Substrate specificity of lysophosphatidic acid acyltransferase β—evidence from membrane and whole cell assays

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Abstract Membranes of mammalian cells contain lysophosphatidic acid acyltransferase (LPAAT) activities that catalyze the acylation of sn-1-acyl lysophosphatidic acid (lysoPA) to form phosphatidic acid. As the biological roles and biochemical properties of the six known LPAAT isoforms have yet to be fully elucidated, we have characterized human LPAAT-β activity using two different assays. In a membrane-based assay, LPAAT-β used lysoPA and lysophosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor, and it preferentially transferred phosphatidylmethanol (lysoPM) but not other lysophosphoglycerides as an acyl acceptor. In contrast, if lysoPM and 14C-labeled 20:4 were added to PC-3 or DU145 cells, 14C-labeled PM was also formed, but the rate of PM formation was higher in cells that overexpressed LPAAT-β and was inhibited by the LPAAT inhibitor CT-32501. We propose that LPAAT-β catalyzes the intracellular transfer of 18:1, 18:0, and 16:0 acyl groups but not 20:4 groups to lysoPA. —Hollenback, D., L. Bonham, L. Law, E. Rossnagle, L. Romero, H. Carew, C. K. Tompkins, D. W. Leung, J. W. Singer, and T. White. Substrate specificity of lysophosphatidic acid acyltransferase β—evidence from membrane and whole cell assays. J. Lipid Res. 2006. 47: 593–604.

Membrane-associated lysophosphatidic acid acyltransferase (LPAAT) activities, identified in bacteria, yeast, plant, and animal cells, catalyze the transfer of acyl groups from acyl-CoA to lysophosphatidic acid (lysoPA) to form phosphatidic acid (PA) (1–3). PA is a component of cell membranes and a key intermediate in the de novo synthesis of phosphoglycerides, the major components of cell membranes, and triacylglycerol, the major form of energy storage in plants and animals. Models that suggest that PA can activate numerous cell functions, including proliferation, cytoskeletal organization, vesicle trafficking and the oxidative burst in neutrophils, have also gained wide acceptance.

Six human LPAAT isoforms (α–ζ), also known as 1-acyl-sn-glycerol-3-phosphate acyltransferase 1–6, have been cloned. Only the α and β isoforms, which share 34% sequence identity, have been shown to have significant LPAAT activity and to complement the growth defect in pbsC, an Escherichia coli strain mutated in its sole LPAAT gene (4). These isoforms also contain two to four predicted transmembrane domains and two highly conserved motifs, H(X)4D and EGTR, which are essential for the catalytic activity of a family of acyltransferases (5–7). Northern blot analysis of human LPAAT-α suggests that it has broad tissue distribution, whereas that of human LPAAT-β is more restricted, primarily to heart, liver, adipose, and pancreas (8–11). The inherited loss of function of LPAAT-β has also been linked to a rare form of congenital, generalized lipodystrophy, characterized by a nearly complete absence of adipose tissue from birth (12, 13).

Although membrane-associated LPAAT activities were first described by Kornberg and Pricer (14) in 1953, progress has been slow to understand their cellular function(s) and to separately define each isozyme’s enzymatic properties. Significant steps could be made if these enzymes were solubilized from the membranes and purified to homogeneity. Unfortunately, attempts to solubilize LPAATs into detergent micelles have resulted in the immediate and complete loss of enzymatic activity. Thus, most information on LPAAT enzymatic properties comes from experiments using cell lysates or membrane preparations containing a recombinant LPAAT isozyme. When the

Supplementary key words phosphatidic acid • lysophosphatidylmethanol • phosphatidylmethanol • acyl-CoA

Abbreviations: GFP, green fluorescent protein; LPAAT, lysophosphatidic acid acyltransferase; lysoPA, lysophosphatidic acid; lysoPC, lysophosphatidylcholine; lysoPM, lysophosphatidylmethanol; PA, phosphatidic acid; PLD, phospholipase D; PM, phosphatidylmethanol.

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potential acyl donors 14:0-CoA, 16:0-CoA, 18:0-CoA, and 20:4-CoA were tested separately against lysates from COS 7 cells containing human recombinant LPAAT-β, all acyl donors were active. When the potential acyl acceptors sn-1-18:1 lysoPA, sn-1-acyl lysophosphatidylcholine (lysoPC), and lysoplatelet-activating factor (sn-1-alkyl lysoPC) were tested separately, only lysoPA was active (10). A separate study showed that 18:1-CoA was also an acceptable acyl donor (8). Studies of human or murine LPAAT-α expressed in JC201, COS 7, or SF21 cells have shown that it has broad preference for acyl-CoAs and can use lysoPA but not lysoPC, lysophosphatidylethanolamine, lysophosphatidylserine, or lysoplatelet-activating factor as an acyl acceptor (9, 15, 16). In spite of these efforts, the LPAATs’ enzymatic properties need a more detailed characterization to fully understand the effect that LPAAT catalysis may have on biological processes requiring PA.

To this end, we studied the substrate specificity of LPAAT-β in more detail using SF9 membrane preparations containing recombinant LPAAT-β and compared it with that of native SF9 membranes and SF9 membrane preparations containing recombinant LPAAT-α. We also developed a novel assay to estimate LPAAT-β activity in whole cells. This assay measured the intracellular formation of lysophosphatidyl ethanolamine (PM) and took advantage of two reagents, lysophosphatidyl ethanolamine (lysoPM) and LPAAT-β chemical inhibitors. LysoPM was shown to be an acyl acceptor for LPAAT-β but not LPAAT-α, and unlike lysoPA, it readily traversed cell membranes. The LPAAT-β chemical inhibitors show submicromolar potency against LPAAT-β but do not inhibit LPAAT-α even at 40 μM and were used to help differentiate the activity of the two isozymes. We used this second assay to test whether the LPAAT-β acyl chain transfer specificity observed in the membrane assay was reciprocated in cells.

EXPERIMENTAL PROCEDURES

Materials

14C-labeled 18:1, 14C-labeled 20:4, 14C-labeled 18:1-CoA, 3H-labeled sn-1-18:1 lysoPA, and γ32P-labeled ATP were from Perkin-Elmer; 32P-labeled sn-1-18:1 lysoPA was prepared using E. coli diacylglycerol kinase (Calbiochem) to phosphorylate 1-monolein (Doosal Serdery Research Laboratories) with γ32P-labeled ATP as described (17); all other lipids were from Avanti Polar Lipids. Silica Gel 60 HP-TLC plates were from Analtech. Organic solvents were American Chemical Society grade or better from J. T. Baker or EM Science. The synthesis of compounds CT-32228 and CT-32212 (18) and of compound CT-32501 (19) were as described. Anti-LPAAT-α and anti-LPAAT-β antibodies have been described (20). All other chemicals were of reagent grade from Sigma.

Preparation of lysoPM

sn-1-18:1 lysoPM was synthesized from sn-1-18:1 lysoPA essentially as described (21). One gram of the protonated form of sn-1-18:1 lysoPA was dissolved in 50 ml of chloroform and mixed with an ethereal solution of diazomethane until a yellow color persisted. The solvent was evaporated, and the dimethylated lysoPA was mixed with 2.0 g of NaI in anhydrous 2-butanone and then refluxed for 90 min. The solvent was removed, and the lysoPM was purified by flash chromatography as the sodium salt by eluting with chloroform-methanol-acetic acid-water (50:30:15:5). The solvent was evaporated, and the lysoPM was reconstituted in chloroform. This yielded 800 mg of lysoPM, whose structure and purity (>95%) were confirmed by TLC, LC-MS, and NMR.

Ectopic expression of LPAAT-β and LPAAT-α in PC-3 and DU145 cells

Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. PC-3, a human prostate tumor cell line (American Type Culture Collection; CRL-1435), was cultured in Ham’s F12K medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, and 10% fetal bovine serum. DU145, a human prostate tumor cell line (American Type Culture Collection; HTB-81), was cultured in Eagle’s minimal essential medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. For the overexpression of LPAATs in these cells, human LPAAT-β cDNA and human LPAAT-α cDNA were cloned separately into a modified version of the LXSN retroviral vector (Clontech) (22). The Moloney murine leukemia virus long terminal repeat drives LPAAT expression, and the SV40 promoter drives the selectable marker (neo) expression. As a further modification of the vector, a recognition site for cre recombinase (23) was introduced into the long terminal repeat regions. Control vectors containing alkaline phosphatase cDNA, green fluorescent protein (GFP) cDNA, or vector alone were also produced using the same method. The retroviral packaging cell lines for LPAAT-β, LPAAT-α, and control vectors were produced and characterized as described (24). Clones producing high-titer virus (0.5–1 × 10^6 G418-resistant colony-forming units/ml) were used for the transduction of PC-3 and DU145 cells in their respective media; clonal cell lines were subsequently generated. None of the transduced cells exhibited any gross morphological or proliferative alterations.

LPAAT activity assay in cell lysates

The magnitude of overexpression of each recombinant LPAAT was determined by assaying total endogenous LPAAT activity in cell lysates essentially as described (25). Briefly, transduced PC-3 and DU145 cells were harvested by trypsinization, suspended in 400 μl of ice-cold PBS, and probe-sonicated with a Branson Sonifier at output = 2, duty cycle = 2 for 10 pulses. The samples were centrifuged at 1,500 g for 2 min at 4 °C to obtain the supernatants, which were quantified for protein. The LPAAT activity in these supernatants was estimated during a 7 min incubation at 37 °C in 12 × 75 mm silanized, borosilicate glass test tubes containing 50 μl of 50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mg/ml fatty acid-free BSA, 0.2 mM 14C-labeled 18:1-CoA (~40,000 cpm/assay), 0.2 mM sn-1-18:1 lysoPA, and 0.005–0.03 mg/ml protein; the reaction was quenched with organic solvent. The 14C-labeled PA was resolved by TLC on Silica Gel 60 plates, and the density of radioactive bands was quantitated by exposing the plate to a phosphor screen overnight and scanning the phosphor screen with a Storm 840 PhosphorImager (GE Healthcare Life Sciences) as described (26). The increase in total LPAAT activity achieved in PC-3 cells was typically 5- to 20-fold for LPAAT-α and 20- to 45-fold for LPAAT-β. One clone from PC-3 cells containing recombinant LPAAT-β was used frequently in this study and is referred to as PC-3/B1 (or B1 in Fig. 3D). The increase in total LPAAT activity achieved in DU145 cells typically was 2- to 5-fold for LPAAT-α and 4- to 10-fold for LPAAT-β.
Whole cell PM formation assay

PM synthesis was estimated in cultured cells by quantitating the conversion of exogenously added lypoPM and 14C-labeled fatty acid to 14C-labeled PM during a 35 min incubation at 37°C. PC-3 and DU145 cells, containing a recombinant protein as indicated, were separately plated onto 96-well plates in 500 µl of the appropriate medium (see above) and grown to 70–80% confluence. On the day of the assay, the medium volume was adjusted to 96 µl. The lypoPM and 14C-labeled fatty acid stocks were prepared separately by drying each solvent under a stream of nitrogen and resuspending the lypoPM in PBS and the 14C-labeled fatty acid in PBS + 20 mg/ml fatty acid-free BSA. The assay was performed by incubating 2 µl of the lypoPM stock with the cells for 5 min, adding 2 µl of the 14C-fatty acid stock, and incubating for an additional 30 min. The final concentrations were 50 µM (Olpa1F) and 50 µCi of 14C-labeled 18:1 in a total volume of 100 µl, unless stated otherwise. Background PM levels were determined by adding PBS without lypoPM. The assay was terminated by removing the incubation mixture, washing the cells three times with ice-cold PBS, discarding the final wash, and then adding 0.25 ml of methanol. The plates were gently swirled, and the methanol was transferred to glass test tubes. This method extracted >98% of the PM from the wells (data not shown). Carrier 18:1-18:1 PM (4 µg), 1.0 ml of chloroform, 0.25 ml of methanol, and 0.25 ml of 0.2 M H3PO4 were added to each tube. The tubes were vortexed for 10 s and centrifuged for 2 min at 500 g to clarify the phases; the upper phase was discarded. The lower phase was dried under a stream of nitrogen, and the sample was resuspended in 100 µl of chloroform-methanol (2:1). 14C-labeled PM was typically resolved by TLC in chloroform/methanol/NH4OH (65:30:4). To help confirm the identity of the PM, in some instances a two-pot system was also used in which the TLC plates were first developed in chloroform/methanol/NH4OH (70:25:3), dried thoroughly, and then developed in chloroform-methanol-acetic acid-water (85:10:8:1). The density of radioactive bands was scanned and quantified with a Storm PhosphorImager as described above. Unless stated otherwise, 14C-labeled PM formation is expressed as a percentage of the lane: the pixel volume of the PM region ÷ the pixel volume of the entire lane × 100, where pixel volume is the signal intensity calculated with the Storm PhosphorImager and is directly proportional to the amount of radioactivity. This calculation provided a relative rate of esterification of radioactive fatty acid into lypoPM versus the entire lipid fraction and served as a loading control. For experiments using LPAAT-β chemical inhibitors, the inhibitors were diluted from a DMSO stock into PBS + 4 mg/ml BSA. The volume of medium was adjusted to 94 µl, and 2 µl of inhibitor stock or excipient alone was added to the cells 5 min before the addition of the lypoPM stock. The final concentration of DMSO was 0.01% (v/v) and did not affect the outcome of the assay (data not shown). IC50 values were determined using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA; www.graphpad.com). Other changes in the assay are noted in the text and figure legends.

Expression of LPAAT-β and LPAAT-α in Sf9 cells

For the construction of Baculovirus expression vectors, the full-length human LPAAT-α and LPAAT-β cDNAs were amplified by PCR from the DNA templates pCE9.LPAAT-α and pCE9.LPAAT-β (8) using the primers 5’-TGATATCCAGAGGATCTC-TATGGATTGTGGCCAGGGCGG-3’ (olpa1F) and 5’-CAGGCTCT-TAGATACCCACCGCAGCAGCTCTC-5’ (olpa1R) for LPAAT-α and the primers 5’-TGATATCCAGAGGATCTC-TATGGATTGTGGCCAGGGCGG-3’ (olpb1F) and 5’-CAGGCTCT-TAGATACCCACCGCAGCAGCTCTC-5’ (olpb1R) for LPAAT-β. The ~870 bp fragments generated were reamplified by PCR using the primers 5’-CCCTACGTCCGACATGGAACAAAAATTTGATACCGAACGATC3’ (olpb2F) and olpa1R for LPAAT-α and the primers olpb2F and olpb1R for LPAAT-β. The ~890 bp fragments generated were then cleaved with SalI and XbaI for insertion into pFastBac™ HTc vector (Invitrogen) between the SalI and XbaI sites. The plasmids pFB.LPAAT-α and pFB.LPAAT-β were further modified by cleaving them with XhoI and SalI, converting the cohesive ends to blunt ends with Klenow DNA polymerase and deoxyribonucleoside triphosphate and recircularizing by ligase to remove the polyhistidine tag coding region. These plasmids were then transformed into E. coli DH10Bac™ (Invitrogen) for the generation of recombinant Bacmid DNAs for transfection into Sf9 insect cells for the production of recombinant Baculovirus stocks using the protocol described in the Bac-to-Bac® Baculovirus Expression System (Invitrogen), a eukaryotic expression system for generating recombinant Baculovirus through site-specific transposition in E. coli. Viral stocks harvested from the transfected cells were then used to infect fresh insect cells for the subsequent expression of LPAAT-α and LPAAT-β proteins.

Preparation of membranes from Sf9 cells

All steps were performed at 0–4°C. Sf9 cell pellets (~106 cells) containing recombinant LPAAT were thawed and resuspended in 5 ml of buffer A [20 mM HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol, 1 mM benzamidine, 1 µg/ml soybean trypsin inhibitor, and 1 µg/ml pepstatin A] with 1 mM Pefabloc. The cells were lysed by sonication using a Branson Sonifier at output = 2, duty cycle = 2, 10 pulses each for 10 s. DTT was then added to 1 mM from a 1 M stock. The samples were centrifuged at 1,500 rpm for 5 min. The resulting supernatant was centrifuged at 100,000 g for 1 h. The resulting pellet, which contained the Sf9 membranes, was resuspended in buffer A + 1 mM DTT and stored at −70°C. LPAAT-β and LPAAT-α activities were stable in this form for >2 years.

LPAAT activity assay in Sf9 membranes

Enzyme assays were performed in 12 × 75 mm siliconized, borosilicate glass test tubes at 37°C for 3–10 min. For the estimation of LPAAT activity in Sf9 membrane preparations, typical assay mixtures (300 µl) consisted of 251 µl of buffer B (50 mM HEPES, pH 7.5, 1 mM EDTA, and 100 mM NaCl), 25 µl of enzyme in buffer B + 10% glycerol, 12 µl of 14C-labeled 18:1-CoA (40,000–100,000 cpm), and 12 µl of sn-1-18:1 lysoPA. To prevent the nonspecific binding of substrates to the tubes, stock solutions of each substrate were prepared separately in DMSO. The final concentration of DMSO in all assays was 8% (v/v) and did not affect LPAAT-β or LPAAT-α activity as determined in separate experiments (data not shown). At the end of the assay, 1 ml of chloroform/methanol/HCl (1:20:0.027), carrier PA and lysoPA (0.3 µg), 0.3 ml of chloroform, and 0.1 ml of water were added. The tubes were vortexed for 10 s and centrifuged for 2 min at 500 g to clarify the phases; the upper phase was discarded. The lower phase was washed with 1 ml of chloroform-methanol-water (10:150:100) and then dried under a stream of nitrogen. 14C-labeled PA was resolved by TLC in chloroform-methanol-acetic acid-water (85:12.5:12.5:3) and quantitated using the Storm PhosphorImager as described above. Product formation was linear with time and protein concentration. Vmax and Km values were determined using GraphPad Prism software. If other potential acyl acceptors were used in the assay, the following TLC solvent systems were used: for lysophosphatidylinositol, lysophosphatidylether, or lysophosphatidylglycerol, chloroform-methanol-acetic acid-water (85:12.5:12.5:3); for lysoPC and...
lysophosphatidylethanolamine, chloroform-methanol-ammonia (65:40:5); for monoolein and diolein, hexane-diethyl ether-acetone (60:40:5); and for glycerol-3-phosphate, chloroform-methanol-acetic acid-water (70:30:4:6).

For competition experiments between two different acyl-CoA species, [3H]sn-1-18:1 lysophosphatidic acid (lysophospha- tidic acid (lysophPA), 14C-labeled 18:1-CoA, and a second, unlabeled acyl-CoA were included in the reaction. After extraction and TLC, the PA band was scraped from the plate and extracted from the silica with 1.3 ml of chloroform-methanol-water (1:1:0.1). The extract was transferred to a glass scintillation vial and dried. Samples were counted in Ultima Gold scintillant (Perkin-Elmer) on a Packard Tri-Carb 1600 Scintillation Counter using the preset 3H/14C channels (pulse height discriminator settings of 0–12 for 3H and 12–156 for 14C) and the tSIE/AEC quench indicating parameter. The amount of 18:1 groups transferred equaled the moles of 14C-labeled PA formed, and the amount of the second acyl group transferred equaled the moles of 3H-labeled PA formed minus the moles of 14C-labeled PA formed. Relative, normalized velocities were calculated from the equation

\[
\frac{v_1}{v_2} = \frac{[S_1]}{[S_2]} \times \frac{(k_{cat1}/K_{m1})}{(k_{cat2}/K_{m2})}
\]

where \(v\) = initial velocity, \([S]\) = substrate concentration, and \(k_{cat}\) and \(K_m\) are catalytic constants for substrates 1 and 2 as indicated (27).

**Other methods**

Protein concentrations were assayed using the bicinchoninic acid method (Pierce) with BSA as the standard. NuPAGE (Invitrogen) was performed according to the manufacturer’s instructions, and Western blotting with anti-LPAAT-β and anti-LPAAT-α monoclonal antibodies was performed as described (28). Error bars in the figures indicate standard deviations unless only two values were obtained, in which cases the standard errors of means are shown. Error bars that are not visible are smaller than the data point symbols.

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**Fig. 1.** Acyl-CoA molecular species preferences of lysophosphatidic acid acyltransferase-β (LPAAT-β) and LPAAT-α. A: Preparations of SF9 wild-type membranes or those containing recombinant LPAAT-β or LPAAT-α were analyzed by NuPAGE and Western blotting, as described in Experimental Procedures. Separate blots were incubated with either the anti-LPAAT-α antibody or the anti-LPAAT-β antibody as indicated. Positions of the molecular mass markers are indicated on the right side of the blot. B: The preferences of the LPAAT enzymes for different molecular species of acyl-CoA were determined by measuring the amount of phosphatidic acid (PA) formed in separate incubation assays (8 min, 37°C) containing buffer B, 8 μM 32P-labeled sn-1-18:1 lysophosphatidic acid (lysophPA), 8 μM of the indicated molecular species of acyl-CoA, and 0.1 μg/ml SF9 membrane preparations; wild-type membranes or those containing recombinant LPAAT-β or LPAAT-α were used as indicated on the abscissa. Two experiments were performed that used all three membrane preparations, and a third experiment was performed that used only the SF9 membrane preparations containing recombinant LPAAT-β. Results from one representative experiment are shown, and all three experiments gave similar results. Error bars represent standard errors of the means from duplicate samples. C: SF9 membrane preparations containing recombinant LPAAT-β were incubated as described for B except with the indicated concentrations of 18:1-CoA. The experiment was performed in duplicate, and the results shown are representative of three experiments.
RESULTS

Expression of LPAAT-β and LPAAT-α in Sf9 cells

We expressed the LPAAT-β and LPAAT-α transgenes in Sf9 cells under the control of the SV40 promoter; membrane preparations were generated as described in Experimental Procedures. Membrane preparations from cells infected with LPAAT-β or LPAAT-α cDNA could catalyze the transfer of oleoyl groups from 18:1-CoA to sn-1-18:1 lysoPA at rates of 205 ± 36 nmol/min/mg protein (n = 5) and 115 ± 48 nmol/min/mg protein (n = 4), respectively, which are 110- and 60-fold higher than the rate observed using wild-type Sf9 membranes. Furthermore, when these membrane preparations were analyzed by Western blotting using anti-LPAAT-β and anti-LPAAT-α monoclonal antibodies, the anti-LPAAT-β antibody recognized a band at the expected molecular mass of 31 kDa only in membranes containing recombinant LPAAT-β, whereas the anti-LPAAT-α antibody recognized a band of similar molecular mass only in membranes containing recombinant LPAAT-α (Fig. 1A). We originally planned to solubilize and purify the LPAAT isoforms before studying their enzymatic properties. Unfortunately, this proved inefficient, as both activities were immediately and completely inactivated in the presence of detergents. On the other hand, it appeared that both isoforms were overexpressed in the membrane fractions and accounted for >98% of the total LPAAT enzymatic activity. Thus, it was possible to use the membrane preparations to separately study each isoform, without the concern for other interfering LPAAT activities in the assay.

Acylation donor and acceptor preferences of LPAAT-β

Several sets of experiments were performed to examine the acyl donor preference of LPAAT-β. One set examined the ability of LPAAT-β to use different molecular species of acyl-CoA. Sf9 membrane preparations containing recombinant LPAAT-β (see Experimental Procedures) were separately incubated with sn-1-18:1 lysoPA and different molecular species of acyl-CoA. The results revealed that LPAAT-β could use all acyl-CoAs tested (Fig. 1B). When the concentration of 18:1-CoA was varied in the assay, LPAAT activity was saturable, with an apparent V_{max} of 210 nmol/min/mg protein and an apparent K_{m} of 0.4 μM (Fig. 1C). The catalytic site specificity for an enzyme is defined as the relative values of the specificity constant (√V_{max}/K_{m}) for different substrates (27). As these values cannot be derived from assays using one concentration of one acyl donor, as shown in Fig. 1B, competition experiments were performed in which Sf9 membrane preparations containing recombinant LPAAT-β were incubated with 3H-labeled sn-1-18:1 lysoPA, 14C-labeled 18:1-CoA, and a second, unlabeled acyl-CoA in the same tube. The amount of each acyl group transferred to lysoPA was determined as described in Experimental Procedures, and the ratio of these two values represents the specificity of 18:1-CoA to the second acyl-CoA. The results shown in Table 1 suggest that, compared with 18:1-CoA, LPAAT-β displays approximately equal preference toward 16:0-CoA and 18:0-CoA, a moderate preference (8.5-fold) compared with 18:2-CoA, and an at least 18-fold greater preference compared with 12:0-CoA, 14:0-CoA, 20:0-CoA, and 20:4-CoA.

<table>
<thead>
<tr>
<th>14C-Labeled Acyl-CoA</th>
<th>3H-Labeled Acyl-CoA</th>
<th>Relative Normalized Velocities</th>
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<tr>
<td>18:1</td>
<td>12:0</td>
<td>&gt;18</td>
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<td>18:1</td>
<td>14:0</td>
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<td>18:1</td>
<td>16:0</td>
<td>1.8 ± 0.9</td>
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<td>18:1</td>
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LPAAT-β, lysophosphatidic acid acyltransferase-β. The ability of LPAAT-γ to catalyze the transfer of 18:1 acyl groups over other acyl groups was measured in a single reaction consisting of Sf9 membranes containing recombinant LPAAT-β, 6 μM 3H-labeled lysophosphatidic acid, 0.6 μM 14C-labeled 18:1-CoA, and either 0.6 μM 20:0-CoA or 1.2 μM of the indicated acyl-CoA for 7 min at 37°C; relative normalized velocities were calculated as described in Experimental Procedures. Results are from two experiments for 14:0-CoA and 20:0-CoA and from three experiments for 20:4-CoA (the same result was observed each time); results are averages ± SD from four separate experiments for all others. The limit of detection in the assay was a ratio of 18.

Mouse LPAAT-α also catalyzes a CoA-dependent transacylase reaction, in which acyl groups are transferred from donor phospholipids to acceptor lysophospholipids in the presence of unesterified CoA, although at a much slower rate compared with its acyltransferase activity (15). When Sf9 membrane preparations containing recombinant human LPAAT-β were incubated with 3H-labeled sn-1-18:1 lysoPA and unesterified CoA, no PA formation was detected. Furthermore, unesterified CoA did not affect the rate of LPAAT-β-catalyzed acylation of sn-1-18:1 lysoPA with 18:1-CoA (data not shown). Thus, LPAAT-β does not appear to display a CoA-dependent transacylase activity in this assay.

Experiments were also performed to elucidate the acyl acceptor preference of LPAAT-β. Sf9 membrane preparations containing recombinant LPAAT-β were incubated separately with 14C-labeled 18:1-CoA and various sn-1-16:0, sn-1-18:0, or sn-1-18:1 lysophospholipids. The results showed that LPAAT-β transferred oleoyl groups to lysoPA; transfer to the other lysophospholipids was never consistently above background levels (Fig. 2A). LPAAT-β was also unable to use glycerol-3-phosphate, 1-monoolein, or dioleoylglycerol as an acyl acceptor (data not shown). When the concentration of sn-1-18:1 lysoPA was varied in the assay, LPAAT activity was saturable, with a representative V_{max} of 200 nmol/min/mg protein and an apparent K_{m} of 2.0 μM (Fig. 2B). We then examined the nature of the head group specificity of LPAAT-β more closely using sn-1-18:1 lysoPM as a substrate, which represents a very small modification to the phosphate moiety of lysoPA but significantly affects the charge and hydrophobicity of the molecule. When sn-1-18:1 lysoPM was substituted for lysoPA in the LPAAT membrane assay, PM was formed at lysoPM concentrations similar to that of lysoPA. At 3 μM
lysoPM, this activity was at least 20-fold higher than the same reaction using wild-type Sf9 membranes (Fig. 2C) but 20-fold lower than the corresponding LPAAT-b reaction using lysoPA (compare Fig. 2B, C). Together, these results suggest that lysoPA is the preferred acyl acceptor for LPAAT-b.

Acyl donor and acceptor preferences of LPAAT-α

Experiments were performed to examine the substrate preferences of the LPAAT-α activity, and the results showed many similarities with LPAAT-β. For example, like LPAAT-β, LPAAT-α activity was able to transfer the fatty acyl groups of all acyl-CoAs tested to sn-1-18:1 lysoPA (Fig. 1B), and LPAAT-α activity used lysoPA exclusively as the acyl acceptor over other lysophospholipids, glycerol-3-phosphate, 1-monoolein, and diolein (Fig. 2A and data not shown). In contrast, LPAAT-α activity was unable to transfer oleoyl groups from 18:1-CoA to sn-1-18:1 lysoPM (Fig. 2C).

Intracellular formation of PM from exogenous lysoPM

When sn-1-18:1 lysoPM and 14C-labeled oleate were added to PC-3 or DU145 cells, a radioactive band was formed that comigrated with authentic 18:1-18:1 PM by...
formation in wild-type, control-transduced, and LPAAT-β-only LPAAT-β compared with wild-type or vector control cells (Fig. 3D), transduced with either LPAAT-β, PM formation was measured. Although lysates from cells transduced with either LPAAT-β or LPAAT-β showed a 5- to 25-fold increase in total LPAAT enzymatic activity compared with wild-type or vector control cells (Fig. 3D), only LPAAT-β-transduced cells showed an increase (3- to 5-fold) in 14C-labeled PM formation (Fig. 3E). In PC-3/β1, a PC-3 clonal cell line transduced with LPAAT-β, PM formation was proportional with lysoPM concentration and time (Fig. 3B, C). Similar results were observed in separate experiments that used DU145 wild-type cells and DU145 cells transduced with LPAAT-β, LPAAT-α, GFP, or vector only (data not shown). In addition, in similar experiments in which sn-1:18:1 lysoPM was added to PC-3 or PC-3/β1 cells, PM formation was also observed (data not shown).

Cell Therapeutics, Inc. has developed triazine-based compounds that inhibit LPAAT-β biochemical activity at sub-200 nM IC50 concentrations but do not inhibit the activities of LPAAT-α, γ, δ, or ε, phospholipase D1 (PLD1), or PLD2 at 40 μM (Table 2) (20, 28). When LPAAT activity was assayed in Sf9 membranes containing recombinant LPAAT-β, CT-32228 inhibited LPAAT-β activity when either lysoPA or lysoPM was used as the acyl acceptor (Fig. 2D), with similar IC50 values. Furthermore, when LPAAT activity was measured in PC-3 or DU145 cell lysates using lysoPA as the acyl acceptor (see Experimental Procedures), CT-32228 at saturating (10 μM) concentrations inhibited 58 ± 6% (n = 4) of the total endogenous LPAAT activity in PC-3 lysates and 66 ± 3% (n = 7) of the total endogenous LPAAT activity in DU145 lysates. As these lysates could potentially contain LPAAT-β, LPAAT-α, and perhaps other LPAAT activities, and the assay, which uses 18:1-CoA and sn-1:18:1 lysoPA as substrates, will not necessarily distinguish between these activities, we interpret these data as indicating that LPAAT-β accounts for ~60% of the total endogenous LPAAT activity in these cells. Similar results were reported by Hideshima et al. (25).

Several experiments were then performed to test the effect of LPAAT-β inhibitors on the incorporation of 14C-labeled 18:1 into lysoPM in cells. Figure 3E shows the effect of CT-32228 on PM formation in PC-3 cells. The results indicate that CT-32228 inhibited ~50% of the PM formation in wild-type, control-transduced, and LPAAT-α-transduced cells and 60–75% of the PM formation in LPAAT-β-transduced cells. The effect of the inhibitors on PM formation appeared to be saturable, as separate experiments showed that the IC50 in PC-3/β1 cells for PM formation by CT-32228 was 321, 353, and 1,940 nM (the reason for the discrepancy in the IC50 values is unknown) and the IC50 for PM formation by CT-32501 was 460 ± 180 nM (n = 5) (Fig. 4). At saturating concentrations, CT-32501 inhibited as much as 95% and CT-32228 inhibited 60–75% of the PM formation in PC-3/β1 cells. One explanation for these differences in maximal inhibition might be the distribution of the inhibitors in the cell. It is important to note that CT-32212, a structurally similar compound to the LPAAT-β inhibitors but a weak inhibitor (Table 1), does not affect PM formation (Fig. 4). In similar experiments using DU145 cells transduced with LPAAT-β, the IC50 for PM formation by CT-32228 was 900 ± 400 nM (n = 3), whereas CT-32212 did not inhibit PM formation at 20 μM (data not shown).

As the LPAAT activity assay in Sf9 membranes suggested that LPAAT-β appears to prefer the transfer of 18:1 over 20:4 acyl groups by at least 18-fold (Table 1), experiments were performed to test whether this specificity was also observed in cells. PC-3 wild-type or PC-3/β1 cells were incubated with sn-1:18:1 lysoPM and with either 14C-labeled 18:1 or 14C-labeled 20:4. 14C-labeled 20:4 was also incorporated into PM in a time-dependent and lysoPM concentration-dependent manner (data not shown). However, unlike 14C-labeled 18:1, there was no significant difference in the incorporation of 14C-labeled 20:4 into PM between the two cell lines (Fig. 5). Furthermore, CT-32501 inhibited the incorporation of 14C-labeled 18:1 into lysoPM but did not inhibit the incorporation of 14C-labeled 20:4 into lysoPM (Fig. 5). These data provide evidence, from a second assay method, for the specificity of LPAAT-β toward the transfer of 18:1 acyl groups over 20:4 acyl groups.

Although the structure of lysoPM suggests that it has amphiphatic properties, it does not appear to damage the cells. Incorporation of 14C-labeled 18:1 into lysoPM and other lipids was linear to at least 100 μM lysoPM and for at least 60 min of incubation time in PC-3 and DU145 cells. If lysoPM was disrupting cellular membranes, ATP would likely be released from the cell, preventing the acyl-CoA synthetase-catalyzed conversion of the exogenously added 14C-labeled 18:1 into 18:1-CoA, a necessary step before the acylation of lysophospholipids. Second, incubation of DU145 or MCF7 cells with 50 μM lysoPM for 1–3 days did not affect their proliferation rates (data not shown). Third, as with lysoPM, 50 μM lysoPC can be added to cells without any noticeable deleterious effects on the membrane integrity of the cells (29).

It is important to note that similar experiments in which we tried to label cellular lipids with exogenously added 33P-labeled lysoPA did not result in any incorporation of 33P label into the lipid fraction (data not shown). This may be attributable in part to the inability of lysoPA to traverse the plasma membrane and/or to the action of one or more lipid phosphate phosphohydrolases that convert the lysoPA to inorganic phosphate and monoacylglycerol (30). In a separate manner, a significant amount of 14C-labeled
Fig. 3. Acylation of sn-1-18:1 lysophosphatidylcholine (LPC) with 14C-labeled 18:1 in PC-3 cells: effects of time, LPC concentration, and total LPAAT activity. A: Separate wells containing wild-type (wt) PC-3 cells or PC-3 cells transduced with LPAAT-β (β1), LPAAT-α (α1), or green fluorescent protein (GFP) cDNAs were incubated with 14C-labeled 18:1 (220,000 cpm) and the indicated concentrations of sn-1-18:1 LPC and CT-32228, as described in Experimental Procedures. After lipid extraction, TLC, and exposure of the plate to a phosphor screen (see Experimental Procedures), the screen was scanned with a Storm PhosphorImager in phosphor mode. The migration of lipid standards is indicated: NL, neutral lipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI/PS, phosphatidylinositol/phosphatidylserine; ori, origin.

B: Effect of LPC concentration on PM formation. PC-3 (squares) and PC-3/β1 (circles) cells were incubated (40 min, 37 °C) in the presence of 0.2 μCi of 14C-labeled 18:1 and the indicated concentrations of sn-1-18:1 LPC. Samples were processed as described for A, and 14C-labeled PM was quantitated and normalized to the total radioactivity detected in the lane. Results are representative of two experiments performed in triplicate. Error bars in all panels indicate SD.

C: Rate of PM formation. PC-3 (squares) and PC-3/β1 (circles) cells were incubated in the presence of 50 μM sn-1-18:1 LPC and 0.2 μCi of 14C-labeled 18:1 for the indicated times at 37 °C. Samples were processed and radioactivity quantitated as described for B, except that the data for this panel are expressed in cpm PM, because PM formation was quantitated and normalized to the total radioactivity detected in the lane. Results are representative of two experiments performed in triplicate.

D: Total endogenous LPAAT activity in PC-3 wild-type (WT) cells and PC-3 cells transduced with retroviral vectors containing no transcript (X1 and X2), GFP (GFP1, GFP2, GFP3), LPAAT-α (α1, α2, α3), or LPAAT-β (β1, β2, β3) cDNA was measured with the LPAAT activity assay in cell lysates (see Experimental Procedures). For reference, total LPAAT activity in the lysates from wild-type cells was 18 ± 5 (n = 8) nmol PA formed/min/mg protein.

E: Effect of the overexpression of LPAAT-β or LPAAT-α on 14C-labeled PM formation in PC-3 cells. PC-3 wild-type cells or cells transduced as described for D were incubated separately (40 min, 37 °C) in the presence of 0.4 μCi of 14C-labeled 18:1 and 50 μM sn-1-18:1 LPC (black bars). In some cases, 10 μM of the LPAAT-β inhibitor CT-32228 was added to the cells 12 min before the addition of the 14C-labeled 18:1 and the LPC (white bars). Approximately 4% of the total radioactivity was incorporated into the lipid fraction for each of the clones. Results are representative of two experiments performed in duplicate. Note that β1 described in A, D, and E refers to the PC-3/β1 clonal cell line.
20:4 is incorporated into PM (Fig. 5). Although LPAAT-β does not appear to be involved, other acyltransferase activities that transfer 20:4 acyl groups to various lysophospholipids (3) may also acylate lysoPM.

**DISCUSSION**

This report describes the enzymatic properties of LPAAT-β and LPAAT-α and introduces a novel assay to estimate intracellular LPAAT-β activity. We designed the LPAAT activity assay in Sf9 membranes under strict guidelines in an attempt to ensure that the assay did not mask the properties of the enzyme in question. First, the assay used membranes from Sf9 cells that overexpressed either human LPAAT-β or human LPAAT-α. Although not their natural membrane, each enzyme was separately expressed with high activity such that other acyltransferase activities were not detected. Second, BSA, often included in assays with acyl-CoA-metabolizing enzymes, was not included because it has submicromolar affinities for long-chain acyl-CoA (31–33) and lysoPA (34) and would most likely influence the substrates’ availability to the LPAAT. Indeed, inclusion of BSA in the membrane assay did not significantly affect the $V_{max}$ of the reaction but did increase the apparent $K_m$ of the substrates (data not shown). Third, the substrate concentrations were kept below reported critical micellar concentration values (35–38), as changes in the physical structure of the substrates during the assay would make interpretation of the data nearly impossible. With these guidelines established, it is interesting that LPAAT-α and LPAAT-β showed subtle differences in their preference for different acyl-CoAs: 14:0-CoA, 16:0-CoA, and 18:2-CoA were most active in the LPAAT-α reaction, whereas 18:1-CoA was most active in the LPAAT-β reaction (Fig. 1B). Furthermore, only LPAAT-β was able to use lysoPM as a substrate (Fig. 2C). Although greater confidence in the biochemical characterization of these isozymes will have to await their purification and insertion into well-defined lipid vesicles, it seems reasonable to assume that the membrane assay displays, at least in part, the properties of each LPAAT and is not a limitation of the system.

To confirm the results observed in the LPAAT activity assay in Sf9 membranes, a second, cell-based assay was used in which LPAAT-β activity was measured by acylation of lysoPM. LPAAT-β appears to acylate lysoPM in cells based on the following observations.

1) Using the membrane assay, lysoPM was shown to be a substrate for LPAAT-β but not LPAAT-α (Fig. 2C). 2) Addition of lysoPM and 14C-labeled 18:1 to PC-3 or DU145 cells resulted in the formation of a 14C-labeled lipid that comigrated with

![Fig. 4](image)

**Table 2.** LPAAT-β inhibitors

<table>
<thead>
<tr>
<th>CT No.</th>
<th>Structure</th>
<th>LPAAT-β IC50 nM</th>
<th>LPAAT-α IC50</th>
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<td><img src="image" alt="Structure" /></td>
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<td>&gt;40,000</td>
<td>19</td>
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<td>36,000</td>
<td>&gt;40,000</td>
<td>20</td>
</tr>
</tbody>
</table>

LPAAT activity was measured in Sf9 membranes containing recombinant LPAAT-β or LPAAT-α, as described previously (20).

![Fig. 5](image)
authentic PM by TLC in two separate solvent systems; the formation of lysoPM (Fig. 3A). 3) Cells transduced with LPAAT-β (5- to 20-fold higher total LPAAT activity as measured in cell lysates) showed greater incorporation of label into PM than the corresponding wild-type cells or cells transduced with LPAAT-α, GFP, or vector only (Fig. 3D, E and data not shown). 4) Addition to cells of isofrom-specific, small molecule LPAAT-β inhibitors decreased the incorporation of 14C-labeled 18:1 into PM (Figs. 3-5). 5) Treatment of DU145 cells, either wild type or containing recombinant LPAAT-β, with LPAAT-β small, interfering RNA resulted in a knockdown of LPAAT-β activity, as measured in cell lysates, and a decrease in the incorporation of 14C-labeled 18:1 into PM, as measured in cells (M. Coon, personal communication).

It is curious that there is an apparent discrepancy between the observed lysoPM acylation rates in the membrane assay and the whole cell assay. In the membrane assay, lysoPM is acylated by LPAAT-β at a much lower rate than lysoPA (compare Fig. 2B, C), but in the whole cell assay, PM formation accounts for as much as 5% and 25% of the total radioactivity incorporated into the lipid fraction of PC-3 and PC-3/β1 cells, respectively (Fig. 3E). There are several possible explanations. First, the membrane assay suggests that both substrates are active at similar concentrations, suggesting similar affinities for LPAAT-β. Second, the whole cell assay has the advantage of measuring LPAAT-β activity within an active membrane, perhaps within a unique microenvironment that is not duplicated in the membrane assay. Third, the whole cell assay may take into account proteins that may affect LPAAT-β activity. For example, the cellular acyl-CoA pool appears to be controlled by acyl-CoA binding proteins and fatty acid binding proteins, and these soluble, 10–15 kDa proteins can affect the activity of enzymes in which acyl-CoA is a substrate, product, or inhibitor (39–41), including LPAAT activities (42–44). Some or all of these factors may account for the robust, LPAAT-β-dependent PM signal in the whole cell assay.

We have tried to show an effect on lipid metabolism in cells that overexpress LPAAT-β or with the use of the LPAAT-β isoform-specific inhibitors. For example, we incubated ECV304, DU145, MCF7, and NIH3T3 cells with 14C-labeled 18:1 or with 14C-labeled glycerol, or we incubated DU145 cells with 32P-labeled phosphate. Surprisingly, we did not detect any change in the pattern of incorporation of the radioactive tracer into PA or the major glycerolipids or any change in the total mass of each lipid between wild-type cells and the LPAAT-β-overexpressing populations (data not shown). Similar observations have been noted for overexpressed PLD isozymes (45) and certain E. coli lipid-metabolizing enzymes (46). Furthermore, the only significant changes caused by the addition of LPAAT-β inhibitors was on the accumulation of lysoPA in 3T3-L1-derived adipocytes incubated with 14C-labeled 18:1, or in DU145 cells incubated with propranolol, a lipid phosphate phosphohydrolase inhibitor, plus 32P-labeled phosphate (data not shown). There may be several possible explanations for why a clear PM signal is observed in the whole cell assay but a change in PA metabolism or mass is not observed in the assays mentioned above. Exogenously added 18:1, glycerol, and phosphate must first be converted to acyl-CoA and/or lysoPA. These substrates can then be used by LPAAT-β, but also by other enzymes. In addition, 18:1, glycerol, and phosphate can each be incorporated into lipids by other pathways. These factors may dilute the signal we are trying to observe from LPAAT-β. On the other hand, the fate of the lysoPM may be more limited, as LPAAT-β and perhaps a few other unidentified acyltransferases are able to acylate it, and the resulting PM may not be metabolized as rapidly as PA.

It would appear that the major product of the LPAAT-β-catalyzed reaction is PA of a restricted molecular species, based on the following observations. First, data from the LPAAT activity assay in Sf9 membranes suggest that lysoPA but not other lysophospholipids, glycerol-3-phosphate, 1-monoooleoylglycerol, or dioleoylglycerol could be used as an acyl acceptor (Fig. 2A and data not shown). Second, JC201 cells, an E. coli strain with a temperature-sensitive mutation within its sole LPAAT gene, can be rescued at a nonpermissive temperature by expression of LPAAT-β (8). Third, LPAAT-β appears to be specific for the transfer of 16:0, 18:0, and 18:1 acyl groups over 12:0, 14:0, and 20 carbon acyl groups (Table 1). Fourth, the high selectivity of LPAAT-β for the transfer of 18:1 groups over 20:4 groups was also observed in the whole cell assay (Fig. 5).

Questions still remain concerning the fate of the PA produced by LPAAT-β. With the cloning of six LPAAT genes, questions arise regarding the role of each isoform’s cellular functions (47). Based on two separate assays in this study, the fact that 20:4 groups are not transferred if 18:1 groups are present strongly suggests that LPAAT-β is not closely involved in synthesizing glycerolipids esterified with 20:4 that are fated for the eicosanoid pathways. On the other hand, LPAAT-β may function within a lipid synthetic pathway. It is widely accepted that de novo-synthesized glycerolipids contain primarily monoenoic and dienoic acyl groups at the sn-2 position (48). Our data suggest that LPAAT-β can contribute to this molecular species profile. Consistent with this, LPAAT-β is highly expressed in adipocytes, and Lu et al. (47) have suggested that mouse LPAAT-β expression is regulated by peroxisome proliferator-activated receptor α, a transcription factor that regulates the expression of lipid-metabolizing enzymes. Patients with a form of congenital generalized lipodystrophy, a disorder characterized by a lack of nonmechanical adipose tissue from birth, contain mutations in the LPAAT-β gene, rendering it nonfunctional (11, 13, 49). Although a simple explanation is that patients without active LPAAT-β are unable to synthesize triglyceride in adipocytes, they have functional LPAAT-α, which is also present in healthy adipocytes, produce high levels of triglyceride in the plasma compartment, and appear to have normal mechanical fat. Therefore, it is of interest that these two isoforms appear not to have entirely redundant biological functions. To make matters more complex, PA of molecular species similar to those described above may
also play a role in signaling (45, 50, 51), and evidence has been presented raising the possibility that LPAAT-β may contribute to tumor progression (20). Further study will be required to address questions regarding the cellular role of LPAAT-β.

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