Phosphatidylethanolamine Has an Essential Role in *Saccharomyces cerevisiae* That Is Independent of Its Ability to Form Hexagonal Phase Structures*

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Two yeast enzymes, Psd1p and Psd2p, catalyze the decarboxylation of phosphatidylserine to produce phosphatidylethanolamine (PtdEtn). Mitochondrial Psd1p provides ~90% of total cellular phosphatidyleserine decarboxylase activity. When the *PSD1* gene is deleted, the resultant strain (*psd1Δ*) grows normally at 30 °C in glucose and in the absence of exogenous choline or ethanolamine. However, at elevated temperature (37 °C) or on the nonfermentable carbon source lactate, the growth of *psd1Δ* strains is minimal without ethanolamine supplementation. The reduced growth and viability correlate with a PtdEtn content below 4% of total phospholipid. These results suggest that there is a critical level of PtdEtn required to support growth. This theory is supported by growth data revealing that a *psd1Δ* *psd2Δ* *dpl1Δ* strain can only grow in the presence of ethanolamine. In contrast, a *psd1Δ* *psd2Δ* strain, which makes low levels of PtdEtn from sphingolipid breakdown, can be rescued by ethanolamine, choline, or the ethanolamine analogue propanolamine. *psd1Δ* *psd2Δ* cells grown in 2 mM propanolamine accumulate a novel lipid, which was determined by mass spectrometry to be phosphatidylpropanolamine (PtdPrn). PtdPrn can comprise up to 40% of the total phospholipid content in supplemented cells at the expense of phosphatidylethanolamine and PtdEtn. The absolute level of PtdEtn required for growth when PtdPrn is present appears to be 1% of the total phospholipid content. The essential function of the PtdEtn in the presence of propanolamine does not appear to be the formation of hexagonal phase lipid, insofar as PtdPrn readily forms hexagonal phase structures detectable by ^{31}P NMR.

In *Saccharomyces cerevisiae* the aminoglycerophospholipids, phosphatidylethanolamine (PtdEtn)1 and phosphatidylcholine (PtdCho), are synthesized by two main pathways. When yeast are grown in the absence of exogenous ethanolamine or choline, the aminoglycerophospholipids are synthesized via the *de novo* pathway shown in Fig. 1. Phosphatidylserine (PtdSer) is first synthesized in the endoplasmic reticulum (ER) and its associated membranes by PtdSer synthase (Pss1p) (1) and is subsequently decarboxylated to form PtdEtn by PtdSer decarboxylase 1 (Psd1p) in the mitochondria (2) and by PtdSer decarboxylase 2 (Psd2p) in the Golgi/vacuole (3). The PtdEtn is exported from the sites of decarboxylation to the ER and subsequently methylated to form PtdCho (2, 4, 5). When ethanolamine and choline are present, PtdEtn and PtdCho are synthesized via the Kennedy pathway, which uses CDP-ethanolamine and CDP-choline intermediates (5). PtdEtn derived from the Kennedy pathway can also be methylated to form PtdCho (4, 5). Phosphoethanolamine that serves as an intermediate in the Kennedy pathway can also be synthesized as a consequence of sphingolipid degradation via dihydrophosphinosine-1-phosphate metabolism using Dpl1p (6, 7).

Early genetic examination of yeast aminoglycerophospholipid synthesis by Atkinson et al. (8) isolated mutants requiring ethanolamine or choline for growth. All of the mutants isolated belonged to a single complementation group designated *cho1* that had defects in Pss1p. The auxotrophic requirements of the *cho1* mutants were not only fulfilled by ethanolamine or choline, but also by monomethylethanolamine or dimethylethanolamine (8). Yeast strains with defects in both PtdSer decarboxylases (*psd1Δ* *psd2Δ*) are also auxotrophic for either ethanolamine or choline (3), but mutants having defects in both methylation enzymes (*mem1/chol mem2/api2*) are stringently auxotrophic for choline (5). These nutrient requirements suggested that PtdCho, rather than PtdSer or PtdEtn, was an essential lipid. All of the described mutants, however, contained a wild-type dihydrophosphinosine-1-phosphate lyase gene (*DPL1*) permitting low levels of PtdEtn synthesis without ethanolamine supplementation.

In designing an experimental approach to study lipid transport in yeast, the *psd1Δ* *psd2Δ* and double *psd1Δ* *psd2Δ* null alleles were constructed. Two assumptions were made based on experimental data: that the *psd1Δ* and *psd2Δ* single null strains grow without auxotrophic requirements (2, 3), and that ethanolamine...
Fig. 1. Schematic diagram of amionoglycerophospholipid biosynthesis in yeast. In the absence of exogenous ethanolamine and choline, amionoglycerophospholipids are synthesized via the de novo pathway. Serine is incorporated into PtdSer by PtdSer synthase (Pss1p) in the ER. The resultant PtdSer is then transported to the location of PtdSer decarboxylase 1 (Psd1p) at the inner mitochondrial membrane or PtdSer decarboxylase 2 (Psd2p) in the Golgi/vacuole, where it is converted to PtdEtn. PtdEtn produced by Psd1p or Psd2p is then transported from the mitochondria or Golgi/vacuole back to the ER. Within the ER, PtdEtn is methylated to PtdCho by the sequential action of PtdEtn methyltransferase 1 and 2 (Pemp1 and Pemp2). PtdEtn can also be synthesized from sphingolipid-derived phosphoethanolamine originating from the activity of dihydrophosphoglycerol-1-phosphate lyase (Dpl1p). Alternatively, when Etn and Cho are present, PtdEtn and PtdCho are synthesized via the Kennedy pathway (not shown in detail).

auxotrophs isolated in screens of mutagenized cells die because of the inability to produce enough PtdCho. However, our recent work with the temperature-sensitive mutants derived from the psd1Δ strain demonstrated that the psd1Δ parental strain is an ethanolamine auxotroph at 36°C (9). Also recently, Birner et al. (10) demonstrated that psd1Δ strains are ethanolamine auxotrophs when grown on the nonfermentable carbon source lactate. In this report, we examined the growth phenotype of a psd1Δ strain and show that it is a conditional ethanolamine auxotroph that does not die because of a lack of PtdCho, but rather because of a depletion of PtdEtn. We also provide evidence that PtdEtn, or one of its metabolites, is essential for growth of S. cerevisiae and this essential function is independent of the ability of PtdEtn to form hexagonal phase structures.

EXPERIMENTAL PROCEDURES

Materials—All simple salts, buffers, amino acids, nutritional supplements, and solvents were purchased from either Sigma or Fisher. Yeast media components (yeast extract, peptone, and nitrogen base without amino acids) were purchased from Difco or American Biorganics Inc. Preparalamin (Pn) and monomethylatedaminolamine (MME) were purchased from Aldrich.

Phospholipid standards for thin layer chromatography (TLC), dioleoyl-PtdCho, and dioleoyl-PtdEtn were obtained from Avanti Polar Lipids or Sigma. Thin layer Silica Gel H plates were purchased from Analtech Corp, and thin layer Silica Gel 60 plates were purchased from Merck. The radiochemical [3H]-serine was purchased from Amersham Biosciences, Inc.

Yeast Strains and Growth Methods—The strains utilized in these studies and their genotypes are shown in Table I. The growth media for yeast, YPD and minimal medium, contained 2% glucose (SC) or 2% lactate (SL) as indicated (11). Adenine (20 mg/liter), uracil (20 mg/liter), and ethanolamine (EtN, 2 mm) were routinely added to the YPD to give YPDAE. Etn, choline (Cho), MME, and Pn were prepared as 0.5 M stocks (pH 6-7) that were filter-sterilized. [3H]-Serine Labeling of Yeast Strains—All yeast strains were initially grown at 30°C in SC or SL containing 2 mm Etn, to mid-log phase. Subsequently, cells were diluted to an A600 nm of 0.3 in SC or SL without serine, containing 10 μCi [3H]-serine/ml. For specific incubation conditions, refer to table legends. After incubation, the cells were precipitated with trichloroacetic acid (10% final concentration) and combined with ~10 mg of carrier cells. Pellets were washed twice with cold water, and the lipids were extracted and analyzed by TLC as previously described (2, 3). The TLC plates were sprayed with 0.2% (w/v) 8-anilino-1-naphthalenesulfonic acid, and the lipids were visualized under UV light. Lipids were scraped from the TLC plates, and radioactivity was quantified by liquid scintillation counting.

Lipid Phosphorus Measurement—Strains were grown under the conditions described in the table legends. The cells were harvested, and the lipids were extracted as described above except carrier cells and trichloroacetic acid were not added. For quantification of mitochondrial lipids, crude and purified mitochondria fractions were isolated using the method of Glick and Pon (12). PtdCho, PtdIns, PtdSer, PtdEtn, cardiolipin, and other lipids were separated by two-dimensional TLC on Silica 60 plates in chloroform/methanol/acidic acid (13/5/2 v/v/v), followed by chloroform/methanol/formic acid (13/2 v/v/v). For analysis of the psd1Δ psd2Δ lipid content, the two-dimensional TLC was performed using chloroform/methanol/ammonium hydroxide (65/35/5 v/v/v) followed by chloroform/acidic acid/methanol/water (75/25/5/2.2 v/v/v). Lipids were visualized by iodine vapor and scraped into glass tubes. Phosphorus was quantified by the Rouser method (13).

Liquid Chromatography/Mass Spectrometry (LC/MS)—LC/MS and tandem mass spectrometry (LC/MS/MS) was carried out in a Sciex API-III+ triple quadruple mass spectrometer (PE-Sciex, Thornhill, Toronto, Canada). This machine was equipped with an electrospray ionization source interfaced to a gradient HPLC system. The electrospray ionization spray voltage was 4500 V, the orifice potential maintained at 75 V, and the collisional offset potential was 20 eV for tandem experiments. For collisional induced decomposition experiments, the addition of 0.5 m EITPA, and the ether was evaporated under a stream of N2 gas. The lipid was extracted by a Bligh and Dyer extraction (16). The dioleoyl-PtdPrn was synthesized by a transphosphatidylidylation reaction using dioleoyl-PtdCho as a substrate (14). 100 mg of dioleoyl-PtdCho was incubated in a two-phase diethyl ether, 165 mM sodium acetate, acidic pH (pH 4.5), with 100 mM calcium chloride solution with 25% propanolamine in the presence of phospholipase D, isolated from Savoy cabbage (15), for 2 h at room temperature. The reaction was stopped by the addition of 0.5 m EITPA, and the ether was evaporated under a stream of N2 gas. The lipid was extracted by a Bligh and Dyer extraction (16). The dioleoyl-PtdPrn was isolated by preparative TLC on Silica 60 plates developed in chloroform/acidic acid/methanol/water (75/25/5/2.2 v/v/v/v). The lipid was eluted from the silica gel with a Bligh and Dyer solvent and then extracted by phase separation. The chloroform phase was recovered and the solvent evaporated under a stream of N2 gas. Residual acetic acid was removed from the isolated lipid by resuspension and evaporation of chloroform. The concentration of dioleoyl-PtdPrn was determined by lipid phosphorus measurement, and the purity was assessed by TLC and mass spectrometry. 31P NMR Spectroscopy—Dioleoyl-PtdEtn, dioleoyl-PtdCho and dioleoyl-PtdPrn (40~50 mg) were hydrated in 500 μl of 20 mM HEPES (pH 7) containing 100 mM sodium chloride and 10% deuterium oxide and vortexed at room temperature. The pH of the lipid mixture was checked.
and adjusted as necessary. The $^{31}$P NMR spectra were recorded on a Varian Inova 600-MHz spectrometer operating at 243 MHz for $^{31}$P. The free induction decays were accumulated by employing 21-$\mu$s 90° pulse, and 320–1600 scans with a 30-kHz sweep width and a 1.0-s interpulse delay. The spectra were collected in the presence of proton decoupling.

RESULTS

Growth Characteristics of the psd1Δ Strain—Yeast express two PtdSer decarboxylase enzymes: Psd1p, which resides in the inner mitochondrial membrane (2); and Psd2p, which co-localizes to the vacuole and Golgi compartments (3). Strains containing a psdΔ mutation are dependent upon Psd2p and Dpl1p activity for growth in the absence of exogenous ethanolamine or choline (3). When the psd1Δ strain was originally characterized, it displayed wild-type growth in the absence of ethanolamine (2). This characterization, however, was only done in liquid glucose medium at 30 °C or on solid medium at 30 °C with glucose. When we were analyzing temperature-sensitive mutants in this genetic background, we discovered that the psd1Δ parental strain is an ethanolamine auxotroph at elevated temperature in liquid medium (9). To further characterize the growth of the psd1Δ strain, we analyzed the auxotrophic requirements in various carbon sources at 30 °C and 37 °C in both liquid and solid media. The growth characteristics of the psd1Δ strain in liquid glucose medium are shown in Fig. 2. At 30 °C (Fig. 2A), as described previously (2), the psd1Δ strain displays wild-type growth regardless of ethanolamine or choline supplementation. However, at 37 °C (Fig. 2B), the psd1Δ strain shows minimal growth without ethanolamine or choline supplementation, but either ethanolamine or choline supplementation restores wild-type growth.

The addition of the dpl1Δ allele to the psd1Δ strain makes the strain strictly dependent upon Psd2p for PtdEtn synthesis in the absence of ethanolamine. The psd1Δ dpl1Δ strain, when pregrown in the presence of ethanolamine and then shifted to ethanolamine/choline-free medium, displays almost wild-type growth at 30 °C (Fig. 3A). However, if the psd1Δ dpl1Δ strain is grown in ethanolamine/choline-free medium for five to seven generations and then diluted to a lower absorbance, it can no longer grow in the same medium (Fig. 3B). These observations indicate that cells accumulate sufficient intracellular ethanolamine pools to support growth for several generations, but the depletion of these pools leads to arrest of growth. At 37 °C the growth characteristics of the psd1Δ dpl1Δ strain are identical to the psd1Δ strain (data not shown).

The observed ethanolamine auxotrophy of the psd1Δ and psd1Δ dpl1Δ is dependent upon how the growth is assessed. psd1Δ and psd1Δ dpl1Δ cells grow on solid synthetic glucose medium in the absence of ethanolamine at both 30 °C and 37 °C (data not shown). This apparent lack of ethanolamine auxotrophy at 37 °C for psd1Δ cells, and at 30 °C and 37 °C for psd1Δ dpl1Δ cells on solid media may be caused by recycling of excreted choline or ethanolamine. Recent evidence (17) demonstrates that choline recycling following PtdCho formation from the de novo pathway can be highly efficient.

Previous work from our laboratory showed that the psd1Δ strain exhibits a greater tendency to produce petite, rho− cells, compared with wild-type cells (2). The production of petite cells is normally the result of loss of mitochondrial function. Because Psd1p is a mitochondrial enzyme and PtdEtn is enriched in this organelle, we examined the growth of the psd1Δ strain on the nonfermentable carbon source lactate, to determine whether a requirement for functional mitochondria alters the nutrient requirements of this strain. We observed almost identical growth characteristics as described by Birner et al. (10) for both the psd1Δ and psd1Δ dpl1Δ strains (data not shown). The psd1Δ strain at both 25 °C and 30 °C does not grow in the absence of ethanolamine or choline, but supplementation with either nutrient restores wild-type growth. At 37 °C the psd1Δ strain does not grow regardless of nutrient supplementation. This ethanolamine auxotrophy was also observed for the psd1Δ strain on solid lactate medium and in liquid glycerol medium at 30 °C. Thus, the psd1Δ strain is an ethanolamine auxotroph under all conditions that require functional mitochondria.

Analysis of the de Novo Aminoglycerophospholipid Synthesis in the psd1Δ Strain—The effects of the psd1Δ mutation upon
aminoglycerophospholipid metabolism were examined by measuring the incorporation of $[^3H]$serine precursor into phospholipids, to determine whether alterations in Psd2p activity could explain the conditional ethanolamine auxotrophy. The data in Table II show the radiolabel accumulation into PtdSer, PtdEtn, and PtdCho by wild-type and psd1Δ cells grown at 30 °C and 37 °C in glucose medium after 4 h of incubation, and in lactate medium after 12 h of incubation. The data are expressed as the percentage of total radiolabel observed in PtdSer, PtdEtn, and PtdCho. The amount of $[^3H]$serine incorporated into PtdEtn in the psd1Δ strain was decreased by 60–70% at 30 °C and 70–80% at 37 °C compared with the wild-type strain in both carbon sources. This decrease was compensated by a 1.5–3-fold increase in $[^3H]$serine incorporation into PtdSer. The changes in PtdCho labeling are difficult to interpret because significant radiolabeling can occur via the one-carbon pathway using preexisting pools of PtdEtn (4).

Analysis of Aminoglycerophospholipid Composition of the psd1Δ Strain—Because the alterations in aminoglycerophospholipid synthesis displayed by the psd1Δ strain were similar in both permissive and nonpermissive conditions, we examined the steady-state levels of the aminoglycerophospholipids to determine the cause of the ethanolamine auxotrophy. The size of the aminoglycerophospholipid pools was determined by phosphorus analysis. Results shown in Table III are the percentage of total lipid phosphorus observed in PtdSer, PtdCho, PtdIns, and PtdEtn from cells grown in glucose medium in the absence or presence of ethanolamine at 30 °C and 37 °C. Under all conditions analyzed, the content of PtdCho in the psd1Δ and psd1Δ dpl1Δ strains were similar or elevated compared with the wild-type strain. The PtdEtn content, however, was decreased by 50–70% in both psd1Δ and psd1Δ dpl1Δ cells compared with wild-type cells at 30 °C, in the absence or presence of ethanolamine. At 37 °C the PtdEtn content was decreased in both psd1Δ and psd1Δ dpl1Δ strains by 80% without nutrient addition, and by 4–11% in the presence of ethanolamine compared with wild-type cells. The decreased PtdEtn content in both strains was compensated for by an increase in PtdIns.

Under these experimental conditions, the lipid composition of the psd1Δ dpl1Δ strain did not vary significantly from that of the psd1Δ strain.

The data in Table IV show the sizes of the aminoglycerophospholipid pools from whole cell homogenates and the crude mitochondria fraction from cells grown in lactate medium at 25 °C. The results we observed displayed similar trends to that described by Birner et al. (10), but the exact percentages of each lipid pool were different. This difference is most likely caused by variation of growth conditions and experimental procedures. We grew cells in minimal lactate medium with controlled amounts of ethanolamine, whereas Birner et al. (10) used YPL medium. YP medium contains enough choline and ethanolamine to rescue the ethanolamine auxotrophy of the psd1Δ strain (data not shown). The phospholipid composition of the crude mitochondrial fraction (Table IV) for both the psd1Δ and wild-type strains is similar to that observed for the whole cell homogenate. The PtdCho pool in psd1Δ cells grown in lactate medium was elevated compared with wild-type cells in the absence or presence of ethanolamine, and the PtdEtn content was decreased by 70–85%. The decrease in PtdEtn levels in psd1Δ cells is compensated for by increases in PtdIns and PtdSer content. The addition of ethanolamine to the growth media does not dramatically alter the aminoglycerophospholipid composition of either strain.

Because alterations in the aminoglycerophospholipid composition of the psd1Δ cells do not explain their inviability at elevated temperature or in lactate medium, we compared the absolute PtdEtn pool sizes from all conditions (Tables III and IV). Under conditions where the psd1Δ strain is an ethanolamine auxotroph (glucose at 37 °C, and lactate at 25 °C), the PtdEtn content of this strain is under 4%; however, under permissive conditions, psd1Δ cells have a PtdEtn content between 8 and 30%. Thus, growth of the psd1Δ strain appears to correlate with a PtdEtn content greater than 4%, when PtdEtn is required for PtdCho synthesis. Only in glucose medium at 37 °C does the presence of ethanolamine restore wild-type PtdEtn content in the psd1Δ strain.

Characterization of the Growth Phenotype of psd1Δ psd2Δ and psd1Δ psd2Δ dpl1Δ Strains—Because the psd1Δ strain appears to have limiting PtdEtn when grown at elevated temperature in glucose medium or in nonfermentable carbon sources, we tested the hypothesis that yeast require a critical level of PtdEtn for growth. Fig. 4 shows the growth characteristics in glucose medium at 30 °C of a psd1Δ psd2Δ strain that makes low levels of PtdEtn from sphingolipid breakdown in the absence of ethanolamine using Dpl1p, and a psd1Δ psd2Δ dpl1Δ strain that cannot make PtdEtn in the absence of ethanolamine. If PtdEtn is essential for growth, then the psd1Δ psd2Δ dpl1Δ strain should not grow in the presence of choline. The psd1Δ psd2Δ strain is an ethanolamine auxotroph (Fig. 4A), but can grow in the presence of either ethanolamine or choline. The psd1Δ psd2Δ dpl1Δ strain also cannot grow in the absence of ethanolamine and, unlike the psd1Δ psd2Δ strains, cannot grow in the presence of choline. The requirement for ethanolamine in psd1Δ psd2Δ dpl1Δ strain cannot be compensated for by either ethanolamine analogue MME or Prn (Fig.
Wild-type (SEY6210) and psd1Δ (RYY52) cells were grown to mid-log phase at 30 °C in SC or SL medium plus ethanolamine (2 mM). Cells were harvested by centrifugation, washed twice with SC, and resuspended at an absorbance of 0.05 to 0.2 in SC or SC plus 2 mM

| Strain | Total cpm | Distribution of radiolabel |
|--------|-----------|-----------------------------|
|        |           | PtdSer | PtdEtn | PtdCho |
| Glucose 30 °C |           |        |        |        |
| Wild-type | 11.1 ± 3.0 | 17.0 ± 2.6 | 48.7 ± 2.0 | 33.0 ± 2.4 |
| psd1Δ | 5.9 ± 1.4 | 25.2 ± 3.7 | 21.1 ± 1.7** | 52.2 ± 4.5* |
| Glucose 37 °C |           |        |        |        |
| Wild-type | 6.9 ± 1.6 | 21.8 ± 3.0 | 24.0 ± 1.5** | 52.2 ± 2.5** |
| psd1Δ | 3.4 ± 1.0 | 32.9 ± 3.0* | 7.1 ± 0.8*** | 57.1 ± 3.3** |
| Lactate 30 °C |           |        |        |        |
| Wild-type | 25.4 ± 11.8 | 6.9 ± 1.5 | 42.4 ± 3.3 | 49.0 ± 4.1 |
| psd1Δ | 6.0 ± 1.1 | 20.1 ± 3.0*** | 11.9 ± 1.4** | 66.3 ± 3.9* |
| Lactate 37 °C |           |        |        |        |
| Wild-type | 13.1 ± 2.7 | 9.9 ± 0.4 | 30.6 ± 3.8 | 58.7 ± 4.5 |
| psd1Δ | 2.7 ± 0.2 | 22.8 ± 1.9** | 7.0 ± 1.4** | 67.5 ± 2.8* |

Wild-type (SEY6210), psd1Δ (RYY52), and psd1Δ dpl1Δ (JCY272) cells were grown to mid-log phase at 30 °C in SC medium plus ethanolamine (2 mM). Cells were harvested by centrifugation, washed twice with SC, and resuspended at an absorbance of 0.05 to 0.2 in SC or SC plus 2 mM ethanolamine and incubated at 30 °C or 37 °C for 8 h. Cells were harvested and lipids quantified. Results are the percentage of total lipid phosphorus in each phospholipid pool. Data are expressed as the mean ± S.E. from three to seven experiments. Significance was determined using a two-tailed t-test compared with wild-type under similar conditions (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**Table II**

**Phosphatidylethanolamine Requirement in Yeast**

**Table III**

**Analysis of lipid content from cell homogenates and crude mitochondria purified from cells grown in lactate at 25 °C**

Wild-type (SEY6210) and psd1Δ (RYY52) cells were grown to mid-log phase at 30 °C in SL medium plus ethanolamine (2 mM). Cells were harvested by centrifugation, washed twice with SL and resuspended at an absorbance of 0.05 to 0.2 in SL or SL plus 2 mM ethanolamine and incubated at 30 °C for 24 h. Cells were harvested, mitochondria were purified, and lipids were quantified. Results are the percentage of total lipid phosphorus in each phospholipid pool. Data are expressed as the mean ± S.E. from three to five experiments. Significance was determined using a two-tailed t-test compared with wild-type under similar conditions (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**Table IV**

**Whole cell homogenate**

| Strain | PtdSer | PtdEtn | PtdCho | PtdIns | Cardiolipin | Other |
|--------|--------|--------|--------|--------|-------------|-------|
| Wild-type | 6.6 ± 0.4 | 22.0 ± 0.5 | 39.0 ± 1.3 | 21.3 ± 0.5 | 6.8 ± 1.0 | 5.3 ± 0.9 |
| Wild-type + Etn | 6.4 ± 0.5 | 31.9 ± 1.2 | 28.0 ± 1.0 | 19.8 ± 0.7 | 8.6 ± 1.6 | 5.3 ± 0.5 |
| psd1Δ | 9.2 ± 0.4*** | 3.6 ± 0.4*** | 48.0 ± 1.9*** | 28.6 ± 0.5*** | 5.2 ± 1.1 | 5.4 ± 0.6 |
| psd1Δ + Etn | 10.0 ± 0.5*** | 9.8 ± 0.2*** | 45.1 ± 0.9*** | 24.8 ± 0.5*** | 6.5 ± 0.5 | 3.8 ± 1.0 |

**Mitochondria**

| Strain | PtdSer | PtdEtn | PtdCho | PtdIns | Cardiolipin | Other |
|--------|--------|--------|--------|--------|-------------|-------|
| Wild-type | 4.0 ± 0.3 | 36.8 ± 0.1 | 36.9 ± 0.2 | 18.8 ± 1.4 | 10.7 ± 0.6 | 2.8 ± 0.2 |
| Wild-type + Etn | 4.0 ± 0.3 | 34.0 ± 1.3 | 29.1 ± 1.8 | 17.8 ± 1.3 | 9.8 ± 0.6 | 5.3 ± 0.9 |
| psd1Δ | 8.4 ± 0.3** | 3.5 ± 0.7** | 48.0 ± 1.9 | 27.7 ± 0.7* | 8.1 ± 0.1 | 4.3 ± 0.6 |
| psd1Δ + Etn | 9.4 ± 1.2* | 9.2 ± 1.1*** | 45.4 ± 1.8** | 22.1 ± 1.2* | 8.2 ± 0.5 | 5.7 ± 0.9 |
concentration dependence in the absence (Fig. 5A) or presence
of either compound. It was surprising that propanolamine
could not rescue the growth of cells grown in propanolamine
with 2 mM propanolamine supplementation, the ethano-
amine requirement can be reduced to 50% of the total lipid
 phosphor. When grown in propanolamine, PtdPnr makes up
40% of the total lipid, with PtdCho reduced to 9.5% and PtdEtn
to 1%. A similar lipid composition was observed in psd1Δ psd2Δ
strain. These data also demonstrate that, under the appropriate
conditions, steady-state PtdEtn levels as low as 1% of total lipid
can support log phase growth.

To confirm the identity of the PtdPnr, we purified the lipid by
two-dimensional TLC and further analyzed it using electro-
spray ionization mass spectrometry. The major positive ion in
the purified lipid sample was [M+H]+ 702, which is consistent with
a phosphopropanolamine moiety, based on a loss of 141 mass
units observed for PtdEtn (18). These data did not exclude the
possibility that the sample was phosphatidylmonomethylethano-
amine (PtdEtn(Me)) because it would be expected to give
identical data. To prove that the lipid synthesized by the psd1Δ

**Fig. 4.** The psd1Δ psd2Δ dpl1Δ strain is an ethanolamine aux-

**Fig. 5.** Propanolamine markedly reduces the ethanolamine
requirement of psd1Δ psd2Δ dpl1Δ cells. Log phase psd1Δ psd2Δ
psd1Δ cells grown in propanolamine or ethanolamine
was quantified by phosphorus analysis (Fig. 6B). For the cells
grown in ethanolamine, PtdEtn comprises 14%, PtdCho comprises
38%, and PtdEtn(Me)₂ comprises 4% of the total lipid
phosphorus. When grown in propanolamine, PtdPnr makes up
40% of the total lipid, with PtdCho reduced to 9.5% and PtdEtn
to 1%. A similar lipid composition was observed in psd1Δ psd2Δ
dpl1Δ cells grown in the presence of 2 mM propanolamine and
50 μM ethanolamine (data not shown). These data show that
the psd1Δ psd2Δ and psd1Δ psd2Δ dpl1Δ strains readily incor-
porate propanolamine into PtdPnr, and suggest that this lipid
may replace some but not all of the cellular requirements for
PtdEtn.-Dpl1Δ
psd1Δ psd2Δ strain in the presence of propanolamine was indeed
PtdPrn, normal phase LC/MS was performed with the isolated
yeast lipid and a PtdEtn(Me) standard. There was a 7-min
difference between the elution time of the PtdPrn compared
with the PtdEtn(Me) standard (data not shown), demonstrat-
ing the yeast lipid is not PtdEtn(Me).

We also analyzed the purified PtdCho spot from the propanol-
amine-treated sample by MS/MS to determine whether it con-
tained any methylated-PtdPrn species. The data indicated that
greater than 90% of the lipid in the PtdCho spot was PtdCho,
and it contained only traces of trimethyl-PtdPrn (data not
shown). These results indicate that the PtdEtn methyltrans-
ferases (Pem1p and Pem2p) can use some PtdPrn as a sub-
strate, but PtdEtn is used more efficiently.

Analysis of the Physical Properties of PtdPrn—Under physi-
ological conditions unsaturated PtdCho, PtdSer, and PtdIns all
form bilayers when hydrated, but only PtdEtn forms a hexag-
onal phase in vitro (19–24). These hexagonal phase structures
have been proposed to be important for membrane fluidity and
membrane fusion (25–27). Thus, one apparent requirement of
cells for PtdEtn may be a consequence of the role of this lipid in
forming hexagonal phases. We next sought to examine whether
PtdPrn could also form hexagonal phases and replace PtdEtn
in this regard. To determine the physical structure PtdPrn
prefers, we performed 31P NMR analysis on synthetic dioleoyl-
PtdPrn, using the method of Cullis et al. (19). The dioleoyl-
PtdPrn was hydrated at neutral pH, and its 31P NMR spectra
were compared with that of dioleoyl-PtdCho and dioleoyl-
PtdEtn (Fig. 8). As described previously (19, 28), dioleoyl-
PtdEtn undergoes a phase shift from the bilayer conformation
to the hexagonal phase with increasing temperature. The mid-
point for the phase shift is near 5°C. We also found that
PtdPrn readily forms hexagonal phase structures and does so
at a lower temperature than PtdEtn. We were not able to define
conditions in which the analog formed only the bilayer phase
because the NMR probe temperature cannot go below 15 °C.
The dioleoyl-PtdCho gave the expected spectra at 10 °C that
indicates the presence of the lamellar-bilayer phase. From
these data we conclude that PtdPrn forms hexagonal phase
structures similar to those of PtdEtn. Thus, the inability of the
analog to replace PtdEtn in the psd1Δ psd2Δ dpl1Δ strains is

![FIG. 6. psd1Δ psd2Δ cells grown in the presence of propanolamine accumulate PtdPrn at the expense of PtdCho and PtdEtn. Log phase psd1Δ psd2Δ cells grown at 30 °C in SC plus 2 mM Etn (open bars) or SC plus 2 mM Prn (light gray bars). Cells were harvested by centrifugation, and lipids were extracted. A, lipids were separated by two-dimensional TLC and visualized by iodine staining. B, the isolated lipids were quantified by a phosphorus assay and the results are expressed as the percentage of total lipid phosphorus. Data are expressed as mean ± S.E. of three experiments. Significance was determined using a two-tailed t test compared with cells grown with Etn (*, p < 0.05; **, p < 0.01; ***, p < 0.001).](image)

![FIG. 7. MS/MS analysis demonstrates that psd1Δ psd2Δ cells grown in the presence of propanolamine synthesize PtdPrn. Log phase psd1Δ psd2Δ cells grown at 30 °C in SC plus 2 mM propanolamine were harvested by centrifugation. Lipids were extracted and separated by two-dimensional TLC. Purified PtdPrn was extracted from the silica gel and used for mass spectrometry analysis. The major ion in the purified PtdPrn sample (m/z 702) was analyzed by MS/MS.](image)
likely caused by a function of PtdEtn that is independent of hexagonal phase formation or by a lower propensity to form bilayer phases. The data also demonstrate that a hexagonal phase lipid, PtdPrn, can comprise 40% of the total membrane phospholipids, and cells still display wild-type growth.

DISCUSSION

We have previously constructed strains containing a null allele for either or both the PSD1 and PSD2 genes (2, 3, 29). The original characterization of these strains showed that either the psd1Δ or the psd2Δ strain remained prototrophic for ethanolamine and retained some PtdSer decarboxylase activity, whereas the double mutant was an ethanolamine auxotroph with no measurable PtdSer decarboxylase activity. The psd1Δ psd2Δ strain, like pss1Δ cho1Δ mutants, could be rescued with either ethanolamine or choline (3, 8). Because either nutrient could rescue cells that lacked the de novo aminoglycerophospholipid synthetic pathway, it was concluded that most probably PtdCho and not PtdSer or PtdEtn was essential for the phospholipid synthetic pathway, it was concluded that most

We measured the size of the phospholipid pools to determine whether alterations in lipid composition explain the conditional ethanolamine auxotrophy. As observed with the [3H]serine labeling, under all conditions examined the lipid profile of the psd1Δ strain was altered compared with the wild-type strain. The content of the PtdEtn pools of the psd1Δ and psd1Δ dpl1Δ strains were decreased compared with the parental strain by 70–80% when measured in the absence of ethanolamine and by 10–70% in the presence of ethanolamine. It appears that the Kennedy pathway-derived PtdEtn cannot fully compensate for the lack of PtdSer activity in these cells. Surprisingly, in the psd1Δ and psd1Δ dpl1Δ strains, the size of the PtdCho pools were equal to or larger than wild-type cells in all cases. This indicates that a lack of PtdCho is not the cause of the ethanolamine auxotrophy.

The overall lipid profiles of the psd1Δ strain grown under the various conditions do not provide a clear explanation for the inviability of cells either at 37 °C, or in lactate, when grown in the absence of ethanolamine. However, an examination of the absolute levels of PtdEtn shows that under permissive conditions the PtdEtn content of the psd1Δ strain is at least 8% of the total phospholipid, but under the nonpermissive conditions the PtdEtn content was around 4% of the total phospholipid. This indicates there may be an essential requirement for PtdEtn in yeast, and that in the psd1Δ strain the Psd2p and Dpl1p enzymes cannot supply enough PtdEtn to fulfill both PtdCho synthesis and this essential requirement when cultured at 37 °C, or when mitochondrial function is required.

Comparison of the growth characteristics and lipid compositions of the psd1Δ and psd1Δ dpl1Δ strains indicates that Dpl1p does not dramatically alter the steady-state PtdEtn levels in the cell. However, the presence of Dpl1p does provide enough additional PtdEtn to sustain long term growth of cells at 30 °C under ethanolamine-free conditions.

To test the hypothesis that PtdEtn is an essential lipid, we analyzed the growth phenotype of the psd1Δ psd2Δ dpl1Δ strain. This strain cannot make PtdEtn via the de novo pathway (3, 29) or from sphingolipid degradation (6, 7) and can only synthesize PtdEtn when supplemented with ethanolamine. The psd1Δ psd2Δ dpl1Δ strain is an ethanolamine auxotroph that cannot be rescued by choline. This clearly demonstrates that either PtdEtn or one of its metabolites is essential. The psd1Δ psd2ΔΔ strain, which can make PtdEtn from sphingolipid degradation via Dpl1p in the absence of ethanolamine, can grow in the presence of ethanolamine, choline, or the ethanolamine analogues propanolamine or monomethylethanolamine. psd1Δ psd2ΔΔ cells grown in the presence of propanolamine accumulate PtdPrn at the expense of PtdCho and PtdEtn. In 2 mM propanolamine, the PtdEtn levels within the cell drop to 1% of the total phospholipid pool, and this may be the minimum level of this lipid necessary for growth. PtdPrn must have the ability to compensate for the low levels of PtdEtn and PtdCho, but there is at least one function for PtdEtn or one of its metabolites that requires the exact structure of the ethanolamine head group.

The various metabolic roles for PtdEtn in the cell are shown in Fig. 9. In addition to serving as a precursor for PtdCho, PtdEtn is involved in the glycosylphosphatidylinositol (GPI) modification of proteins. The GPI anchor contains three phosphoethanolamine moieties that are thought to be transferred from PtdEtn (30–32) by the action of Mcd4p, Gpi13p, and Gpi7p (33–35). The GPI anchor is linked to proteins through the amine group of one of the phosphoethanolamine moieties, and a second phosphoethanolamine group appears to be essen-
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Fig. 9. The pathways for PtdEtn synthesis and metabolism. PtdEtn is synthesized by the de novo pathway from PtdSer using Ptd1p or Ptd2p, from sphingolipids using Ptd1p and by the Kennedy pathway from ethanolamine. PtdEtn is used within the cell to synthesize PtdCho (via Pem1p and Pem2p), glycosylphosphatidylinositol anchors (GPI, via Gpi7p, Mcd4p, and Gpi13p) and for the direct modification of proteins (Appg8p by Appg7p and Appg3p).

Ethanolamine

PtdEtn

PtdSer

Phosphoethanolamine

Glycosylphosphatidylinositol anchors

Sphingolipids

Dpl1p

Appg7p

McD4p

Gpi13p

Pem1p

Pem2p

Appg3p

Ketl1p

Direct modification of protein (Appg8p)

Metabolism

Synthesis

strains from 0.5 mM to 50 μM. These data provide compelling evidence that PtdEtn plays an essential function in cells that is independent of the physical properties of this lipid.

REFERENCES

1. Gaigg, B., Simbeni, R., Hrusnak, C., Paltaluf, F., and Daum, G. (1995) Biochim. Biophys. Acta 1234, 214–220
2. Trotter, P. J., Pedretti, J., and Voelker, D. R. (1999) J. Biol. Chem. 265, 21416–21424
3. Trotter, P. J., Pedretti, J., Yates, R., and Voelker, D. R. (1995) J. Biol. Chem. 270, 6071–6080
4. Atkinson, K., Fogel, S., and Henry, S. A. (1980) J. Biol. Chem. 255, 6653–6661
5. Paltaluf, F., Kuhlwein, S. D., and Henry, S. A. (1992) in The Molecular and Cellular Biology of the Yeast Saccharomyces (Jones, E. W., Pringle, J. R., and Broach, J. R., eds) Vol. 2, pp. 415–506, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
6. Saba, J. D., Nara, F., Bielawska, A., Garrett, S., and Hannun, Y. A. (1997) J. Biol. Chem. 272, 26087–26090
7. Gottlieb, D., Heideman, W., and Saba, J. D. (1999) Mol. Cell. Biol. Res. Commun. 1, 66–71
8. Atkinson, K. D., Jensen, B., Kolat, A. I., Storm, E. M., Henry, S. A., and Fogel, S. (1988) J. Bacteriol. 169, 255–264
9. Storey, M. K., Wu, W. I., and Voelker, D. R. (2001) Biochim. Biophys. Acta 1532, 234–247
10. Birner, R., Burgeon, M., Schreier, R., and Daum, G. (2001) Mol. Biol. Cell 12, 1007–1017
11. Sherman, P. (1991) Methods Enzymol. 194, 3–21
12. Glick, B. S., and Pun, L. A. (1995) Methods Enzymol. 260, 213–223
13. Rouxer, S., Siakatos, A. N., and Fleisher, S. (1966) Lipids 1, 85–86
14. Comfurius, P., and Zwaal, R. F. (1977) Biochim. Biophys. Acta 488, 36–42
15. Farquhar, R. F. (1977) Biochim. Biophys. Acta 488, 36–42
16. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 39, 911–917
17. Xie, Z., Fang, M., and Bankaitis, V. A. (2001) Mol. Biol. Cell 12, 1117–1129
18. Murphy, R. (1983) Mass Spectrometry of Lipids, pp. 232–239, Plenum Press, New York
19. Cullis, P. R., and de Krijff, B. (1978) Biochim. Biophys. Acta 513, 31–41
20. Gruner, S. M., Cullis, P. R., Hope, M. J., and Tilcock, C. P. (1992) Annu. Rev. Biophys. Biochem. 14, 211–238
21. Trivedi, A., Ghare, S., Singhgal, G. S., and Prasad, R. (1982) Biochim. Biophys. Acta 692, 202–209
22. Yeagle, P. L. (1989) FASEB J. 3, 1833–1843
23. Huang, C., and Li, S. (1989) Biochim. Biophys. Acta 1422, 273–307
24. Sreevali, C., Yaburak, G., Goubern, M., Vincent, M., and Gallay, J. (1990) Biochim. Biophys. Acta 1023, 263–289
25. Cullis, P. R., Fenske, D. B., and Hope, M. J. (1996) in Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D. E., and Vance, J., eds) Vol. 31, pp. 1–33, Elsevier, Paris
26. Verkleij, A. J., Leunissen Bijleveld, J., de Krijff, B., Hope, M. J., and Cullis, P. R. (1984) CIBA Found. Symp. 103, 45–59
27. Verkleij, A. J., van Echteld, C. J., Gerritsen, W. J., Cullis, P. R., and de Krijff, B. (1980) Biochim. Biophys. Acta 600, 620–624
28. Cullis, P. R., de Krijff, B., Verkleij, A. J., and Hope, M. J. (1986) Biochem. Soc. Trans. 14, 242–245
29. Trotter, P. J., and Voelker, D. R. (1995) J. Biol. Chem. 270, 6062–6070
30. Kamitani, T., Menon, A. K., Hallax, Y., Warren, C. D., and Yeh, E. T. (1991) J. Biol. Chem. 266, 24611–24619
31. Menon, A. K., Eppinger, M., Mayor, S., and Schwarz, R. (1993) EMBO J. 12, 1907–1914
32. Menon, A. K., and Stevens, V. L. (1992) J. Biol. Chem. 267, 15277–15280
Phosphatidylethanolamine Requirement in Yeast

33. Gaynor, E. C., Mondesert, G., Grimme, S. J., Reed, S. I., Orlean, P., and Emr, S. D. (1999) *Mol. Biol. Cell* **10**, 627–648
34. Hong, Y., Maeda, Y., Watanabe, R., Ohishi, K., Mishkind, M., Riezman, H., and Kinoshita, T. (1999) *J. Biol. Chem.* **274**, 35099–35106
35. Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivenc-Gansel, E., Vionnet, C., Conzelmann, A., and Benghezal, M. (1999) *J. Biol. Chem.* **274**, 15251–15261
36. Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P., and Latge, J.-P. (2000) *J. Biol. Chem.* **275**, 14882–14889
37. Mondesert, G., Clarke, D. J., and Reed, S. I. (1997) *Genetics* **147**, 421–434
38. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000) *J. Cell Biol.* **151**, 263–276
39. Komatsu, M., Tanida, I., Ueno, T., Ohsumi, M., Ohsumi, Y., and Kominami, E. (2001) *J. Biol. Chem.* **276**, 9846–9854
40. Lang, T., Schaeffeler, E., Berreuehler, D., Bredaenderer, M., Wolf, D. H., and Thumm, M. (1998) *EMBO J.* **17**, 3597–3607
41. Logue, J. A., de Vries, A. L., Fodor, E., and Cossins, A. R. (2000) *J. Exp. Biol.* **203**, 2105–2115
42. Mikhaleva, N. I., Golovastov, V. V., Zolov, S. N., Bogdanov, M. V., Dowhan, W., and Nesmeyanova, M. A. (2001) *FEBS Lett.* **493**, 85–90
43. Schatz, G., and Dobberstein, B. (1996) *Science* **271**, 1519–1526
44. Bogdanov, M., Umeda, M., and Dowhan, W. (1999) *J. Biol. Chem.* **274**, 12339–12345
45. Emoto, K., and Umeda, M. (2000) *J. Cell Biol.* **149**, 1215–1224
46. Umeda, M., and Emoto, K. (1999) *Chem. Phys. Lipids* **101**, 81–91