A new yeast strain, designated pstB2, that is defective in the conversion of nascent phosphatidylserine (PtdSer) to phosphatidylethanolamine (PtdEtn) by PtdSer decarboxylase 2, has been isolated. The pstB2 strain requires ethanolamine for growth. Incubation of cells with [3H]serine followed by analysis of the aminoglycero-phospholipids demonstrates a 50% increase in the labeling of PtdSer and a 72% decrease in PtdEtn formation in the mutant relative to the parental strain. The PSTB2 gene was isolated by complementation, and it restores ethanolamine prototrophy and corrects the defective lipid metabolism of the pstB2 strain. The PSTB2 gene is allelic to the pleiotropic drug resistance gene, PDR17, which encodes a phosphatidylinositol/phosphatidylcholine transfer protein. The protein, PstB2p, displays phosphatidylinositol but not PtdSer transfer activity, and its overexpression and is homologous to SEC14, which encodes a phosphatidylinositol/phosphatidylcholine transfer protein. The protein, PstB2p, displays phosphatidylinositol but not PtdSer transfer activity, and its overexpression causes suppression of sec14 mutants. However, overexpression of the SEC14 gene fails to suppress the conditional lethality of pstB2 strains. The transport-dependent metabolism of PtdSer to PtdEtn occurs in permeabilized wild type yeast but is dramatically reduced in permeabilized pstB2 strains. Fractionation of permeabilized cells demonstrates that the pstB2 strain accumulates nascent PtdSer in the Golgi apparatus and a novel light membrane fraction, consistent with a defect in lipid transport processes that control substrate access to PtdSer decarboxylase 2.

The biogenesis of organelle membranes requires the coordinated transport of proteins and phospholipids from their sites of synthesis to their final locations. Considerable molecular information is available regarding protein sorting for assembly into membranes (1, 2). In contrast, little is known about the mechanisms required for lipid transport within eukaryotic cells. Multiple mechanisms for lipid transport have been proposed including (a) vesicle packaging and routing, (b) phospholipid transfer proteins, and (c) zones of apposition between donor and acceptor organelle compartments (3, 4). Some experimental evidence exists in support of each of the above proposed mechanisms. However, the current results suggest considerable diversity, rather than a simple set of unifying principles, is involved in the process of lipid transport.

In an effort to address the problem of lipid transport, this laboratory has focused upon the metabolism of PtdSer in the yeast Saccharomyces cerevisiae (5–9). The lipid, PtdSer, provides a number of advantages for examining transport because the multiple events in its conversion to phosphatidylycholine (PtdCho) occur within different organelle domains and provide discrete biochemical indicators of transport steps. The topology of PtdSer metabolism and the basis of the genetic strategy used in this study are outlined in Fig. 1. PtdSer is synthesized in the endoplasmic reticulum or closely related membranes by PtdSer synthase (10). Following its synthesis, the lipid is disseminated throughout the cell. Upon arrival at the mitochondria or the Golgi/vacuole, PtdSer is decarboxylated to form PtdEtn (8, 11). The mitochondrial decarboxylase is Psc1p, and the Golgi/vacuole enzyme is Psc2p (5, 7). Following its formation in either the mitochondria or the Golgi/vacuole, PtdEtn is transported to the endoplasmic reticulum for the synthesis of PtdCho (5, 12). The methyltransferase enzymes Pem1p and Pem2p catalyze the formation of PtdCho from PtdEtn using S-adenosylmethionine as the methyl donor (13).

In this study, we have used cells harboring the null allele, pscD::TRP1, to force all PtdSer metabolism through the Golgi/vacuolar compartments (5, 9). In this genetic background, we reason that it should be possible, after mutagenesis, to isolate new strains with defects in metabolic steps between PtdSer formation and PtdEtn synthesis. Our approach follows from the observations that cells with inactivating mutations in PtdSer synthase (PSS) or both PSD1 and PSD2 genes remain viable if supplemented with ethanolamine (EtN) (7, 8, 12). EtN rescues the aforementioned strains by providing PtdEtn via the CDP-ethanolamine pathway. These latter findings imply that specific defects in PtdSer and PtdEtn methylation. Included in these steps are vectorial lipid transport events between the endoplasmic reticulum and the Golgi/vacuole for PtdSer and between the Golgi/vacuole and endoplasmic reticulum for PtdEtn. Our approach follows from the observations that cells with inactivating mutations in PtdSer synthase (PSS) or both PSD1 and PSD2 genes remain viable if supplemented with ethanolamine (EtN) (7, 8, 12). EtN rescues the aforementioned strains by providing PtdEtn via the CDP-ethanolamine pathway. These latter findings imply that specific defects in PtdSer and PtdEtn transport may be identifiable and amenable to rescue by EtN supplementation. This general approach has been applied in a previous study and has identified the PtdIns 4-kinase, Stt4p, as an important component in PtdSer metabolism in steps between

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its synthesis in the endoplasmic reticulum and decarboxylation in the Golgi/vacuole (9). Our goals in this study were to: 1) identify new Etn auxotrophs among mutated populations of strains with a pslD::TRP1 allele, 2) characterize the Etn auxotrophs genetically and biochemically, and 3) identify and characterize the gene complementing the defect.

In this report we identify a new strain, designated pstB2, with interesting properties in aminoglycerophospholipid metabolism. The strain is an Etn auxotroph with pronounced defects in phospholipid metabolism. The defects in lipid metabolism include the accumulation of high levels of PtdSer in the Golgi fraction and a light membrane fraction. The gene complementing the growth and biochemical defect encodes a protein of previously unknown biochemical function with structural similarity to the PtdIns/PtdCho transfer protein, Sec14p.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals, including amino acids for yeast media, were purchased from either Sigma or Fisher Scientific. Other components for yeast and bacterial growth media were purchased from Difco. Phospholipids were obtained from Avanti Polar Lipids. Thin layer silica gel H plates were purchased from Analtech Corp. The radioisotopes [3-3H]serine and [1-14C]serine were from Amersham Pharmacia Bio- tech, Inc. 

The lipid [14C]-diacylglycerol was synthesized from [1-14C]serine and NBD-CDP-diacylglycerol using Escherichia coli PtdSer synthase (14). Reagents for protein determination were either from Pierce or Bio-Rad. Pre-cast SDS-polyacrylamide gels were purchased either from NOVEX or FMC. Mouse monoclonal antibodies against the V5 epitope tag of the PstB2p fusion protein were obtained from Invitrogen. Other reagents used for ligand blotting were obtained from Bio-Rad and Sigma.

Cells, Plasmids, and Libraries—Yeast were cultured in synthetic complete or YPD (standard YPD medium plus additional adenine, uracil, and ethanolamine) media (9). When the PstB2p fusion protein was overexpressed in yeast under GAL1 promoter regulation, the cells were grown in uracil-free synthetic medium containing ethanolamine, with 2% galactose and 1% raffinose as the carbon sources. The parental strain, RYYS2 (MATa lys2 trp1 ura3 his3 leu2 suc2 pslD1::TRP1), was constructed from the wild type strain, SEY6210 (MATa lys2 trp1 ura3 his3 leu2 suc2), as described previously (5). The pstD2a strain, PTTY43 (MATa ura3 leu3 his3 trp1 ade2 suc2 psd2Δ1::HIS3), used to examine complementation by the ethanolamine auxotroph was constructed directly from wild type strain SEY6211 (MATα ura3 leu2 his3 trp1 ade2 psd2Δ1::HIS3), as described previously (5). The psl mutant strain, RYYS7 (MATα his3-1), used in the identification of the yeast carrying a defect in PSS by complementation analysis was obtained from a cross of KAI101, generously provided by Dr. Susan A. Henry (Carnegie Mellon University, Pittsburgh, PA) (12), with X1049 (Yeast Genetic Stock Center). In this paper we use the simplifying terminology PSS and psd, respectively, to identify the wild type and mutant alleles of phosphatidylerine synthase in place of the original CHO1 and cho1 designations (15–17). The PstB2 mutant strain, WWY57, was isolated as an ethanolamine auxotroph from the ethylmethane sulfonate-mutagenized (18) parental strain RYYS2. RYYS7 and WWY71 are two pslD1::TRP1 strains used to outcross the original pstB2 mutant. WWY71 was obtained from a cross between PTTY40 (MATa ura3 his3 trp1 met14 psd1Δ1::TRP1) and PTTY41 (MATa ura3 leu3 trp2 his3 lys2 pslD1::TRP1). The sec14 strain used was CTY1–1A (MATa ura3–52 his3–200 lys2–501 s14–3). The YCp50 yeast genomic library was generously provided by Dr. Vytas Bankaitis (University of Alabama, Birmingham, AL). The YEp352 plasmid was obtained from Dr. Alex Franzushoff (University of Colorado Health Sciences Center, Denver, CO). The pUC18-HIS3 construct was a gift from Dr. Rodney Rothstein (Columbia University, NY).

Preparation of the Yeast Total Membranes and Microsomes—Yeast cell membranes were prepared as described (1). The total membranes were collected from the cell-free extract by ultracentrifugation at 100,000 × g for 1 h at 4 °C. The resulting membranes were resuspended using a Potter-Elvehjem device. The microsomes were also prepared from the cell-free extract by centrifugation at 15,000 × g for 20 min to remove dense membranes followed by ultracentrifugation at 100,000 × g for 1 h at 4 °C to sediment the low density membranes. The protein concentrations of the total membranes and the microsomes were determined using a bicinchoninic acid method (Pierce) or the Bradford method (19).

Enzyme Assays—PSD2 activity was measured as described previously (8). Briefly, the activity was assayed by trapping [14C]CO2 released by the hydrolysis of [1-14C]serine using 4 M KOH-impregnated filter paper at 30 °C. PtdSer synthase activity was determined by measuring the incorporation of either [3H]serine or [14C]serine into PtdSer as described by Carman and Bae-Lee (20).

Radiolabeling and Phospholipid Analysis of Cells—All strains were grown in synthetic complete medium plus ethanolamine at 30 °C until reaching mid-log phase. Cultures were then washed and diluted to an A600 of 0.2 to 0.4 with synthetic complete medium (SC) containing 10 μg/ml L-[1-14C]serine (50 μCi/ml). These cultures were incubated at 30 °C with shaking for 4 h. Trichloroacetic acid (5% w/v) final concentration was added to each culture at the end of the incubation, and cells were washed twice with ice-cold water. Lipids were isolated by ethanol extraction (21) and analyzed by thin layer chromatography (TLC) on Silica Gel H plates using a solvent system containing chloroform, methanol, 2-propanol, 0.25% KCl, triethylamine (90:25:6:18, v/v). Phospholipids were identified by co-chromatography with authentic standards and visualized by staining the TLC plates with 0.1% anilino-1-naphthalel sulfonic acid and exposure to ultraviolet light. Individual lipid spots were scraped from the TLC plates into 0.5 ml of water plus 4.5 ml of scintillation mixture (Fisher Scientific). The radioactivity of each lipid sample was determined in a liquid scintillation counter.

Isolation of the PSTB2 Gene—The Etn auxotrophic mutant, pstB2, was transformed with a YCp50 yeast genomic library using the YEASTAKER kit purchased from CLONTECH. Cells harboring plasmids were first selected for the presence of the plasmid marker, URA3, by growth on synthetic complete medium plus ethanolamine lacking uracil. The transformants were then screened for ethanolamine prototrophy on SC medium containing uracil. The plasmids were recovered from the positive transformants and amplified in E. coli as described previously (22). Amplified plasmids were isolated from the E. coli and retransformed into the pstB2 mutant to confirm their ability to complement the growth defect of the mutant strains. Further confirmation of the activity of the complementing plasmid was obtained from experiments that demonstrated concordant loss of Etn prototrophy and the linked plasmid uracil prototrophy. For plasmid loss studies, the pstB2 mutant carrying complementing plasmids was cultured under nonselective conditions for greater than 30 generations and then monitored for both ethanolamine and uracil auxotrophy.

DNA Sequencing and Plasmid Constructs—The original YCp50 P57/B2 clones were sequenced from both ends of the insert using primers that annealed to the plasmid sequences adjacent to opposite ends of the insert site. Sequencing was performed utilizing the ABI prism Ready Dye Deoxy Terminator Cycle Sequencing Kit. Cycle sequencing products were purified with Centricip Spin Columns (Princeton Separations, Adelphia, NJ) and then analyzed on an ABI 377 automated sequencer at the Molecular Science Resource Center of the National Jewish Medical and Research Center, Adelphia, NJ. The resulting sequences were used to design primer sets for the artificial location of the insert using the S. cerevisiae Genome Data base (23). Predicted open reading frames (ORFs) within the insert were either individually subcloned or subcloned as a cluster containing two to three ORFs into a YCp50 or YEp352 vector depending on the available restriction sites. The subclones were retransformed into the pstB2 mutant to test their complementation of the Etn auxotrophy.

The pYES plasmid encoding a galactose inducible, V5His6 epitope-tagged form of PstB2p (PstB2V5His6) was obtained from Invitrogen and used for expression in yeast. A plasmid encoding the epitope-tagged version of the protein for expression in insect cells was constructed by excising the DNA encoding PstB2V5His6 from the pYES plasmid using SspI and XhoI restriction enzymes and ligating the fragment into PBIR 2.1. Ligation of the DNA into PBIR 2.1 was performed after A-tailing the insert with Taq polymerase. The desired insert was excised by EcoRI digestion and subsequently ligated into a PV1L1392 vector for expression in Sf9 cells.

Disruption of Chromosomal P57/B2 Allele—The P57/B2 gene was disrupted in a YEp352 vector by inserting a 1.8-kb BamHI DNA fragment containing the BST1 deletion (24). The pYES plasmid carrying BST1 was subcloned into pYES plasmid sequence. The pstB2–HIS3 allele was disrupted from YEp352 by SacI/XhoI digestion and then subcloned into the E. coli vector pGEM4Z. The chromosomal P57/B2 gene was disrupted by one-step gene replacement (24) using the YEASTAKER system (CLONTECH) with the linear SacI/XhoI fragment containing pstB2–HIS3 from the pGEM4Z plasmid construct. Recombinants were selected for the presence of His' prototrophy.

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The disruption of the chromosomal PSTB2 gene was confirmed by PCR (25). Primers flanking the disruption or complementing the HIS3 gene were generated such that the primer pairs annealed to the 5′- and 3′-end of the PSTB2 gene would yield a 1.9-kb fragment for the wild type allele, whereas the HIS3 internal primer and the 3′-flanking primer would yield a 0.4-kb fragment. The primer set was designed to overlap the disrupted allele. The transforming DNA did not contain the sequence corresponding to the 5′- or the 3′-PCR primer to ensure that the appropriate PCR products could only be generated after integration into the PSTB2 locus. Genomic DNA was prepared from strains by standard methods (26), and the PCR reaction was performed using the Taq DNA polymerase in a Perkin-Elmer DNA ThermalCycler. PCR amplified fragments were visualized by agarose gel electrophoresis and staining with ethidium bromide (27).

**PtdIns and PtdSer Transfer Assays—** Both Sec14p and PstB2p were overexpressed as amino-terminal His6 fusion proteins under control of the lac operator in E. coli using a pQE30 vector (Qiagen). An E. coli lysate containing the fusion protein was prepared from isopropyl-1-thio-β-D-galactopyranoside-induced cells by sonication in lysis buffer (50 mM sodium phosphate, pH 7.4, 0.3 mM NaCl, 2 mM β-mercaptoethanol, 1 mM NaNO2, 7 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, and 7 μg/ml DNase I) on ice for a total of 3 min in 1 min bursts with 1 min pauses on ice between each burst. The resulting homogenate was centrifuged at 3000 × g for 10 min followed by a 30,000 × g centrifugation for 20 min. The supernatant (cytosol) was collected, and the centrifugation was repeated five times resulting in the suspension becoming translucent.

**RESULTS**

**Isolation and Characterization of the pstB2 Mutant—** Strains containing a psl1Δ::TRP1 allele must synthesize virtually all of their PtdEtn by transporting nascent PtdSer to the locus of the Psl2p in the Golgi/vacuole, when grown in the absence of Etn (see Fig. 1). Mutagenesis of these strains can yield new strains that are Etn auxotrophs with mutations in steps between PtdSer synthesis and decarboxylation that are likely to be defective in lipid transport. We mutagenized the strain RY952 (MATa lys2 trp1 ura3 his3 leu2 suc2 psl1Δ::TRP1) and screened 150,000 survivors for the presence of Etn auxotrophs. We obtained 32 new Etn auxotrophs, 9 of which were psl2 mutants, 2 of which were pss mutants, and 23 of which belonged to a new category. Outcrossing and complementation analysis revealed that three strains belonging to a single complementation group had a strong growth phenotype and some of the biochemical properties expected for putative pstB mutations. One of these strains was designated pstB2 and further characterized.

The growth properties of the pstB2 mutant were studied and the findings are shown in Fig. 2A. The parental strain and the pstB2 strain exhibit similar growth kinetics when maintained
on minimal medium containing Etn. In contrast, removal of Etn from the medium has little effect upon the parental strain but is lethal for the \( \text{pstB}^2 \) strain. The \( \text{pstB}^2 \) strain appears to divide twice before growth arrest. After prolonged incubation the cells lyse. These results demonstrate that the \( \text{pstB}^2 \) strain is an Etn auxotroph and that the growth phenotype observed in solid medium is also apparent in liquid medium.

The \( \text{pstB}^2 \) strain and its parental strain were examined for defects in lipid metabolism by following the incorporation of \(^{[3}H\)serine into aminoceroglycerophospholipids and the results are shown in Fig. 2B. In parental cells, \(^{[3}H\)serine is readily incorporated into PtdSer, PtdEtn, and PtdCho. In the \( \text{pstB}^2 \) strain, \(^{[3}H\)serine is also incorporated into all three lipids, but the pattern of labeling is markedly altered. Relative to the parental strain, the mutant shows a 50% increase in the labeling of the PtdSer pool and a 72% decrease in the labeling of the PtdEtn pool. This finding is consistent with a defect in the formation of PtdEtn from PtdSer in the mutant. There is also a modest change in the labeling of the PtdCho pool, but analysis of this lipid pool is not straightforward because \(^{[3}H\)serine can significantly label this lipid via the one carbon pathway (8, 12).

A trivial explanation of the reduced PtdEtn formation in the \( \text{pstB}^2 \) strain is that it is a consequence of defective Ptd2p. However, genetic experiments in which the \( \text{pstB}^2 \) strain is crossed with a \( \text{psd}^1\)::\( \text{TRP1} \) \( \text{psd}^2\)::\( \text{HIS3} \) double mutant reveal that the activity of Psd2p within the \( \text{pstB}^2 \) strain is sufficient to support normal growth in the absence of Etn. Direct biochemical measurement of Ptd2p catalytic function further demonstrates that the enzyme activity in the mutant is 75% of that found in the parental strain (Fig. 3). In addition, increased expression of Psd2p by transformation of \( \text{pstB}^2 \) mutant strains with a centromeric plasmid harboring the \( \text{PSD2} \) gene does not rescue the mutants. These findings make it unlikely that a mutation in the structural gene for PSD2 is responsible for the alteration in lipid metabolism. The biochemical phenotype of the \( \text{pstB}^2 \) mutant is also consistent with a defect in PtdSer synthase, as genetic crosses demonstrate that the \( \text{pstB}^2 \) and \( \text{pss} \) strains can complement each other. The PtdSer synthase activity of the \( \text{pstB}^2 \) mutant is also normal (120% relative to parental strain). We also used genetic manipulation of \( \text{PSD1} \) to investigate whether the defect in \( \text{pstB}^2 \) was directly linked to PtdSer metabolism. If the defect in the \( \text{pstB}^2 \) mutant is genuinely coupled to the cell's need for PtdEtn, then provision of this lipid by the action of Psl1p, instead of exogenous Etn, should bypass the mutation. Transformation of the \( \text{pstB}^2 \) strain with a plasmid harboring the \( \text{PSD1} \) gene effects complete bypass of the \( \text{pstB}^2 \) mutation. Collectively, the characteristics of the \( \text{pstB}^2 \) mutant described above are those expected for strains with defects in the transport of nascent PtdSer to the locus of Ptd2p.

**Cloning and Characterization of the \( \text{PSTB2} \) Gene**—The strong growth phenotype of the \( \text{pstB}^2 \) mutant in the context of a \( \text{psd}^1\)\Delta mutation enabled the cloning of a complementing gene. The \( \text{pstB}^2 \) strain was transformed to Ura\(^+\) prototrophy using a YCp50 vector harboring a yeast genomic library. ~1.3 \times 10^5 Ura\(^+\) transformants were further examined for Etn prototrophy by replica plating onto minimal medium lacking both Ura and Etn. We identified 63 strains that displayed the ability to grow in the absence of both Ura and Etn. From the pool of 63 transformants, we identified 53 that coordinately lost both Ura and Etn prototrophies after growth for 30 generations in nonselective medium. These plasmid loss experiments demonstrated genetic linkage between vector-encoded and genomic insert-encoded functions. Five yeast strains containing the plasmid that conferred Etn prototrophy were randomly selected, and the plasmid was recovered by shuffling into \( \text{E. coli} \) (22). Reinroduction of these plasmids into the \( \text{pstB}^2 \) mutant restored the growth to wild type levels in the absence of Etn. Restriction endonuclease analysis revealed that all five of the strains contained a plasmid with identical or partially overlapping genomic inserts.

The recovered plasmid was subjected to DNA sequence analysis that revealed the nucleotide sequence at both ends of the genomic insert. The genomic insert was identified as a 10-kb pair piece of DNA derived from chromosome XIV that included 7 complete and 2 partial ORFs. Each of the ORFs was subcloned and examined for complementation of the growth defect of the \( \text{pstB}^2 \) mutant. Only one of the ORFs, designated \( \text{PDR17} \) in the \( \text{S. cerevisiae} \) Genomic Data base, complemented the mutant strain.

The \( \text{PSTB2}/\text{PDR17} \) gene has recently been reported by van den Hazel \textit{et al.} (36) to be involved in hypersensitivity to multiple drugs when its closest homologue, \( \text{PDR16} \) gene, is absent. Although the function of \( \text{PSTB2}/\text{PDR17} \) in multiple drug resistance of yeast was not elucidated in that report, they demonstrated that a \( \text{pdr17::HIS3} \) containing a wild type copy of \( \text{PSD1} \) in its genome has a moderate accumulation of PtdSer and a decrement of PtdEtn levels. This is consistent with our result that \( \text{PSTB2}/\text{PDR17} \) plays a role in PtdSer metabolism.

The characteristics of the \( \text{pstB}^2 \) strain grown without Etn either in the presence or the absence of the plasmid harboring the \( \text{PSTB2}/\text{PDR17} \) gene are shown in Fig. 4A. The \( \text{PSTB2}/\text{PDR17} \) gene restores the growth of the mutant strain to levels that are equivalent to that of the parental strain. The ability of the genomic insert to correct the biochemical phenotype of the mutant is shown in Fig. 4B. Both the accumulation of PtdSer and the markedly reduced levels of PtdEtn found in the \( \text{pstB}^2 \) strain are rectified by the \( \text{PSTB2}/\text{PDR17} \) gene present on either low copy (YCP50) or high copy (YEp5352) plasmids. A convenient way to compare strains with defects in aminocerogleryosphospholipid metabolism is to express the activity as a ratio of product (PtdEtn) to precursor (PtdSer) as shown in Fig. 4C. In parental strains the PtdEtn/PtdSer ratio is 0.44, whereas in the mutant strain the value is 0.07. In contrast, mutant strains with the \( \text{PSTB2}/\text{PDR17} \) gene present on high copy or low copy plasmids...
incubated at 30 °C, and the presence of plasmids harboring wild type (D) circles was confirmed by Southern analysis. PSD2 activities were measured as described under “Experimental Procedures.” Data are expressed as the percentage of label in individual phospholipid over total radiolabel in each strain. PtdOH, phosphatidic acid. Other abbreviations are the same as Fig. 1. The results are the average of four independent experiments performed in duplicate. Values are mean ± S.E.

FIG. 2. The pstB2 strain is an Etn auxotroph with defective lipid metabolism. A, the Etn-glucose growth phenotype. Both the parental strain (psd1Δ, circles) and the pstB2 strain (psd1Δ/pstB2, triangles) were grown in SC medium plus Etn at 30 °C to mid-log phase. Cultures were then washed once and diluted to an A600 nm of 0.02 in SC medium either with (solid symbols) or without (open symbols) Etn. The diluted cultures were incubated at 30 °C, and the A600 nm of each culture was monitored at the indicated time points throughout the experiment. The results represent the average of three independent experiments performed in duplicate. B, aminoglycerophospholipid metabolism of the pstB2 strain. The aminophospholipid composition of the pstB2 strain was determined by following the incorporation of L-[1-14C]serine as described under “Experimental Procedures.” Data are expressed as the percentage of label in individual phospholipid over total radiolabel in each strain. PtdEtn, phosphatidyl ethanolamine; PtdSer, phosphatidyl serine; PtdCho, phosphatidyl choline; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid.

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FIG. 3. PSD2 activity of the pstB2 strain in the absence or presence of plasmids harboring wild type PSD2 or PSTB2 genes. All strains were harvested at mid-log phase in SC or Etn medium with or without uracil at 30 °C. Cell-free extracts were prepared, and PSD2 activities were measured as described under “Experimental Procedures.” PSD2 activities are presented as the percentages relative to the activity of parental control (100%). The results represent at least four independent experiments performed in duplicate. Values are mean ± S.E.

have PtdEtn/PtdSer ratios that are essentially the same as that of the parental strain. These findings establish that the PSTB2/PDR17 gene complements the biochemical as well as the growth defect of the pstB2 mutant.

The effects of PSTB2/PDR17 gene expression on PtdSer activity are shown in Fig. 3. Transformation of the pstB2 strain with the wild type gene has no significant effect upon PtdSer decarboxylase activity measured in cell extracts. These findings suggest that PstB2p does not directly modulate PtdSer decarboxylase activity. We have also constructed null alleles of the PSTB2/PDR17 gene using the single step disruption procedure of Rothstein (24). The construct used for the disruption is shown in Fig. 5A. Verification of the integration site of the construct was determined by PCR using primers that only recognize the disrupted gene present in the correct locus. The results shown in Fig. 5B demonstrate the presence of the null and wild type alleles in two different diploid strains. The diploid strains were induced to sporulate, and the resultant tetrads were dissected. Of 20 tetrads analyzed, all showed 2:2 segregation of His+ and His− phenotypes. All the His− strains from the wild type diploid (SEYd) are viable and have no apparent growth defect. This result agrees with the previous report that the PSTB2/PDR17 gene is not essential (36). However, all His− strains from diploid WWYd9 carrying double psd1Δ null alleles were Etn auxotrophs, demonstrating the synthetic lethality between pstB2::HIS3 and psd1Δ. The PtdEtn/PtdSer ratio of strains containing the pstB2::HIS3 allele is shown in Fig. 4B along with that of the parental and the pstB2 mutant strains. The pstB2::HIS3 allele reproduces the growth and biochemical phenotype of the pstB2 mutant derived by mutagenesis. The results strongly suggest that PstB2/Pdr17p function is biochemically linked to PtdSer-dependent metabolism of PtdSer.

Sequence analysis of the PSTB2/PDR17 gene establishes that it is related to the PtdIns/PtdCho transfer protein encoded by the SEC14 gene. The PSTB2/PDR17 gene has also been reported as a member (SFH4) of five yeast Sec14p homologues (SFHs) (37). The sequence alignment of the coding regions of the genes is shown in Fig. 6. The deduced protein sequences demonstrate a central region of homology between Sec14p and PSTB2/PDR17p comprising ~27 kDa. The sequence similarity in the central region is 58%. In contrast the amino- and carboxyl-terminal regions of the two proteins are markedly divergent with little similarity. The nonhomologous amino-terminal region of PstB2p is 11 kDa in size, and the carboxyl-terminal region is 3 kDa in size.

Properties of the PstB2/Pdr17p—The discovery that the PstB2/Pdr17p is related to the Sec14p raises the question of whether the protein functions as a lipid transfer protein, perhaps specific for PtdSer. To examine this question we expressed the PstB2p using a baculovirus vector and Sf9 cells. For these studies we employed both normal and carboxyl-V5His, epitope-tagged PstB2/Pdr17p. From genetic studies with constructs encoding the PstB2/Pdr17-V5Hisp, we know that this form of the protein restores the growth of pstB2 mutants (data not shown). Overexpression of PstB2/Pdr17-
V5His6p in yeast and Sf9 cells yields only weak phospholipid transfer activity detectable above the normal background activity of Sf9 cells. In contrast, expression a His6-PstB2/Pdr17p in *E. coli* provides a source of crude protein with significant transfer activity for PtdIns as shown in Fig. 7. Comparison of immunoblots that detect the epitope tag on PstB2p expressed in bacteria and Sf9 cells surprisingly demonstrates that the latter contain ~10 times the amount of the recombinant protein. We also compared the level of Sec14p expression to that of PstB2p in *E. coli*. The Sec14p was expressed at ~25 times the level of PstB2p. Thus the specific transfer activity of recombinant PstB2p appears to be nearly five times greater than that for Sec14p. We also investigated the activity of the *E. coli* derived protein with PtdSer as a substrate and were unable to detect any transfer of this lipid. Thus, the recombinant PstB2/Pdr17p clearly has *in vitro* activity related to the Sec14p.

We next examined the reciprocal complementation of *sec14*ts and *pstB2* strains by their wild type genes. Overexpression of the PSTB2/PDR17 gene can complement the sec14ts growth defect. In contrast overexpression of the SEC14 gene fails to rescue the pstB2 mutant.

Examination of the subcellular distribution of PstB2/Pdr17p reveals that it is both soluble and membrane bound as shown in Fig. 8A. Part of the membrane-bound population can be removed by 2 M KCl washing of the membranes (Fig. 8A). However, a significant portion of the membrane-bound population remains resistant to removal by high salt washing. Soluble preparations of the epitope-tagged PstB2/Pdr17p derived from recombinant baculovirus-infected Sf9 cells were used to examine the reversibility of the membrane association. The results shown in Fig. 8B demonstrate that the soluble form of PstB2/Pdr17p will readily associate with yeast microsomal membranes. In additional experiments (data not shown) we verified that the cytosolic form of the protein did not nonspecifically precipitate under the conditions used for membrane binding. The nature of the membrane association is currently not understood. However, liposome binding experiments using membranes composed of PtdCho alone or in binary or ternary mixtures with PtdSer, PtdIns, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate do not reveal any high affinity interactions (data not shown). The results suggest that the association of PstB2/Pdr17p with membranes may be mediated by resident proteins.

**Lipid Accumulation in pstB2 Mutants Occurs in the Golgi and a Light Membrane Fraction**—When examined at the whole cell level, the pstB2 mutant strain exhibits a relative accumulation of PtdSer and reduced formation of PtdEtn (Figs. 2B and 4B). We examined subcellular fractions derived from both mutant and parental strains to determine where in the cell the PtdSer accumulates. For these experiments we conducted labeling studies and performed subcellular fractionation on permeabilized cells. We have established that permeabilized cells faithfully recapitulate the lipid transport processes of wild type cells and the lipid accumulation defect of mutant cells. The detailed properties of the permeabilized cells will be described in another publication. We have found it necessary to use permeabilized yeast cells because we are unable to obtain good separation of organelles from intact cells when they are grown on minimal medium. In addition, our procedure (and most others) used spheroplasts as the starting point for isolation of organelles. The time required for spher-
plasting cells at the end of a labeling experiment adds a confounding and unwanted variable into the experiments that is eliminated by using permeabilized cells. The results in Fig. 9, A and B, show the distribution of PtdSer synthase, Kex2p protease, and vacuolar ATPase using our procedure. By these criteria the resolution of elements of the endoplasmic reticulum specific for PtdSer synthesis, the Golgi apparatus, and the vacuolar compartment is exceptional. The Ptd2p, which metabolizes PtdSer to PtdEtn, is found in both the Golgi and vacuolar compartments (Fig. 9C). The amount of Ptd2p found in the Golgi compartment from permeabilized cells is low but significant.

We examined the subcellular distribution of nascent PtdSer and PtdEtn by labeling permeabilized cells with [^3H]serine and subsequently using the gradient analysis procedures depicted in Fig. 10. The data presented in Fig. 10 reveal several important details about lipid translocation and metabolism in both wild type and mutant yeast strains. In wild type strains clear evidence is presented demonstrating nascent PtdSer and PtdEtn in the Golgi and light membrane fractions. The ratio of PtdEtn/PtdSer is 0.3 for the Golgi and 0.3 for the light membranes of wild type cells but less than 0.04 for the corresponding fractions from the mutant cells. The results are consistent with PstB2/Pdr17p playing an integral role in controlling the distribution of PtdSer and PtdEtn in the Golgi and light membrane fractions. The amount of PtdSer synthase to a specific subcompartment of the Golgi.

We next sought to determine the distribution of PstB2p in the subcellular fractions derived from the permeabilized cells. Constructs expressing PstB2p with a V5His<sub>6</sub> epitope tag were used for localization. Genetic experiments demonstrate that the epitope-tagged version of PstB2/Pdr17p complements the null allele of the PSTB2/PDR17 gene. Fig. 11A is an immunoblot analysis of equivalent quantities (adjusted for volume differences) of the major fractions derived from the permeabilized cells. The PstB2/Pdr17p found in the cell homogenate resides in the S30 and P30 fractions. Only minor amounts of PstB2/Pdr17p are recovered in the interface fraction (used for the isolation of Golgi and vacuole membranes) that is derived from the S30 fraction. More detailed analysis of the distribution of
PstB2/Pdr17p in the Golgi and vacuole fractions is shown in Fig. 11B. The membranes were recovered from these latter fractions by 10-fold dilution of the sorbitol and sedimentation at 280,000 × g. The PstB2/Pdr17p is found associated with light membranes, the Golgi, and vacuole fraction. A significant proportion of the PstB2/Pdr17p found in the denser vacuole fraction shows evidence of proteolytic degradation. These findings indicate that the subcellular distribution of PstB2/Pdr17p is broad and not restricted to either the cytosol or a specific organelle membrane.

DISCUSSION

The mechanisms by which phospholipids are transported from their sites of synthesis to the multiplicity of organelles found in eukaryotic cells are poorly understood. We have approached this problem by devising a genetic screen, outlined in Fig. 1, to identify new yeast strains defective in the transport-dependent metabolism of PtdSer. A new strain, \textit{pstB2}, was identified; its complementing gene, \textit{PSTB2/PDR17}, was cloned, partially sequenced, and mapped, and the corresponding null allele was created.

Both the original \textit{pstB2} mutant and strains engineered to contain \textit{pstB2/pdr17::HIS3} null alleles exhibit the same growth characteristics, consisting of conditional synthetic lethality with \textit{psd1} mutations and markedly reduced formation of PtdEtn from a PtdSer precursor. The defect in PtdEtn formation is related to the transport-dependent metabolism of PtdSer. In the \textit{psd1} mutant background, PtdSer must be transported to Golgi and vacuolar compartments for decarboxylation. Examination of aminoglycerophospholipid metabolism at the whole cell level clearly shows an accumulation of the precursor, PtdSer, and a significant decrement in the formation of PtdEtn. Genetic and enzymatic analysis eliminate specific lesions in Ptd2p function as a cause of the reduction in PtdEtn.
formation. In addition, we can not find any evidence for mislocalization of Psd2p (Fig. 9C). Consistent with the latter findings is the normal localization of Kex2p and the vacuolar H^+-ATPase (Fig. 9B), indicating that the pstB2 lesion does not cause general protein sorting defects.

The growth phenotype and the abnormal lipid metabolism of the pstB2 strains are complemented by a gene we name PSTB2. This gene has recently been reported as PDR17, which causes hypersensitivity of yeast to multiple drugs when deleted from the genome along with its closest homologue, PDR16 (36). However, deletion of the PSTB2/PDR17 gene alone did not increase drug sensitivity (36), and this strain showed perturbations in lipid metabolism much milder than the pstB2 mutant in a psd1D genetic background. Because the synthetic lethality between pstB2/pdr17 and psd1D suggests that PSTB2/PDR17 gene is involved in the Ptd2p-dependent PtdSer metabolism, it is likely that the wild type allele of PSD1 in the pstB2::HIS3/pdr17Δ cell provides adequate amounts of PtdEtn to maintain membrane permeability as well as viability.

The PSTB2/PDR17 gene is structurally related to the SEC14 gene encoding a PtdIns/PtdCho transfer protein. The deduced amino acid sequences for PstB2/Pdr17p and Sec14p consist of a central core region of 27 kDa and 58% homology.

**Fig. 8.** The PstB2/Pdr17p is amphitropic. A, expression and cellular distribution of PstB2/Pdr17p. V5His6 epitope-tagged PstB2/Pdr17p was expressed in a pstB2 strain grown in minimal medium containing galactose and raffinose as the carbon sources. Cell-free extract (CE), cytosol (Cyt), membranes before KCl wash (M), KCl washed membranes (M*), and KCl washing supernatant (W) were prepared as described under "Experimental Procedures." All fractions (2.3 µg of protein) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Epitope-tagged PstB2/Pdr17p was detected using mouse anti-V5 epitope monoclonal antibody and visualized by colorimetric reaction of horseradish peroxidase. MW, molecular mass standards. B, association of cytosolic PstB2/Pdr17p with yeast microsomes. SF9 cytosol (10 µg of protein) from cells expressing the epitope-tagged PstB2/Pdr17p was mixed with 22 µg of protein of KCl washed yeast microsomes from wild type PstB2 yeast as described under "Experimental Procedures." In lane a and b the 100,000 × g pellet was examined and in lane d the 100,000 × g supernatant was examined for the presence of the V5 antigen. SF9 cells expressing yeast Dpp1p were used as an SF9 infected control.

**Fig. 9.** Subcellular fractionation of permeabilized yeast. A, sedimentation of the endoplasmic reticulum by 30,000 × g centrifugation. The PSS activity was used as the endoplasmic reticulum marker. PSS activity is expressed as the percentage of the enzyme activity in the 30,000 × g supernatant (S30) or pellet (P30) over the combined activities from both fractions. The data are mean ± S.E. from three independent experiments. B, separation of the Golgi and the vacuole compartments. The Golgi and the vacuolar membranes resolved on sorbitol gradients were identified by following the Golgi marker, Kex2p peptidase activity, and vacuolar H^+-ATPase reactivity to the antibodies against either the 60- or the 100-kDa subunit of vacuolar H^+-ATPase. C, distribution of PSD2 activity on sorbitol gradients. The PSD2 activity of each fraction of the sorbitol gradient was expressed as the percentage of the total PSD2 activity applied on the gradient. The data shown in B and C are representative results from at least four separate gradients for each strain.
A New Gene Involved in Transport-dependent PtdSer Metabolism

Relative to Sec14p, PstB2/Pdr17p has a divergent amino-terminal region of 11 kDa and a divergent carboxyl-terminal region of 3 kDa. The PstB2/Pdr17p is amphitropic and is found in both cytosolic and total membrane fractions of yeast cells. Similar amphitropic properties have been described for Sec14p (38). There does not appear to be any specificity in the membrane association of PstB2/Pdr17p. Recombinant forms of the PstB2/Pdr17p produced in S9 cells also exhibit the amphitropic character, and the soluble form will readily bind to yeast microsomal membranes. The structural similarity between Sec14p and PstB2/Pdr17p raises the question of whether the latter has lipid transfer activity. In the original reports of lipid transfer activity in cytosol from sec14ts mutants, almost no activity in addition to that ascribable to Sec14p could be detected. We also find that lipid transfer activity attributable to PstB2/Pdr17p is minimal in yeast cells or S9 cells overexpressing the protein. At present, we do not know if our inability to measure phospholipid transfer effected by PstB2p derived from eukaryotes is because of the presence of inhibitory factors or post-translational modification. It is unlikely that the V5His6 epitope tag renders the protein inactive, because this structural variant effectively rescues strains with pstB2Δ alleles. However, overexpression of PstB2/Pdr17p in E. coli yields protein preparations in which PtdIns transfer activity is easily measured. Thus, it is clear that PstB2/Pdr17p has intrinsic lipid transfer activity in vitro. The transfer activity is presumably related to its function in vivo, but it remains unclear as to whether this is a true lipid transfer function for the purpose of membrane biogenesis via a soluble carrier within the cell. Direct tests of PstB2/Pdr17p as a cofactor or stimulator of Ptds2p demonstrate that it does not enhance the catalytic activity of the PtdSer decarboxylase in vitro. High level expression of the PSTB2/PDR17 gene can also function to suppress sec14 mutations. This surprising result implicates PstB2/Pdr17p in some aspects of Golgi function.

We have further established that the defect in the transport dependent metabolism of PtdSer observed at the whole cell level is also seen in permeabilized cells. The fidelity of the permeabilized cell system to the intact cell system, with respect to lipid transport has now provided the means to assess the location of PtdSer accumulation in mutant cells relative to their wild type counterparts. Previously, this has been a difficult problem to overcome insofar as the subcellular fractionation schemes typically used to isolate Golgi, vacuoles, and plasma membrane from cells grown on rich medium have not been successful with cells grown on synthetic medium. Using permeabilized yeast cells as the starting point for subcellular fractionation we demonstrate that we can resolve the Golgi, vacuoles, endoplasmic reticulum, and a novel light membrane fraction extremely well. Examination of the distribution of newly synthesized aminoglycerophospholipids among the subcellular fractions provides significant new information about the site of action of PstB2/Pdr17p and Ptds2p. The results presented in Fig. 10 demonstrate that significant amounts of the decarboxylated PtdSer reside within the Golgi apparatus and a light membrane fraction of permeabilized cells. Surprisingly, little detectable decarboxylation of PtdSer occurs in the vacuolar compartment, even though this is where the majority of the decarboxylation is located. The results suggest that most of the decarboxylation in the permeabilized cells occurs in the Golgi. However, we can not completely rule out that a rapidly transported pool of PtdSer is decarboxylated elsewhere.

The PstB2/Pdr17p appears to play a critical function in regulating the decarboxylation of PtdSer. Precisely how PstB2/ Pdr17p functions is not yet known. The data clearly indicate that the protein binds membranes in vitro and has an affinity for PtdIns as measured by the lipid transfer assay. However, based upon our liposome binding studies, the high affinity binding of the protein to membranes requires components other than phospholipids. The role of the membrane binding in PstB2p function is currently unknown. However, previous studies (9) have identified Stt4p as another protein involved in the PstB pathway. It is feasible that PstB2p could bind and modulate the activity of Stt4p.

Empirically our data support a role for PstB2/Pdr17p in regulating the access of PtdSer to the decarboxylase. It is noteworthy that the pstB2 mutation does not prevent the transport of PtdSer to the Golgi or the appearance of PtdSer in the light membrane fraction (see Fig. 10). The nature of the light membrane fraction is also not known. The light membranes are of very low density as they do not sediment in 40% sorbitol at 150,000 × g. Possible candidates for these membranes include retrograde vesicles, intra-Golgi vesicles, or an-

**FIG. 10.** The pstB2 strain accumulates PtdSer in the Golgi and a light membrane fraction. Permeabilized wild type (psdl/psl/PSTB2, A) and pstB2 (psdl/pstB2, B) cells were labeled with [3H]serine and then subjected to subcellular fractionation as described under “Experimental Procedures.” The gradient in A was loaded with interface membranes containing 2.3 × 10^5 cpm lipid. The gradient in B was loaded with interface membranes containing 1.5 × 10^5 cpm lipid. LM indicates the location of the light membrane fraction. The result is a representative one of three separate gradients for each strain. Open circle, PtdSer; solid circle, PtdEtn.
terograde vesicles exiting the trans Golgi. We currently have a bias that these are retrograde vesicles, because PtdEtn formed by the action of Psd2p must be transported to the endoplasmic reticulum to synthesize PtdCho.

In summary, this report provides evidence that PstB2/Pdr17p is involved in the Ptd2p-dependent PtdSer metabolism to PtdEtn. The similarity between PstB2/Pdr17p and Sec14p suggests that PstB2/Pdr17p may play a role in regulating PtdSer transport vesicles. The amphitropic nature of PstB2/Pdr17p may be part of the regulatory mechanisms to control its function.

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A New Gene Involved in the Transport-dependent Metabolism of Phosphatidylserine, *PSTB2/PDR17*, Shares Sequence Similarity with the Gene Encoding the Phosphatidylinositol/Phosphatidylcholine Transfer Protein, *SEC14*

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