Control of lipid composition of membranes is crucial to ensure normal cellular functions. *Saccharomyces cerevisiae* has two different phosphatidylserine decarboxylase enzymes (Psd1 and Psd2) that catalyze formation of phosphatidylethanolamine. The mitochondrial Psd1 provides roughly 70% of the phosphatidylethanolamine (PE) biosynthesis in the cell with Psd2 carrying out the remainder. Here, we demonstrate that loss of Psd2 causes cells to acquire sensitivity to cadmium even though Psd1 remains intact. This cadmium sensitivity results from loss of normal activity of a vacuolar ATP-binding cassette transporter protein called Ycf1. Measurement of phospholipid levels indicates that loss of Psd2 causes a specific reduction in vacuolar membrane PE levels, whereas total PE levels are not significantly affected. The presence of a phosphatidylinositol transfer protein called Pdr17 is required for Psd2 function and normal cadmium tolerance. We demonstrate that Pdr17 and Psd2 form a complex in vivo that seems essential for maintenance of vacuolar PE levels. Finally, we refine the localization of Psd2 to the endosome arguing that this enzyme controls vacuolar membrane phospholipid content by regulating phospholipids in compartments that will eventually give rise to the vacuole. Disturbance of this regulation of intracellular phospholipid balance leads to selective loss of membrane protein function in the vacuole.

INTRODUCTION

The biochemical nature of integral membrane proteins demands that their polypeptide backbones are in direct contact with the membrane bilayer. This physical proximity supports the view that changes in the lipid content of membranes would naturally influence the activity of these integral membrane proteins. However, testing this idea is complicated by the multiple roles played by membrane lipids in terms of cell physiology. This problem is exacerbated in eukaryotic organisms with multiple membrane-defined compartments, each with a unique lipid composition (reviewed in Maxfield and Tabas, 2005; van Meer, 2005). To approach the contribution of particular lipids to membrane function in eukaryotic cells, mutant strains of *Saccharomyces cerevisiae* have been used with specific defects in biosynthesis of phospholipids (recently reviewed in Carman and Han, 2009) or sterols (reviewed in Sturley, 2000). These types of experiments have been quite useful in determining the importance of sterols in permease association with lipid rafts (Bagnat *et al.*, 2000; Dupre and Hagnauer-Tsapis, 2003; Hearn *et al.*, 2003; Umebayashi and Nakano, 2003) and in implicating the essential nature of the phospholipid phosphatidylethanolamine (PE) in delivery of nutrient transporters to the plasma membrane (Opekarova *et al.*, 2005).

The primary route of PE production in *S. cerevisiae*, like most eukaryotic cells, is via the mitochondrially localized phosphatidylserine (PS) decarboxylase enzyme (Psd1) 1 (Achleitner *et al.*, 1995). However, cells that lack the *PSD1* are still capable of growth in the absence of exogenous ethanolamine due to the presence of a second phosphatidylserine decarboxylase called Psd2 (Trotter *et al.*, 1995; Trotter and Voelker, 1995). Psd2 has been argued to enrich in fractions corresponding to the Golgi/vacuolar region of the cell and to contain a C2 domain associated with membrane binding (Kitamura *et al.*, 2002). Other than its role in maintaining ethanolamine prototrophy in a *psd1*Δ cell, no specific phenotypes have been associated with loss of Psd2.

In this report, we provide evidence that cells lacking Psd2 exhibit a cadmium hypersensitive defect. Importantly, although these *psd2Δ* cells are cadmium sensitive, *psd1Δ* strains are no more cadmium sensitive than a wild-type strain. Our earlier work determined that overproduction of Psd1 (Gulshan *et al.*, 2008), even in a catalytically inactive form, was able to elevate expression of a plasma membrane multidrug transporter called Pdr5 (Balzi *et al.*, 1994; Bisssinger and Kuchler, 1994; Hirata *et al.*, 1994). Overexpression of Psd2 elevated cadmium tolerance but only if the protein was proteolytically matured. The action of Psd2 on cadmium tolerance is mediated by the vacuolar membrane-localized ATP-binding cassette (ABC) transporter protein Ycf1, a well-known determinant of cadmium resistance (Szczyzypka *et al.*, 1994). Fluorescence microscopy and biochemical fractionation experiments demonstrate that Psd2 is localized to the endosomal system of *S. cerevisiae*. Together, these data indicate that Psd2 influences PE content in the endocytic system and the vacuole. Changes in vacu-
Table 1. Strains used in this study

| Strain       | Genotype                                      | Reference          |
|--------------|-----------------------------------------------|--------------------|
| SEY6210      | MATa leu2-3, 112ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mde- | S. Emr (Cornell University, Ithaca, NY) |
| BY4742       | MATA ade2-101 ura3-52 his3-Δ200, trp1-Δ63 leu2Δ1lys2-801 | Open Biosystems    |
| YPH499       | MATa leu2-3, 112ura3-52 his3-Δ200 try2-801, ade2-101 ura3-52 his3-Δ200, trp1-Δ63 leu2Δ1lys2-801 | A. K. Bachhawat (IMTECH, Chandigarh, India) |

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MATERIALS AND METHODS

Yeast Strains and Media

Yeast strains used in this study are listed in Table 1. Cells were grown in cultures containing YPD (2% yeast extract, 1% peptone, and 2% glucose) under nonselective conditions or appropriate CSM media (Bio 101, Vista, CA) under selective conditions (Sherman et al., 1979). Drug resistance was measured by the spot test assay on plates with either a single concentration of drug or gradient plates (Katzmann et al., 1999). Transformation was performed using the lithium acetate technique (Ito et al., 1983).

Strain Construction

The open reading frames (ORFs) PDR17, PDR16, and YCF1 were disrupted in SEY6210 by polymerase chain reaction (PCR)-mediated gene disruption using the KanMX4 cassette, yielding KGS50, KGS51, and KGS52, respectively. The following primers were used for amplification of KanMX4 deletion cassettes of YCF1, PDR17, and YCF1-De1, YCF1-De2, Pdr17-For, Pdr17-Rev, Pdr16-For, and Pdr16-Rev. The PDS2 ORF and PDR17 ORF were also disrupted in the YPH499 background by using PCR-mediated gene disruption with the psd2Δ::MX4 and pdr17::KanMX4 cassettes to yield KGS67 and KGS68. The primers used to amplify the deletion cassettes for psd2Δ::MX4 and pdr17::KanMX4 were Pds2 Del-For, Psd2 Del-Rev, and Pdr17-For, Pdr17-Rev, respectively. A strain carrying deletions in the YCF1 and BPT1 genes was constructed by transforming the ycf1::KanMX4 cassette into a strain carrying the ycf1::KanMX4 allele, yielding KGS53. The ycf1::KanMX4 deletion cassette was amplified using primers Bpt1-For and Bpt1-Rev, yielding Bpt1Δ::KanMX4. The 1X hemagglutinin (HA)-tagged allele of Psd2 (obtained from Ypr352-PSD2-1XHA (Kitamura et al., 2002) was first generated in psd2Δ::kanMX cassette by recombining in the PS2D-1XHA allele to restore ethanolamine prototrophy. The 1X HA-tagged version was then exchanged with 3xHA::TRP1 and GFP::TRP1 cassettes amplified from plasmids pFA6a-3XHA and pFA6a-GFP (Longtine et al., 1998), yielding KGS 55 and KGS 57.

The C-terminal 3X HA tag and green fluorescent protein (GFP) tag fusions of PDS2 were also constructed in SEY6210 by transforming this strain with a 3XHA::TRP1 or GFP::TRP1 cassette amplified from the plasmids strain KGS55 and KGS57, yielding KGS61 and KGS62. To generate strains carrying tandem affinity purification (TAP) tag fusions of PDR17 and PDR17 in wild-type cells, SEY6210 strain was transformed with a TAP-HIS3MX6 cassette, amplified by using primers Pdr17TAP-For, Pdr17TAP-Rev, Pdr17TAP-For, and Pdr17TAP-Rev, from the TAP tag strain collection (Open Biosystems, Huntsville, AL), yielding KGS69 and KGS68. The TAP-HIS3MX6 cassette for PDR17 for making C-terminal TAP-tagged fusions was also transformed in strains PSY4 and KGS54, yielding KGS63 and KGS62, respectively. Strains carrying C-terminal GFP and TAP tag fusions of YCF1 were constructed in SEY6210 and pds2Δ (PSY14) by transforming these strains with a GFP::HIS3MX6 and TAP-HIS3MX6 cassette amplified from GFP tag strain collection (Open Biosystems), yielding KGS63, KGS 64, KGS 65, and KGS66. All the strains were confirmed by PCR. Primer sequences are available on request.

Plasmids

A high-copy-number plasmid carrying PDS2 with a single HA epitope at its C terminus was provided by Dennis Voelker (Kitamura et al., 2002). A pGBG105 (2-µm YIRA3) clone carrying a GAL1-PDS2 fusion gene was purchased from Open Biosystems. A 1.053-kb PCR fragment containing the PDR17 ORF was amplified as a BamHI fragment from SEY6210 (wild-type) genomic DNA. This fragment was then cloned under control of the strong PGK1 promoter in pXTZ188 (Zhang and Moye-Rowley, 2001) to generate pKGS8. The plasmid pBG105 carrying the PDS2 with a C-terminal HA tag was cut with SacI restriction enzyme to release the GAL1 promoter as well as 250 base pairs of N-terminal PDS2 ORF sequence. A NcoI and EcoRV fragment carrying 600 base pairs of the PDS2 promoter and 250 base pairs of the PDS2 ORF starting from ATG was amplified as SacI fragment from wild-type genomic DNA and cloned in plasmid pBG105-GAL1-PDS2-HA.
tag was generated by transforming PSD2 ORF by using primers PSD2-ClaI-200-For and PSD2-GGST-Rev. Both fragments were then used as template to amplify 64 bp of mutated C-terminal PSD2 ORF by using primers PSD2-Clal-200-For and PSD2-GGST-Rev. Primer sequences are available on request. The 64-bp pair fragment was then transformed into a PSD2Δ plasmid containing as well as retested for defective ethanolamine complementation CSM-URA plates containing 1 mM ethanolamine. Multiple transformants ORF while leaving the GGST motif intact. The transformants were recovered and the GGST motif intact. The transformants were recovered from the colony that exhibited the expected ethanolamine auxotrophy and transformed pKGE37. This plasmid was then sequenced as well as retested for defective ethanolamine complementation of psd1Δ psd2Δ strain. The plasmid carrying the PSD2-3X HA C-terminal tag was generated by transforming psd1Δ psd2Δ cells with plasmid YEp552 2 μm URA3 PSD2-HA with PSD2-3X HA-TRP1 cassette amplified from plasmid pFa6a-3X HA-TRP1 (Longtine et al., 1998). TRP1 transformants were recovered and the ability of the 3X HA-tagged Psd2 to normally confer ethanolamine auxotrophy verified. The physical structures of all plasmids were confirmed by restriction digest and DNA sequencing. Multiclonal vectors expressing Tgl1-mCherry and Tgl2-mCherry were obtained from David Katzmann (Mayo Medical School, Rochester, MN) and the clone expressing Stn1-RFP was from Kazuma Tanaka (Hakkoaido University, Sapporo, Japan).

Monochlorobimane (MCB) Transport
This assay was adapted from previous work (Li et al., 1996). Yeast cultures were grown in rich YPD medium until saturation. Cells were resuspended in fresh YPD media with a starting O.D. of 0.1. After allowing cell growth for 2 h, 14,000 rpm for 5 min in an Eppendorf microcentrifuge. For immunoprecipitation assays were performed using lysed spheroplasts. In an additional control, extracts were made from untransformed cells with glass beads. After washing, these cells were centrifuged to CSM media containing excess adenine. Cells were then harvested on a coverslip, and examined under a fluorescent microscope. For quantification of ade pigment was analyzed by Western blotting with anti-TAP and anti-HA antibodies. Protein extracts were probed with either anti-HA or anti-TAP antibodies. To visualize immunoreactive proteins, the nitrocellulose membrane, blocked with 5% nonfat dry milk in phosphate-buffered saline, and then probed with various antibodies. Proteins were detected using peroxidase-conjugated secondary antibody.
RESULTS

Psd2 Is Required for Tolerance to Heavy Metals

In the model eukaryote *S. cerevisiae*, three different routes exist to support the biosynthesis of the phospholipid PE (recently reviewed in Carman and Han, 2009). The major source of PE biosynthesis comes through the decarboxylation of PS in the mitochondria catalyzed by the Psd1 phosphatidylserine decarboxylase enzyme (Clancey et al., 1993; Trotter et al., 1993). A second nonmitochondrial phosphatidylserine decarboxylase enzyme called Psd2 is sufficient to retain ethanolamine independent growth in *psd1*Δ cells (Trotter et al., 1995). The final avenue for PE biosynthesis, the c-terminal processing of phosphatidylserine decarboxylase enzymes. Detailed biochemical analyses in *E. coli* and mammalian cells revealed that this processing is required to generate the active site of the enzyme (Li and Dowhan, 1990; Kuge et al., 1996). The serine residue that is exposed upon cleavage between the glycine and serine residues is required for phosphatidylserine decarboxylase activity (recently reviewed in Schuiki and Daum, 2009). Similarly, mutation of an LGST sequence corresponding to the presumptive processing site in the mitochondrial Psd1 of *S. cerevisiae* eliminated the ability of the resulting mutant protein to confer ethanolamine prototrophy on cells (Gulshan et al., 2008). Psd2 contains a related sequence (GGST) (Trotter et al., 1993) that is located at an analogous position in the polypeptide chain. A site-directed mutant from of Psd2 was prepared in which the GGST was changed to AAAT. Wild-type and GGS1041AAA mutant forms of Psd2 were expressed as epitope-tagged proteins in a *psd1*Δ background. Transformants were analyzed for the expression level of Psd2 by Western blotting and tested for their ability to complement the cadmium hypersensitivity of the *psd2Δ* strain (Figure 2).

The mutant GGS1041AAA Psd2 protein failed to complement cadmium hypersensitivity of the *psd2Δ* strain, whereas the wild-type protein restored tolerance to this heavy metal. Western blot analysis indicated that although the expected C-terminal product was seen in cells expressing the wild-type protein, the mutant GGS1041AAA Psd2 did not generate any C-terminal fragment. Similarly, the GGS1041AAA Psd2 was unable to support ethanolamine independent growth when present in a *psd1Δ psd2Δ* cell (data not shown). These observations argue that normal processing and catalytic activity of Psd2 are required for this protein to carry out nearly as severe as a *ycf1*Δ mutant, indicative of a pronounced defect in the ability to tolerate this heavy metal.

Because Psd2 and Ycf1 seem to cause similar effects on cadmium resistance, we wanted to determine the relationship between the two genes encoding these proteins. A high-copy-number plasmid containing the wild-type *PSD2* gene was introduced into isogenic wild-type and *ycf1*Δ *bpt1*Δ cells. Bpt1 is a homologue of Ycf1 (Klein et al., 2002; Sharma et al., 2002), and the double mutant was used to eliminate the background activity of cadmium resistance otherwise supported by Bpt1. Similar results were obtained when using *ycf1*Δ single mutants (data not shown). Transformants were placed on media containing a gradient of either HgBr or cadmium. After several days' growth, these plates were photographed (Figure 1).

Two important findings emerged from this experiment. First, overproduction of Psd2 led to an increase in resistance to both heavy metals. These heavy metals have previously both been established as substrates of Ycf1 but Bpt1 observed to influence cadmium tolerance only (Gueldry et al., 2003). Second, this resistance is not seen in *ycf1*Δ *bpt1*Δ cells. Together, these data support the view that Ycf1 function responds in a dose-dependent manner to levels of Psd2.

Catalytic Function of Psd2 Is Required to Support Cadmium Resistance

Our previous work with the mitochondrial Psd1 enzyme produced the unexpected finding that the catalytic function of this protein was not required to exert the effect of this protein on drug resistance (Gulshan et al., 2008). To determine whether catalytic activity of Psd2 was required for the enzyme to influence cadmium tolerance, a mutant form of this gene was constructed. Earlier work in *Escherichia coli* (Li and Dowhan, 1990), and our more recent studies in *S. cerevisiae* (Gulshan et al., 2008), demonstrated the necessity for the C-terminal region of the phosphatidylserine decarboxylase enzyme to influence cadmium tolerance, a mutant form of Ycf1 (Klein et al., 2002; Gueldry et al., 2002) and the double mutant was used to influence cadmium tolerance only (Gueldry et al., 2003). Second, this resistance is not seen in *ycf1*Δ *bpt1*Δ cells. Together, these data support the view that Ycf1 function responds in a dose-dependent manner to levels of Psd2.

**Figure 1.** Genetic analysis of Psd2 interaction with Ycf1 substrates. (A) The indicated mutant strains and isogenic wild-type were grown to mid-log phase, and then 1000 cells of each placed on solid medium containing a gradient of the compound listed at the bottom of the panel (Cyh, cycloheximide). Gradient plates were prepared as described previously (Katzmann et al., 1999), and the relative increase in drug concentration was indicated by the bar of increasing width. Plates were incubated at 30°C and photographed once growth was visible. (B) Isogenic wild-type or *ycf1*Δ *bpt1*Δ cells were transformed with the high-copy-number vector plasmid carrying wild-type *PSD2* (2 μm *PSD2*) or the empty vector (Vector). Transformants were grown to mid-log phase with selection for the plasmid and tested for resistance as described above.
of the psd2Δ strain. Because this PE pool acts via influencing Ycf1, then ethanolamine supplementation would be unable to suppress the cadmium sensitivity caused by loss of the YCF1 gene. We also tested the ability of the Kennedy pathway to suppress the cadmium sensitivity of a strain lacking the phosphatidylinositol transfer protein (PITP) homologue Pdr17. Pdr17 was previously shown to be required for PE biosynthesis by Psd2 (Wu et al., 2000), suggesting the possibility that loss of this PITP might similarly cause a reduction in cadmium resistance. An isogenic series of disruption strains was tested for cadmium tolerance on plates containing or lacking supplementation with ethanolamine (Figure 2).

Loss of either Psd2 or Pdr17 causes a large reduction in cadmium resistance, but this sensitivity can be suppressed with the addition of ethanolamine to cadmium-containing medium. This behavior was not reproduced by a ycf1Δ strain that was cadmium sensitive irrespective of the presence of ethanolamine. These data support the view that both Psd2 and Pdr17 are required for normal cadmium tolerance through their roles in PE biosynthesis. Furthermore, both Psd2 and Pdr17 act through the vacuolar ABC transporter Ycf1.

Unique Contributions of Psd2 and Pdr17 to Cadmium Tolerance

Because loss of either Psd2 or Pdr17 caused cadmium sensitivity, we tested the ability of increased dosage of each protein to suppress the cadmium defect caused by loss of the other. A high-copy-number plasmid that overproduced Psd2 or a fusion gene between the strong PGK1 promoter and PDR17 was used to drive elevated levels of each protein. These plasmids were introduced, along with a vector control, into isogenic psd2Δ and pdr17Δ strains. Transformants were then analyzed for the ability to confer cadmium resistance as well as the steady-state level and the membrane association of each protein (Figure 3).

Overproduction of Psd2 was unable to suppress the cadmium sensitivity of a pdr17Δ strain, although it was able to complement the same phenotype in a psd2Δ strain. Similarly, PGK-driven expression of Pdr17 complemented the cadmium sensitivity of a pdr17Δ strain but not of the isogenic psd2Δ cell. These data argue that, as seen previously for PE biosynthesis (Wu et al., 2000), Pdr17 and Psd2 carry out unique, essential roles in conferring normal cadmium resistance on cells.

The mutual dependence of cadmium tolerance shown here and PE biosynthesis shown previously on the presence of both Psd2 and Pdr17 suggested that these proteins might exhibit biochemical phenotypes in the absence of the other. To determine the basis of the defects caused by loss of either one of these partner proteins, steady-state levels of both Psd2 and Pdr17 were compared in the presence and absence of the other subunit. The transformants described above were grown to mid-log phase and levels of both proteins determined by Western blot analysis (Figure 3).

Steady-state levels and processing of Psd2 were independent of the presence of Pdr17. Similarly, Pdr17 was equally expressed in isogenic wild-type and psd2Δ cells. These data demonstrate that the steady-state levels of these two proteins are controlled independently.

Previous experiments determined that the membrane association of Psd2 requires the presence of a C2 domain present in the amino terminus of this protein (Kitamura et al., 2002). Removal of the C2 domain retained catalytic activity but eliminated the ability of the resulting mutant to function in vivo. To determine whether membrane associa-
tion of either Psd2 or Pdr17 might require the presence of the other, the association of these two phospholipid biosynthetic enzymes was assessed in isogenic wild-type or mutant strain lacking potential partner protein. This analysis was carried out using integrated epitope-tagged alleles of each gene in order to avoid possible complications from overproduction. Whole cell protein extracts were prepared under native conditions and then centrifuged at 10,000 × g to generate a 10K pellet and supernatant fraction. Equal amounts of protein were analyzed by Western blotting.

Both Psd2 and Pdr17 were found in the 10K pellet fractions exclusively, consistent with membrane localization. Extraction of these pellet fractions with Na2CO3 but not Triton X-100 released Psd2 from the pellet fractions. Pdr17 exhibited very similar fractionation properties (data not shown). This biochemical behavior is consistent with peripheral membrane association via these factors. However, association of these phospholipid biosynthetic enzymes with their target membranes is independent of the presence of the other. These experiments argue that although function of Psd2 and Pdr17 depends on the presence of both proteins, their expression and membrane association do not.

Psd2 and Pdr17 Are Physically Associated

Because both cadmium resistance and PE biosynthesis required the presence of both proteins, we wondered whether these factors might form a heteromeric complex. A large-scale analysis of interacting proteins in S. cerevisiae has also provided support for the idea that Psd2 and Pdr17 interact (Krogan et al., 2006). To directly assess the possibility that these proteins might physically interact, a coimmunoprecipitation assay was carried out. A control PITP (Pdr16) was used to evaluate the specificity of any potential Psd2:Pdr17 interaction. We examined the cadmium and fluconazole resistance profiles of strains lacking the related PITPs. Pdr16 and Pdr17. PDR16 and PDR17 were initially identified as downstream targets of the Pdr1/Pdr3 transcription factors (van den Hazel et al., 1999). To evaluate the phenotypes caused by loss of these different PITPs, we constructed isogenic wild-type, pdr16Δ and pdr17Δ mutants. These strains were tested for their ability to tolerate challenge by cadmium or fluconazole (Figure 4). Loss of Pdr16 was found to cause azole sensitivity, a finding reported previously in a different genetic background (van den Hazel et al., 1999), whereas loss of Pdr17 alone caused cadmium sensitivity as detailed above. These data indicate that, although Pdr16 is related to Pdr17 by sequence conservation (reviewed in Gric, 2007), their functions are distinct.

To evaluate interaction of Psd2 with Pdr17, two TAP-tagged strains were generated by integrating the TAP cas-
Psd2 Action Enhances Ycf1-dependent Transport Activity

Psd2 function could trigger increased Ycf1 activity in a variety of ways. A YCF1-lacZ plasmid we have characterized previously (Wemmie et al., 1994) was used to allow a facile readout for YCF1 gene expression. Ycf1-TAP and Ycf1-GFP fusion proteins obtained from large-scale collections of tagged proteins (Ghaemmaghami et al., 2003; Huh et al., 2003) were used to examine steady-state levels and vacuolar localization, respectively. These different assays for Ycf1 expression and localization failed to show any significant differences when compared in wild-type and isogenic psd2Δ cells (data not shown). These results suggested that any changes in Ycf1 function were more likely to be due to effects on the transport activity of this protein. To test this idea, we examined vacuolar accumulation of two different Ycf1 substrates, MCB and an endogenous red pigment that accumulates in adenine biosynthetic mutant strains (Chaudhuri et al., 1997).

MCB has been demonstrated previously to accumulate in the vacuole in a Ycf1-dependent manner (Li et al., 1996). Wild-type and psd2Δ cells were incubated with MCB for 0, 2, or 4 h, and then they were examined by fluorescence microscopy. These cells were also labeled with the dye FM4-64 to visualize the vacuolar membranes (Figure 5).

As can be seen in Figure 5, after 2 h of incubation, MCB fluorescence was clearly seen in the lumen of the vacuole of wild-type cells. Conversely, no detectable fluorescence was found in the vacuolar lumen of a psd2Δ strain at this same time point. After extended incubations (4 h), both wild-type and psd2Δ cells showed luminal fluorescence indicative of MCB accumulation.

This finding supported the view that loss of Psd2 depressed the level of Ycf1 transport activity present in the vacuolar membrane and that this reduction was probably the cause of the cadmium hypersensitivity of the psd2Δ strain. Because the MCB accumulation assay requires the dye to first enter the cells, the reduced vacuolar accumulation seen in psd2Δ strains could potentially be due to defects in MCB uptake. To address this issue, we carried out a second Ycf1 transport assay using the endogenous adenine pigment as the substrate for Ycf1. In some adenine mutants, biosynthetic intermediates accumulate upstream of the enzymatic block in the pathway that are modified and produce an intense red autofluorescence (referred to as the ade pigment) (Smirnov et al., 1967). The red color requires the transport of these intermediates into the lumen of the vacuole, a process catalyzed by Ycf1 and related ABC transporters (Chaudhuri et al., 1997). Because this pigment is produced endogenously, no concerns exist with the ability of the substrate to present equally to Ycf1 in wild-type or psd2Δ cells. This assay was performed using isogenic ade2Δ cells containing (wt) or lacking (psd2Δ) the PSD2 gene were grown on limiting adenine to induce formation of the ade pigment (Ade). Microscopy was as above with the exception that the presence of the autofluorescent ade pigment was detected.
global loss of vacuole function in the absence of Psd2, wild-type and psd2Δ cells were stained with the dye quinacrine, which is accumulated on the basis of the pH gradient of the vacuole and serves as an indicator for function of the vacuolar ATPase activity. No differences in quinacrine staining were detected when comparing wild-type and psd2Δ cells (data not shown).

Psd2 Localizes to the Endosome

Previous experiments have reported localization of Psd2 to a region termed the Golgi/vacuole (Wu and Voelker, 2001). Because the experiments above indicated that Psd2 function had an important impact on both Ycf1 transport activity and PE levels in the vacuole, we revisited the question of Psd2 localization. A more refined identification of the membrane likely to be targeted by Psd2 is important to the understanding of how this enzyme regulates intracellular phospholipid homeostasis.

To examine Psd2 localization, we first constructed a strain in which GFP was integrated at the wild-type PSD2 locus to form a Psd2-GFP fusion protein. This strain expressed a fluorescent form of Psd2 at levels modulated by its authentic chromosomal locus. This PSD2-GFP fusion gene was able to confer ethanolamine independent growth when introduced into a psd1Δ background indicating that the fusion protein retained enzymatic function (data not shown). Fluorescent microscopic analysis of the Psd2-GFP–expressing cells exhibited punctate fluorescence at structures within the cytoplasm. These punctate structures did not colocalize with the labeling seen for Ycf1-GFP (vacuolar membrane) or Anp1-RFP (Golgi) (data not shown). To determine whether Psd2-GFP might be localized within the endocytic system, we used several different marker proteins known to be associated with this membrane compartment including a Snc1-RFP fusion protein, known to localize to the plasma membrane and early endosomes (Gurunathan et al., 2000; Lewis et al., 2000), as well as Tlg1- and Tlg2-mCherry fusion proteins as markers for this compartment (Kama et al., 2007). The strain expressing Psd2-GFP was transformed individually with expression plasmids driving accumulation of Snc1-RFP, Tlg1-mCherry, or Tlg2-mCherry. Transformants were grown to mid-log phase and analyzed microscopically as described above (Figure 6).

We found that Psd2-GFP formed only a few punctuate structures in the cytoplasm and that these structures were also labeled by the fluorescent Snc1, Tlg1, and Tlg2 proteins. To determine if Pdr17 colocalized to the endocytic system as did Psd2, a Pdr17-GFP fusion gene was constructed. The Snc1-RFP plasmid was introduced into cells expressing Pdr17-GFP and distribution of these two fluorescent proteins compared (Figure 6C). As seen for Psd2-GFP and Snc1-RFP, colocalization of Pdr17 and Snc1 was detected. These fluorescent microscopic data supported the conclusion that both Psd2 and Pdr17 are localized to the endocytic system rather than either Golgi network or vacuole as suggested previously (Wu and Voelker, 2001).

To further validate our identification of the endocytic system as the site of Psd2 localization, we performed indirect immunofluorescent localization using a Psd2-3X HA-expressing strain. The presence of Psd2 was detected using a mouse anti-HA antibody. We also visualized a component of the vacuolar ATPase, the Vma2 protein using an antibody directed against this V1 subunit (Yamashiro et al., 1990). Cells were also stained with DAPI to provide a reference for nuclear and mitochondrial DNA (Figure 6B).

As seen when using the Psd2-GFP fusion protein, Psd2-3X HA was found to be present in a limited number of cytoplasmic structures. These structures were clearly resolvable from the location of the vacuolar membrane indicated by anti-Vma2 staining. Together with the colocalization with the fluorescent endosomal marker proteins above, these data argue against vacuolar localization of Psd2 and rather that this phosphatidylserine decarboxylase activity is found in the endocytic system.

Further support for our hypothesis that Psd2 localized to endosomes came from a FM4-64 chase experiment. FM4-64 is a widely used vacuolar membrane dye that reaches the vacuole via the endocytic pathway (Vida and Emr, 1995). At early times after labeling with FM4-64, the early endocytic system can be visualized but this disappears over the time course of labeling as the dye makes it way through the endocytic pathway. We labeled cells expressing the Psd2-GFP fusion protein with FM4-64, washed away excess dye...
and then visualized both the dye and Psd2-GFP with increasing time (Figure 7).

Immediately after labeling, FM4-64 can be seen along with Psd2-GFP but there was no overlap in these fluorescent signals. After 5 min of incubation, double labeling was detected in this cells indicating that the dye had arrived in the Psd2-GFP–positive compartment. This double labeling was no longer seen as the incubation was continued to 10 min. Note that the limiting membrane of the vacuole can be seen at 10 min and that this structure is clearly distinct from the Psd2-GFP signal. This experiment is consistent with Psd2 being localized to an endosomal compartment that can be labeled with FM4-64. We performed these same analyses with cells expressing Pdr17-GFP. The expression of Pdr17-GFP was found to be quite low with only few cells showing punctate cytoplasmic fluorescence (data not shown). To circumvent this problem we placed Pdr17-GFP under the strong TDH3 promoter. Using this elevated level of Pdr17-GFP expression, this fusion protein was localized to the plasma membrane, cytoplasm as well as in endosomes (Figure 7B). These data support the view that both Psd2 and Pdr17 are localized to the endosomal system.

To confirm these microscopic analyses, we carried out biochemical fractionation of intracellular membranes using sucrose gradient analysis (Katzmann et al., 1999). To carry out this experiment, we used a strain containing a version of PSD2 containing an integrated 3X HA tag at its C terminus. Similarly, we integrated a TAP epitope tag at the C terminus of PDR17 in the same strain which allowed detection of both Psd2 and Pdr17 in the same gradient fractions. This duallly epitope-tagged strain was grown to mid-log phase, gently lysed and membranes fractionated on a 10–60% sucrose gradient. Aliquots of each fraction were resolved on SDS-PAGE and subjected to Western blot analysis using the antibodies indicated (Figure 8).

Both Psd2-3X HA and Pdr17-TAP were enriched in overlapping aliquots from the center of this sucrose gradient. These proteins were also enriched in the same fractions containing the highest levels of the endosomal marker protein Pep12 (Gerrard et al., 2000) and distinct from either a plasma membrane protein (Pma1) (Serrano et al., 1986) or an endoplasmic reticulum marker (Kar2) (Rose et al., 1989).

We also carried out Ficoll gradient analysis of membrane proteins which clearly resolves vacuolar proteins from other membrane components as previously demonstrated (Vida et al., 1990; Wemmie and Moye-Rowley, 1997). The Psd2-3X HA and Pdr17-TAP–expressing strain was grown to mid-log phase and gently lysed. Lysates were separated by centrifugation in a Ficoll step gradient and membrane fractions collected at each interface. Proteins were recovered by TCA precipitation and subjected to Western blotting analysis using antibodies to the polypeptides indicated (Figure 8B). We also carried out Ficoll gradient analysis of membrane proteins which clearly resolves vacuolar proteins from other membrane components as previously demonstrated (Vida et al., 1990; Wemmie and Moye-Rowley, 1997). The Psd2-3X HA and Pdr17-TAP–expressing strain was grown to mid-log phase and gently lysed. Lysates were separated by centrifugation in a Ficoll step gradient and membrane fractions collected at each interface. Proteins were recovered by TCA precipitation and subjected to Western blotting analysis using antibodies to the polypeptides indicated (Figure 8B).
Loss of Psd2 Specifically Reduces Endosomal and Vacuolar PE Content

Because loss of Psd2 leads to a decrease in Ycf1-dependent transport activity, we hypothesized that a change in PE content of the vacuolar membrane could explain this reduction. Previous work has demonstrated that Psd1 provides the major route for PE biosynthesis with Psd2 carrying out the remainder (Birner et al., 2001). This finding suggested that loss of Psd2 might cause a decrease in the vacuolar pool of PE. To test this idea, we prepared vacuolar membranes from wild-type, psd1Δ, and psd2Δ cells by using Ficoll gradient centrifugation. Enrichment for vacuoles was confirmed by Western blotting for Vph1 (data not shown). We also analyzed phospholipid content in whole cell lipid extracts. Phospholipids were analyzed using an assay for lipid phosphorous content (Chalvardjian and Rudnicki, 1970) and quantitated (Figure 9).

PE levels measured in vacuole-enriched membrane fractions were reduced from 40% in wild-type or psd1Δ cells to 25% in psd2Δ strains. Consistent with this observed decrease in PE found in psd2Δ cells, levels of PS increased. PS levels were not significantly different when comparing wild-type and psd1Δ cells. Control experiments measuring these same phospholipids present in total cellular lipids reproduced the findings of others (Birner et al., 2001) as loss of Psd1 caused a drop in total PE and an increase in PS, whereas psd2Δ cells showed no significant difference from the isogenic wild-type strain.

Although the reduction in vacuolar PE levels would explain the observed decrease in Ycf1-mediated function, the lowered vacuolar PE was unlikely to result from a direct effect of Psd2 or Pdr17 because these proteins were found on the endosome (Figures 6–8). Together, these data suggest the possibility that the action of the endosomally localized Psd2:Pdr17 complex acts to regulate the PE content of the vacuole. To test this suggestion, we prepared endosome-enriched fractions using differential centrifugation (Singer-Kruger et al., 1993) and assessed phospholipid and protein levels (Figure 9, B and C).

The most endosome-enriched fraction was represented by the P3 samples. P3 was found to have very little Vph1, consistent with this purification separating vacuolar membranes from endosomal fractions. Some endoplasmic reticulum contamination was still present as evidenced by the detection of the luminal Kar2 protein in this fraction. However, when P3 fractions were generated from cells lacking Psd2 and analyzed for phospholipid content, PE levels were found to be reduced. This reduction did not occur when the same fraction was produced from either wild-type or psd1Δ cells. PS levels were not significantly different in any of these samples. P2 fractions exhibited a reduction in PE levels in samples from psd1Δ cells but no other changes of note. These assays are consistent with the interpretation that PE levels are lowered in endosomes from psd2Δ strains and that this reduction is linked to a similar decrease in vacuolar PE levels. This depletion in vacuolar membrane PE is coupled to the observed reduction in Ycf1 activity in cells lacking Psd2.

DISCUSSION

Biosynthesis of phospholipids is of central importance in the normal functioning of biological membranes. S. cerevisiae has served as an outstanding model system allowing the fundamental mechanisms of eukaryotic membrane production to be defined. While genetics have clearly indicated the existence of both a mitochondrial and a extra-mitochondrial route for de novo PE production, clear phenotypes only existed for the mitochondrially localized Psd1 pathway (Birner et al., 2001). Here, we provide evidence that the second route of PE production catalyzed by the phosphatidylserine decarboxylase Psd2 is required for normal function of a vacuolar membrane protein. This functional requirement is mediated by the need for correct PE levels in the vacuolar membrane that support normal activity of Ycf1 and possibly other proteins.

Psd2 represents a relatively unusual type of phosphatidylserine decarboxylase enzyme and is found in plants but not in bacteria or mammals (Voelker, 1997). Psd1 homologues are present in mammalian mitochondria and at the inner membrane of bacteria (reviewed in Vance, 2003). Proteins exhibiting high sequence similarity to Psd2 have been described in plants (Nerlich et al., 2007), suggesting the
Possibility that a common role for control of internal membrane PE exists in these organisms. Ycf1 homologues have been described in plants that localize to internal membranes and are required for cadmium resistance (Tommasini et al., 1996).

Although Psd1 and Psd2 catalyze the same reaction, the PS substrate is apparently presented to each enzyme quite differently. Psd1 is located close to the site of PS synthesis, closely resembles the E. coli Psd enzyme and has not been shown to require any other enzyme to carry out its role. Conversely, Psd2 is nearly twice the size of Psd1 and not as closely related to the bacterial enzyme (Trotter et al., 1995). Psd2 also requires the presence of the Pdr17 PITP to be able to productively interact with PS as even elevated dosage of PSD2 is unable to bypass a pdr17Δ mutation (Figure 3). Previous work has demonstrated that membrane association of Psd2 requires the presence of an amino-terminally located C2 domain (Kitamura et al., 2002). Together, these data suggest that although Psd2 and Pdr17 form a complex on the membrane, the independent membrane association of both proteins is likely required to permit complex formation.

The finding that Psd2 was localized to the endosomes was unexpected. Previous assignment of Psd2 to a Golgi/vacuole distribution (Trotter and Voelker, 1995) was based strictly on biochemical fractionation experiments which did not provide the resolution to define the endosomal location we show here. We anticipated finding Psd2 on the vacuolar membrane because we found that loss of this enzyme triggered a cadmium-sensitive defect centered on reduction of Ycf1 function. The endosomal enrichment of Psd2/Pdr17, coupled with the demonstration that loss of this complex lowered vacuolar PE levels, indicates that phospholipid levels of the vacuolar membrane may be controlled by the direct action of this enzyme complex on the endosome. This suggestion is directly supported by our measurements of PE content of internal membranes. Modulation of endosomal PE levels is eventually communicated to the vacuolar membrane through vesicular transport. The indirect control of phospholipid content by Psd2 is reminiscent of the effect of the P-type ATPase Drs2 on plasma membrane asymmetry (Chen et al., 2006). Drs2 is localized to the Golgi membranes (Chen et al., 1999), yet its loss causes defects in distribution of phospholipids on the plasma membrane.

Changes in PE levels have been extensively documented to lead to problems in folding of membrane proteins (recently reviewed in Dowhan and Bogdanov, 2009). Most of these studies have been carried out using reconstituted membrane proteins in an in vitro setting. Our experiments provide in vivo demonstration of the importance of phospholipid composition in regulating membrane protein function. An alternative view of the consequences of loss of Psd2 on Ycf1 function is that elevated PS levels act to inhibit transporter activity. Based on the extensive documentation of the stimulatory effect of PE on folding and subsequent activity of membrane proteins (Bogdanov et al., 1999, 2002; Zhang et al., 2003; Hakizimana et al., 2008), we favor the positive effect of PE levels increasing Ycf1 activity rather than high PS levels inhibiting function of this ABC transporter.

Another important distinction between previous work carried out primarily on prokaryotic permeases and stimulation of their folding by PE (Wang et al., 2002), comes from the fundamental differences in the biogenesis of prokaryotic and eukaryotic membrane proteins. The exceptionally well-studied prokaryotic lactose permease, in which the positive effect of PE on folding has been demonstrated (Bogdanov et al., 1999, 2002), is inserted into its final membrane destination as it is being synthesized. This is quite different from Ycf1 that follows the typical itinerary of eukaryotic membrane proteins consisting of initial biosynthesis and insertion into the membrane of the endoplasmic reticulum, transit through the Golgi and only then reaching its vacular membrane site of function (Wemmie and Moye-Rowley, 1997). Our data suggest a possible means of regulation of Ycf1 as the unique phospholipid composition of the vacuole compared with other internal membranes is evidently required for full activity of this transporter. This would have the result of keeping Ycf1 activity relatively low as it transits the secretory pathway en route to its functional residence in the vacuolar membrane.

Our previous work on Psd1 demonstrated that this phosphatidylserine decarboxylase protein has another function that in mitochondrial-nuclear signaling in addition to its enzymatic activity (Gulshan et al., 2008). Psd1 also closely resembles its bacterial counterpart Psd (Clancey et al., 1993; Trotter et al., 1993) that has been carefully studied in enzymological terms. Although Psd1 and Psd2 carry out the same conversion of PS to PE, they execute this reaction in dramatically different ways. All available data indicate that Psd1 requires no other protein to function as a phosphatidylserine decarboxylase, whereas Psd2 clearly requires Pdr17 (Wu et al., 2000). Additionally, loss of Psd1 C-terminal processing prevented enzymatic function but had no detectable effect on steady-state level of the resulting mutant protein (Gulshan et al., 2008). Conversely, introducing this same C-terminal processing block into Psd2 led to a reduction in the level of mutant protein. Perhaps the different subcellular distributions of Psd1 and Psd2 explain the differential dependence on normal C-terminal processing for protein expression.

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