Lysophosphatidylcholine Metabolism in Saccharomyces cerevisiae

THE ROLE OF P-TYPE ATPases IN TRANSPORT AND A BROAD SPECIFICITY ACYLTRANSFERASE IN ACYLATION*

Received for publication, August 13, 2007, and in revised form, October 12, 2007 Published, JBC Papers in Press, October 21, 2007, DOI 10.1074/jbc.M706718200

Wayne R. Riekhof‡1, James Wu†1, Miguel A. Gijón§, Simona Zarini, Robert C. Murphy, and Dennis R. Voelker‡2

From the Department of Medicine, Program in Cell Biology, National Jewish Medical and Research Center, Denver, Colorado 80206 and Department of Pharmacology, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado 80045

We recently described a new route for the synthesis of phosphatidylethanolamine (PtdEtn) from exogenous lyso-PtdEtn, which we have termed the exogenous lysolipid metabolism (ELM) pathway. The ELM pathway for lyso-PtdEtn requires the action of plasma membrane P-type ATPases Dnf1p and Dnf2p and their requisite β-subunit, Lem3p, for the active uptake of lyso-PtdEtn. In addition, the acyl-CoA-dependent acyltransferase, Ale1p, mediates the acylation of the imported lysolipid to form PtdEtn. We now report that these components of the lyso-PtdEtn ELM pathway are also active with lyso-1-acyl-2-hydroxy-sn-glycero-3-phosphocholine (PtdCho) as a substrate. Lyso-PtdCho supports the growth of a choline auxotrophic pem1Δ pem2Δ strain. Uptake of radiolabeled lyso-PtdCho was impaired by the dnf2Δ and lem3Δ mutations. Introduction of a lem3Δ mutation into a pem1Δ pem2Δ background impaired the ability of the resulting strain to grow with lyso-PtdCho as the sole precursor of PtdCho. After import of lyso-PtdCho, the recently characterized lyso-PtdEtn acyltransferase, Ale1p, functioned as the sole lyso-PtdCho acyltransferase in yeast. A pem1Δ pem2Δ ale1Δ strain grew with lyso-PtdCho as a substrate but showed a profound reduction in PtdCho content when lyso-PtdCho was the only precursor of PtdCho. Ale1p acylates lyso-PtdCho with a preference for monounsaturated acyl-CoA species, and the specific LPCAT activity of Ale1p in yeast membranes is >50-fold higher than the basal rate of de novo aminoglycerophospholipid biosynthesis from phosphatidylserine synthase activity. In addition to lyso-PtdCho, lyso-PtdEtn, and lyso-phosphatidic acid, Ale1p was also active with lysophosphatidylserine, lysophosphatidylglycerol, and lysophosphatidylinositol as substrates. These results establish a new pathway for the net synthesis of PtdCho in yeast and provide new tools for the study of PtdCho synthesis, transport, and remodeling.

The exogenous lysolipid metabolism (ELM) pathway of yeast, which has been the subject of recent studies by our laboratory (1, 2), involves two processes that are essential to eukaryotic cell biology but which remain poorly characterized at the molecular level; that is, phospholipid translocation across membranes and the acylation of lysophospholipids. The ELM pathway enables yeast to use lyso-PtdEtn as the sole source of PtdEtn and PtdCho required for cell growth. In this system lyso-PtdEtn is imported by the action of the plasma-membrane aminophospholipid translocases Dnf1p and Dnf2p, which require Lem3p as an accessory factor for their proper function. Once imported, lyso-PtdEtn is converted to PtdEtn by an acyl-CoA-dependent acyltransferase. The resulting PtdEtn rescues ethanolamine (Etnt) auxotrophic psd1Δ psd2Δ strains much more efficiently than free Etnt, and lyso-PtdEtn restores the mitochondrial pool of PtdEtn in a psd1Δ strain that cannot be replenished by PtdEtn derived from Psd2p or the Kennedy pathway.

The acyltransferase responsible for the acylation of lyso-PtdEtn in yeast is encoded by the YOR175C locus and was named Ale1p (acyltransferase for lyso-PtdEtn). Ale1p has a very high specific activity relative to other anabolic processes of lipid metabolism in yeast such that the rate of PtdEtn synthesis from exogenous lyso-PtdEtn exceeds that required for membrane biogenesis. The enzymatic activity is highly enriched in the mitochondria-associated ER membrane (MAM) and persists in purified mitochondria, indicating that it is a major factor of MAM that is tightly associated with the outer mitochondrial membrane. Ale1p also acts as the major lyso-PtdOH acyltransferase (LPAAT) in yeast and shows a synthetic genetic interaction with Scl1p, which was the only LPAAT previously known in yeast (3, 4).

Given the lysophospholipid substrate promiscuity of Ale1p and the potential for the plasma membrane aminophospholipid translocases to transport a wide range of phospholipids and

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of the Breast Cancer Research Program and the American Cancer Society Great West Division Postdoctoral Fellowship Award.

‡ Department of Pharmacology, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado 80045

§ Recipient of American Cancer Society Great West Division Postdoctoral Fellowship Grant PF-06-288-01-CSR through the generous support of the Generations Cancer Foundation, Denver, CO.

1 The abbreviations used are: ELM, exogenous lysolipid metabolism pathway; Cho, choline; P-Chol, phosphoryl-Chol; Gro-P-Chol, sn-glycero-3-phospho-Chol; PtdCho, phosphatidyl-Chol; lyso-PtdCho, 1-acyl-2-hydroxy-sn-glycero-3-phospho-Chol; CDP, cytidine diphosphate; Etnt, ethanolamine; PtdEtn, phosphatidyl-Etn; P-Etn, phosphonyl-Etn; lyso-PtdEtn, 1-acyl-2-hydroxy-sn-glycero-3-phospho-Etn; LPCAT, lyso-PtdCho acyltransferase; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; YPD, yeast extract-peptone-dextrose; SC, synthetic complete; ER, endoplasmic reticulum; LC, liquid chromatography; MS, mass spectrometry; AU, absorbance unit.
Lyso-PtdCho Metabolism in Yeast

TABLE 1
Strains used in this study

| Strain | Genotype | Source |
|--------|-----------|--------|
| PTY44  | Mat a trpl 1 ura3 lys2 his3 suc2 pad1::TRP1 pad2::HIS3 | Ref. 24 |
| WRY36  | Mat a trpl 1 ura3 lys2 his3 suc2 pad1::TRP1 pad2::HIS3 dnf1::KLEI12 | Ref. 1 |
| WRY44  | Mat a trpl 1 ura3 lys2 his3 suc2 pad1::TRP1 pad2::HIS3 dnf2::KANm | Ref. 1 |
| WRY39  | Mat a trpl 1 ura3 lys2 his3 suc2 pad1::TRP1 pad2::HIS3 dnf1::KLEI12 dnf2::KANm | Ref. 1 |
| WRY28  | Mat a trpl 1 ura3 lys2 his3 suc2 pad1::TRP1 pad2::HIS3 lem3::KANm | Ref. 1 |
| BY4742 | Mat a his4Δ1 leu2Δ20 lys2Δ0 ura3Δ0 ale1Δ::KANm | Research Genetics |
| nte1Δ  | Mat a his4Δ1 leu2Δ20 lys2Δ0 ura3Δ0 ntelΔ::KANm | Research Genetics |
| JWY41  | Mat a his4Δ1 leu2Δ20 lys2Δ0 ura3Δ0 ntelΔ::HYGm ntelΔ::NATm | This Study |
| JWY46  | Mat a his4Δ1 leu2Δ20 lys2Δ0 ura3Δ0 pen1Δ::KANm pen2Δ::KANm | This Study |
| JWY49  | Mat a his4Δ1 leu2Δ20 ura3Δ0 pen1Δ::KANm pen2Δ::KANm lem3Δ::NATm | This Study |
| JWY48  | Mat a his4Δ1 leu2Δ20 ura3Δ0 pen1Δ::KANm pen2Δ::KANm ale1Δ::HYGm | This Study |

lyosphospholipid-like compounds (5–7), we designed experiments to test other lysophospholipids as ELM pathway substrates. We now report that lyso-PtdCho is a substrate for the ELM pathway, with genetic and biochemical requirements similar but not identical to the lyso-PtdEtN ELM pathway. We also directly demonstrate that lyso-PtdCho is a substrate for the Ale1 acyltransferase and that the acyl-CoA specificity is similar to that of lyso-PtdEtN as a substrate.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise noted, all chemicals, solvents, and amino acids were purchased from Sigma or Fisher. Yeast extract, peptone, and yeast nitrogen base were from Difco. Silica-60 thin layer chromatography plates were from EM Sciences. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL), except acyl-CoA substrates, which were from Sigma. Lyso-PtdCho was purchased as a 20 mg/ml chloroform solution, and this solvent was removed under vacuum followed by repeated addition and evaporation of methanol to eliminate traces of chloroform. The dried lyso-PtdCho was dissolved in either 10% v/v Tergitol Nonidet P-40 or ethanol at a concentration of 25 mM and stored at −20 °C until use.

Synthesis of Radiolabeled Lyso-PtdCho—To synthesize lyso-Ptd[methyl-14C]Cho, we developed a method based on the procedure of Sohlenkamp et al. (8). We used the plasmid pTB2559 in Escherichia coli strain BL21(DE3) (kindly provided by Dr. Otto Geiger, Center for Genomic Sciences, Universidad Nacional Autónoma de México, Cuernavaca, Mexico), which expresses the Sinorhizobium melloti PtdCho synthase (pcs) gene under the control of a T7 promoter to produce SmPcs in E. coli cells. Cultures were pre-grown to mid-log phase (A600 = 0.5) at 37 °C in LB medium containing 50 mg/liter kanamycin and followed by a 10-min incubation to remove cellular debris. The cells were collected by centrifugation and washed in 50 mM Tris–HCl, pH 8.0. Cells were suspended at 1000 × g for 10 min to remove cellular debris, and membranes were collected by centrifugation at 30,000 × g for 1 h. The Pcs-containing membranes were suspended in 50 mM Tris–HCl, pH 8.0, 15% (v/v) glycerol at a protein concentration of 5 mg/ml and frozen in aliquots at −80 °C until use. Synthesis of Ptd[methyl-14C]Cho was carried out in a 400-μl reaction consisting of 25 mM Tris–HCl, pH 8.0, 0.2% Triton X-100, 5 mM MnCl2, 0.25 mM CDP-diacylglycerol, 0.182 mM Cho (10 μCi total, 55 Ci/mol). 1 μl (5 μg of protein) of the SmPcs-containing membrane preparation was then added, and the reaction was initiated at 30 °C for 30 min. This was followed by the addition of another 1 μl of enzyme and an additional 0.25 mM equivalent of CDP-diacylglycerol in 0.2% Triton X-100 and a further 30-min incubation. Under these conditions, ~40–50% of the labeled Cho was converted to PtdCho in a typical synthesis. The reaction was stopped by lipid extraction, and the resulting Ptd-[methyl-14C]Cho was treated with bee venom phospholipase A2 as described previously for lyso-PtdEtN synthesis (1) to synthesize lyso-Ptd[methyl-14C]Cho.

Yeast Culture and Genetic Manipulations—Yeast strains used in this study and their associated genotypes are provided in Table 1. Strains with deletions of specific open reading frames were constructed by standard methods involving one-step gene replacement (9). Gene replacements were carried out by transformation of PCR fragments containing an appropriate marker gene (as indicated in Table 1) flanked by at least 40 base pairs of DNA identical to the 5’ and 3’ regions outside the start and stop codons of the open reading frame of interest. The eviction of target genes from the resultant drug-resistant colonies was confirmed by PCR amplification of the 5’ and 3’ recombination junctions using appropriate combinations of marker-gene- and target-gene-specific primers. Strains were routinely maintained on standard YPD medium (1% yeast extract, 2% peptone, 2% glucose, 1.5% Bacto agar). For media containing lyso-PtdCho, 1% (v/v) Tergitol Nonidet P-40 was included, and lyso-PtdCho was added to the desired concentration from a sterile 25 mM stock solution in 10% (v/v) Tergitol Nonidet P-40 or ethanol. In some experiments a defined medium was used consisting of yeast nitrogen base (Difco) at 6.7 g/liter, complete amino acid mix, and 2% (w/v) glucose to give synthetic complete (SC) medium. In the lyso-PtdCho uptake and metabolism experiments, 1% Tergitol Nonidet P-40 was included, and this medium is denoted SCGT. For determination of growth requirements, 5-fold serial dilutions of yeast cultures were spotted onto plates as described previously (1).

Lysophospholipid Acytransferase Assays—Microsomes were isolated from the BY4742 wild-type and the isogenic ale1Δ as a 100,000 × g microsomal membrane fraction isolated from a post-mitochondrial supernatant of wild-type BY4742 strain and its isogenic ale1Δ derivative, prepared by the method of
Glick and Pon (10). The protein concentration was measured using Bradford reagent (Bio-Rad). Acyltransferase activity was determined in 200-μl reactions containing 50 mM HEPES, pH 6.0, 1 mM EDTA, 150 mM NaCl, 100 μM 1-oleoyl-2-hydroxy-3-sn-glycerophos-photophospho-[methyl-14C]Cho (5 × 10^4 cpm), 0–100 μM acyl-CoA, and 5–20 μg of microsomal protein. Assays were conducted for 5–20 min at 30 °C depending on the specific activity of the preparation toward the given substrate. The reactions were stopped by the addition of 500 μl of chloroform/methanol (2:1, v/v) followed by vigorous vortexing and centrifugation in a microcentrifuge tube to separate the phases. Radioactive PtdCho was identified by thin-layer chromatography on silica-60 TLC plates in the solvent system chloroform/methanol/water (65/25/4, v/v/v) and quantified on a PhosphorImager. For kinetic experiments, calculation of the K_m and V_max for the acyl-CoA substrates was carried out by direct fitting of a hyperbola to the kinetic data with the program “Hyper.”

Lyso-PtdIns, lyso-PtdGro, and lyso-PtdSer acyltransferase assays were conducted using the same buffer compositions as for the lyso-PtdEnt acyltransferase assays (2). Lyso-PtdIns acyltransferase and lyso-PtdGro acyltransferase assays were conducted with 100 μM unlabeled lysophospholipid and 100,000 cpm of [1-14C]-oleoyl-CoA (American Radiolabeled Chemicals) diluted to 50 μM final concentration with unlabeled compound. Lyso-PtdSer acyltransferase activity was determined using 50,000 cpm of lyso-Ptd[U-14C]Ser diluted to 100 μM with unlabeled compound (Avanti), and 50 μM oleoyl-CoA served as acyl donor. Lyso-Ptd-[U-14C]Ser, lyso-PtdGro, and lyso-PtdIns were synthesized by digestion of the corresponding diacyl compounds with bee venom phospholipase A_2 (Sigma) as previously described for the synthesis of lyso-PtdEnt (1).

**Lyso-PtdCho Supplementation and Lipid Analysis of Strains**—The pem1Δ pem2Δ and pem1Δ pem2Δ ale1Δ strains were pregrown in YPD and used to inoculate 100-ml cultures in SC+Cho (2 mM) or SC+lyso-PtdCho (0.5 mM) at an initial A_600 of 0.05. Cultures were grown for 16 h to early stationary phase, and the cells were harvested by centrifugation. Lipid extraction and quantification of lipid classes was as previously described (1).

**Acyl-CoA Substrate Choice Assay**—Acyltransferase activity was assayed with a mixture of fatty acyl-CoAs by an adaptation of a previously described method (11). Yeast microsomes (24 μg, isolated as described above) were incubated in a total volume of 200 μl of assay buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 12.5 μM bovine serum albumin, 50 μM 1-[2H_3]palmitoyl-2-hydroxy-sn-glycerol-3-phosphocholine (≥97 atom % H; Avanti Polar Lipids, Alabaster, AL), and 30 μM each caproyl-CoA, myristoyl-CoA, palmityl-CoA, palmitoleoyl-CoA, stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA, and arachidonyl-CoA (Sigma-Aldrich). After 30 min at 37 °C, lipids were extracted by the method of Bligh and Dyer (12), dried under N_2, and resuspended in 60 μl of high performance liquid chromatography solvent A (1 mM ammonium acetate in 60/20/20, CH_3OH/CH_3CN/H_2O), and an aliquot (30 μl) was injected into the LC/MS/MS system. A gradient from 100% solvent A to 100% solvent B (1 mM ammonium acetate in CH_3OH) was utilized to elute a C18 column (Columbus 150 × 2 mm, 5 μm, Phenomenex) at a flow rate of 200 μl/min. Solvent B was increased from 0 to 100% in 42 min and kept at 100% for 23 min.

Identification of the different species formed was carried out using a Sciex API 2000 triple quadrupole mass spectrometer. Mass spectrometric analyses were performed in the positive ion mode using multiple reaction monitoring of the following specific transitions: m/z 681→184 for [2H_3]_16:0a/10:0a-GPCho, m/z 737→184 for [2H_3]_16:0a/14:0a-GPCho, m/z 765→184 for [2H_3]_16:0a/16:0a-GPCho, m/z 763→184 for [2H_3]_16:0a/16:0a-GPCho, m/z 793→184 for [2H_3]_16:0a/18a:0a-GPCho, m/z 791→184 for [2H_3]_16:0a/18a:1a-GPCho, m/z 789→184 for [2H_3]_16:0a/18:2a-GPCho; m/z 813→184 for [2H_3]_16:0a/20:4a-GPCho. The synthesis of the different phosphatidylcholines is expressed as the integrated area of the corresponding intensity peaks on LC/MS/MS.

**Labeling of Cells with Radioactive Lyso-PtdCho**—We carried out lyso-PtdCho supplementation studies with strains BY4742 (wild type) and isogenic ale1Δ, nte1Δ, and ale1Δ nte1Δ mutant strains. These strains were initially grown overnight in 5 ml of YPD medium, and a 2-ml aliquot of culture was diluted into 50 ml of fresh YPD medium. Upon reaching an A_600 of 0.5, the cultures were centrifuged, and the cells were washed twice with SCGT media and suspended in 10 ml of SCGT (final A_600 = 2.5). 2 ml of this cell preparation was incubated with 100,000 cpm of lyso-Ptd[methyl-14C]Cho (diluted with unlabeled compound to 250 μCi) for 3 h. The cells were recovered by centrifugation and washed twice with cold 2% (w/v) Triton X-100, and once in water. Cells were suspended in 400 μl of H_2O, and a 200-μl aliquot was taken for liquid scintillation spectrometry before lipid extraction to determine the total amount of radioactivity taken up by the cells.

Another 200-μl aliquot of the cells was subjected to lipid extraction by a modified Bligh-Dyer protocol involving the addition of 300 μl of EtOH and incubation in a sealed tube in a boiling water bath for 45 min. This was followed by the addition of chloroform/methanol (2:1, v/v) and centrifugation to separate the phases. Radioactive PtdCho was identified by thin-layer chromatography on silica-60 TLC plates in the solvent system chloroform/methanol/water (65/25/4, v/v/v) and quantified on a PhosphorImager.
of 4 ml of chloroform/methanol (1:1, v/v) and 1.6 ml of water. The tubes were vigorously mixed and centrifuged to separate the phases, and the upper aqueous phase was removed to a separate tube and lyophilized. This material was dissolved in 0.2 ml of water, and the radioactivity of an aliquot of the aqueous phase was measured by liquid scintillation spectrometry. The remainder of this aqueous fraction was resolved by TLC on Silica 60 plates (EM Sciences) in the solvent 0.5% (w/v) aqueous NaCl, ethanol, 28% ammonium hydroxide (10:10:1, v/v/v/v). Excess unlabeled Cho, P-Cho, CDP-Cho, and Gro-P-Cho were added as carrier to facilitate the identification of radiolabeled compounds, and the bands were identified by spraying the TLC plate with Dragendorff’s reagent (13), which gives a color reaction with quaternary amines such as Cho. The radioactive products in the organic phase were resolved by Silica-60 TLC in the system chloroform, methanol, isopropanol, 0.25% (w/v) aqueous KCl, triethylamine (30:9:25:6:18, v/v/v/v/v) and quantified on a PhosphoImager screen (GE Healthcare).

RESULTS

Dnf2p and Lem3p Are Required for Lyso-PtdCho Uptake by Yeast—Our previous results obtained by examining the uptake of lyso-PtdEtn and its dependence on the plasma-membrane aminophospholipid translocase components Dnf1p, Dnf2p, and Lem3p (1) led us to test the genetic requirements for lyso-PtdCho uptake. The lem3Δ, dnf1Δ, dnf2Δ, and dnf1Δ dnf2Δ strains were previously constructed in the Etn auxotrophic PTY44 (psd1Δ psd2Δ) genetic background. The Etn auxotrophy is irrelevant for these experiments, and Etn was supplied in the medium used for lyso-PtdCho uptake. Yeast rapidly took up lyso-PtdCho at a rate (25 nmol/h/AU of cells) similar to that described previously for lyso-PtdEtn (1). As detailed in Fig. 1, the ability to import lyso-Ptd[methyl-14C]Cho was significantly diminished in the dnf2Δ and lem3Δ mutants. The largest decrement in transport occurred upon deletion of the LEM3 gene, which reduced the rate of lyso-PtdCho uptake to ~5% that of the wild type. The dnf2Δ mutation also caused a large decrease in lyso-PtdCho uptake, and this strain had an uptake rate of ~35% that of the wild type. The dnf1Δ mutation produced a minimal defect in lyso-PtdCho uptake in either the wild-type or dnf2Δ genetic backgrounds. These results identify a major role for Dnf2p and Lem3p in regulating lyso-PtdCho transport and indicate that Dnf1p contributes to no more than 10% of the lyso-PtdCho transport activity.

Lyso-PtdCho Supports the Growth of Choline Auxotrophs in a Lem3p-dependent Manner—The observed rate for lyso-PtdCho transport reported above is sufficient to support all of the cellular requirements for PtdCho synthesis. The physiological relevance of this rate of lyso-PtdCho transport was tested using lem3Δ pem1Δ pem2Δ mutant strains that require either Cho or lyso-PtdCho for growth. The data presented in Fig. 2 demonstrate that 0.5 mM lyso-PtdCho supports cell growth as well as 3 mM Cho in the lem3Δ pem2Δ strain. The effects of a lem3Δ mutation, which blocks lyso-PtdCho uptake, are also included in Fig. 2 and demonstrate that the lyosphospholipid must be transported into the cell to restore the growth of lem3Δ pem2Δ strains. An additional conclusion obtained with the lem3Δ pem2Δ lem3Δ strain is that no significant extracellular degradation of lyso-PtdCho occurs to produce Cho, which could be imported and used by the cell for PtdCho synthesis.

Experiments were also conducted to assess the transport and metabolism of lyso-PtdSer and lyso-PtdIns. The results of these experiments4 were negative, and it appears that these compounds are not taken up and utilized in the same way as lyso-PtdEtn and lyso-PtdCho. Specifically, we were unable to demonstrate a temperature- or time-dependent uptake of lyso-PtdSer. This finding was corroborated by the inability of lyso-PtdSer or short-chain PtdSer species to support the growth of a cho1Δ strain (lacking PtdSer synthase) on minimal medium. Nearly identical experiments were conducted with lyso-PtdIns. This compound failed to support the growth of an ino1Δ strain (deficient in de novo inositol synthesis) or a strain bearing a pis1Δ conditional allele of the Pis1 gene encoding PtdIns synthase.

Ale1p Is a Lyso-PtdCho Acyltransferase—We next investigated the metabolism of the lyso-PtdCho that is taken up by the Dnf2p/Lem3p-dependent mechanism. Previous work on the yeast Ale1p demonstrated that the enzyme exhibited both lyso-PtdEtn acyltransferase and lyso-PtdOH acyltransferase activities (2). We examined whether Ale1p was involved in lyso-PtdCho metabolism by measuring LPCAT activity in an ale1Δ mutant. The result of this experiment is shown in Fig. 3A, and demonstrated that the ale1Δ mutant membranes were devoid of LPCAT activity. This finding revealed that Ale1p acts promiscuously, providing the major acyltransferase activity to reacylate both lyso-PtdEtn and lyso-PtdCho. We next used yeast microsomes as a source of native, Ale1p-derived LPCAT activity in further experiments. Fig. 3B shows the results of enzyme kinetic experiments aimed at determining the acyl-CoA substrate specificity of Ale1p when lyso-PtdCho is the acyl-acceptor. Similar to the results we obtained with lyso-PtdEtn as the acyl-acceptor substrate, the Ale1p LPCAT activity showed a much higher Vmax with the unsaturated acyl-CoA substrates. Contrary to the lyso-PtdEtn acyltransferase kinetic assays, in which the Km values for the unsaturated substrates were ~5–10-fold higher than the saturated substrates, the Km values for all acyl-substrates in the LPCAT assays, either saturated or

4 W. R. Riekof, J. Wu, and D. R. Voelker, unpublished observations.
Lyso-PtdCho Metabolism in Yeast

Ale1p is a lyso-PtdCho acyltransferase. A, the specific lyso-PtdCho acyltransferase activity was measured using cell homogenates of the wild-type strain BY4742 and its isogenic ale1Δ derivative. Data represent the mean ± S.D. of three independent experiments conducted in triplicate. B, enzyme kinetic experiments were performed as described under “Experimental Procedures” by conducting the LPCAT assay using a fixed concentration (100 μM) of lyso-PtdCho and varying the concentrations of the individual acyl-CoA species. The data presented are a representative dataset from three independent experiments. FA, fatty acid. C, a “substrate choice” experiment was performed by conducting the LPCAT assay using 1-[^3]H_1]-palmitoyl-lyso-PtdCho (50 μM) as the acyl-acceptor for an equimolar mixture (30 μM each) of the acyl-CoA species indicated below the abscissa. Reactions were conducted for 30 min followed by lipid extraction to stop the reaction. The products were separated and quantified using an LC/MS/MS system as described under “Experimental Procedures.” The most abundant species was arbitrarily defined as 100%, and other species were expressed as a percentage of the maximum. Data represent the mean ± S.D. of three independent experiments.

The ale1Δ Mutation Impairs PtdCho Biosynthesis via the ELM Pathway—To further define the role of Ale1p in the biosynthesis of PtdCho from exogenous lyso-PtdCho, we grew the Cho auxotrophic pem1Δ pem2Δ and pem1Δ pem2Δ ale1Δ strains with Cho or lyso-PtdCho as the source of PtdCho and then measured the relative content of this lipid. Incorporation of Cho into PtdCho occurs via the Kennedy pathway, which is functional in both strains. We anticipated little if any change in PtdCho content between the pem1Δ pem2Δ and pem1Δ pem2Δ ale1Δ strains grown with Cho as the source of PtdCho. As detailed in Fig. 4, this prediction was proven true insofar as the relative amounts of PtdCho in the two strains are the same regardless of the presence or absence of Ale1p. Conversely, even though the pem1Δ pem2Δ ale1Δ strain was fully viable when grown with exogenous lyso-PtdCho as the PtdCho precursor (data not shown), we expected that the net content of PtdCho would be markedly decreased in the ale1Δ strain relative to the AEL1 parent when grown under these conditions. This latter prediction was verified by the finding that the pem1Δ pem2Δ ale1Δ strains showed a >60% decrement in the content of PtdCho relative to the pem1Δ pem2Δ strain after being cultured overnight with lyso-PtdCho as the sole source of PtdCho. The relative PtdSer content remained roughly the same, whereas the relative content of PtdIns and PtdEtn were increased in the ale1Δ mutant (data not shown). This result

unsaturated, were similar. This results in a strongly enhanced specificity constant ($V_{max}/K_m$) for the unsaturated substrates.

As an independent verification of the preference for unsaturated acyl-CoA species, we performed an experiment in which equimolar amounts of various acyl-CoA species were incubated in an LPCAT assay with deuterated palmitoyl-lyso-PtdCho as the acyl acceptor. This allows the Ale1p LPCAT activity to “choose” its favored acyl-CoA substrate, and in practice the substrate with the highest initial velocity at the given concentration will give the fastest reaction rate and the most diacyl product. The acyl-CoAs used were caproyl (10:0), myristoyl (14:0), palmitoyl (16:0), palmitoleoyl (16:1Δ9), stearoyl (18:0), oleoyl (18:1Δ9), linoleyl (18:2Δ9,12), and arachidonoyl (20:4Δ5,8,11,14). The products of the reaction were detected by LC/MS/MS, and the deuterated palmitoyl-lyso-PtdCho substrate enabled discrimination of the assay products from the endogenous PtdCho molecular species present in the yeast microsome. As shown in Fig. 3C, the preferred acyl-CoA substrates are the 16- and 18-carbon unsaturated species palmitoleoyl, oleoyl, and linoleoyl. The saturated species gave very weak product formation relative to the others, as expected from the kinetic data in Fig. 3B, and arachidonoyl-CoA also seemed to be a relatively poor substrate. This latter finding may indicate that the acyl-CoA binding pocket of Ale1p contains binding determinants for both the length and degree of unsaturation of the fatty acyl chain.

...
Lyso-PtdCho Metabolism in Yeast

FIGURE 4. The PtdCho content of pem1Δ pem2Δ ale1Δ strain is markedly reduced when lyso-PtdCho is the exogenous PtdCho precursor. The pem1Δ pem2Δ and pem1Δ pem2Δ lem3Δ strains were grown overnight in SC media supplemented with either 2 mM Cho or 0.5 mM lyso-PtdCho. Cultures were harvested and subjected to lipid extraction, and individual lipid classes were separated by thin-layer chromatography and quantified as described under "Experimental Procedures." For clarity, only the relative content of PtdCho is shown. Data represent the mean ± S.D. of three independent experiments conducted in duplicate.

establishes that, whereas Ale1p is not absolutely essential for the utilization of lyso-PtdCho by a pem1Δ pem2Δ strain, it is required for the efficient use of this compound in maintaining bulk PtdCho content in cells.

Nte1p Is Partially Responsible for Lyso-PtdCho Degradation—

The growth of the pem1Δ pem2Δ ale1Δ strain with lyso-PtdCho necessitates that the imported lipid be broken down and re-incorporated by the Kennedy pathway (see Fig. 8). The neuropathy target esterase homolog of yeast (Nte1p (14)) is a likely candidate for lysophospholipase-mediated deacylation of lyso-PtdCho. We examined the metabolism of imported lyso-PtdCho in a wild-type strain and in isogenic ale1Δ, nte1Δ, and ale1Δ nte1Δ mutant strains. Cultures of these four strains were incubated with lyso-Ptd[1-14C]Cho for 3 h followed by washing and lipid extraction of the cells. In the two-phase Bligh-Dyer lipid extraction system, lyso-PtdCho and PtdCho are present in the organic phase, and any Cho metabolites derived from deacylation and metabolism of lyso-PtdCho (i.e. Cho, P-Chol, CDP-Chol, or Gro-P-Chol) are present in the aqueous phase. As shown in Fig. 5, we measured the amount of label from lyso-PtdCho that is present in the aqueous phase of the cellular lipid extracts as a means of estimating the in vivo lysophospholipase activity. All of these strains took up comparable amounts of lyso-PtdCho. However, we found that the wild-type strain and the ale1Δ mutant converted ~25% of the total label into water-soluble metabolites of Cho. Introduction of the nte1Δ mutation reduced this amount by about half regardless of the presence or absence of Ale1p. This result indicates that Nte1p is at least partly responsible for the degradation of imported lyso-PtdCho. The other PLB isoforms (15) or a lyso-phospholipase C or D could provide the additional redundant catabolic activity. Taken together, we conclude that lyso-PtdCho degradation occurs regardless of the capacity for acylation by Ale1p and that Nte1p activity accounts for ~50% of the flux through this catabolic pathway.

The ale1Δ Mutation Partially Suppresses the Cho Auxotrophy of a pem1Δ pem2Δ Strain at High Temperature—Fig. 6 shows an unexpected growth phenotype that we observed when the pem1Δ pem2Δ ale1Δ strain was grown at a high temperature. The wild-type strain grows with or without Cho at either 30 or 35 °C, as expected. Also expected was the Cho auxotrophy exhibited by the pem1Δ pem2Δ strain grown under these conditions. However, we observe that when the ale1Δ mutation is introduced into the pem1Δ pem2Δ strain, there is a partial suppression of the need for Cho at high temperature. This phenotype does not result in the continued growth of this strain, as
Lyso-PtdCho Metabolism in Yeast

FIGURE 7. Ale1p is promiscuous with regard to the lysophospholipid substrate. Acyltransferase assays were performed with microsomes isolated from the BY4742 wild-type (WT) strain and its isogenic ale1Δ derivative as described under “Experimental Procedures.” The wild-type specific activities for each lysophospholipid are listed, and the mutant value is referenced as a percentage of the wild type. The corresponding wild-type values for lyso-PtdGro and lyso-PtdCho as substrate are ~40 and 80 nmol/min/mg of protein, respectively. The data represent the mean ± S.D. of two independent experiments conducted in triplicate.

FIGURE 8. Model for the lyso-PtdCho ELM pathway. In this scheme lyso-PtdCho is imported into the cell via the P-type ATPase complex Dnf2p/Lem3p present at the plasma membrane. Once internalized, lyso-PtdCho is predominantly acylated to form PtdCho by the membrane-bound O-acyltransferase family member Ale1p, present in the ER and mitochondria (Mito)-associated membrane (MAM) fractions of the cell. Lyso-PtdCho is also a substrate for degradation by Nte1p and possibly other lysophospholipase/phospholipase B (PLB) isoforms. The involvement of a glycerophosphodiesterase (GDE) is also inferred in this scheme. After lyso-PtdCho catabolism, the free Cho is incorporated into PtdCho by the Kennedy pathway. Either the Ale1p acylation pathway or the coupled catabolism/Kennedy pathway is sufficient to support the growth of a pem1Δ pem2Δ mutant. However, the degradative pathway is incapable of restoring cellular PtdCho pools to wild-type levels, indicating that the acyltransferase pathway is predominant.

DISCUSSION

The results presented in this report describe new genetic and biochemical pathways for the assimilation and metabolism of lyso-PtdCho in yeast. This route appears to be part of a broader strategy used by eukaryotes for the synthesis of phospholipids that we propose be called the ELM pathway (Fig. 8). Recent work from our laboratory has described the participation of many of the same molecular components in the utilization of exogenous lyso-PtdEtn (1, 2). Evidence for the operation of this system in mammals appeared more than 40 years ago (18, 19), but the contribution of this pathway to specific defined genes and enzymes has been lacking until now.

The data presented in Fig. 1 clearly identify the genetic basis for lyso-PtdCho transport in yeast as being dependent upon DNF2 and LEM3 genes, with perhaps a very minor contribution from the DNF1 gene. The encoded gene products, Dnf1p and Dnf2p, are P-type ATPases that reside in the plasma membrane and have previously been shown to effect the transport of fluorescent phospholipid analogs at this locale (7). The protein Lem3p is thought to be a chaperone that functions as a β subunit for the ATPases. It is not yet entirely clear how Lem3p is required for the correct localization and activity of Dnf1p and Dnf2p at the plasma membrane. The combination of Dnf1p, Dnf2p, and Lem3p is now also known to be essential for lyso-PtdEtn transport (1). Although the gene products identified in lyso-PtdEtn and lyso-PtdCho transport are the same, there are important differences, most notably in the contribution by PtdIns acyltransferase and lyso-PtdSer acyltransferase activities (17).
Lyso-PtdCho Metabolism in Yeast

Dnf1p. Previously, as much as 30% of the lyso-PtdEtn transport was shown to be attributable to Dnf1p and greater than 60% of the transport was due to Dnf2p. In the present work the contribution of Dnf1p to lyso-PtdCho transport is minimal. These findings suggest the existence of important structural differences between Dnf1p and Dnf2p that are relevant to specific phospholipid recognition and transport activity. The data for both lyso-PtdEtn and lyso-PtdCho uptake definitively demonstrate that Lem3p is an indispensable component of the transport machinery.

Both transport measurements and genetic experiments reveal that the flux of lyso-PtdCho through the transport system is high and biologically relevant. The transport rate for lyso-PtdCho (25 nmol/h/AU) greatly exceeds the rate of PtdCho synthesis required for a cell doubling (~6–7 nmol/h/AU). In addition, the supplementation of pem1Δ pem2Δ strains with lyso-PtdCho restores cell growth. This demonstrates that both the plasma membrane transport and metabolism and intracellular transport of PtdCho derived from the lysophospholipid precursor are sufficient for bypass of the methylation-deficient genetic background. The formation of PtdCho from lyso-PtdCho also provides an immense energy savings for the cell, with each transported lysophospholipid molecule conserving the equivalent of up to 55 ATP molecules that would otherwise be used in the synthesis of fatty acid, the generation of the glycerol backbone, and the synthesis and attachment of the polar headgroup of the lipid.

In contrast to our findings for lyso-PtdCho and lyso-PtdEtn, we could find no evidence for lyso-PtdSer or lyso-PtdIns transport. In biochemical experiments, lyso-Ptd[14C]Ser was not taken up by yeast cells. In genetic experiments, neither lyso-PtdSer nor short-chain species of PtdSer were able to support the growth of the psslΔ cho1Δ mutant yeast strains. This result was unanticipated given the Lem3p-dependent uptake of NBD-PtdSer that was described by Pomorski et al. (7). This discrepancy may indicate a difference in the transport properties of lyso-PtdSer versus its fluorescent NBD analog. Further experiments will be required to resolve this issue. Genetic experiments testing for the presence of lyso-PtdIns transporters in pis1 and ino1Δ strains also failed to provide evidence for the transport and metabolism of this lipid.

Subsequent to transport into the cell, lyso-PtdCho is acylated by the action of Ale1p using an acyl-CoA substrate. The deletion of Ale1p eliminates the acylation of lyso-PtdCho. Previous data has shown that the same enzyme acylates lyso-PtdEtn and lyso-PtdOH (2). Kinetic analyses demonstrate that the Ale1p LPCAT activity utilizes unsaturated acyl-CoAs in preference to saturated acyl-CoAs. This is essentially the same substrate preference that was previously described for the action of Ale1p upon lyso-PtdEtn substrates. In experiments where the enzyme was simultaneously presented with equimolar concentrations of a series of acyl CoAs (C10:0, C14:0, C16:0, C18:0, C16:1, C18:1, C18:2, and C20:4), the acylation of lyso-PtdCho proceeded with preferential esterification of unsaturated fatty acids of 16- and 18-carbon chain length. Although arachidonic acid is among the most efficient fatty acids in rescuing the unsaturated fatty acid auxotrophy of ole1 strains (20, 21), the relatively poor utilization of arachidonoyl-CoA indicates that Ale1p may not play a major role in the esterification of this polyunsaturated fatty acid when supplied to yeast. However, it is noteworthy that the absolute rate of arachidonoyl-CoA esterification, although slow relative to the preferred substrates, is comparable with other anabolic processes of yeast lipid metabolism; therefore, further investigation will be required to delineate the relative contributions of Ale1p and other acyltransferases in the utilization of arachidonic acid and other polyunsaturated fatty acids.

Additional experiments in this study clearly demonstrate that Ale1p has broad specificity for multiple lysophospholipids and functions as the major acyltransferase not only for lyso-PtdCho, lyso-PtdEtn, and lyso-PtdOH but also for lyso-PtdSer, lyso-PtdIns, and lyso-PtdGro. Thus, Ale1p functions as the major lysophospholipid acyltransferase for yeast. A recently published study (17) has also identified lyso-PtdOH, lyso-PtdIns, and lyso-PtdSer as substrates for both Ale1p (referred to as Slc4p in Benghezal et al. (17)) and Slc1p.

The genetic elimination of Ale1p activity renders yeast cells incapable of using lyso-PtdCho for the direct synthesis of PtdCho. Under such conditions the lyso-PtdCho imported into the cell is degraded to form Cho and P-Chol, which can be shunted into the Kennedy pathway that utilizes P-Chol and CDP-Chol as intermediates in PtdCho synthesis. The degradation of the lyso-PtdCho appears to be partly controlled by the lysophospholipase activity of Nte1p, the yeast ortholog of the mammalian neuropathy target esterase. Thus, it appears that lyso-PtdCho transport is fully operative in the absence of Ale1p and that degradative mechanisms prevent the potentially toxic accumulation of lyso-PtdCho from occurring under these circumstances. This also allows for indirect utilization of exogenous lyso-PtdCho. Energetically, this strategy appears to be beneficial since direct esterification of lysophospholipids has the greatest energetic advantage to the organism, but partial catabolism of lysophospholipids and resynthesis also provides significant energetic savings compared with de novo synthesis of all the substituents of a phospholipid.

The regulation of Ale1p is currently not understood. However, the broad lysophospholipid substrate specificity of the enzyme and preference for unsaturated fatty acids suggest it plays a major role in remodeling reactions of yeast phospholipids. It is also an ideal candidate as a founding family member for other eukaryotic acyltransferases involved in remodeling phospholipids. Especially important members of this family will be those enzymes involved in esterifying arachidonic acid in mammalian systems (11, 22, 23). Because arachidonic acid serves as an important precursor for prostanoids, leukotrienes, thromboxanes, and hydroxyeicosatetraenoic acids, the action of Ale1p orthologs is likely to play a significant role in mammalian eicosanoid biology. It is likely that Ale1p orthologs are involved in the synthesis of phospholipid precursors used for arachidonic acid storage and mobilization. It is further plausible that Ale1p orthologs are critical for controlling the size and lifetime of the arachidonic acid pool that is released in response to stimuli (11).

In summary, the data presented in this paper provide three important new findings. The first major finding is that lyso-PtdCho transport is regulated by specific genes and transport-
ers at the plasma membrane. This information extends the recent findings regarding lyso-PtdEtn transport at the plasma membrane. These two observations also establish important new genetic and biochemical tools for studying the structure and function of mammalian P-type ATPases that have been implicated in regulating membrane asymmetry in numerous eukaryotes. The second major finding is that lyso-PtdCho transport is coupled to an acyltransferase activity that works broadly on many phospholipids. The identification of the acyltransferase is likely to have far-reaching implications for membrane phospholipid structure, remodeling, and turnover and eicosanoid signaling. The third major finding is that lysophospholipid transport and acylation constitutes a novel pathway for phospholipid synthesis in eukaryotes that is regulated by several specific genes and capable of bypassing de novo synthetic routes. The lysophospholipid metabolic pathways are likely to facilitate new genetic and biochemical strategies for manipulating other eukaryotic pathways of phospholipid synthesis.

Acknowledgments—We thank Dr. Michael Vasil (University of Colorado Health Sciences Center, Aurora, CO) and Dr. Otto Geiger (Universidad Nacional Autónoma de México, Cuernavaca, Mexico) for providing E. coli strains expressing bacterial PtdCho synthase. We also thank Dr. Susan Henry (Cornell University, Ithaca, NY) for providing a pis1ts strain.

Note added in proof—During the review of this work, two studies (25, 26) have described similar results regarding the activity and lyso-phospholipid promiscuity of the Alelp acyltransferase. The product of the YOR175C gene (Alelp) is referred to in these works as Lpt1p.

REFERENCES
1. Riekhof, W. R., and Voelker, D. R. (2006) J. Biol. Chem. 281, 36588–36596
2. Riekhof, W. R., Wu, J., Jones, J. L., and Voelker, D. R. (2007) J. Biol. Chem. 282, 28344–28352
3. Athenstaedt, K., and Daum, G. (1997) J. Bacteriol. 179, 7611–7616
4. Nagiec, M. M., Wells, G. B., Lester, R. L., and Dickson, R. C. (1993) J. Biol. Chem. 268, 22156–22163
5. Hanson, P. K., Malone, L., Birchmore, J. L., and Nichols, J. W. (2003) J. Biol. Chem. 278, 36041–36050
6. Iwamoto, K., Kobayashi, S., Fukuda, R., Umeda, M., Kobayashi, T., and Ohta, A. (2004) Genes Cells 9, 891–903
7. Pomorski, T., Lombardi, R., Riezman, H., Devaux, P. F., van Meer, G., and Holthuis, J. C. (2003) Mol. Biol. Cell 14, 1240–1254
8. Sohlenkamp, C., de Rudder, K. E., Rohrs, V., Lopez-Lara, I. M., and Geiger, O. (2000) J. Biol. Chem. 275, 18919–18925
9. Gietz, R. D., and Woods, R. A. (2002) Methods Enzymol. 350, 87–96
10. Glick, B. S., and Pon, L. A. (1995) Methods Enzymol. 260, 213–223
11. Zarini, S., Gijon, M. A., Folco, G., and Murphy, R. C. (2006) J. Biol. Chem. 281, 10134–10142
12. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
13. Benning, C., Huang, Z. H., and Gage, D. A. (1995) Arch. Biochem. Biophys. 317, 103–111
14. Fernandez-Murray, J. P., and McMaster, C. R. (2006) Biochim. Biophys. Acta 1774, 331–336
15. Merkel, O., Oskolkova, O. V., Raab, F., El-Toukhhy, R., and Paltauf, F. (2005) Biochem. J. 387, 489–496
16. Schuldiner, M., Collins, S. R., Thompson, N. J., Denic, V., Bhamidipati, A., Punna, T., Ihmels, J., Andrews, B., Boone, C., Greenblatt, J. F., Weissman, J. S., and Krogan, N. J. (2005) Cell 123, 507–519
17. Benghezal, M., Roubaty, C., Veepuri, V., Knudsen, J., and Conzelmann, A. (2007) J. Biol. Chem. 282, 30845–30855
18. Stein, O., and Stein, Y. (1965) Life Sci. 4, 203–208
19. Stein, Y., and Stein, O. (1966) Biochim. Biophys. Acta 116, 95–107
20. Walenga, R. W., and Lands, W. E. (1975) J. Biol. Chem. 250, 9130–9136
21. Walenga, R. W., and Lands, W. E. (1975) J. Biol. Chem. 250, 9121–9129
22. Chilton, F. H., Hadley, J. S., and Murphy, R. C. (1987) Biochim. Biophys. Acta 917, 48–56
23. Chilton, F. H., and Murphy, R. C. (1986) J. Biol. Chem. 261, 7771–7777
24. Trotter, P. J., Pedretti, J., Yates, R., and Voelker, D. R. (1995) J. Biol. Chem. 270, 6071–6080
25. Jain, S., Stanford, N., Bhagwat, N., Seiler, B., Costanzo, M., Boone, C., and Oelkers, P. (2007) J. Biol. Chem. 282, 30562–30569
26. Tamaki, H., Shimada, A., Ito, Y., Ohta, M., Takase, J., Miyashita, M., Miyagawa, H., Nozaki, H., Nakayama, R., and Kumagai, H. (2007) J. Biol. Chem., 282, 34288–34298