPase β-subunits also modulate intrinsic kinetic parameters of the Na\(^+\)/K\(^+\)-pump (Geering, 2008), and a similar role for Cdc50-like proteins in P\(_2\)-ATPases cannot be excluded.

In sum, the combination of cell biological and genetic data presented here demonstrate that Cdc50-like proteins are essential for ER exit of plant P\(_2\)-ATPases and in this way show a striking similarity to the role of the Cdc50 proteins and P2C-ATPase β-subunits appear to be functionally similar. Hence, it seems that Cdc50 proteins function as chaperones responsible for the proper maturation of P\(_2\)-ATPases, rather than serving as translocases or sympotyers themselves. P2C-ATPase β-subunits also modulate intrinsic kinetic parameters of the Na\(^+\)/K\(^+\)-pump (Geering, 2008), and a similar role for Cdc50-like proteins in P\(_2\)-ATPases cannot be excluded.

In sum, the combination of cell biological and genetic data presented here demonstrate that Cdc50-like proteins are essential for ER exit of plant P\(_2\)-ATPases and in this way show a striking similarity to the role of the β-subunit in P2C-ATPases. A surprising finding has been that Cdc50-like proteins do not contribute with specific ligands for intracellular targeting and lipid specificity and that these properties reside in the catalytic P\(_2\)-ATPase subunit. These results mark an important step forward in understanding how P\(_2\)-ATPases have been adapted to contribute to lipid flipping across membranes. Understanding the physiological roles of diverse plant P\(_2\)-ATPase complexes remains a major challenge for the future.

ACKNOWLEDGMENTS

The authors acknowledge Prof. Chris Hawes (Oxford Brookes University), Dr. Federica Brandizzi (Michigan State University), and Prof. Liwen Jiang (The Chinese University of Hong Kong) for providing DNA constructs for coloization experiments. This work was supported by the Danish National Research Foundation (M.G.F.), The Danish Governments Globalisation Fund (L.R.P.), the Carlsberg Foundation (T.G.P.), and the Deutsche Forschungsgemeinschaft Grant Po748/10 (T.G.P.).

REFERENCES

Alder-Baerens, N., Lisman, Q., Luong, L., Pomorski, T., and Holthuis, J. C. (2006). Loss of P\(_2\)-ATPases Drs2p and Dnf3p disrupts aminophospholipid translocation across the plasma membrane phosphatidyserine asymmetry. Science 320, 528–531.

Earley, K. W., Haag, J. R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C. S. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. 45, 616–629.

Ebashi, F., and Ebashi, S. (1962). Removal of calcium and relaxation in actomyosin systems. Nature 192, 378–379.

Farge, E., Occhi, D. M., Subtil, A., and Dautry-Varsat, A. (1999). Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells. Am. J. Physiol. 276, C725–C733.

Furuta, N., Fujimura-Kamada, K., Saito, K., Yamamoto, T., and Tanaka, K. (2007). Endocytic recycling in yeast is regulated by putative phospholipid translocons and the Ypt31p/32p-Rcy1p pathway. Mol. Biol. Cell 18, 295–312.

Gall, W. E., Geething, N. C., Hua, Z., Ingram, M. F., Liu, K., Chen, S. L., and Graham, T. R. (2002). Drs2p dependent formation of exocytic clathrin-coated vesicles in vivo. Curr. Biol. 12, 1623–1627.

Geering, K. (2001). The functional role of β-subunits in oligomeric P-type ATPases. J. Bioenerg. Biomembr. 33, 425–438.

Geering, K. (2008). Functional roles of Na,K-ATPase subunits. Curr. Opin. Nephrol. Hypertens. 17, 526–532.

Gietz, R. D., and Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polynucleotide glycol method. Methods Enzymol. 350, 87–96.

Gomes, E., Jakobsen, M. K., Axelsen, K. B., Geisler, M., and Palmgren, M. G. (2000). Chilling tolerance in Arabidopsis involves ALA1, a member of a new family of putative aminophospholipid translocons. Plant Cell 12, 2441–2454.

Gottardi, C. J., and Caplan, M. J. (1993). Delivery of Na\(^+\),K\(^+\)-ATPase in polarized epithelial cells. Science 260, 552–554.

Graham, T. R. (2004). Flippases and vesicle-mediated protein transport. Trends Cell Biol. 14, 670–677.

Hanson, P. K., Malone, L., Birchmore, J. L., and Nichols, J. W. (2003). Lem3p is essential for the uptake and potency of alkylphosphocholine drugs, edelfosine and miltefosine. J. Biol. Chem. 278, 50402–50408.

Hawes, C., Saint-Jore, C., Martin, B., and Zheng, H. Q. (2001). ER confirmed as the location of mystery organelles in Arabidopsis plants expressing GFP! Trends Plant Sci. 6, 245–246.

Hua, Z., Fatheddin, P., and Graham, T. R. (2002). An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. Mol. Biol. Cell 13, 3162–3177.

Hua, Z., and Graham, T. R. (2003). Requirement for Neo1p in retrograde transport from the Golgi complex to the endoplasmic reticulum. Mol. Biol. Cell 14, 4971–4983.

Jorgensen, P. L., Hakansson, K. O., and Karlish, S. J. (2003). Structure and mechanism of Na,K-ATPase: functional sites and their interactions. Annu. Rev. Physiol. 65, 817–849.

Katoh, Y., and Katoh, M. (2004). Identification and characterization of Cdc50A, Cdc50B and Cdc50C genes. Oncol. Rep. 12, 939–943.

Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A., and Ecker, J. R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell 72, 447–451.

Koecho, C., and Schell, J. (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of Agrobacterium binary vector. Mol. Gen. Genet. 204, 383–396.

Kotzer, A. M., Brandizzi, F., Neuman, U., Paris, N., Moore, I., and Hawes, C. (2004). ArfBabF2B (Arf7) acts on the vacuolar trafficking pathway in tobacco leaf epidermal cells. J. Cell Sci. 117, 6377–6389.

Kuhlbrandt, W. (2004). Biology, structure and mechanism of P-type ATPases. Nat. Rev. Mol. Cell Biol. 5, 282–295.

Lenoir, G., Williamson, P., and Holthuis, J. C. (2007). On the origin of lipid asymmetry: the flip side of ion transport. Curr. Opin. Chem. Biol. 11, 654–661.

Lenoir, G., Williamson, P.,uts, C. F., and Holthuis, J. C. (2009). Cdc50p plays a major role in the ATPase reaction cycle of the putative aminophospholipid transporter Drs2p. J. Biol. Chem. 284, 17956–17967.
Liu, K., Surendranath, K., Nothwehr, S. F., and Graham, T. R. (2008). P_{4,5,6,7}-ATPase requirement for AP-1/clathrin function in protein transport from the trans-Golgi network and early endosomes. Mol. Biol. Cell 19, 3526–3535.

Märki, F., Hänni, E., Fredenhagen, A., and van Oostrom, J. (1991). Mode of action of the lanthionine-containing peptide antibiotics duramycin, duramycin B and C, and cinnamycin as indirect inhibitors of phospholipid A2. Biochem. Pharmacol. 42, 2027–2035.

Morth, J. P., Pedersen, B. P., Toustrup-Jensen, M. S., Sørensen, T. L., Petersen, J., Andersen, J. P., Vilsen, B., and Nissen, P. (2007). Crystal structure of the plasma membrane proton pump. Nature 450, 1043–1049.

Muller, P., Pomorski, T., and Herrmann, A. (1994). Incorporation of phospholipid analogues into the plasma membrane affects ATP-induced vesiculation of human erythrocyte ghosts. Biochem. Biophys. Res. Commun. 199, 881–887.

Palmgren, M. G., and Harper, J. F. (1998). Pumping with plant P-type ATPases, putative aminophospholipid translocases with a role in endocytosis. Mol. Biol. Cell 9, 1240–1254.

Parsors, A. B., et al. (2006). Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. Cell 126, 611–625.

Paulusma, C. C., Folmer, D. E., Ho-Mok, K. S., de Waart, D. R., Hilarious, P. M., Verhoeven, A. J., and Oude Elferink, R. P. (2008). ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flipase activity. Hepatology 47, 268–278.

Paulusma, C. C., and Oude Elferink, R. P. (2005). The type 4 subfamily of P-type ATPases, putative aminophospholipid translocases with a role in human disease. Biochim. Biophys. Acta 1741, 11–24.

Pedersen, B. P., Buch-Pedersen, M. J., Morth, J. P., Palmgren, M. G., and Nissen, P. (2007). Crystal structure of the plasma membrane proton pump. Nature 450, 1111–1114.

Pérez-Victoria, F. J., Sánchez-Cañete, M. P., Castanys, S., and Gamarro, F. (2006). Phospholipid translocation and miflfo sine potency require both L. donovani miflfo sine transporter and the new protein LdRok3 in Leishmania parasites. J. Biol. Chem. 281, 23766–23775.

Pomorski, T., Lombardi, R., Riezman, H., Devaux, P. F., van Meer, G., and Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. Mol. Biol. Cell 14, 1240–1254.

Poulsen, L. R., López-Marqué, R. L., McDowell, S. C., Okkeri, J., Licht, D., Schulz, A., Pomorski, T., Harper, J. F., and Palmgren, M. G. (2008a). The Arabidopsis P_{4,5,6,7}-ATPase ALA3 localizes to the Golgi and requires a β-subunit to function in lipid translocation and secretory vesicle formation. Plant Cell 20, 658–676.

Poulsen, L. R., López-Marqué, R. L., and Palmgren, M. G. (2008b). Flipases: still more questions than answers. Cell. Mol. Life Sci. 65, 3119–3125.

Puts, C. F., and Holthuis, J. C. (2009). Mechanism and significance of P_{4,5,6,7} ATPase-catalyzed lipid transport: Lessons from a Na^{+}/K^{+}-pump. Biochim. Biophys. Acta 1791, 603–611.

Riekhof, W. R., and Voelker, D. R. (2009). The yeast plasma membrane P_{4,5,6,7}-ATPases are major transporters for lysophospholipids. Biochim. Biophys. Acta 1791, 620–627.

Rose, A. B., and Broach, J. R. (1990). Propagation and expression of cloned genes in yeast-2 microns circle-based vectors. Methods Enzymol. 185, 234–279.

Rauad, A. F., Nilsen, L., Richard, F., Larsen, M. K., Bessereau, J. L., and Tuck, S. (2009). The C. elegans P_{4,5,6,7}-ATPase TAT-1 regulates lysosome biogenesis and endocytosis. Traffic 10, 88–100.

Saint-Jore, C. M., Evins, J., Batoko, H., Brandizzi, F., Moore, I., and Hawes, C. (2002). Redistribution of membrane proteins between the Golgi apparatus and endoplasmic reticulum in plants is reversible and not dependent on cytoskeletal networks. Plant J. 29, 661–678.

Saito, K., Fujimura-Kamada, K., Furuta, N., Kato, U., Umeda, M., and Tanaka, K. (2004). Cdc35p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in Saccharomyces cerevisiae. Mol. Biol. Cell 15, 3418–3432.

Seigneuret, M., and Devaux, P. F. (1984). ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. Proc. Natl. Acad. Sci. USA 81, 3751–3755.

Shinoda, T., Ogawa, H., Cornelius, H., and Toyoshima, C. (2009). Crystal structure of the sodium-potassium pump at 2.4 Å resolution. Nature 459, 446–450.

Siegmund, A., Grant, A., Angeletti, C., Malone, L., Nichols, J. W., and Rudolph, H. K. (1998). Loss of Drs2p does not abolish transfer of fluorescence-labeled phospholipids across the plasma membrane of Saccharomyces cerevisiae. J. Biol. Chem. 273, 34399–34405.

Sparkes, I. A., Ruinons, J., Kearns, A., and Hawes, C. (2006). Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat. Protoc. 1, 2019–2025.

Tang, X., Halleck, M. S., Schlegel, R. A., and Williamson, P. (1996). A subfamily of P-type ATPases with aminophospholipid transporting activity. Science 272, 1495–1497.

Toyoshima, C., Nakasaki, M., Nomura, H., and Ogawa, H. (2000). Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. Nature 405, 647–655.

Tse, Y. C., Mo, B., Hillmer, S., Zhao, M., Lo, S. W., Robinson, D. G., and Jiang, L. (2004). Identification of multivesicular bodies as prevacuolar compartments in Nicotiana tabacum BY-2 cells. Plant Cell 16, 672–693.

Villalba, J. M., Palmgren, M. G., Berberian, G. E., Ferguson, C., and Serrano, R. (1992). Functional expression of plant plasma membrane H^{+}-ATPase in yeast endoplasmic reticulum. J. Biol. Chem. 267, 12341–12349.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33, 949–956.

Wang, J., Cai, Y., Miao, Y., Lam, S. K., and Jiang, L. (2009). Wortmannin induces homotypic fusion of plant prevacuolar compartments. J. Exp. Bot. 60, 3075–3083.

Wicky, S., Schwarz, H., and Singer-Krueger, B. (2004). Molecular interactions of yeast Neu1p, an essential member of the Drs2 family of aminophospholipid translocases, and its role in membrane trafficking within the endomembrane system. Mol. Cell. Biol. 24, 7402–7418.

Zhang, X., and Oppenheimer, D. G. (2009). Irregular Trichome Branch 2 (TBB2) encodes a putative aminophospholipid translocase that regulates trichome branch elongation in Arabidopsis. Plant J. 60, 195–206.

Zhou, X., and Graham, T. R. (2009). Reconstitution of phospholipid translocation activity with purified Drs2p, a type-IV P-type ATPase from budding yeast. Proc. Natl. Acad. Sci. USA 106, 16586–16591.

---

Function of Flippase α-Subunits

---

Vol. 21, March 1, 2010 801