Phosphatidylglycerol (PG) is an important membrane polyglycerolphospholipid required for the activity of a variety of enzymes and is a precursor for synthesis of cardiolipin and bis(monoacylglycerol) phosphate. PG is subjected to remodeling subsequent to its de novo biosynthesis to incorporate appropriate acyl content for its biological functions and to prevent the harmful effect of lysosphatidylglycerol (LPG) accumulation. The enzymes involved in the remodeling process have not yet been identified. We report here the identification and characterization of a human gene encoding an acyl-CoA:lysophosphatidylglycerol acyltransferase (LPGAT1). Expression of the LPGAT1 cDNA in SF9 insect and COS-7 cells led to a significant increase in LPG acyltransferase activity. In contrast, no significant acyltransferase activities were detected against glycerol 3-phosphate or a variety of lysophospholipids, including lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinoisitol, and lysophosphatidylserine. The recombinant human LPGAT1 enzyme recognized various acyl-CoAs and LPGs as substrates but demonstrated clear preference to long chain saturated fatty acyl-CoAs and oleoyl-CoA as acyl donors, which is consistent with the lipid composition of endogenous PGs identified from different tissues. Kinetic analyses of LPGAT1 expressed in COS-7 cells showed that oleoyl-LPG was preferred over palmitoyl-LPG as an acyl receptor, whereas oleoyl-CoA was preferred over lauroyl-CoA as an acyl donor. Consistent with its proposed microsomal origin, LPGAT1 was localized to the endoplasmic reticulum by subcellular fractionation and immunohistochemical analyses. Northern blot analysis indicated that the human LPGAT1 was widely distributed, suggesting a dynamic functional role of the enzyme in different tissues.

The anionic phospholipid phosphatidylglycerol (PG) is a common polyglycerolphospholipid required for many cellular functions (1). PG represents ~1% of total phospholipids in most mammalian tissues, except for lung, and is found in many subcellular locations, such as microsomal, mitochondrial, and nuclear membranes (2, 3). In lung, PG represents ~5% of total phospholipids and is a major component of lung surfactant (2, 3). PG plays an important role in maintaining the normal function of lung, and absence of PG during fetal development is associated with a high risk of neonatal respiratory distress syndrome (4, 5). Biochemical evidence indicates that PG is a potential activator of members of the protein kinase C family, including protein kinase C-θ (6) and nuclear protein kinase C-βII (7). PG is also a precursor for the biosynthesis of bis(monoacylglycerol) phosphate that plays an important role in liposome formation and endosome organization (8, 9). Bis(monoacylglycerol) phosphate is also a predominant phospholipid of macrophages (10–12) and a target antigen for human antibodies associated with antiphospholipid syndrome characterized by increased risk of thrombosis, recurrent fetal loss, and thrombocytopenia (13).

The de novo biosynthesis of PG occurs via the cytidine diphosphate-diacylglycerol (CDP-DAG) pathway that begins with formation of CDP-DAG from phosphatidic acid catalyzed by phosphatidic acid:CTP cytidylyltransferase. CDP-DAG is then converted to PG by sequential action of phosphatidylglycerol phosphate (PGP) synthase and PGP phosphatase (Fig. 1) (14). PG is an important precursor for the synthesis of cardiolipin, a polyglycerolphospholipid that is required for the activity of a large number of mitochondrial enzymes and carrier proteins (15). Consequently, disruption of phosphatidylglycerol phosphate synthase (PGS) gene in yeast causes PG and cardiolipin deficiency and inhibition of growth on nonfermentable carbon sources (16). In yeast cells, PG appears to substitute for cardiolipin functions (17), as evidenced by a significant increase in PG content resulted from disruption of the CRDI gene that encodes cardiolipin synthase (15, 18). PG and cardiolipin deficiency in Chinese hamster ovary cells caused by a mutation in the PGS gene results in mitochondrial morphological and functional abnormalities manifested by increased glycolysis, reduced oxygen consumption, stringent temperature sensitivity for cell growth in glucose-deficient medium, and reduced ATP production. In support for a possible role of PG in oxidative phosphorylation, decreased cardiac PG levels were reported in streptozotocin-induced diabetic rats (19, 20).

Phospholipids are known to undergo a rapid deacylation-reacylation recycling process to incorporate appropriate fatty acyl composition back to their parent molecules. The re-acyla-

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The abbreviations used are: PG, phosphatidylglycerol; GPAT, glycerol-3-phosphate acyltransferase; LPG, lysophosphatidylglycerol; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; LPA, lysophosphatic acid; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinoisitol; LPS, lysophosphatidylserine; DAPI, 4,6-diamidino-2-phenylindole; LPGAT1, acyl-CoA:lysophosphatidylglycerol acyltransferase; CDP-DAG, cytidine diphosphate-diacylglycerol; PGP, phosphatidylglycerol phosphate.
A Gene Encoding a Human ER-associated LPG Acyltransferase

MATERIALS AND METHODS

Cloning of the Full-length Lysophosphatidylglycerol Acyltransferase cDNA—A human cDNA clone (NCBI accession number BC034621) was identified in NCBI data bases based on its conserved sequence motifs with the human glycerol-3-phosphate acyltransferase (GPAT) (36) as a candidate gene for a novel GPAT. This GPAT cDNA clone was initially named hGPAT4, and subsequently changed its name to acyl-CoAlysophosphatidylglycerol acyltransferase (LPGAT1) after functional characterization. A PCR primer pair (forward, 5′-CGGAGTCCA-GTTGGAATGGCTA-3′, and reverse, 5′-ACGGTGACCTTGAACAGGGTCCACTG-3′) was used to amplify the full-length coding region of the human LPGAT1 gene from Marathon-Ready cDNA prepared from the human liver (clonette). Amplification was performed by PCR using Promega DNA polymerase (Invitrogen) and a thermal cycling condition of 35 cycles (94 °C for 30 s, 62 °C for 30 s, and 68 °C for 2 min), resulting in a 1.2 kb cDNA product that was cloned into the SacI site of pcDNA3.1 vector (Stratagene, La Jolla, CA) and sequenced.

Northern Blot Analysis—To analyze the tissue distribution pattern of human LPGAT1 mRNA, multiple human tissue poly(A) RNA Northern Blots (Clontech) were hybridized with [α-32P]dCTP (3000 Ci/mmol, ICN Radiochemicals)-labeled probes prepared from full-length cDNA of human LPGAT1 gene using a Prime-It RNeasy Random Primer Labeling Kit (Stratagene, La Jolla, CA). Hybridization was carried out in ULTRAHyb (Ambion, Austin, TX) at 55 °C overnight, followed by three washes at 55 °C in 2× SSC buffer containing 0.1% SDS and 1× EDTA. Blots were stripped with boiling 1× SDS to remove radiolabeled probe and re-probed with human radiolabeled GPAT cDNA as an internal control. The blots were exposed to a PhosphorImager Screen to visualize signals and were quantified by ImageQuant (Amersham Biosciences).

Expression of LPGAT1 in Insect Cells—Expression of LPGAT1 in insect cells was performed by using a Bac-to-Bac Baculovirus Expression System (Invitrogen). The human LPGAT1 cDNA that carries the entire coding region was subcloned into EcoRI and NotI sites of the pFastBac vector (Invitrogen), which was subsequently transformed into DH10Bac™ Escherichia coli cells to generate a recombinant bacmid that carries the insertion of the LPGAT1 cDNA. High titer recombinant baculovirus was generated by transfecting the bacmid DNA into Spodoptera frugiperda 9 (Sf9) insect cells followed by several rounds of amplification to increase viral titer. After infection with recombinant baculoviruses for 65 h, Sf9 cells were harvested in ice-cold phosphate-buffered saline (PBS), pelleted by centrifugation, lysed, and assayed immediately for enzyme activity or frozen in liquid N2 for later use. Cell pellets were homogenized in 20 mM NaCl with 20 up-and-down strokes in a motor-driven Dounce homogenizer (Heidelberg, Germany) followed by three passages through a 27-gauge needle. The protein concentration in homogenate was determined by a BCA Protein Assay Kit (Pierce) according to the manufacturer’s instructions.

Expression of LPGAT1 and FLAG-LPGAT1 in Mammalian Cells—A mammalian expression vector for full-length human LPGAT was engineered by subcloning the 1.2 kb cDNA fragment from the pcDNA3.1 vector into the NotI and XhoI sites of pcDNA3.1(+), pcDNA3.1(−)Hygrom (Invitrogen). A FLAG-tagged version of LPGAT1 was engineered by PCR amplification with 5′-TACGTTACANCCGATCGACGCTATAACGCTTAGAAGTCCACGT-3′ (referred to as a FLAG tag) and 5′-ACGGTGACCTTGAACAGGGTCCACTG-3′ (reverse) to add a FLAG tag to the N terminus of LPGAT1 and a thermal cycling condition of 35 cycles (94 °C for 30 s, 53 °C for 30 s, and 68 °C for 2 min). The amplified DNA fragment was cloned into the pcDNA3.1 vector (Stratagene, La Jolla, CA) and verified by sequencing. The insert was then subcloned into the HindIII and NotI sites of pcDNA3.1(+), pcDNA3.1(−)Hygrom vectors for transient expression in COS-7 cells. COS-7 cells were maintained under the conditions recommended by American Tissue Culture Collection (ATCC, Manassas, VA). A day before transfection, two million cells were subcultured onto a 100×20-mm plate resulting in ~70% confluence. Cells were transfected with 10 μg of DNA premixed with FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. Forty four hours after the transfection, cells were harvested and washed PBS (+), pelleted by centrifugation, lysed, and assayed immediately or frozen in liquid N2 for later use.

In Vitro Assays for GPAT Activity—GPAT activity was determined by conversion of glycerol 3-phosphate to 1-acyl-sn-glycerol 3-phosphate in the presence of acyl-CoA. The reaction was conducted at room temperature in a final volume of 200 μl as described previously (37, 38). The reaction mixture contained 75 mM Tris/HCl, pH 7.4, 4 mM MgCl2, 1 mM/ml bovine serum albumin free fatty acids, 8 mM NaF, 50 μM palmitate, 100 μM acyl-CoA, 5 μM linoleic acid, 200 μM [14C]linoleic acid, and 20 mM MgCl2. After 10 min, the reaction was stopped by adding 1 ml of methanol:water (1:1) and the lipid extracts were then extracted with benzene:chloroform (3:1) for subsequent analysis of lipid composition of the rat liver phosphatidylethanolamine after injection of [14C]linoleic acid indicates that 95% of the linoleic acid in 1-stearoyl-2-linoleoylphosphatidylethanolamine was incorporated by means of deacylation and reacylation (21). The deacylation-reacylation cycle for the remodeling of phosphatidylcholine in mammals has been extensively investigated (22, 23).

The molecular and biochemical nature of the enzyme has not been characterized in the present study. We report the identification and characterization of a human gene encoding polypeptide possessing activities of LPGAT, designated as LPGAT1. The recombinant LPGAT1 expressed in both Sf9 insect cells and COS-7 cells catalyzed efficiently the reacylation of LPG to PG with various LPGs and acyl-CoAs as substrates but preferred long saturated fatty acyl-CoAs as acyl donors. Subcellular localization analyses indicated that LPGAT1 was localized in the ER, which is consistent with its proposed palmosomal origin.
toyl-CoA, 3 mM glycerol 3-phosphate, 1–2.5 µCi of [3H]glycerol 3-phosphate (20 Ci/mmol, American Radiolabeled Chemicals Inc, St. Louis, MO), and 50 µg of cellular homogenate. The reaction was initiated by adding protein homogenate, incubated at room temperature for 10 min, and terminated by adding 0.5 ml of water-saturated 1-butanol. The product from the GPAT enzyme assay was extracted and quantified as described previously (37).

In Vitro Assays for Lysophospholipid Acyltransferase Activities—The human LPGAT1 was assayed for lysophospholipid acyltransferase activities by measuring the incorporation of radiolabeled acyl moieties of acyl-CoAs (acyl donors) into phospholipids in the presence of relevant lysophospholipids (acyl acceptors). The lysophospholipids used in the experiment include lysophosphatidic acid (LPA), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS) (Sigma), and lysophosphatidylglycerol (LPG) (Avanti Polar Lipids, Inc., Alabaster, AL). The reaction mixture contained 80 mM Tris/HCl, pH 7.4, 200 µM lysophospholipid, 20 µM [14C]acyl-CoA (50 mCi/mmol, American Radiolabeled Chemicals Inc), and cell homogenate (50 µg) that contained recombinant LPGAT1 expressed in Sf9 or COS-7 cells, in a total volume of 200 µl. The reaction was initiated by addition of the protein homogenate and was terminated by adding 1 ml of chloroform/methanol (2:1, v/v), after 10 min of incubation at room temperature. The phospholipid product was extracted and separated on TLC plate as described previously (39).

In Vitro Assays for LPGAT Activity—Enzymatic reaction was initiated by addition of 50 µg of cell homogenates that contained the recombinant LPGAT1 expressed in COS-7 cells and incubated for 10 min at room temperature in a 200-µl reaction mixture that contained 80 mM Tris/HCl, pH 7.0, 200 µM [14C]acyl-CoA (50 mCi/mmol), and 200 µM of various LPGs, including 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)](sodium salt), 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)](sodium salt), 1-stearyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)](sodium salt), and 1-oleoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)](sodium salt) (Avanti Polar Lipids, Inc., Alabaster, AL). Radiolabeled acyl-CoAs used in the studies were n-octanoyl-CoA, lauroyl-CoA, palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA, and arachidonyl-CoA (American Radiolabeled Chemicals Inc., St. Louis, MO). The reaction was terminated by adding 1 ml of chloroform/methanol (2:1, v/v), followed by addition of 0.4 ml of 0.9% KCl to facilitate phase separation. After vigorous vortex for 10 s to extract lipids, phase separation was performed by a brief centrifugation. Aliquots of the organic phase containing phospholipids were dried under a N2 stream and separated by the Linear-K Preadsorbent TLC Plate (Waterman Inc., Clifton, NJ) with the chloroform/methanol/water (65:25:4, v/v). Individual lipid moieties were identified by standards with exposure to I2 vapor.

Statistical Analysis—Differences in enzyme activities between different substrates were analyzed by one-way analysis of variance and Student t test.

Western Blot Analysis—COS-7 cells transfected with FLAG-tagged LPGAT1 expression vector or empty vector were harvested in Bug Buster HT buffer (Novagen) in the presence of 1× Complete protease...
inhibitors (Roche Diagnostics) and incubated for 10 min at room temperature with 1% SDS, followed by centrifugation at 20,000 x g for 5 min. The supernatant was used to detect the expression of LPGAT1 by Western analyses. The protein lysate was denatured by boiling for 3 min in 1x loading buffer containing NuPAGE reducing agent, resolved on 4–20% NOVEX Tris-glycerin SDS-PAGE (Invitrogen), and transferred to a nitrocellulose membrane. The membrane was incubated for 2 h at room temperature in washing buffer (0.9% NaCl, 20 mM Tris/HCl, pH 7.5, 0.1% Tween 20) containing 5% nonfat milk to block nonspecific binding. The blots were then incubated with mouse monoclonal anti-FLAG M2 antibody (1.0 μg/ml, Sigma) overnight at 4 °C in the washing buffer. After four washes (5 min each), the membrane was incubated with anti-mouse IgG horseradish peroxidase conjugate (1:5000, Amersham Biosciences) for 1 h at room temperature. The blots were washed four times (5 min each) and visualized with ECL Plus (Amersham Biosciences). The signal was scanned by a PhosphorImager (Amersham Biosciences) and quantified by ImageQuant software.

Subcellular Fractionation—Subcellular fractionation analysis was carried out to localize human LPGAT1 transiently expressed in COS-7 cells. Cell pellets were homogenized with a Dounce homogenizer in 10 volumes (w/v) of solution consisting of 0.25M sucrose, 0.01M Tris/HCl, pH 7.4, and 1 mM EDTA. The homogenate was first centrifuged at 800 x g for 10 min to remove cell debris and nuclear fractions. The mitochondrial fraction was obtained by centrifuging the supernatant at 8,000 x g for 10 min. Microsomal fraction was prepared from the postmitochondrial supernatant by sedimentation at 100,000 x g for 60 min. The mitochondrial and microsomal fraction was suspended in PBS buffer and stored in aliquots at –80 °C.

Immunocytohistochemistry—Cells were grown and transfected on a coverslip (BD Biosciences). Forty eight hours after transfection, cells were first incubated in the growth medium with 100 nM MitoTracker Red CMX Ros for 10 min at 37 °C to achieve the specific staining for mitochondria. The cells were then washed two times with PBS (2 min each) and fixed with freshly prepared 4.0% paraformaldehyde prewarmed at 37 °C. The samples were rinsed twice with PBS (5 min each) and permeabilized with 0.2% Triton X-100 in PBS, followed by incubation for 1 h in 5% normal donkey serum to block nonspecific binding. The samples were then incubated for 2 h at room temperature with mouse monoclonal anti-FLAG M2 antibody (5.0 μg/ml, Sigma) or rabbit anti-calnexin N-terminal polyclonal antibody (1.0 μg/ml, StressGen Biotechnologies Corp, Victoria, Canada). After brief wash with PBS three times, the samples were incubated for 1 h at room temperature with Cy2-conjugated donkey anti-mouse IgG or Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The cells were also counterstained with DAPI (Molecular Probes, Eugene, OR) to visualize nuclei. The samples were washed four times with PBS and analyzed with a confocal fluorescence microscope (Olympus BX61, Nashua, NH).

RESULTS

Identification and Cloning of the Human LPGAT1 Gene—A human LPGAT1 cDNA clone (NCBI accession number BC034621) was initially identified in NCBI data bases based on its conserved sequence motifs with the human GPAT (36) as a candidate gene for a novel GPAT. A 1.2-kb cDNA fragment encoding the full-length human LPGAT1 enzyme was cloned by PCR amplification using a cDNA library from human liver and a primer pair designed from the 5’- and 3’-untranslated regions, respectively, of the predicted human LPGAT1 open reading frame. Sequence analysis of the 1.2-kb cDNA suggests a
full-length coding region for the enzyme as evidenced by an in-frame stop codon located 110-bp 5‘-upstream of the predicted AUG start site embedded within a Kozak consensus sequence for translation initiation. The human LPGAT1 gene predicts a 370-amino acid protein of 43 kDa that carries a 23-amino acid signal-anchor domain at the N terminus and a potential N-linked glycosylation site at position 209 (Fig. 2). The predicted LPGAT1 peptide sequence carries motifs that match all the four conserved domains among members of the GPAT family (36), suggesting a functional role as a potential GPAT enzyme. The human LPGAT1 gene is localized on the human chromosome 1 at 1p36.13-q42.3. Results from blast analyses of the public genomic and EST data bases suggest that the LPGAT1 gene is highly conserved throughout eukaryotic species, from fungi to mammals. An ortholog from Cae- norhabditis elegans (NCBI accession number NM_073010) shares 38.4% identity and 54.4% similarity, respectively, with the predicted human LPGAT1 peptide sequence.

**Tissue Distribution of the Human LPGAT1 mRNA**—The tissue distribution of the human LPGAT1 mRNA was analyzed by Northern blot analysis. Two mRNA splice isoforms of 7.5 and 9.5 kb in length, respectively, were detected in most human tissues examined including peripheral blood, liver, lung, placentas, kidney, and brain (Fig. 3). When normalized with the mRNA level of G3PDH, the expression level was the highest in liver and placenta. In contrast, very low level of expression was detected in human colon.

**Analysis of Acyltransferase Activity of the Recombinant LPGAT1 Expressed in Sf9 Cells toward Glycerol 3-Phosphate and Various Lysophospholipids**—A baculovirus expression system was developed to achieve high level expression of the recombinant LPGAT1 in Sf9 insect cells for functional characterization of the enzyme. An enzymatic assay was initially carried out with glycerol 3-phosphate and oleoyl-CoA as substrates to de-
termine whether the polypeptide encoded by LPGAT1 cDNA possesses GPAT activity with the recombinant human GPAT1 enzyme expressed in Sf9 cells as a positive control, because the LPGAT1 enzyme carries motifs that match all the four conserved domains among members of the GPAT family (36). In contrast to an 8-fold increase in GPAT activity from the recombinant human GPAT1, no significant increase in GPAT activity was detected from the recombinant LPGAT1 relative to the negative control (data not shown). By using oleoyl-CoA as an acyl donor, we next analyzed the recombinant LPGAT1 enzyme for acyltransferase activities against a variety of lysophospholipids, including LPA, LPC, LPE, LPI, LPS, and LPG. As shown in Fig. 4A (quantified in Fig. 4B), a 3-fold increase in acyltransferase activity was observed from the recombinant LPGAT1 expressed in Sf9 cells relative to the negative controls (Sf9 cells infected with the wild-type baculovirus or with recombinant baculovirus expressing an unrelated protein, URP, a novel acyltransferase with unknown function) when oleoyl-LPG was used as a substrate. In contrast, no significant increase in acyltransferase activity was detected against LPA, LPC, LPE, LPI, and LPS as a substrate, respectively, suggesting that the human LPGAT1 gene encodes an LPG acyltransferase.

Acylation Selectivity of the Recombinant LPGAT1 Expressed in COS-7 Cells toward Different Acyl-CoAs and LPGs—To provide further evidence for enzymatic features of LPGAT1, we analyzed acyl selectivity of the recombinant LPGAT1 transiently expressed in COS-7 cells using sn-1-palmitoyl-LPG as an acyl acceptor and various acyl-CoAs as acyl donors, including n-octanoyl-CoA (C8:0), lauroyl-CoA (C12:0), palmitoyl-CoA (C16:0), stearoyl-CoA (C18:0), oleoyl-CoA (18:1), linoleoyl-CoA (18:2), and arachidonyl-CoA (C20:4). The experiments were conducted under conditions where 200 μM sn-1-palmitoyl-LPG was incubated with 20 μM each of the [14C]acyl-CoAs for 10 min in the presence of 50 μg of protein homogenate from COS-7 cells transiently transfected with the LPGAT1 expression vector or the empty vector. As shown by Fig. 5A (quantified in Fig. 5C), the recombinant LPGAT1 expressed in COS-7 cells demonstrated clear preference to long chain fatty acyl-CoAs and oleoyl-CoA as acyl donors, as evidenced by significantly higher (\(*, p < 0.05\)) LPGAT1 activity than other acyl-CoA species. In contrast, the recombinant LPGAT1 enzyme exhibited poor activity toward medium chain saturated and long chain polyunsaturated fatty acyl-CoAs, as compared with the negative control. The LPGAT1 activity was dependent on the presence of LPG, because the acyltransferase activity was totally abolished in the absence of LPG when assayed with palmitoyl-CoA as the acyl donor (Fig. 5A, compare lane 6 with 15).

We also examined the preference of the recombinant LPGAT1 to various LPGs that differ in fatty acyl chain length and degree of saturation at sn-1 position, including myristoyl (C14:0), palmitoyl (C16:0), stearoyl (C18:0), and oleoyl (C18:1) using palmitoyl-CoA as the acyl donor under the same assay conditions. As shown in Fig. 5B (quantified in Fig. 5D), the LPGAT1 enzyme recognized each of the LPGs as a substrate, as demonstrated by a significant increase in acyltransferase activity relative to the negative control. Although the four LPGs differed in both acyl chain length and degree of saturation, there is no significant difference between them in catalytic activity by the LPGAT1, suggesting a lack of strict acyl selectivity toward acyl acceptors by the LPGAT1 enzyme with palmitoyl-CoA as the acyl donor (Fig. 5, B and D). A lack of commercially available LPGs with more divergent acyl structures has precluded further testing on acyl selectivity toward LPGs with short chain or polyunsaturated fatty acyl groups.

Effect of Acyl Composition on Kinetics of the Recombinant LPGAT1 Expressed in COS-7 Cells—To further characterize acyl preference of LPGAT1, we next analyzed the kinetics of the recombinant LPGAT1 enzyme against palmitoyl-LPG and oleoyl-LPG, the two most common endogenous LPGs, in combination with two selective acyl-CoAs: oleoyl-CoA and lauroyl-CoA, the well and poorly represented acyl species of natural PGs, respectively. The initial rate of the enzyme was established by incubating 200 μM each of the LPGs with 20 μM each of the [14C]acyl-CoAs for 1, 3, 5, 10,15, 20, and 30 min, respectively, in the presence 50 μg of COS-7 cell homogenate as a source of LPGAT1 enzyme. Kinetic analysis was carried out by incubating 200 μM each of the LPGs with an increasing concentration (6, 12, 30, 60, and 120 μM) of each acyl-CoAs, respectively, for 10 min under the same assay condition. All enzyme activity data were derived from at least three independent experiments and were shown as mean ± S.E. A significant difference in LPGAT1 activity (p < 0.05) from lauroyl-CoA as the acyl donor with each LPG was indicated with an asterisk.

Fig. 6. Effects of acyl composition on enzyme kinetics of the recombinant human LPGAT1 expressed in COS-7 cells. The LPGAT1 kinetics was analyzed using palmitoyl-LPG and oleoyl-LPG, the most common endogenous LPGs, in combination with oleoyl-CoA and lauroyl-CoA, the well and poorly represented acyl species of natural PGs, respectively. The initial rate of the enzyme was determined by incubating 200 μM each of the LPGs with 20 μM each of the [14C]acyl-CoAs for 1, 3, 5, 10,15, 20, and 30 min, respectively, in the presence 50 μg of COS-7 cell homogenate as a source of LPGAT1 enzyme. The results established the first 10 min as the initial rate for LPGAT1 against these substrates (data not shown). Kinetic analysis was carried out by measuring the LPGAT activity with increasing concentrations (6, 12, 30, 60, and 120 μM) of acyl-CoA in the presence of 200 μM each of the LPGs. As shown in Fig. 6, oleoyl-CoA was preferred over lauroyl-CoA as an acyl donor with each LPG as the acyl receptor, whereas oleoyl-LPG was preferred over palmitoyl-LPG as the acyl acceptor by the recombinant LPGAT1 enzyme expressed in COS-7 cells. Thus, the recombinant LPGAT1 demonstrated the highest activity toward oleoyl-CoA and oleoyl-LPG as substrates (Fig. 6).

Subcellular Localization of LPGAT1 Transiently Expressed in COS-7 Cells—To facilitate Western blot and immunocyto-histochemical detections of the recombinant human LPGAT1, we engineered a mammalian expression vector by attaching a FLAG tag to the N terminus of LPGAT1. The FLAG-LPGAT1 enzyme expressed in COS-7 cells migrated on SDS-PAGE with apparent molecular mass of 43 kDa (Fig. 7A), which is consistent with the molecular weight predicted from the open reading frame of the human LPGAT1 gene, suggesting no major glycosylation of the enzyme in mammalian cells. The attachment of the FLAG tag to the N terminus of LPGAT1 did not affect LPGAT1 enzyme activities, because the recombinant FLAG-LPGAT1 expressed in COS-7 exhibited a similar level of acyltransferase activity and acyl selectivity as those observed from...
the nontagged LPGAT1 expressed in COS-cells when tested against a variety of acyl-CoAs as acyl donors (data not shown).

To investigate the subcellular localization of LPGAT1, COS-7 cells transiently transfected with FLAG-LPGAT1 expression vector or empty vector were homogenized and fractionated into mitochondrial and microsomal fractions by differential sedimentation. The fractions were analyzed by Western blot analysis using antibodies to FLAG, calnexin, and prohibitin, which were used as markers for LPGAT1, ER, and mitochondria, respectively. The results demonstrated that LPGAT1 was significantly enriched in the microsomal fraction (Fig. 7B, Micro) as compared with the total cell lysate (Homo) and the mitochondrial fraction (Mito). Furthermore, the LPGAT protein showed a very similar subcellular distribution pattern with calnexin, but not prohibitin, suggesting that LPGAT1 is a microsomal enzyme. Although a low level of LPGAT1 was also detected in the mitochondrial fraction, this was likely caused by microsomal contamination, as evidenced by a similar level of calnexin detected in the mitochondrial fraction (Fig. 7B).

We next performed immunocytohistochemical analyses of the recombinant FLAG-LPGAT1 transiently expressed in COS-7 cells to define its subcellular localization in intact cells.
A Gene Encoding a Human ER-associated LPG Acyltransferase

Forty eight hours after transfection, cells were processed for mitochondria staining with MitoTracker Red CMXRos or indirect immunofluorescence staining with antibodies specific for the FLAG epitope (green) as well as calnexin (red). Cells were also counterstained with DAPI to visualize nuclei (blue). The FLAG-LPGAT1 protein expressed in COS-7 cells displayed a perinuclear and punctuated pattern (Fig. 7C, a and d). The FLAG-LPGAT1 protein was not co-localized with MitoTracker Red CMXRos-stained mitochondria (Fig. 7C, b), as demonstrated by the well separated green (FLAG-LPGAT1) and red (mitochondria) colors in the merged image (Fig. 7C, c). In contrast, the FLAG-tagged LPGAT1 protein was clearly co-localized with the ER marker, calnexin (Fig. 7C, e), as demonstrated by the yellow color in the merged image (Fig. 7, f). As a negative control of the immunostaining process, no significant staining was observed in mock-transfected cells stained with anti-FLAG antibody or with normal mouse IgG (data not shown). The results demonstrated conclusively that LPGAT1 was an ER-associated protein.

DISCUSSION

The presence of microsomal LPGAT activities in various mammalian tissues has been well documented (32–35). The reacylation process is believed to play an important role for attaining an appropriate fatty acyl composition because the mitochondrial enzymes involved in the synthesis of PG exhibit no acyl selectivity for their substrates (40). The reacylation of LPG back to the PG also prevents the accumulation of LPG that is known to have harmful effects (41). In this study, we identified a human gene encoding an enzyme that efficiently catalyzed the reacylation of LPG and was therefore assigned as acyl-CoAlyso phosphatidylglycerol acyl transferase (LPGAT1).

Evidence that the LPGAT1 gene encodes an LPGAT enzyme is provided by the findings that the recombinant human LPGAT1 enzyme expressed in Sf9 and COS-7 cells catalyzed efficiently the reacylation of LPG with various LPGs and acyl-CoAs as substrates. The LPG activity was dependent on the presence of both acyl-CoAs and LPGs, and the absence of the acyl donor or substitution of acyl-CoA with free fatty acid resulted in loss of this activity. Additionally, the acyltransferase activity was specific for LPG as evidenced by a lack of acyltransferase activity when tested against either glycerol 3-phosphate or a variety of lysophospholipids including LPA, LPC, LPE, LPI, and LPS as substrates. Furthermore, consistent with the microsome being the major site for LPG acyltransferase activity (32–35), the LPGAT1 enzyme was localized in the ER when analyzed by both subcellular fractionation and immunohistochemical analyses.

The substrate specificity of endogenous LPGAT enzymes has been subjected to debate. Two of the early reports indicated that LPGATs from rat alveolar type II cells demonstrated clear preference to palmitate (33, 42), whereas dog pulmonary microsomal LPGAT exhibited preference to oleyl-CoA (34). In contrast, LPGAT enzymes associated with isolated rat heart microsomes preferred medium chain fatty acyl-CoAs as substrates (35). Consistent with the lipid composition of the endogenous PGs (43–47), the recombinant LPGAT1 expressed in COS-7 cells demonstrated clear preference to long chain saturated fatty acyl-CoAs as well as oleyl-CoA as substrates. Furthermore, the recombinant LPGAT1 enzyme preferred oleyl-LPG over palmitoyl-LPG when oleyl-CoA, but not palmitoyl-CoA, was used as the acyl donor as demonstrated by the kinetic analyses. Thus, the recombinant LPGAT1 demonstrated the highest activity toward the re-synthesis of sn-1,2-diacyl PG, a phospholipid previously shown to be more effective in reconstituting the GPAT activity than the corresponding dipalmitoyl-PG (48). Most intriguingly, the recombinant LPGAT1 expressed in SF9 cells showed a different acyl selectivity profile from that expressed in the COS-7 cells and preferred lauroyl-CoA as an acyl donor (data not shown).

PG is primarily synthesized in mitochondria where phosphatidylglycerophosphate (PGP) synthase is localized (Fig. 1). PG is a precursor for the synthesis of cardiolipin, a polyglycerophospholipid that plays an important role in maintaining mitochondrial function. However, neither the microsomal enzyme(s) reported in previous studies (34, 35) nor the human LPGAT1 in the current report exhibited acyl selectivity toward C18:1 and C18:2, the predominant acyl groups found in cardiolipin. Thus, LPGAT is unlikely to be responsible for maintaining the right acyl composition of cardiolipin. Indeed, we have recently identified and characterized a gene encoding a lysocardiolipin acyltransferase that displayed acyl selectivity toward monounsaturated and diunsaturated chains of C18 as its substrates (31).

Defective remodeling of PG and cardiolipin is part of the pathophysiology associated with Barth syndrome (24–26), an X-linked cardiосkeletal myopathy and neutropenia caused by mutations of an acyltransferase gene (27). In yeast cells, disruption of the PGP synthase gene resulted in no detectable levels of PG and cardiolipin (16). Mutation of the PGP synthase gene in Chinese hamster ovary cells caused defective biosynthesis of both PG and cardiolipin, which resulted in mitochondrial dysfunction, which includes defects in the respiratory electron transport chain (14, 49, 50). Cardiolipin is a predominant glycerophospholipid found in the mitochondria of heart (15, 51) and is believed to play an important role in maintaining normal heart function (52). Hence, a reduction in cardiolipin level is associated with heart diseases caused by hyperthyroidism and aging, and development of anti-cardiolipin antibody is associated with onset of thrombocytopenia and recurrent thrombosis (15). In support for a role of LPGAT1 in maintaining normal cellular function, the LPGAT1 gene is ubiquitously expressed in human tissues, and the gene is conserved throughout all eukaryotic organisms, from yeast to C. elegans. Thus, the cloning and characterization of the human LPGAT1 gene has laid a foundation for future studies in defining a regulatory role of the enzyme in health and diseases, such as Barth and neonatal respiratory distress syndromes (4, 5).

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