Saccharomyces cerevisiae lysophospholipid acyltransferase, Lpt1, requires Asp146 and Glu297 for catalysis

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Running title: S. cerevisiae Lpt1p requires Asp146 and Glu297 for catalysis

Abbreviations:
acyl-CoA cholesterol acyltransferase (ACAT), acyl-CoA diacylglycerol acyltransferase (DGAT), 4,4V-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), grehin O-acyltransferase (GOAT), acyl-CoA lysophospholipid acyltransferase acyl-CoA (LPCAT), lysophosphatidylcholine acyltransferase (LPLAT), membrane bound O-acyltransferase (MBOAT), N-ethylmaleimide (NEM), phenylgloxyl (PG), phenylmethylsulfonfylfluoride (PMSF), phosphatidylcholine (PC), Workman’s Reagent K (WRK)
ABSTRACT

The esterification of lysophospholipids contributes to phospholipid synthesis, remodeling, and scavenging. Acyl-CoA dependent lysophospholipid acyltransferase activity with broad substrate utilization is mediated by S. cerevisiae Lpt1p. We sought to identify Lpt1p active site amino acids besides the histidine conserved among homologs and repeatedly found to be required for catalysis. In vitro Lpt1p assays with amino acid modifying agents implicated aspartate, glutamate, and lysine as active site residues. Threonine and tyrosine were not ruled out. Aligning the primary structures of functionally characterized LPT1 homologs from fungi, plants, and animals identified eleven conserved aspartate, glutamate, lysine, threonine, and tyrosine residues. Site directed mutagenesis of the respective codons showed that changing D146 and E297 abolished activity without abolishing protein expression. The mechanism of Lpt1p was further analyzed using monounsaturated acyl-CoA species with different double bond positions. Delta 6 species showed the highest catalytic efficiency. We propose that D146 and E297 act in conjunction with H382 as nucleophiles which attack the hydroxyl group in lysophospholipids in a general acid/base mechanism. This sequential mechanism provides a precedent for other members of the membrane bound O-acyltransferase family. Also, Lpt1p optimally orients acyl-CoA substrates with 7.5 angstroms between a double bond and the thioester bond.

Supplementary keywords

enzymology/enzyme mechanisms, phospholipids/biosynthesis, phospholipids/metabolism, fatty acid/transferase, DGAT, active site; reaction mechanism, membrane bound O-acyltransferase
INTRODUCTION

Acyl-CoA dependent acyltransferases mediate the formation of ester bonds between acyl chains and the hydroxyl groups of acyl acceptors. Acyl-CoA species range in length and degree of saturation and acyl acceptors range from lipids such as lysophospholipids and diacylglycerol to proteins such as grehlin and Wnt. In the mouse genome, there are three distinct phylogenetic clusters of acyltransferase genes including 32 genes in all (1). Whether this array evolved primarily to address the many possible combinations of substrates remains to be determined. Whether a common reaction mechanism is shared among the phylogenetic clusters is also not known since to date, no acyl-CoA dependent acyltransferase has had its reaction mechanism unequivocally determined. We postulate that within each cluster, the acyltransferase reaction mechanism was established in the common ancestor. In that case, establishing the mechanism of one member of a cluster will likely predict the mechanism for all members of that cluster.

One acyltransferase phylogenetic cluster is the membrane bound O-acyltransferase (MBOAT) (2) family, sometimes called the acyl-CoA cholesterol acyltransferase (ACAT) family for its founding member (3). Alignments of the primary structure of family members and subsequent site directed mutagenesis have identified only a single, strictly conserved histidine with a likely, direct role in catalysis. Mutation of this histidine abolished activity in human ACAT1 (H460) (4), African green monkey ACAT2 (H438) (5), yeast acyl-CoA lysophospholipid acyltransferase (Lpt1p) (H382) (6), murine acyl-CoA diacylglycerol acyltransferase (DGAT1) (H426) (7), and mouse grehlin O-acyltransferase (GOAT) (H338) (8). Mutation of a conserved asparagine (N307) was also critical for mouse GOAT activity (8) but altering the conserved residue (N306) in mouse porcupine, a homolog that esterifies the Wnt protein, only decreased activity by 20% (9). The mouse lysophosphatidylcholine acyltransferase 3 (LPCAT3) required a strictly conserved phenylalanine (F384) for activity (10). However, the hydrophobicity of phenylalanine makes it an unlikely catalytic residue.

We chose to use one of the S. cerevisiae members of the MBOAT gene family, LPT1 (also named ALE1, SLC4, and LCA1), as a convenient and interesting representative for identifying additional, catalytically important residues. Lpt1p is an acyl-CoA dependent lysophospholipid acyltransferase
(LPLAT; EC 2.3.1.23) with broad substrate specificity regarding the lysophospholipid head group (6, 11-14). This provides for a role in de novo phospholipid synthesis, via esterification of lysophosphatidic acid, and in phospholipid remodeling and the scavenging of lysophospholipids. Dual topology reporter analysis supports that Lpt1p has 13 transmembrane domains with the catalytically important H382 facing the ER lumen (15). Deletion of LPT1 in S. cerevisiae slowed phosphatidylethanolamine (PE) remodeling (16) and deletion of the homologous gene in C. albicans resulted in slowed remodeling in all phospholipids after cellular PL species were skewed with palmitate feeding (17). The most similar homolog in higher eukaryotes, LPCAT3, also has a role in maintaining membrane composition. In vitro, siRNA knock-down of LPCAT3 expression in HeLa cells increased the abundance of saturated acyl chains in cellular phospholipids (18). The unfolded protein response was also enhanced in those cells. In vivo, shRNA mediated reduction in mouse hepatic LPCAT3 expression increased very low density lipoprotein secretion (19).

The studies presented here aimed to further understand the reaction mechanism of S. cerevisiae Lpt1p with a larger goal of establishing a precedent for its homologs.
MATERIALS AND METHODS

Materials

Chemicals were mainly obtained from Sigma, Fisher and Sunrise (yeast media). Acyl-CoA and lysophosphatidylcholine (lysoPC) were obtained from Avanti Polar Lipids. [\(^{14}\)C]oleyl-CoA and [\(^{14}\)C]lysoPC were from Perkin Elmer.

Insertion of Flag tag epitope and site-directed mutations into pRS423GP/LPT1.

Addition of the eight amino acid Flag-tag (DYKDDDDK; (20)) onto the C-terminus of the LPT1 open reading frame in pRS423GP/LPT1 (12) was achieved by PCR using oligos 423-1 and LPT1-3tag, digestion with SpeI and SacI, and ligation into pRS423GP which contains the Gal 1, 10 promoter. This pRS423GP/LPT1-flag construct was the template for PCR-based generation of site directed mutants by overlap extension (21) using the primers in Table 1.

In vitro, microsomal lysophosphatidylcholine acyltransferase (LPCAT) assays

S. cerevisiae strain ODY545 (W303-1a, mat. a, lpt1\(\Delta::URA3\) (12) was transformed with pRS423GP/-, pRS423GP/LPT1, or pRS423GP / LPT1-flag variants and cultured into log phase in synthetic complete (SC) media minus histidine with 2% (w/v) galactose. Microsomes were isolated as described previously (22) and protein concentration measured by Lowry assay (23). In vitro LPCAT assays included 1 µg microsomal protein, 0.83 - 80 µM acyl CoA, 2.5 - 50 µM [\(^{14}\)C]lysoPC (38,500 or 15,400 dpm/nmol) in 175 mM Tris-HCl, pH 7.4 in 100 µl. Prior to [\(^{14}\)C]lysoPC addition, reaction mixtures were warmed at 30 °C for 2 minutes. When applicable, amino acid modifying agents were included during this pre-warming and those reactions had 1% (v/v) isopropanol. After substrate addition and 2 minutes of reaction at 30 °C, 2 ml chloroform: methanol (2:1) was added. Lipids were extracted, resolved using a TLC buffer system of 57:33:7:3 chloroform: methanol: acetic acid: water, and PC removed and quantified by scintillation counting. Relative specific activity in pRS423GP/- microsomes (2.2 nmol / min / mg) was subtracted from all other values unless otherwise stated. Statistical analysis was performed in Minitab 16.0.

Significant difference from wild type was detected using a two sample, two-tailed t-test and from zero
using a one sample t-test. Apparent $K_m$ and $V_{max}$ were determined by Eadie-Hofstee transformation (24).

**Immunoblotting**

12 µg of microsomes were denatured in Laemmli sample buffer (30 mM Tris, pH=6.8, 3% (w/v) SDS, 10 mM EDTA, 5% (v/v) glycerol, 2.5% (v/v) $\beta$-mercaptoethanol) with 2M urea, 1 mM PMSF, and 1:30 dilution of protease inhibitor cocktail (Sigma). After resolution by SDS-PAGE, proteins were transferred to nitrocellulose (Westran, Whatman) and sequentially probed with the monoclonal M2 anti-Flag epitope antibody (Thermo Scientific, 1:1000) and a secondary, anti-rabbit IgG coupled to horse radish peroxidase (Sigma, 1:1000) in blotting buffer (5% (w/v) non-fat milk, 50 mM Tris, pH=8.0, 80 mM NaCl, 2 mM CaCl$_2$, 0.01% (v/v) antifoam, 0.2% (v/v) igepal CA-630). Visualization was achieved using a chemiluminescent HRP substrate (Pierce) with detection by C-DIGIT imager (LI-COR).
RESULTS

Identification of catalytically important Lpt1p amino acid residues by chemical modification.

We sought to identify Lpt1p active site amino acid residues that may function in conjunction with the conserved histidine (H382) previously shown to be required for catalysis (6). Accordingly, candidate residues were those with polar or charged R-groups: aspartate, glutamate, lysine, arginine, serine, threonine, tyrosine, and cysteine. Few entries in the Mechanism, Annotation and Classification in Enzymes (MACiE) database directly involve asparagine or glutamine (25). Amino acid modifying agents specific to the respective residues were included in in vitro microsomal lysophosphatidylethanol acyltransferase (LPCAT) assays. Microsomes were used since there are no precedents for purifying an MBOAT with activity retained. Since microsomes contain an array of acyltransferases, lysoPC was the chosen substrate as Lpt1p is by far the major LPCAT in yeast. Microsomes isolated from lpt1Δ::URA3 yeast expressing LPT1 from a plasmid displayed about thirty times greater LPCAT activity than background (lpt1Δ::URA3 with empty plasmid). The low amount of background activity allows for the activity observed to be attributed solely to Lpt1p. Concentrations of the modifying agents were at or higher than the IC50 determined for other enzymes (26, 27).

Both 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and Workman’s reagent K (WRK) inhibited activity by more than 80% (Fig. 1), suggesting that lysine, aspartate, and glutamate residues are in or close to the Lpt1p active site. The moderate (40%) inhibition by NEM indicates a possible role for cysteine(s). However, no cysteine is conserved among Lpt1p and functionally characterized homologs in fungi, plants and animals (Fig. 2). The inability of phenylglyoxylate (PG) and phenylmethylsulfonyl-fluoride (PMSF) to inhibit removed arginine and serine from being candidate catalytic residues. Threonine and tyrosine were not modified and thus still considered as candidates.

Identification of catalytically important residues in Lpt1p by conservation

To identify amino acids important in catalysis, alignment of biochemically characterized homologous LPLATs from three kingdoms was performed (Fig. 2). The choice of homologs provided for a great deal of evolutionary distance and thus opportunity for sequence variation within the structural constraints of
using similar lysophospholipid substrates. Other MBOATs, such as ACAT1 and DGAT1, were excluded because such inclusions distinctly reduced the number of conserved residues. Perhaps the dimensions needed to accommodate the differently shaped acyl acceptors requires customized active site architecture, requiring different spacing among the active site residues and therefore preventing detection by alignment. Accordingly, Lpt1p was aligned with functional homologs from one fungus (Candida albicans (14)), two plants (Arabidopsis thaliana (28) and Brassica rapa (29)), two paralogs each, and two animals (Homo sapiens (30-32) and Mus musculus (1)), three paralogs each. Aspartate / glutamate, serine / threonine, and lysine / arginine substitutions were considered to be equivalent.

One aspartate (residue 146), two glutamates (297, 331), two threonines (127, 374), three tyrosines (277, 289, 388), and four lysines (40, 136, 288, 403) were absolutely conserved in all twelve homologs. A second alignment performed with 61 uncharacterized fungal homologs found that of the 12 candidate residues, all but one, Y277, were conserved (data not shown).

**Site directed mutagenesis of eleven conserved codons in LPT1-flag; protein production.**

To determine if the conserved residues were critical for catalysis, site directed mutants were generated in pRS423GP / LPT1-flag. Substitute codons were chosen to remove the R group’s electrophilic or nucleophilic character while maintaining the approximate size. Galactose induced expression of the wild type and mutant LPT1-flag in lpt1Δ::URA3, microsome isolation, and immunoblotting of microsomes identified a single band of the expected size, 73 kDa. While varying levels of band intensity were observed, all constructs conferred qualitatively similar expression.

**Site directed mutagenesis of eleven conserved codons in LPT1-flag; LPCAT activity.**

The effect of the site directed mutations on LPCAT activity was determine by in vitro microsomal assays. Oleoyl-CoA, shown previously to be an optimal substrate (12), and lysoPC were provided at near saturating concentrations (50 µM each). For two mutants, D146L and E297L, the measured activity was not significantly different than zero (Fig. 4). This absence of activity supports that D146 and E297 are directly involved in catalysis.
The finding of two critical amino acids having carboxylate R-groups suggested a role for these in binding a divalent cation. Depletion of zinc has been shown to inhibit Lpt1 activity by 80% (33) and 25 µM of zinc increased Lpt1p activity by 50% (11). To address the possibility that D146L and E297L cause loss of activity due to weakened zinc binding, the ability of 25 µM and 50 µM zinc to restore activity was tested. No stimulation was observed in either mutant (data not shown). Higher concentrations of zinc were not assayed since zinc inhibits Lpt1p at 100 µM and above (11).

E331L and Y388F also had profound effects on activity, reducing it by 75% and 66%, respectively (Fig. 4). Since the remaining activity was significantly above zero, barring a neighboring glutamate or tyrosine being used as a secondary active site, a direct role for E331 and Y388 in catalysis is not supported. Active site residue redundancy was also a possibility regarding lysines since mutating the four conserved lysines independently showed no significant reduction in activity. To address this, a construct harboring mutations of all four conserved lysines was generated. This 4K to 4Q mutant also showed no significant reduction in activity (Fig. 4) and was still inhibited by over 90% by DIDS. Therefore, the target of DIDS inhibition was not detected by the sequence alignment. Lpt1p contains 54 lysines making the systematic mutation of lysines unfeasible. Interestingly, the K403Q mutant mediated triple the normal Lpt1p activity. This may be useful in future biotechnology applications involving LPT1 over-expression.

Effect of monounsaturated acyl-CoA double bond position on Lpt1p reaction kinetics

Several kinetic studies of Lpt1p have shown a preference for unsaturated acyl-CoA species compared to saturated acyl-CoA species (6,12,14). This suggests a need for a kink or contortion in the acyl-CoA’s hydrocarbon chain to optimally fit into the catalytic site. We utilized 18-carbon, monounsaturated acyl-CoA species with double bonds at delta 6, 9, and 11 to probe the catalytic site. In vitro microsomal LPCAT assays with substrate concentration series for each acyl-CoA species found that 18:1Δ6 and 18:1Δ11 had lower apparent K_m values than the common, monounsaturated 18-carbon (oleoyl) species, 18:1Δ9 (Fig. 5). Since substrate preference is best expressed as catalytic efficiency (k_cat / K_m) and since all
of the reactions had the same, although undetermined, enzyme concentration, apparent \( V_{\text{max}} / K_m \) should be proportional to the catalytic efficiency. The catalytic efficiency was highest for 18:1\( \Delta^6 \) even though 18:1\( \Delta^9 \) had the highest apparent \( V_{\text{max}} \). Given that every two carbons in acyl chains are about 2.5 angstroms apart, this data suggests that the contortion conferred by a cis double bond about 7.5 angstroms from the thioester allows for binding with positioning of the thioester into the active site. Binding and positioning were less optimal when that distance increased by only 3.8 angstroms.

**Characterization of the type of BiBi mechanism.**

Since Lpt1p mediates a reaction with two substrates and two products (i.e. BiBi), we next sought to determine if the mechanism was sequential or ping pong. Accordingly, a series of *in vitro* LPCAT assays with all combinations of five sub-saturating oleoyl-CoA and five lysoPC concentrations was performed. Double reciprocal plots of lysoPC concentration versus reaction velocity generated a family of lines that have unique slopes and intersect (with the exception of 20 \( \mu \)M oleoyl-CoA) to the left of the \( y \)-axis (Fig. 6). This is consistent with Lpt1p sequentially binding acyl-CoA and lysoPL substrates to form a ternary complex (34).
DISCUSSION

The acyl-CoA dependent lysophospholipid acyltransferase reaction mediated by Lpt1p is at its basis a transferase reaction. Transferase reactions often involve acid / base chemistry and specifically, nucleophilic substitution (25). For Lpt1p, a possible substitution mechanism involves a catalytic residue donating an electron (i.e. acting as a nucleophile or Lewis base) to the lysoPL’s hydroxyl group, making it a better nucleophile to attack the carbonyl carbon of the thioester bond in acyl-CoA. We sought to determine what, if any, residues in addition to H382 are critical for catalysis by Lpt1p and if a nucleophilic attack mechanism was consistent with the contribution of those residues.

Inhibition by Workman’s Reagent K, multiple-sequence alignment and site directed mutagenesis identified D146 and E297 as critical for catalysis. The corresponding residues were not chosen for analysis in two previous studies that sought to identify critical residues in MBOATs (5, 10). Both studies performed site directed mutagenesis of codons in regions of clustered sequence conservation. Analysis of African green monkey ACAT1 found that mutation of D400, which aligns with S. cerevisiae Lpt1p D320, abolished activity (5). However, mutation of D400 also dramatically reduced expression and mutating the conserved aspartate, D378, in African green monkey ACAT2 had no effect on activity. These data leave the catalytic role of D400 in question. Within the clusters of amino acid conservation in human LPCAT3, E186 and D314 were identified and mutated but full or 20% activity remained, respectively (10). Therefore, the present study is the first to identify carboxylate-containing residues as essential for catalysis by an MBOAT.

How D146 and / or E297 function in conjunction with H382 remains to be determined. Topology analysis of S. cerevisiae Lpt1p using SUC2 (invertase) - HIS4C chimeras identified H382 to be on a short loop that faces the ER lumen (15). However, that study focused on the C-terminal third of Lpt1p. No data directly ascertained the location of D146 and E297. Hydrophobicity plots predicted that D146 faces the cytosol while the location of E297 varied among the five topology prediction algorithms (15).

One precedent for combined catalytic contributions by histidine and aspartate is Haemophilus influenzae serine acetyl transferase. Crystal structure determination with substrate bound identified two
histidines and one aspartate in proximity to the substrate. Site directed mutagenesis of each dramatically decreased activity. The proposed mechanism has H154 acting as a general base to promote the deprotonation of the substrate serine’s hydroxyl group which subsequently attacks the carbonyl of the acetyl-CoA thioester. D139 is proposed to form a dyad linkage with H154 and increase the basicity of H154. H189 is proposed to aid in substrate orientation (35).

Another precedent is *Salvia splendens* malonyl-CoA:anthocyanin 5-O-glucoside-6’"-O-malonyltransferase. Alignment of this enzyme with homologs and site directed mutagenesis found it to require H167 and D390. Further experiments supported that enzyme-promoted deprotonation of the carbohydrate substrate’s hydroxyl group created a nucleophile which attacked the malonyl-CoA thioester bond (36). Whether histidine or aspartate functioned as the primary nucleophile was not determined.

Glutamate has also been shown to act as a base in the *S. cerevisiae* GCN5, an acetyl-CoA dependent histone acetyl transferase. In that enzyme, E174 deprotonates a lysine in the histone substrate, activating that lysine for nucleophilic attack of the thioester carbonyl in acetyl-CoA (37). All of these mechanisms lack a modified enzyme intermediate and therefore require formation of a ternary complex. Formation of such a complex is supported by the two-substrate concentration-series kinetic analysis reported here.

Aspartate, glutamate, and histidine may also function in *S. cerevisiae* Lpt1p to bind zinc. These are three of the four residues which are most commonly used to coordinate a catalytic zinc atom (38). In *S. cerevisiae* extracts, LPCAT activity was inhibited by over 80% in the presence of 2 o-phenanthroline, a zinc chelator (30). Provision of 25 µM zinc to microsomes from *LPT1* overexpressing yeast increased *in vitro* LPLAT activity by 50% (11). Partial (40%) inhibition of Lpt1p by NEM, a cysteine modifying agent, also lends support to the involvement of zinc binding as cysteine is the fourth amino acid commonly used to coordinate zinc (35). However, since zinc commonly functions as an electrophile, its involvement in an acyltransferase mechanism is not easily hypothesized. Electrostatically interacting with phosphates in coenzyme A to orient the substrate is one possible role for zinc.

Acyl-CoA binding and orientation by Lpt1p are clearly influenced by the shape of the hydrocarbon moiety. Among 18-carbon monounsaturated acyl-CoA species, the closer the double bond was to the
thioester, the higher the catalytic efficiency ($V_{\text{max}} / K_m$). This agrees with previous work that showed Lpt1p uses arachidonyl-CoA, $20:4\Delta^5,8,11,14$ with a catalytic efficiency double that of oleoyl-CoA, 52 vs. 25 min$^{-1}$mg$^{-1}$ (12). Having an angular binding pocket complementary to acyl-CoAs with a bend about 7.5 nm from the active site may explain how LPCATs can preferentially utilize substrates with a double bond at position $\Delta^5$ or $\Delta^6$. For *S. cerevisiae*, such substrates would derive from exogenous fatty acids since only $\Delta^9$ species are synthesized. Considering the ternary complex formed during the reaction, acyl-CoA orientation may be important to provide space for lysoPL.

Our main conclusions are that two carboxylate R groups, D146 and E297, are critical to acyl-CoA lysophospholipid acyltransferase activity mediated by *S. cerevisiae* Lpt1p. Also important in Lpt1p-mediated catalysis is the positioning of the acyl-CoA. Substrate binding and turnover were greatly affected by moving the cis double bond in monounsaturated acyl-CoA species by only a few carbons.
ACKNOWLEDGEMENTS

P.R. was supported by an Undergraduate Fellowship sponsored by the University of Michigan - Dearborn Office of Research and Sponsored Programs. Funding was also provided as a seed grant from the same office to P.O.
REFERENCES


FIGURE LEGENDS

Fig. 1. Effect of amino acid modifying agents on Lpt1p activity.

W303-1A lpt1Δ::URA3 yeast transformed with pRS423GP/LPT1 were induced in galactose containing synthetic media. Microsomes were isolated and lysophosphatidylcholine acyltransferase (LPCAT) relative specific activity measured using non-saturating substrate concentrations: 20 µM [14C]lysoPC and 20 µM oleoyl-CoA in 175 mM Tris, pH 7.4 for 2 minutes. Protein modifying agents in isopropanol or isopropanol alone (isopr.) were added to the reaction 2 minutes prior to initiation with [14C]lysoPC.

Inhibitors (concentration, target) were DIDS, 4,4V-diisothiocyanato-stilbene-2,2'-disulfonic acid (1 mM, lysine); NEM, N-ethylmeleimide (1 mM, cysteine); PG, phenylglyoxyxl (5 mM, arginine); PMSF, phenylmethylsulfonylfluoride (1 mM, serine), WRK, Workman’s Reagent (4 mM, aspartate, glutamate).

n=4, error bars are standard deviation, * indicates significantly different than isopropanol treated, p<0.05.

Fig. 2. Alignment of characterized LPLATs from three kingdoms, two species each.

Clustal Omega aligned sequences of twelve MBOAT family members previously shown to esterify lysoPC and/or lysoPE. Absolute conservation is boxed, with D/E, K/R, and S/T substitutions considered conserved. Black boxes are conserved aspartate, glutamate, lysine, threonine, and tyrosine residues also conserved in an alignment of all fungal homologs. Red box is a conserved tyrosine not conserved in fungi. Blue box is the conserved H382. Green boxes are conserved arginine and serine residues. * stop codon. S.c. Saccharomyces cerevisiae; C.a. Candida albicans; A.t. Arabidopsis thaliana, B.n. Brassica napus; H.s. Homo sapiens, M.m. Mus musculus. Gene numbers and Genbank protein accession numbers are: S.c. LPT1 (YOR175C, 74583731), C.a. LPT1 (C2_07480W_A, KT750332), A.t. LPLAT1 (AT1G12640, NP_172724.2), A.t. LPLAT2 (AT1G63050, Q9CAN8.1), B.n. LPCAT1 (BnaA06g08200D, 674935400), B.n. LPCAT2 (BnaCnng41640D, 674871331), M.m. LPCAT3 (14792, AAH06753.2), M.m. LPCAT4 (99010, NP_080313.2), M.m. LPEAT1 (218121, Q8BH98.1), H.s. LPCAT3 (10162, Q6P1A2.1), H.s. LPCAT4 (254531, Q643R3.1), H.s. MBOAT1 (154141, Q6ZNC8.1).
Fig 3. **Effect of site directed mutagenesis on Lpt1p expression.**

Site directed mutagenesis generated the twelve indicated mutants in pRS423GP/LPT1-Flag. These constructs, wild type LPT1-Flag, and empty vector were transformed into lpt1Δ::URA3 S. cerevisiae. Expression was induced by galactose and microsomes isolated. 12 µg of microsomal protein were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the M2 anti-Flag antibody (1:1000). Incubation with a horseradish peroxidase (HRP) conjugated, anti-rabbit secondary antibody (1:1000) was followed by enhanced chemiluminescence. Detection was performed using the C-DIGIT Western Blot scanner (LI-COR). Bar to the left indicates migration of the 67 kDa marker protein (BSA).

Fig 4. **Effect of site directed mutagenesis on Lpt1p activity.**

*In vitro* LPCAT activity was measured by incubating 1µg of microsomes (isolated as in Fig. 3), 50 µM [14C]lysoPC, 50 µM oleoyl-CoA, in 175 mM Tris, pH 7.4 for two minutes. Relative specific activity is reported. n = 3-6, error bars are standard deviation * indicates significantly less than wild type, p<0.05; # indicates significantly more than wild type, p<0.05.

Fig 5. **Lpt1p activity with monounsaturated acyl-CoA species with different double bond positions.**

*In vitro* LPCAT activity was measured by incubating 1µg microsomes from pRS423GP/LPT1 transformed lpt1Δ::URA3 (isolated as in Fig. 1), 50 µM [14C]lysoPC, and a series of acyl-CoA concentrations from 0.83 to 80 µM in 175 mM Tris, pH 7.4 for two minutes. Acyl-CoA species are: A) 18:1Δ⁶ B) 18:1Δ⁹ and C) 18:1Δ¹¹. n=3-5, error bars are standard deviation. Curve fitting was performed by SigmaPlot using a four-parameter logistic algorithm. Kinetic parameters determined by Eadie-Hofstee transformation (not shown). cat. eff. = catalytic efficiency (apparent Vmax / apparent Km).

Fig. 6. **Two-substrate concentration series kinetic analysis.**

*In vitro* LPCAT activity was measured as in Fig. 1 by incubating 1µg microsomes from pRS423GP/LPT1 transformed lpt1Δ::URA3 and all combinations of five oleoyl-CoA concentrations (5 µM (●), 7.5 µM (○), 10 µM (▲),15 µM (△), 20 µM (■)) and five [14C]lysoPC concentrations (2.5, 5, 7.5, 10, 15 µM). n=4, error bars are standard error.
Table 1. Oligonucleotides used in this study.

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<td>K40Q SDM</td>
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<td>Y388F SDM</td>
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<td>GCGTCTGTAGATTGACCACATGTTTGGT</td>
<td>K403Q SDM</td>
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<tr>
<td>LPT1-3tag</td>
<td>AACCAACCGAGCTCTATCATATCTCGTCATCGTCCTTGTAATCC</td>
<td>addition of Flag</td>
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<tr>
<td></td>
<td>TCTTC</td>
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Figure 3

The image shows a gel electrophoresis with bands labeled with various amino acid substitutions: wild type, K40Q, T127V, K136Q, D146L, K288Q, Y289F, E297L, E331L, T374V, Y388F, K403, 4K-4Q vector.
Figure 4

![Bar graph showing LPCAT activity (nmol/min/mg) for different LPT1 genotypes. The graph compares wild type (wt) and various mutants such as K40Q, T127V, K136Q, D146L, K288Q, Y289L, E297L, E331L, T127V, Y388F, K403Q, 4K4Q, and vector. The graph includes error bars and symbols (# and *) to indicate statistical significance.](image-url)