Uptake and Utilization of Lyso-phosphatidylethanolamine by Saccharomyces cerevisiae*

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Phosphatidylethanolamine (PtdEtn) is synthesized by multiple pathways located in different subcellular compartments in yeast. Strains defective in the synthesis of PtdEtn via phosphatidylserine (PtdSer) synthase/decarboxylase are auxotrophic for lyso-PtdEtn. Lyso-PtdEtn supports growth and replaces the mitochondrial sn-glycero-3-phosphoethanolamine (lyso-PtdEtn) decarboxylases, import and acylate exogenous 1-acyl-2-aminophospholipid-dependent Kennedy pathway. We now demonstrate that yeast strains with psd1Δ psd2Δ mutations, devoid of PtdSer decarboxylases, import and acylate exogenous 1-acyl-2-hydroxyl-sn-glycero-3-phosphoethanolamine (lyso-PtdEtn). Lyso-PtdEtn supports growth and replaces the mitochondrial pool of PtdEtn much more efficiently than and independently of PtdEtn derived from the Kennedy pathway. Deletion of both the PtdSer decarboxylase and Kennedy pathways yields a strain that is a stringent lyso-PtdEtn auxotroph. Evidence for the specific uptake of lyso-PtdEtn by yeast comes from analysis of strains harboring deletions of the aminophospholipid translocating P-type ATPases (APLTs). Elimination of the APLTs, Dnf1p and Dnf2p, harboring deletions of the aminophospholipid translocating P-type ATPases (APLTs). Elimination of the APLTs, Dnf1p and Dnf2p, blocked the import of radiolabeled lyso-PtdEtn and resulted in growth inhibition of lyso-PtdEtn auxotrophs. In cell extracts, lyso-PtdEtn is rapidly converted to PtdEtn by an acyl-CoA-dependent acyltransferase. These results now provide 1) an assay for APLT function based on an auxotrophic phenotype, 2) direct demonstration of APLT action on a physiologically relevant substrate, and 3) a genetic screen aimed at finding additional components that mediate the internalization, trafficking, and acylation of exogenous lyso-phospholipids.

The yeast Saccharomyces cerevisiae is a powerful model organism for understanding basic processes of lipid metabolism and membrane biogenesis. The major pathways of lipid biosynthesis have been elucidated in this organism, and our knowledge of the genes and proteins that play a direct role in glycerolipid and sterol synthesis is extensive. The biosynthetic reactions for the aminoglycerophospholipids, PtdSer, PtdEtn, and PtdCho, are particularly well characterized, and the pathways for their synthesis and certain aspects of their intracellular transport have been extensively reviewed (1, 2). There are two major pathways ultimately leading to the production of PtdCho, which is the major phospholipid component of yeast membranes. First, PtdCho can be synthesized de novo by reactions catalyzing formation of PtdSer by the Psd1p enzyme, followed by decarboxylation of PtdSer by Psd1p in the mitochondrion or Psd2p in the Golgi, and finally methylation of PtdEtn by the ER localized Pem1p and Pem2p methyltransferase enzymes. Additionally, PtdEtn and PtdCho can be synthesized from exogenous Etn or Cho by the action of the Kennedy pathways, which utilize phosphorylcholine/igmethylphosphotyldimethylethanolamine or phosphorylcholine/igmethylphosphotyldimethylethanolamine-choline as biosynthetic intermediates, respectively. Given the redundancy of multiple pathways leading to the same final products, the de novo and Kennedy pathways are not individually essential for growth, providing that at least one of the alternate pathways remains intact. For example, strains disrupted in the PSS1 (CHO1) (3, 4) gene or double mutants of the PSD1 and PSD2 genes (5) are auxotrophic for Etn or Cho, indicating a reliance on the Kennedy pathway for lipid synthesis. This fact has been exploited in genetic screens aimed at uncovering lipid transport mutants (6–9).

However, the Kennedy and de novo pathways are only partially redundant, and the pools of PtdEtn and PtdCho produced by these different pathways are not functionally equivalent in all respects. For example, in mutants of the mitochondrial branch of the de novo pathway (i.e. psd1Δ strains), the lipid composition of mitochondria is greatly altered relative to the wild type cells, with a mitochondrial PtdEtn deficiency that is not fully restored by PtdEtn produced from exogenous Etn (10, 11). Additionally, recent work using molecular species determina-

tion and flux analysis by mass spectrometry has shown that the different pools of PtdEtn and PtdCho, produced by the Kennedy or de novo pathways, contain different sn-1,2-diacylglycerol molecular species profiles (12, 13). These latter findings reveal that in addition to spatially segregated pools of PtdEtn and PtdCho, there are also chemical distinctions between the products of these pathways.

To circumvent the issues of molecular species and pathway-specific pools of spatially and chemically distinct lipids, it would be preferable to find a way to supply biosynthetic and transport mutants with a source of PtdEtn that restores all cellular pools of these lipids back to wild type levels. To this end, and in addition to the Kennedy and de novo pathways, we now describe the phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtn(Me)2; phosphatidyl(dimethylethanolamine); ER, endoplasmic reticulum.
Lyso-PtdEtn Uptake in Yeast

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise noted, all chemicals, solvents, and amino acids for media were purchased from Sigma or Fisher. Yeast extract, peptone, and yeast nitrogen base were from Difco (Detroit, MI). CI-976 (Tocris Bioscience, Ellisville, MO) was dissolved as a 25 mM stock solution in Me2SO and stored at 20 °C until use. Lipid Extraction and Analysis

**TABLE 1**

<table>
<thead>
<tr>
<th>Strain</th>
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characterization of a third pathway of PtdEtn and PtdCho synthesis in yeast, in which lyso-PtdEtn is specifically imported and used for PtdEtn synthesis by an acyl-CoA-dependent acyltransferase. Lyso-PtdEtn can serve as the sole source of PtdEtn and PtdCho in yeast and is used preferentially (relative to that derived from the Kennedy pathway) for supporting the structure and function of mitochondrial membranes. Uptake of lyso-PtdEtn is mediated by the plasma membrane aminophospholipid translocases Dnf1p and Dnz2p and their noncatalytic subunit Lem3p. Following uptake, lyso-PtdEtn is converted to PtdEtn by the action of an acyl-CoA-dependent acyltransferase activity. This system allows for the direct auxotrophic assay of lyso-PtdEtn internalization, trafficking, and acylation and thus provides a new tool for the study of glycerophospholipid biosynthesis and transport in yeast.
Lyso-PtdEtn Uptake in Yeast

Phospholipids were either resolved by one-dimensional TLC on Silica-60 plates in the solvent system chloroform/methanol/iso-propanol/0.25% KCl/triethylamine (30:9:25:6:18, v/v/v/v/v) or resolved in two dimensions with chloroform/methanol/28% (w/v) ammonium hydroxide (65:25:4, v/v/v) in the first dimension, chloroform/glacial acetic acid/methanol/water (90:30:6:2.6, v/v/v/v) in the second dimension. After lightly spraying the plate with 0.05% (w/v) anilinonapthalene-sulfonic acid in water, the fluorescent bands were visualized under ultraviolet light, and the lipid spots were scraped from the plate and either analyzed for phosphorus content as described (21) or scraped into vials for liquid scintillation spectrometry.

Preparation of Homogenates and Acyltransferase Assays—The yeast strain PTY44 (22) was grown to an A₆₀₀ of 1.0–1.5 in YPDAUE medium and washed with homogenization buffer (50 mM Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.25 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, and 10 µg/mL each of antipain, aprotinin, and leupeptin). The cells were suspended in 2-ml aliquots and disrupted in a Mini-Bead-Beater-8 apparatus (Biospec, Bartlesville, OK) at setting 6, for 6 bursts of 30 s, with cooling on ice between pulses. Homogenates were centrifuged at 20,000 × g, with 5 min to remove unbroken cells and debris, and the protein concentration of the cell homogenate was measured using Bradford reagent (Bio-Rad). The cell homogenates were assayed for acyltransferase activity in 200-µl reactions containing 0.2 mM oleic acid, 0.2 mM CoA, 0.4 mM MgATP, 150 mM NaCl, 1 mM EDTA, 10 mM Tris-Cl, pH 7.4, 100,000 cpm oleoyl-lyso-Ptd[1-¹⁴C]Etn. In this system the endogenous long chain acyl-CoA synthetase is used to generate acyl-CoA in situ. The assays were conducted for 1 h at 37 °C, and the reactions were stopped by the addition of 250 µL of methanol and 250 µL of chloroform, followed by vigorous vortexing and centrifugation in a microcentrifuge tube to separate the phases. The chloroform phase was recovered and subjected to TLC and quantification of radioactivity by exposure to a PhosphorImager screen (Molecular Dynamics, Inc.).

RESULTS

Yeast Strains Auxotrophic for Etn Preferentially Use Exogenous Lyso-PtdEtn as a Precursor of PtdEtn and PtdCho—Previous studies have demonstrated that Etn is a relatively poor precursor for replenishment of the mitochondrial PtdEtn pool in strains harboring a psd1Δ mutation (10, 11). Our initial experiments tested whether lyso-PtdEtn is transported and metabolized by yeast, and whether it efficiently restores the mitochondrial pool of PtdEtn. As shown in Fig. 1A, the growth of PtdEtn deficient strains on agar plates is supported equally well by either 0.5 mM lyso-PtdEtn or 5 mM Etn. As demonstrated previously (5, 22), a psd1Δ psd2Δ strain displays a stringent requirement for Etn (or Cho) in the growth medium. However, this strain still produces PtdEtn via the Kennedy pathway in the absence of Etn, by generating P-Etn from dihydrophosphoglycerol through action of the Dpl1p enzyme (9, 23). Deletion of the DPL1 gene in the psd1Δ psd2Δ background blocks this pathway, resulting in a stringent Etn auxotrophy that is not rescued by Cho (10, 11). Fig. 1A demonstrates that lyso-PtdEtn rescues this psd1Δ psd2Δ dpl1Δ triple mutant, implying that this compound can serve as the precursor of all PtdEtn and PtdCho biosynthesis needed to support the growth of the strain.

To determine whether lyso-PtdEtn supports growth more efficiently than Etn, we next determined the minimum effective concentration of these compounds required for growth of psd1Δ psd2Δ in liquid medium containing either a respiratory (lactate) or fermentable (glucose) carbon source. As shown in Fig. 1B, the effectiveness of lyso-PtdEtn and Etn for supporting growth on glucose is essentially identical, with saturating growth being achieved at ~0.25 mM initial concentration for both supplements. However, for growth under respiratory conditions (with lactate as carbon source) the dose–response curves indicate that Etn is a poorer substrate (≥2 mM required for optimum growth) for generating a critical pool of PtdEtn relative to lyso-PtdEtn (≥0.5 mM required for optimum growth). Mitochondrial membranes contain a large mole fraction of PtdEtn (11), and mitochondrial number and size increase substantially during growth on a nonfermentable carbon source such as lactate. Thus, the results in Fig. 1 clearly demonstrate that lyso-PtdEtn gives rise to a pool of PtdEtn that is better able to support mitochondrial function than PtdEtn produced via the Kennedy pathway.

The Rate of Lyso-PtdEtn Uptake Is Sufficient to Supply All PtdEtn and PtdCho Biosynthesis in Rapidly Dividing Cells—To determine the rate of uptake of lyso-PtdEtn, we measured the time-dependent incorporation of radiolabeled lipid into cells of the psd1Δ psd2Δ strain. A culture of strain PTY44 was incubated with [9,10-³H]palmitoyl-lyso-PtdEtn for 2 h, and at 30-min intervals, an aliquot of cells was diluted and washed in
cold bovine serum albumin/detergent solution to stop further uptake and also remove nonspecifically bound lipid. The result in Fig. 2 demonstrates that the total amount of radioactivity associated with cells increases in a linear fashion up to at least 120 min. These assays (determined at 0.25 mM lyso-PtdEtn) give a value for the uptake rate of $\mu$mol/2 h/$A_{600}$ cells. The starting cell number corresponds to 15 nmol of total PtdEtn and PtdCho. Thus, the rate of lyso-PtdEtn uptake is severalfold more than that needed to support growth of a strain with a 1.5–2-h doubling time.

Lyso-PtdEtn Utilization Is Independent of the Kennedy Pathway—From the data presented in Figs. 1 and 2, we considered two biochemical pathways as the routes for PtdEtn synthesis to mechanistically explain these initial results. First, yeast may import and subsequently degrade lyso-PtdEtn, releasing either Etn or P-Etn that could then be reassimilated via the Kennedy pathway. Second, yeast might take up and acylate the intact lyso-PtdEtn directly by the action of a lysophospholipid acyltransferase (24, 25). To test the first possibility, we constructed a yeast strain with deletions of the PSD1, PSD2, and ECT1 genes, resulting in an absolute inability to form PtdEtn by the PSD pathways and a very limited ability to use Etn for PtdEtn synthesis. The use of Etn by the triple deletion strain could potentially occur as a consequence of low activity of Cct1p toward P-Etn. If the mechanism of lyso-PtdEtn utilization involves degradation and recycling of Etn or P-Etn, then we would expect this triple mutant to fail to grow on either Etn or lyso-PtdEtn. However, a direct acylation mechanism would allow for growth with lyso-PtdEtn supplementation but not Etn supplementation. These predictions were tested by analysis of the growth phenotype of this psd1Δ psd2Δ ect1Δ triple mutant. The experiment shown in Fig. 3A reveals that the triple mutant strain is incapable of using Etn to fulfill its requirements for PtdEtn and/or PtdCho biosynthesis. However, the triple mutant is fully viable with lyso-PtdEtn supplementation and shows an absolute auxotrophic requirement for this lipid.

In addition to evaluating the growth phenotype, we tested the ability of the psd1Δ psd2Δ ect1Δ strain to import and convert Etn and lyso-PtdEtn to PtdEtn and PtdCho. Fig. 3B provides...
further direct evidence that the Kennedy pathway is not required for the utilization of lyso-PtdEtn because the absence of a functional Cct1p enzyme did not affect incorporation of label from lyso-Ptd[U-14C]Etn into PtdEtn and PtdCho. This is in contrast to the incorporation of [1,2-3H]Etn into PtdEtn and PtdCho, presented in Fig. 3C, which shows a 70% decline in Etn incorporation into phospholipids. The low level of incorporation of [1,2-3H]Etn into phospholipids is most likely due to a limited ability of the phosphorylcholine preferring cytidylyltransferase Cct1p to use P-Etn as a substrate. It should be appreciated that this residual level of activity is insufficient to support cell growth. Taken together, the physiological and biochemical phenotypes of the psd1Δ psd2Δ cct1Δ strain provide strong evidence supporting the direct uptake and acylation of lyso-PtdEtn as the primary mechanism by which yeast uses this compound.

The Majority of Cellular Lyso-PtdEtn Acyltransferase Activity Is Acyl-CoA-dependent—We next wanted to determine what type of acyl transfer activity is involved in the acylation of lyso-PtdEtn. We tested two major possibilities, the first being a transacylation mechanism in which the fatty acid of one lyso-PtdEtn molecule is transferred to another, forming PtdEtn and glycerophospho-Etn (26, 27) and the second being a more direct mechanism involving acyl-CoA-dependent acyltransferase(s). Initially, we tested the ability of an inhibitor of acyl-CoA-dependent acyltransferases to block lyso-PtdEtn utilization.

The acyltransferase inhibitor CI-976 (28) has recently been shown to inhibit a Golgi-localized acyl-CoA-dependent lyso-PtdCho acyltransferase activity in mammalian cells (29, 30). This finding led us to test whether CI-976 decreased the efficiency with which yeast used lyso-PtdEtn. As shown in Fig. 4, this compound has no significant effect on the maximum effective concentration of Etn required for growth, and therefore it is neither toxic at this concentration nor directly involved in Etn utilization. However, the addition of 0.5 mM CI-976 modestly increased the concentration of lyso-PtdEtn required for halffmaximal growth. CI-976 is a competitive inhibitor and structural mimic of acyl-CoA (28). The results suggest that a portion of the total lyso-PtdEtn acyltransferase activity is acyl-CoA-dependent and CI-976-sensitive. However, this effect is much weaker than expected based on data derived from mammalian cells (29). This result could indicate that the yeast cell is relatively impermeable to CI-976 or that the bulk of the acyltransferase activity is insensitive to the compound.

We also directly tested the rate of lyso-PtdEtn acylation in yeast homogenates in the absence and presence of an acyl-CoA-generating system. This provided a direct test of the activity of the transacylation mechanism versus the acyl-CoA-dependent mechanism. The transacylation reaction is CoA-independent, whereas the acyltransferase reaction is CoA-dependent. As presented in Fig. 5, the acylation of lyso-PtdEtn is almost entirely acyl-CoA-dependent, as judged by the >98% conversion of the substrate to PtdEtn in the presence of oleic acid, MgATP, and CoA (Fig. 5). This experiment provides another insight into the major catalytic activities present in the system, insofar as incubation of the labeled lyso-PtdEtn with crude yeast homogenate gave no significant loss of radiolabel. This latter result demonstrates that the in vitro rate of lyso-PtdEtn degradation by phospholipases is negligible relative to the rate of acyl-CoA-dependent acylation. Collectively, these findings further argue against a prominent role for lyso-PtdEtn degradation to form Etn or P-Etn for the synthesis of PtdEtn.

PtdEtn and PtdCho Derived from Lyso-PtdEtn Are Not Subject to Extensive Acyl Chain Remodeling—To study the rate of remodeling (if any) of the products of lyso-PtdEtn incorporation, we added lyso-[9',10'-3H]palmitoyl-PtdEtn and lyso-Ptd[U-13C]Etn to yeast at a fixed ratio (3H:13C = 2.5:1) and measured this same ratio in the products PtdEtn, PtdEtn(Me)2, and PtdCho isolated by two-dimensional TLC. The results shown in Fig. 6 provide clear evidence that PtdEtn and PtdEtn(Me)2 have essentially the same ratio of 3H:13C, indicating that remodeling of these species during the 1.5-h incubation either does not occur or is too slow to be resolved. However, PtdCho does show a small decrease in the amount of 3H relative to 13C, and therefore we estimate that the sn-1 palmitoyl moiety of PtdCho derived from lyso-PtdEtn is exchanged with a half-
Considering that the doubling time of the strain at the beginning of the experiment is 1.5–2 h, the remodeling of PtdCho proceeds at a slow rate relative to other metabolic processes.

The other major phospholipids, PtdSer and phosphatidylinositol, are not derived directly from lyso-PtdEtn; however these lipids were significantly labeled in their fatty acid moiety, and there was also a pool of labeled free fatty acid (data not shown). These results indicate that in vivo, lyso-PtdEtn follows at least two routes. In one route, a portion of the total lyso-PtdEtn is used directly for PtdEtn synthesis and then methylated to form PtdCho. In a second route, a portion of the lyso-PtdEtn is deacylated, and the free fatty acid is reincorporated into PtdOH for use in PtdSer and phosphatidylinositol synthesis. This is in contrast to the data presented in Fig. 5, which demonstrates that there is a negligible rate of lyso-PtdEtn hydrolysis in crude extracts. This may reflect a regulatory mechanism that controls cellular esterases such as Nte1p (31) or PLB family members (32) that is active in intact cells but that becomes disrupted in cellular extracts.

PtdEtn Derived from Lyso-PtdEtn Restores Mitochondrial Lipid Content in psd1Δ-containing Strains—We next sought to determine whether the greater efficacy of lyso-PtdEtn relative to Etn in supporting respiratory growth of psd1Δ strains correlated with a higher concentration of mitochondrial PtdEtn. As shown in Fig. 7, the relative amount of PtdEtn was increased 3–4-fold, in both the whole cell lipid extracts and in extracts shown). These results indicate that in vivo, lyso-PtdEtn follows at least two routes. In one route, a portion of the total lyso-PtdEtn is used directly for PtdEtn synthesis and then methylated to form PtdCho. In a second route, a portion of the lyso-PtdEtn is deacylated, and the free fatty acid is reincorporated into PtdOH for use in PtdSer and phosphatidylinositol synthesis. This is in contrast to the data presented in Fig. 5, which demonstrates that there is a negligible rate of lyso-PtdEtn hydrolysis in crude extracts. This may reflect a regulatory mechanism that controls cellular esterases such as Nte1p (31) or PLB family members (32) that is active in intact cells but that becomes disrupted in cellular extracts.

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Lyso-PtdEtn Uptake in Yeast

prepared from crude mitochondria. In this experiment, the cultures were pregrown in YPLac medium and then transferred to SCLac medium containing either 2 mM Etn or 0.5 mM lyso-PtdEtn. The cultures were harvested in late log/early stationary phase (A600 = 1.2–1.6) and either processed directly for lipid extraction or subjected to bead beating and differential centrifugation to isolate a mitochondrial fraction from which lipids were extracted and analyzed. The results presented in Fig. 7A show that, as a fraction of total cellular phospholipids, PtdEtn levels are restored by lyso-PtdEtn to a level much higher than that achieved by Etn supplementation. The same is true of the phospholipid content of purified mitochondria (Fig. 7B), whose lipid composition closely mirrors that of the whole cell. Lyso-PtdEtn supplementation of psd1Δ psd2Δ strains restores both the cellular and mitochondrial levels of PtdEtn to values nearly identical to those of wild type cells as determined previously (11). Taken together, these results indicate that PtdEtn derived from lyso-PtdEtn is able to fill a role that PtdEtn derived from the Kennedy pathway cannot and may indicate a specific compartment or trafficking step to which lyso-PtdEtn has access but to which PtdEtn derived from the Kennedy pathway does not. Additionally, the absence of detectable amounts of lyso-PtdEtn in these lipid extracts (data not shown) further indicates that the acylation of lyso-PtdEtn is rapid relative to uptake of this compound.

The Plasma Membrane P-type ATPase Components Dnf1p, Dnf2p, and Lem3p Facilitate the Transport of Lyso-PtdEtn into the Cell—The cumulative results thus far argue for a model involving the direct uptake and metabolism of lyso-PtdEtn as an intact molecule. From this finding, we took a candidate gene-based approach to search for proteins that might act as lyso-PtdEtn transporters. The first candidates examined were the plasma membrane-localized P-type ATPases Dnf1p and Dnf2p and their noncatalytic β-subunit Lem3p (also known as Ros3p) (33–35). These genes were disrupted individually in the PTY44 (psd1Δ psd2Δ) background, and a dnf1Δ dnf2Δ double mutant was also constructed in this background. The growth phenotypes and corresponding lyso-PtdEtn uptake rates are shown in Fig. 8 and clearly indicate that Dnf1p and Dnf2p are responsible for the bulk of lyso-PtdEtn import activity. The single dnf1Δ and dnf2Δ alleles result in little if any growth defect when lyso-PtdEtn is the sole source of PtdEtn. However, lyso-PtdEtn uptake rates were decreased by ~30% in the dnf1Δ strain and ~65% in the dnf2Δ mutant (Fig. 8B). In the dnf1Δ dnf2Δ double mutant and the lem3Δ mutant, a strong growth phenotype was observed when these strains were grown on a low concentration (0.25 mM) of lyso-PtdEtn, but the growth defect was less severe when the strains were grown with a higher concentration (0.5 mM) of lyso-PtdEtn in the medium. This suggests that Dnf1p, Dnf2p, and Lem3p are the major components facilitating high affinity lyso-PtdEtn uptake and suggest the presence of a second, lower affinity system that is operative at higher lyso-PtdEtn concentrations. The results in Fig. 8B demonstrate that the high affinity system accounts for ~85% of the lyso-PtdEtn transport.

This work was undertaken to explore a possible means of supplying yeast Etn auxotrophs with a source of PtdEtn that restores all cellular pools of this lipid, with special emphasis on mitochondria. This emphasis on the mitochondria derives from the finding that psd1Δ strains have markedly reduced levels of PtdEtn that cannot be rectified by pools of this lipid produced by either the Kennedy pathway or the Psd2p pathway. We reasoned that lyso-PtdEtn might be rapidly imported into cells because of its low molecular weight and detergent properties or via facilitated transport. Once inside the cell the lyso-phospholipid might also have sufficient solubility to partition into membranes harboring acyltransferases that convert it to PtdEtn. This prediction was at least partially borne out by experiments presented in Figs. 1 and 7, which directly demonstrate that lyso-PtdEtn is much more efficient than free Etn at restoring the growth and PtdEtn content of a psd1Δ psd2Δ strain on lactate medium. Specifically, lyso-PtdEtn more efficiently replenished the mitochondrial pool of PtdEtn, leading to improved growth on lactate medium, which requires full mitochondrial function for optimal growth.

One potential route for lyso-PtdEtn metabolism is degradation by extracellular phospholipases B (32) or by the ER-localized PLB enzyme Nte1p (31), with the resulting breakdown...
products being used for PtdEtn synthesis via the Kennedy pathway. The data presented in Figs. 3 and 6 clearly address the issue of lyso-PtdEtn degradation and provide strong evidence against such a pathway for PtdEtn synthesis. However, although we show that the PLB pathway is not necessary for lyso-PtdEtn metabolism, it could be involved in the process of lysolipid metabolism in nature. One could envision a scenario in which yeast use PLB activity to scavenge phospholipids from decaying organic matter, thus generating the relatively more soluble lysophospholipids and fatty acids. This would be followed by the uptake of these components and incorporation into cellular phospholipids as described in the present work. This lysolipid salvage pathway would also be energetically favorable, because the assembly of phospholipids requires expenditure of multiple ATP and CTP molecules (e.g. activation of fatty acids by acyl-CoA synthetases and the cytidylyltransferases of the Kennedy pathways.)

More detailed analysis of lyso-PtdEtn uptake and incorporation into lipids reveals that the process proceeds at a rate 2–3-fold in excess of the rate of PtdEtn synthesis required for a cell doubling. This result indicates that the organism is extremely efficient at recovering lyso-PtdEtn from the environment. Double isotope studies using $[9^{3}H,10^{-14}C]$-palmitoyl-lyso-Ptd[$U^{-14}C]$Etn also reveal that the lipid is largely transported into the cell in intact form and rapidly metabolized to PtdEtn and PtdEtn(Me)$_{2}$ with $^{3}H/^{14}C$ ratios that are identical to the starting lyso-phospholipid. The major end product of the metabolic pathway is PtdCho, and this has a $^{3}H/^{14}C$ ratio that is 70% of the value of the starting lyso-PtdEtn. These results are consistent with rapid acylation of lyso-PtdEtn and minor levels of remodeling of the PtdCho by deacylation-reacylation reactions.

Additional evidence for rapid acylation of transported lyso-PtdEtn comes from measurements of enzymatic activity in whole cell extracts shown in Fig. 5. These results demonstrate nearly quantitative (>98%) acylation of 8 μM lyso-PtdEtn in a 1-h incubation. In the absence of an exogenous acyl-CoA-generating system, the conversion of lyso-PtdEtn to PtdEtn is negligible (<5%), and this low level of conversion is most likely due to the endogenous acyl-CoA pool present in the homogenate. Both the results from enzymatic measurements and the double isotope studies make it unlikely that transesterification of two lyso-PtdEtn molecules to form one PtdEtn is significant. Although the experiments cannot completely rule out the transesterification of a fatty acid from another phospholipid or lyso-phospholipid class, the fact that PtdEtn formation is stringently dependent on an acyl-CoA-generating system makes this possibility remote.

The lyso-PtdEtn acyltransferase responsible for the formation of PtdEtn in our experiments is unknown, and the catalytic activity may be comprised of one or more enzymes found on multiple organelles. Obviously, if the acyltransferases reside in the mitochondria, then transport of the lyso-PtdEtn to this organelle would constitute one important route for PtdEtn synthesis. Alternatively, the acyltransferase could reside in the plasma membrane or elsewhere in the cell, and the restoration of the mitochondrial pool of PtdEtn would require transport of this lipid from that location to the mitochondria. The biochemical and genetic tools we have assembled will enable us to examine this process in more detail.

With the initial characterization of lyso-PtdEtn transport and metabolism, we also undertook a reverse genetic analysis and screened several candidate genes for their role in the process. The genes for two plasma membrane P-type ATPases, Dnf1p and Dnf2p, that require Lem3p for proper localization and activity. The imported lyso-PtdEtn is converted to PtdEtn in an acyl-CoA-dependent reaction, by unknown gene products at unknown locations within the cell. Likely sites for the acyltransferase event include the ER, followed by transfer of PtdEtn to the mitochondrial (pathway A), or the mitochondrion, followed by transfer of PtdEtn to the ER (pathway B), or a combination of these or other pathways. In any case, the resulting PtdEtn is sufficient to meet both the structural needs of the mitochondria for respiration and the biosynthetic needs of the ER for PtdCho synthesis.
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However, lyso-PtdEtn imported by the plasma membrane aminophospholipid translocase complexes provides PtdEtn for the mitochondria and ER and probably the Golgi. Evidence for PtdEtn repletion in the mitochondria comes from direct measurement, and evidence for PtdEtn residence in the ER comes from metabolism of the lipid to PtdCho. The sites of acylation of lyso-PtdEtn are presently unknown but could occur in all membranes or just selected membranes. Studies aimed at elucidating the identities and sites of action of the yeast lyso-PtdEtn acyltransferases are currently underway.

In conclusion, in this paper we demonstrate that exogenous lyso-PtdEtn is efficiently utilized as a precursor for PtdEtn and PtdCho in yeast. The uptake of lyso-PtdEtn occurs by high affinity transport of the lipid, primarily by plasma membrane aminophospholipid translocase complexes. The imported lyso-PtdEtn is rapidly esterified by acyl-CoA-dependent acyltransferases, and the resultant PtdEtn is accessible to methyltransferases for the synthesis of PtdCho.

Acknowledgments—We thank Drs. Charles Rock and Susanne Jackowski (St. Jude Children’s Research Hospital, Memphis, TN) for the gift of E. coli strain Sf201.

REFERENCES

Membrane Transport, Structure, Function, and Biogenesis: Uptake and Utilization of Lyso-phosphatidylethanolamine by *Saccharomyces cerevisiae*

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