Identification and Characterization of the Major Lyso-Phosphatidylethanolamine Acyltransferase in *Saccharomyces cerevisiae*

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Running Title: Lyso-PtdEtn Acyltransferase from Yeast

We recently demonstrated that yeast actively import lyso-phosphatidylethanolamine (lyso-PtdEtn) through the action of plasma membrane P-type ATPases, and rapidly acylate it to form PtdEtn. The predominant lyso-PtdEtn acyltransferase (LPEAT) activity present in cellular extracts is acyl-CoA dependent, but the identity of the gene encoding this activity was unknown. We now demonstrate that a previously uncharacterized open reading frame, YOR175C, encodes the major acyl-CoA dependent LPEAT activity in yeast, and henceforth refer to it as *ALE1* (acyltransferase for lyso-PtdEtn). Ale1p is an integral membrane protein, and is highly enriched in the mitochondria-associated ER membrane (MAM). It is a member of the membrane-bound O-acyltransferase (MBOAT) family, and possesses a dibasic motif at its C-terminus which is likely responsible for Golgi retrieval and retention in the endoplasmic reticulum. An *ale1Δ* strain retains only trace amounts of acyl-CoA dependent LPEAT activity, and strains lacking the capacity for PtdEtn synthesis via the phosphatidylserine decarboxylase and Kennedy pathways show a stringent requirement for both exogenous lyso-PtdEtn and a functional *ALE1* gene for viability. Ale1p catalytic activity has a pH optimum between pH 7-7.5, and a strong preference for unsaturated acyl-CoA substrates.

The yeast *Saccharomyces cerevisiae* has served as a valuable model system for understanding the structural and regulatory aspects of eukaryotic membrane biogenesis. As a consequence, our knowledge of the central pathways of glycerolipid synthesis, transport, and degradation in this organism is quite extensive. However, gaps still remain in the identification of genes encoding proteins that execute key enzymatic steps in yeast lipid metabolism. The lysophospholipid acyltransferases are an example of one such set of enzymes. In eukaryotic systems, these activities are critical for remodeling of membrane lipids for specific purposes, such as the synthesis of dipalmitoyl-PtdCho for lung surfactant (1,2). Recent reports also demonstrate that re-acylation of lysolipids generated by the action of phospholipase A$_2$ enzymes is an important mechanism in the regulation of free arachidonate levels for the production of eicosanoids in neutrophils (3). Despite the importance of lysolipid acyltransferases in membrane lipid remodeling and signaling events, the identities of many of the genes encoding these activities remain unknown. In the yeast system, only a lyso-PtdOH acyltransferase (Slc1p) (4), an acyltransferase involved in glycosyl-phosphatidylinositol anchor remodeling (Gup1p) (5), and a mitochondrial enzyme involved in cardiolipin remodeling (Taz1p) (6,7) have been unequivocally described in both a biochemical and genetic context.

A specific lyso-PtdEtn acyltransferase (LPEAT) protein has not been identified at the molecular level from any organism. However, recent work from our laboratory has revealed a role for LPEAT as part of a new pathway for PtdEtn biosynthesis, which we henceforth refer to as the exogenous lysolipid metabolism (ELM) pathway (8). In this pathway, lyso-PtdEtn is imported by P-type ATPases at the plasma membrane, followed by trafficking of this lipid...
to the site(s) of LPEAT action. The PtdEtn thus formed is capable of fully supplying both the structural requirements of the mitochondria for respiratory growth, and the structural and biosynthetic requirements of the endoplasmic reticulum for PtdCho synthesis. Furthermore, the flux through the ELM pathway and quantities of PtdEtn and PtdCho thus produced are sufficient to satisfy the requirements of rapidly dividing cells for membrane biogenesis, even in the absence of all other PtdEtn synthesis pathways. In this report, we have focused upon: 1) identifying the gene encoding the major LPEAT in yeast, 2) characterizing the biochemical properties of the enzyme, 3) determining the subcellular localization of the enzyme, and 4) elucidating the conditions under which the LPEAT activity is essential for growth. We now present data showing that an uncharacterized yeast gene, YOR175C, which we name ALE1 (acyltransferase for lyso-PtdEtn 1), encodes the major LPEAT activity in yeast. Deletion of this gene abolished essentially all LPEAT activity in cell extracts. Ale1p enzymatic activity is enriched in the mitochondria-associated ER membrane (MAM) and the kinetic properties of this enzyme lead to the preferential placement of an unsaturated fatty acid at the sn-2 position of lyso-PtdEtn. The LPEAT activity of Ale1p is also required for efficient Kennedy pathway-independent utilization of lyso-PtdEtn.

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). Silica-60 TLC plates were from EM Sciences. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL), except acyl-CoA substrates, which were from Sigma. Lyso-PtdEtn (Avanti) 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-PtdEtn was dissolved in 10% (v/v) Tergitol NP-40, filter sterilized, and stored at –20 °C until use. Synthesis of radiolabeled lyso-PtdEtn was as previously described (8).

Yeast culture and genetic manipulations - Yeast strains and their associated genotypes are provided in Tables 1 and 2. Yeast strains with deletions of specific open reading frames (ORFs) 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 ORF of interest. The eviction of target genes from the resultant drug-resistant or prototrophic 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 1% yeast extract, 2% peptone (YP) medium containing either 2% lactate (YPL) or 2% glucose (YPD) as a carbon source, with addition of 40 mg/l adenine, 40 mg/l uracil, and 2 mM Etn (YPDAUE or YPLAUE media). For media containing lyso-PtdEtn, 1% (v/v) Tergitol NP-40 was included, and lyso-PtdEtn was added to the desired concentration from a sterile 25 mM stock solution in 10% (v/v) Tergitol NP-40. In some experiments, defined media was used, consisting of yeast nitrogen base (Difco) at 6.7 g/l, complete amino acid mix, and either glucose to give “SCG” medium, or lactate to give “SCL”. In the lysolipid uptake experiments, 1% Tergitol NP-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 (8).

Directed screening of candidate acyltransferase mutants for loss of LPEAT activity- We identified candidate ORFs in the yeast genome encoding known and putative acyltransferase enzymes (selection of candidates is described in the Results section), and screened their cognate deletion strains (Table 1) for LPEAT activity. Culture of these mutant strains, preparation of homogenates, and LPEAT assays were conducted as previously described (8).

Labelling of wild-type and ale1Δ mutant cells with radioactive lyso-PtdEtn- We carried out lyso-PtdEtn supplementation studies with strains PTY44 (ALE1) and JWY89 (ale1Δ). The strains were initially grown overnight in 5 ml YPDAUE, and a 2 ml aliquot of culture was diluted into 50 ml of fresh YPDAUE medium. Upon reaching an A600 of 0.5, the cultures were
centrifuged and the cells washed twice with SCGT media and suspended in 10 ml of SCGT (final A₆₀₀=2.5). 2 ml of this cell preparation was incubated with 100,000 cpm of 1-oleoyl-2-hydroxyl-3-sn-glycerophospho-[U-¹⁴C]-Etn (diluted with unlabeled compound to 250 µM) for 2 hours. The cells were recovered by centrifugation and washed twice in SCGT and once in water. An aliquot of the washed cells was taken for liquid scintillation spectrometry prior to lipid extraction to determine the total amount of radioactivity taken up by the cells.

Another aliquot of the cells was subjected to lipid extraction by addition of 200 µl water and 300 µl of ethanol in a sealed tube, incubation in a boiling water bath for 45 min, and followed by addition of 4 ml chloroform/methanol (1:1, v/v) and 1.6 ml 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 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 system chloroform/methanol/water (30:9:25:6:18, v/v/v/v/v) and followed by addition of 4 ml chloroform/methanol/water (65/25/4, v/v/v), and quantified as described above. Lyso-PtdOH acyltransferase assays were conducted using the same buffer compositions as for the LPEAT, with 100 µM lyso-PtdOH (Avanti), and 100,000 CPM of 1-¹⁴C]-oleoyl-CoA (American Radiolabeled Chemicals).

LPEAT kinetic assays- Data for pH dependence and acyl-CoA kinetics were generated using a 100,000 xg microsomal membrane fraction isolated from PTY44 cells after lysis by bead-beating in a Bio-Spec apparatus as described previously (8). Homogenization buffer and assay conditions were the same as those used for the determination of LPEAT activity in subcellular fractions. Calculation of the Kᵣ and Vₘₐₓ for the acyl-CoA substrates was carried out by direct fitting of a hyperbola to the kinetic data with the program Hyper (http://www.liv.ac.uk/~jse/software.html).

RESULTS

YOR175C (ALE1) encodes the major lyso-PtdEtn acyltransferase activity in yeast-Our recent work examining the uptake and metabolism of lyso-PtdEtn by yeast (8) prompted us to investigate the acyl-CoA dependent lyso-PtdEtn acyltransferase (LPEAT) activity of yeast cells. We took a reverse-genetic, candidate-gene based approach toward finding the gene(s) specifying the LPEAT activity. Our candidate genes were selected on the basis of similarity to the known acyltransferases Taz1p, Gup1p, Slc1p, Dga1p, and Are1p, and the complete list of knockout strains and corresponding ORF designations that were tested for loss of LPEAT activity are listed in Table 1. Homogenates of these yeast strains harboring deletions in the candidate ORFs were tested for LPEAT activity, and as shown in Fig. 1, deletion of the uncharacterized membrane-bound O-acyltransferase (MBOAT) family member, YOR175C, produces a >95% depletion
in the LPEAT activity of the homogenate relative to the wild-type BY4742 strain. All of the other candidates we tested had LPEAT activities comparable to the wild-type (not shown). This led us to designate the YOR175C gene as ALE1, due to its activity as an acyltransferase for lyso-PtdEtn. We next constructed a strain for functional analysis of the ALE1 gene by introducing an ale1Δ allele into the PTY44 background (psd1Δ psd2Δ) to give strain JWY89 (psd1Δ psd2Δ ale1Δ). The LPEAT activity of PTY44 was essentially identical to that of BY4742 (Fig. 1) and as with the BY4742 background, deletion of the ALE1 gene in the psd1Δ psd2Δ mutant essentially abolished the LPEAT activity of the extract. This series of experiments also revealed that, when an acyl-CoA generating system is used to drive the reaction, the SEY6210 wild-type background has a 2-3 fold higher LPEAT specific activity relative to the BY4742 background, and that the PTY44 (psd1Δ psd2Δ) background was slightly higher still (data not shown), perhaps indicating an increased expression of acyl-CoA synthetase activity. For this reason, further experiments were conducted in strains derived from the PTY44 background, except where noted. PTY44 also serves as the Etn/lyso-PtdEtn auxotrophic background for the genetic and physiological studies of lyso-PtdEtn metabolism that are described below.

In silico and genome-scale analyses of Ale1p: After establishing that YOR175C (ALE1) encodes the major LPEAT activity in yeast, we examined its primary sequence using various prediction algorithms to gain information about its putative localization and function. TargetP (13) (http://www.cbs.dtu.dk/services/TargetP/) failed to predict a secretory signal peptide or a mitochondrial targeting sequence, however the genome-scale GFP tagging localization analysis of Huh et al. (14) indicated that Ale1p is localized to the endoplasmic reticulum. This preliminary localization analysis was corroborated by the highly sensitive TM-HMM algorithm (http://www.cbs.dtu.dk/services/TMHMM-2.0/) that predicts at least 7 transmembrane helices, and by the presence of a dilysine motif at the C-terminus of Ale1p, which typically acts as a Golgi-retrieval and ER retention signal for membrane proteins through interaction with the coatomer complex (15). This data, as well as a multiple sequence alignment with other acyltransferases, is given in Fig. 2. Additional evidence regarding the function of Ale1p comes from the epistatic mini-array profiling data of Schuldiner et al. (16). This study detected a profound growth defect in the ale1Δ slc1Δ double mutant, the implications of which are described below.

Biochemical properties of ALE1: We characterized the basic biochemical and kinetic parameters of the enzyme with lyso-PtdEtn as substrate, including a pH versus activity profile, and determination of the Km and Vmax for various acyl-CoA substrates. As shown in Fig. 3A, the enzymatic activity is relatively insensitive to changes in pH, and the rate versus pH profile shows a broad maximum between 6.5-7.5. We next examined the acyl-CoA substrate preference of Ale1p by conducting kinetic assays with various acyl-CoA species. We determined the Km and Vmax for the acyl-donors by varying the substrate concentrations and measuring initial rates of the conversion of lyso-PtdEtn to PtdEtn, using the most active microsomal fraction as a source of enzyme. We examined oleoyl- (18:1), palmitoleoyl- (16:1), palmitoyl- (16:0), and myristoyl- (14:0) CoA substrates, given that these are the most abundant fatty-acyl species in yeast phospholipids (17). As shown in Fig. 3B, the Km values for the saturated acyl-CoA substrates were significantly lower than the unsaturated substrates. For palmitoyl and myristoyl-CoA, these values were 0.9 and 0.4 μM respectively, and for oleoyl- and palmitoleoyl-CoA species, the Km values were 10 and 17 μM, respectively. Conversely, the Vmax values were much higher for the unsaturated species (38 and 44 nMol/min/mg protein for oleoyl- and palmitoleoyl-CoA, versus 3.7 and 1.2 nMol/min/mg protein for palmitoyl- and myristoyl-CoA. The figure clearly shows that, at any given acyl-CoA concentration, the initial rate with the unsaturated substrates was significantly higher than the saturated substrates. While the specificity constant (Km/Vmax) for all substrates was similar, the kinetic data clearly indicate that, given equivalent concentrations of the different acyl-CoA species, Ale1p preferentially esterifies an unsaturated acyl-chain at the sn-2 position of lyso-PtdEtn.

Ale1p acts as the major lyso-PtdOH acyltransferase in yeast: The product of the SLC1 gene has been shown to act as a lyso-PtdOH acyltransferase (LPAAT) (4,18).
However, haploid slc1Δ mutants are viable, which necessitates that another, redundant LPAAT activity be present in the cell. The epistatic mini-array profile of Schuldiner et al. (16) revealed a synthetic-lethal interaction between the ale1Δ and slc1Δ mutations. One explanation for this result is that Ale1p provides the redundant lyso-PtdOH acyltransferase activity in the slc1Δ mutant, thus rendering the double mutant incapable of making PtdOH. To test this hypothesis, we assayed LPAAT activity in the BY4742 wild-type, and the isogenic slc1Δ and ale1Δ mutants. As shown in Fig. 4, relative to the wild-type parent, the ale1Δ mutant shows a ~85% decrease in the total LPAAT activity of a cellular homogenate. Surprisingly, we detected no loss of LPAAT activity in the slc1Δ mutant, which may indicate that the Ale1p LPAAT activity is up-regulated in the absence of the Slc1p activity. Taken together with the synthetic lethality data (16), this result strongly suggests that Ale1p and Slc1p act as redundant LPAAT activities, with Ale1p accounting for the majority of the catalytic units.

**Subcellular distribution of Ale1p activity** The TM-HMM prediction described above indicated the presence of at least 7-transmembrane helices, indicating an extremely high probability that Ale1p is a membrane bound enzyme. Analysis with the localization prediction algorithm TargetP (13) indicated the lack of either a canonical mitochondrial or secretory targeting signal, while the presence of a C-terminal di-basic motif was indicative of an endoplasmic reticulum protein (15). These results suggested that Ale1p activity might be associated with an ER-derived microsomal fraction. Given the ability of PtdEtn derived from lyso-PtdEtn to preferentially replenish the mitochondrial PtdEtn pool, we also entertained the possibility that Ale1p, like some other enzymes of phospholipid biosynthesis, would be enriched in the ER-derived mitochondria-associated membranes (MAM). To test this hypothesis, we prepared sucrose-gradient purified mitochondria, MAM, microsomes, and cytosol, and measured the specific activity of the Ale1p LPEAT in these fractions. Data from fractions isolated from isogenic wild-type and ale1Δ mutant strains were compared, and in these purified fractions, the LPEAT activity of the ale1Δ mutant was <2% of the wild-type level. This level of activity is at the limit of detection under our assay conditions, hence only data for fractions from the wild-type type is given. Fig. 5C shows that the MAM fraction is the most highly enriched for LPEAT activity, and gives 2-3 fold higher specific-activity than the microsomal and mitochondrial fractions, which are similar in their LPEAT specific activity. Essentially no activity was detectable in the cytosolic fraction. To verify the purity of the respective preparations, the purified membrane fractions were also assayed for PtdSer- and PtdIns-synthase (PSS and PIS, respectively) activities, and these results are also presented in Fig. 5. PSS and PIS activities are known to be enriched in the MAM fraction (19), with the remaining activity present in microsomes and only traces of activity in gradient-purified mitochondria. Our data recapitulate these published activity profiles for PSS and PIS (Fig. 5A and B.), and thus establish the relative purity of our subcellular fractions. Given that there is substantial LPEAT activity in the purified mitochondrial fraction, the PSS and PIS data also show that the Ale1p LPEAT activity that we detect in the mitochondrial fraction does not arise from contaminating microsomes. This fact provides strong evidence that the Ale1p enzyme is preferentially localized in a sub-fraction of the MAM that is tightly bound to the mitochondrial outer membrane, such that it co-purifies with mitochondria on sucrose gradients.

**In the absence of Ale1p, lyso-PtdEtn is degraded to water-soluble products.** The data presented in Fig. 6 demonstrate the catabolism of lyso-PtdEtn in the absence of Ale1p. PtdSer decarboxylase deficient yeast strains either containing (PTY44), or lacking (JWY89), the Ale1p acyltransferase were incubated for 2 hours with labeled lyso-Ptd[14C]Etn. The cells were harvested and washed extensively, followed by lipid extraction and partitioning of the radiolabeled products between aqueous and organic phases. As shown in Fig. 6A and B, the ale1Δ mutation results in a 4- to 5-fold higher proportion of the imported label being present in the aqueous fraction. For the ALE1 wild-type strain, only about 6-7 % of the total label associated with the cells was present in the aqueous fraction, compared with ~30-35% in the ale1Δ mutant.

These findings are consistent with lyso-PtdEtn entering either of two pathways after internalization (see Fig. 8); specifically, acylation via Ale1p to form PtdEtn, or degradation to form water-soluble products.
Upon fractionation of these water soluble compounds (Fig 6C), the majority of the radiolabel (~70%) was found in glycerophosphoethanolamine (Gro-P-Etn), and phosphoethanolamine (P-Etn; ~20%) with the remainder split between Etn and CDP-Etn. This indicates that a phospholipase B activity deacylates the lyso-PtdEtn in the absence of the Ale1p enzyme, producing Gro-P-Etn. This compound is then hydrolyzed by a glycerophosphodiesterase to form Etn, followed by re-incorporation of the Etn into PtdEtn via the CDP-Etn dependent Kennedy pathway (Fig. 8). Consistent with the other radiolabeling data, the rate of PtdEtn formation in the ale1Δ mutant (Fig. 6D) is significantly slower than in the wild-type ALE1 strain. This provides additional evidence that the ale1Δ mutant must first degrade lyso-PtdEtn through a catabolic pathway and subsequently reincorporate the label by the Kennedy pathway. This would lead to the observed slower rate of PtdEtn synthesis and larger pool-sizes for the water-soluble metabolic intermediates. This would also make the Kennedy pathway essential for the growth of a psd1Δ psd2Δ ale1Δ strain. We designed specific genetic experiments to test this latter hypothesis.

Ale1p is essential for Kennedy-pathway independent utilization of lyso-PtdEtn- To further characterize the in vivo function of the ALE1 gene, we examined whether the presence of Ale1p is necessary for the utilization of lyso-PtdEtn by Etn auxotrophs. Fig. 7 addresses this issue through growth assays of the appropriate strains on SC lactate plates containing either no supplement, 5 mM Etn, or 0.25 mM LPE. As shown previously (8), the wild-type strain SEY6210 shows no growth impairment on any media. The Etn auxotrophic psd1Δ psd2Δ strain requires supplementation with either Etn or lyso-PtdEtn. When an ect1Δ mutation is introduced to the psd1Δ psd2Δ background, there is a stringent requirement of lyso-PtdEtn for growth on minimal medium. Conversely, the psd1Δ psd2Δ lem3Δ mutant uses Etn, but is defective in using lyso-PtdEtn, due to the requirement for Lem3p in the lyso-PtdEtn transport process (8). Since Lem3p and Ale1p act sequentially in the ELM pathway for PtdEtn synthesis, we expected Ale1p to also be essential for the efficient utilization of lyso-PtdEtn. However, deletion of ALE1 in a psd1Δ psd2Δ background had no effect on the ability of this strain to grow with lyso-PtdEtn as the sole PtdEtn source.

We entertained two possibilities to explain this unexpected result: either 1) a redundant acyltransferase, of relatively low catalytic activity can acylate lyso-PtdEtn to form PtdEtn independently of Ale1p, or 2) blockage of the LPEAT reaction by the ale1Δ mutation results in the degradation of lyso-PtdEtn and re-assimilation of the Etn and/or P-Etn by the Kennedy pathway. We tested these possibilities by deleting the ECT1 gene in the psd1Δ psd2Δ ale1Δ background. Ect1p functions as the P-Etn cytidylyltransferase, an essential step in the Etn branch of the Kennedy pathway (20). Deletion of ECT1 would block the recycling of lyso-PtdEtn degradation products (i.e. Etn or P-Etn) back into PtdEtn as described in the previous section. We previously showed that Ect1p is not required for the growth of strains supplemented with lyso-PtdEtn as the sole source of PtdEtn (8). When the ect1Δ mutation was introduced into the psd1Δ psd2Δ ale1Δ background, the strain phenocopied the psd1Δ psd2Δ lem3Δ mutant, and failed to grow on lyso-PtdEtn as the major PtdEtn and PtdCho precursor. This indicates that a functional Kennedy pathway is necessary for the growth of the psd1Δ psd2Δ ale1Δ strain on lyso-PtdEtn. From this latter finding, we conclude that Ale1p is the sole acyltransferase activity necessary for Kennedy pathway-independent utilization of lyso-PtdEtn, and that in the absence of Ale1p, lyso-PtdEtn is degraded and re-assimilated by the Kennedy pathway.

Although the psd1Δ psd2Δ ale1Δ ect1Δ mutant is inviable on minimal media with lyso-PtdEtn or Etn supplementation, the strain is viable on YPLAUE medium containing 5 mM Etn, 5 mM Cho, and 0.25 mM lyso-PtdEtn (Fig. 7, “YPL++”). Under these conditions, the bulk of PtdCho synthesis would come from the Cho specific Kennedy pathway, and the minimal amount of PtdEtn needed to support growth of the cell would likely come from the action of the Cct1p enzyme, which exhibits low but significant levels of activity with P-Etn as a substrate (21). While the total amount of PtdEtn thus synthesized would be only a small fraction of that normally present in the cell, previous studies (22,23) have shown that only a trace amount of PtdEtn is necessary for growth, provided that PtdCho is produced from exogenous Cho provided in the medium. The
conditional lethality of this quadruple mutant strain provides a new tool for the heterologous expression and functional characterization of acyltransferases from other species, as discussed below.

DISCUSSION

The lysophospholipid acyltransferases have long been regarded as critical enzymes in several processes of significance to human health and physiology. For example, remodeling of PtdCho associated with lung surfactant synthesis (1,2) and the regulation of free arachidonate levels for eicosanoid synthesis (3) are directly linked with lysophospholipid acyltransferase activities. As part of our broader investigation of interorganellar lipid trafficking, a recent report from this laboratory (8) implicated an uncharacterized lyso-PtdEtn acyltransferase (LPEAT) activity as part of the “exogenous lysolipid metabolism” (ELM) pathway for the net synthesis of PtdEtn. The initial report, coupled with this work, provides a clear picture of the lyso-PtdEtn branch of the ELM pathway. A current model, set in the broader context of cellular PtdEtn and PtdCho metabolism, is given in Fig. 8. In this scheme, yeast cells import lyso-PtdEtn via the plasma-membrane P-type ATPases Dnf1p and Dnf2p, in consort with their non-catalytic β-subunit Lem3p. Once internalized, the lysolipid is rapidly acylated to form PtdEtn by the acyl-CoA dependent LPEAT. Since this latter enzymatic activity was not directly associated with a specific gene in yeast or any other organism, we sought to identify and study the gene(s) encoding the LPEAT of the yeast ELM pathway.

We took a reverse-genetic approach to address the problem of identification of the LPEAT, and focused on genes encoding acyltransferase-like proteins. We used mutants from the whole-genome deletion collection to measure the specific activity of LPEAT in the various acyltransferase deletion backgrounds. The strain bearing a deletion of the uncharacterized MBOAT family member YOR175C gave a nearly total depletion of LPEAT activity, leading us to refer to this gene as ALE1 (acyltransferase for lyso-PtdEtn).

In silico analysis and high-throughput experiments from other laboratories previously showed that Ale1p was likely to be an integral membrane protein of the ER (14). To test this hypothesis, we measured the specific Ale1p activity in purified mitochondria, mitochondria-associated ER membranes (MAM), microsomes, and cytosol. The specific activity in the MAM fraction was greatest, and was approximately 2-3 fold higher than that found in microsomes and mitochondria, which had roughly equivalent specific activities. The cytosol (100,000 g supernatant) contained no detectable activity. This distribution indicates that, like the PtdSer and PtdIns synthases, Ale1p is preferentially located in an ER fraction that associates with the mitochondrion. The specific activity of Ale1p in purified mitochondria was relatively high compared with PSS and PIS activities, which may indicate that Ale1p is localized in a sub-domain of MAM that co-sediments with mitochondria during sucrose gradient purification. The tight mitochondrial association of Ale1p that we observe fits well with our previous biochemical and genetic data, which showed that lyso-PtdEtn is much more efficient than free Etn at restoring the mitochondrial PtdEtn pools of psd1Δ mutants to wild-type levels (8).

After defining the enzymatic activity and localization of Ale1p, we tested for conditions under which its activity is essential for growth. Initially we examined whether a strain bearing the psd1Δ psd2Δ ale1Δ genotype would grow on lyso-PtdEtn as the sole PtdEtn source. To our surprise, the strain grew, indicating either the presence of a redundant LPEAT activity that is capable of producing PtdEtn directly, or the action of a catabolic pathway whereby lyso-PtdEtn is degraded to produce Etn that is re-incorporated by the Kennedy pathway. This latter pathway was active as deduced from the finding that the psd1Δ psd2Δ ale1Δ ctt1Δ strain was not viable under conditions where lyso-PtdEtn was the major source of PtdEtn and PtdCho. Together, the data support a model (shown in Fig. 8) for the ELM pathway in which lyso-PtdEtn can be directed into one of two pathways: acylation by Ale1p, or degradation by unknown catabolic enzymes. The degradative pathway is likely carried out by the ER localized PLB enzyme Nte1p or one of the other PLB enzymes. A more precise mechanistic definition of the degradative pathway will require additional studies with psd1Δ psd2Δ ale1Δ strains. Under standard conditions, either of these pathways
(direct acylation or degradation and re-synthesis by the Kennedy pathway) is sufficient to support growth of a psd1Δ psd2Δ strain with lyso-PtdEtn supplementation. However, based on our previous radiolabeling data measuring the relative rate of turnover of lyso-PtdEtn (and the PtdEtn and PtdCho thus derived) (8), it appears that the Ale1p branch is the preferred route for lyso-PtdEtn incorporation, with the catabolic branch being activated in its absence.

Similar to some other acyl-CoA dependent acyltransferases, Ale1p is a member of the membrane bound O-acyltransferase (MBOAT) family of enzymes (24). Other members of this family include the cardiolipin remodeling enzyme, Tafazzin, which is ubiquitous in the mitochondria of eukaryotes (6,7), as well as the recently characterized GPI-anchor remodeling enzyme, Gup1p (5), which is the closest yeast homolog of Ale1p. Other yeast MBOAT members include Are1p and Are2p (25), enzymes which esterify free sterols with long chain fatty acids, thus producing steryl esters which are stored in the lipid-droplet. Interestingly, a family of uncharacterized MBOAT family members is annotated in the human genome. These genes are designated as MBOAT1-5, with homologs of these being found in all mammalian genomes. Of these, human MBOAT2 (included in the multiple sequence alignment of Fig. 2) is most similar to Ale1p, and may represent the functional ortholog of the yeast LPEAT. Further studies to elucidate the function of these uncharacterized mammalian proteins will be aided by the availability of the yeast ale1Δ mutant as an LPEAT-deficient expression host. In addition, the conditional inability of the psd1Δ psd2Δ ect1Δ ale1Δ mutant to use lyso-PtdEtn as a growth substrate will also allow for the functional characterization of animal and plant homologs based on an auxotrophic assay. Specifically, complementation of the lyso-PtdEtn non-utilization phenotype will serve as a powerful indicator of gene function.

In summary, we have isolated and characterized the gene encoding the major acyl-CoA dependent lyso-PtdEtn acyltransferase in the yeast ELM pathway. This report provides the first molecular identification of a gene encoding this activity from any organism. The enzyme, Ale1p, shows a peculiar dual-localization to microsomal and mitochondrial membranes, prefers unsaturated acyl-CoA species, and is essential for the Kennedy-pathway independent use of lyso-PtdEtn as a growth substrate for Etn auxotrophic strains.
REFERENCES

1. Chen, X., Hyatt, B. A., Mucenski, M. L., Mason, R. J., and Shannon, J. M. (2006) Proc Natl Acad Sci U S A 103(31), 11724-11729
2. Nakanishi, H., Shindou, H., Hishikawa, D., Harayama, T., Ogasawara, R., Suwabe, A., Taguchi, R., and Shimizu, T. (2006) J Biol Chem 281(29), 20140-20147
3. Zarini, S., Gijon, M. A., Folco, G., and Murphy, R. C. (2006) J Biol Chem 281(15), 10134-10142
4. Nagiec, M. M., Wells, G. B., Lester, R. L., and Dickson, R. C. (1993) J Biol Chem 268(29), 22156-22163
5. Bosson, R., Jaquenoud, M., and Conzelmann, A. (2006) Mol Biol Cell 17(6), 2636-2645
6. Testet, E., Laroche-Traineau, J., Noubhani, A., Coulon, D., Bunoust, O., Camougrand, N., Manon, S., Lessire, R., and Bessoule, J. J. (2005) Biochem J 387(Pt 3), 617-626
7. Xu, Y., Malhotra, A., Ren, M., and Schlame, M. (2006) J Biol Chem 281(51), 39217-39224
8. Riekhof, W. R., and Voelker, D. R. (2006) J Biol Chem 281(48), 36588-36596
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. Carman, G. M., and Bae-Lee, M. (1992) Methods Enzymol 209, 298-305
12. Carman, G. M., and Fischl, A. S. (1992) Methods Enzymol 209, 305-312
13. Emanualsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) J Mol Biol 300(4), 1005-1016
14. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Nature 425(6959), 686-691
15. Cossen, P., and Letourneur, F. (1994) Science 263(5153), 1629-1631
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(3), 507-519
17. Choi, J. Y., Stukey, J., Hwang, S. Y., and Martin, C. E. (1996) J Biol Chem 271(7), 3581-3589
18. Athenstaedt, K., and Daum, G. (1997) J Bacteriol 179(24), 7611-7616
19. Gaigg, B., Simbeni, R., Hrastnik, C., Paltauf, F., and Daum, G. (1995) Biochim. Biophys. Acta 1234, 214-220
20. Nakashima, A., Hosaka, K., and Nikawa, J. (1997) J Biol Chem 272(14), 9567-9572
21. Tsukagoshi, Y., Nikawa, J., and Yamashita, S. (1987) Eur J Biochem 169(3), 477-486
22. Birner, R., Burgermeister, M., Schneiter, R., and Daum, G. (2001) Mol Biol Cell 12(4), 997-1007.
23. Storey, M. K., Clay, K. L., Kutateladze, T., Murphy, R. C., Overduin, M., and Voelker, D. J. (2001) J. Biol. Chem 276(51), 48539-48548
24. Hofmann, K. (2000) Trends Biochem Sci 25(3), 111-112
25. Yang, H., Bard, M., Bruner, D. A., Gleson, A., Deckelbaum, R. J., Aljinovic, G., Pohl, T. M., Rothstein, R., and Sturley, S. L. (1996) Science 272(5266), 1353-1356
26. Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El Bakkoury, M., Foury, F., Friend, S. H., Gentalen, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D. J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J. L., Riles, L., Roberts, C. J., Ross-MacDonald, P., Scherens,
B., Snyder, M., Sookhai-Mahadeo, S., Storms, R. K., Veronneau, S., Voet, M., Volckaert, G., Ward, T. R., Wysocki, R., Yen, G. S., Yu, K., Zimmermann, K., Philippsen, P., Johnston, M., and Davis, R. W. (1999) *Science* 285(5429), 901-906

27. Trotter, P. J., Pedretti, J., Yates, R., and Voelker, D. R. (1995) *J. Biol. Chem.* 270, 6071-6080
FOOTNOTES

*This work was supported by National Institutes of Health Grants 2R37-GM32453 (to D.R.V) and 1F32-GM076798 (to W.R.R.)

2The abbreviations used are: CDP-Cho, cytidinediphosphate-choline; CDP-DAG, cytidinediphosphate-diacylglycerol; CDP-Etn, cytidinediphosphate-ethanolamine; Cho, Choline; CoA, Coenzyme A; DAG, sn-1,2-diacylglycerol; ELM, exogenous lysolipid metabolism pathway; Etn, ethanolamine; Gro-P-Etn, sn-glycero-3-phosphoethanolamine; lyso-PtdEtn, 1-acyl-2-hydroxyl-sn-glycero-3-phosphoethanolamine; LPEAT, lyso-PtdEtn acyltransferase; MBOAT, membrane bound O-acyltransferase; P-Cho, phosphorylcholine; P-Etn, phosphorylethanolamine; PtdCho, phosphatidylecholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

ACKNOWLEDGMENT

We thank Jae-Yeon Choi for helpful discussions and advice on subcellular fractionation.
Table 1. **Strains used for directed genetic screening for defects in LPEAT activity.** All strains were generated as part of a genome-wide functional analysis study (26) and were purchased from Open Biosystems (Huntsville, AL).

| Strain   | Genotype                                      |
|----------|-----------------------------------------------|
| BY4742   | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*           |
| ybr042cΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ybr042cΔ::KAN^R* |
| ypr140wΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 taz1Δ::KAN^R* |
| yor298wΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mum3Δ::KAN^R* |
| ydl052cΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 slc1Δ::KAN^R* |
| ydr018cΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ydr018cΔ::KAN^R* |
| ygl084cΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gup1Δ::KAN^R* |
| ypl189wΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gup2Δ::KAN^R* |
| yor175cΔ | *Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ale1Δ::KAN^R* |

Table 2. **Strains used for functional characterization of Ale1p.**

| Strain   | Genotype                                      | Source                  |
|----------|-----------------------------------------------|-------------------------|
| SEY6210  | *Mat α trpl leu2 ura3 lys2 his3 suc2*          | Scott Emr, UCSD         |
| PTY44    | *Mat α trpl leu2 ura3 lys2 his3 suc2 psd1Δ::TRP1 psd2Δ::HIS3* | (27)                    |
| WRY8     | *Mat α trpl leu2 ura3 lys2 his3 suc2 psd1Δ::TRP1 psd2Δ::HIS3 ect1Δ::KAN^R* | (8)                     |
| JWY89    | *Mat α trpl leu2 ura3 lys2 his3 suc2 psd1Δ::TRP1 psd2Δ::HIS3 ale1Δ::HYG^R* | This study              |
| WRY90    | *Mat α trpl leu2 ura3 lys2 his3 suc2 psd1Δ::TRP1 psd2Δ::HIS3 ale1Δ::HYG^R ect1Δ::KAN^R* | This study              |
| WRY28    | *Mat α trpl leu2 ura3 lys2 his3 suc2 psd1Δ::TRP1 psd2Δ::HIS3 lem3Δ:: KAN^R* | (8)                     |
Fig. 1- **YOR175C (ALE1)** encodes the major lyso-PtdEtn acyltransferase in yeast. Homogenates from the wild-type BY4742 strain and an isogenic strain harboring a deletion of the *YOR175C* gene (*ale1Δ*) were tested for LPEAT activity as described in Experimental Procedures. In order to confirm this in a genotype relevant for functional characterization, we introduced an *ale1Δ* mutation into the PTY44 (*psd1Δ psd2Δ*) background and repeated the measurement. Data presented are a triplicate determination (± S.D.) from a representative one of four experiments.

Fig. 2- **YOR175C is a member of the MBOAT family.** A. The uncharacterized membrane protein encoded by *YOR175C* aligned with representative members of the membrane-bound *O*-acyltransferase family. Included in the analysis are an uncharacterized human MBOAT protein (HsMBOAT2), the GPI anchor remodeling enzyme Gup1p, and the yeast sterol acyltransferase Are1p. Identities and similarities between two or more proteins are highlighted as white text on a black background. B. A model for the topology of Ale1p based on the TM-HMM algorithm. This reflects the proposed active site orientation in which the loops, which presumably provide the substrate binding and catalytic functions, are oriented toward the cytosol.

Fig. 3- **Enzymatic properties of Ale1p.** A. The activity of Ale1p was measured across a pH range of 5.5 to 8 using HEPES as buffer for pH 5.5-7 (diamonds), and Tris-Cl as buffer for pH 7-8 (squares). B. Michaelis-Menten kinetics of Ale1p were determined by varying the concentration of acyl-CoA substrates and determining the initial rate for the formation of PtdEtn. *K_M* and *V_max* values were determined by direct fitting of a hyperbola to the experimental data. Data presented in A and B are from a representative one of three experiments conducted with washed microsomal membranes.

Fig. 4- **Lyso-PtdOH assays.** Homogenates of the wild-type BY4742 and isogenic *ale1Δ* and *slc1Δ* strains were assayed for lyso-PtdOH activity as described in the text. Data presented are the average of three separate experiments conducted in triplicate, and error bars represent standard deviation.

Fig. 5- **Subcellular distribution of Ale1 activity.** Cells of the wild-type strain BY4742 were fractionated into microsomes, purified mitochondria, and mitochondria-associate membranes. A. PtdSer synthase and B. PtdIns synthase activities of these fractions were determined as marker enzymes of known distribution. Assays of LPEAT activity (C.) were conducted as described in the text, and specific enzyme activities are shown. Data presented are a triplicate determination (± S.D.) of a representative one of three fractionation experiments.

Fig. 6- **Catabolism of lyso-PtdEtn is enhanced in the ale1Δ mutants.** Cultures of a representative Etn auxotrophic wild-type strain (PTY44) and its isogenic *ale1Δ* mutant (JWY89) were incubated with radiolabeled lyso-PtdEtn for 2 h, followed by lipid extraction and liquid scintillation counting of the aqueous phase, and quantification of both the water-soluble and lipid products by TLC. A. Radioactivity associated with the washed cells at the end of the uptake period (“Total”) and the aqueous phase after lipid extraction (“Aqueous fraction.”) B. The data in (A.) expressed as the percentage of the total cell-associated radioactivity that was present as aqueous Etn metabolites. C. Water soluble metabolites of lyso-PtdEtn were fractionated by TLC and quantified, and expressed as a percentage of the total radioactivity in the aqueous fraction. D. Quantification of PtdEtn synthesis from radiolabeled lyso-PtdEtn by lipid extraction and TLC. All data are from a representative one of three experiments conducted in triplicate, and the values are means ± standard deviation.

Fig. 7- **Ale1p is required for lyso-PtdEtn utilization in the absence of the Kennedy pathway.** Fivefold serial dilutions of the indicated strains were spotted onto solid media as described in Experimental Procedures. Plates were incubated for 3 to 4 days and photographed. *YPL++* medium is YPLAUE (See Experimental Procedures) supplemented with 1% Tergitol NP-40, 5 mM Etn, 5 mM Cho, and 0.25 mM lyso-PtdEtn.
Fig. 8- Model of the ELM pathway in the context of PtdEtn and PtdCho metabolism. In the current model of the exogenous lysolipid metabolism (ELM) pathway: 1) Lyso-PtdEtn is imported through the action of the plasma membrane P-type ATPases Dnf1p and Dnf2p, in complex with their non-catalytic subunit Lem3p. 2) Ale1p is the lyso-PtdEtn acyltransferase of the yeast ELM pathway. 3) Ale1p activity is localized to both ER membranes and MAM. 4) In the absence of Ale1p, a catabolic pathway is operative that releases Etn via the action of phospholipase B ("PLB") and glycerophosphodiesterase ("GDE") enzymes. The free Etn from this catabolic pathway is subsequently re-incorporated into PtdEtn by the Kennedy pathway.
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Identification and characterization of the major lyso-phosphatidylethanolamine acyltransferase in Saccharomyces cerevisiae
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J. Biol. Chem. published online July 24, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M705256200

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