Isolation of a cDNA Encoding Human Lysophosphatidic Acid Phosphatase That Is Involved in the Regulation of Mitochondrial Lipid Biosynthesis*

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In this study, we isolated cDNA encoding lysophosphatidic acid (LPA) phosphatase (LPAP). The amino acid sequence deduced from the cDNA encoding LPAP had 421 residues including a putative signal peptide and was homologous to acid phosphatase, especially at the active site. Human LPAP had 28.5% amino acid identity to human prostatic acid phosphatase. Northern blot analysis showed a ubiquitous expression of LPAP, which was marked in kidney, heart, small intestine, muscle, and liver. Human chromosome map obtained by fluorescence in situ hybridization showed that the gene for LPAP was localized to chromosome 1q21. The mutant in which histidine was replaced with alanine at the active site and the putative signal peptide-deleted LPAP had no LPA phosphatase activity. In the putative signal peptide-deleted LPAP showed no mitochondrial localization. The site of intracellular localization of endogenous LPAP was also mitochondria in MDCK cells and differentiated C2C12 cells. The LPAP homologous phosphatase, human prostatic acid phosphatase, also has LPA phosphatase activity. LPAP-stable transfects NIH 3T3 cells showed less phosphatidic acid, phosphatidylglycerol, and cardiolipin. These results suggested that LPAP regulates lipid metabolism in mitochondria via the hydrolysis of LPA to monoacylglycerol.

Lysophosphatidic acid (LPA)† is known as a bioactive phospholipid. It has been shown that LPA induces a wide range of functions such as enhancement of cell growth, stimulation of neurite retraction, chemotaxis of fibroblasts, and membrane depolarization in quiescent fibroblasts (1). This variety of activities is induced by LPA-specific receptors (2). The binding of LPA to the receptors that are present on the cell surface causes the activation of trimeric G-protein-coupled pathways, resulting in the activation of intracellular signaling molecules including phospholipase C, Ras, and Rho (3). LPA also plays important roles in phospholipid metabolism inside of cells. It is an intermediate lipid in the pathway of phosphatidic acid (PA) synthesis. LPA is synthesized either from sn-glycerol-3-phosphate (G-3-P) or acylglycerol 3-phosphate. The acylation of G-3-P proceeds in two steps: the uptake of one fatty acyl moiety, which results in the formation of either 1-acyl- or 2-acyl-sn-glycerol 3-phosphate (LPA), and the subsequent conversion into PA by the incorporation of a second fatty acid (4, 5). In another LPA synthesis pathway, acylglycerol 3-phosphate, which is synthesized from dihydroyacetone phosphate by acylation, is reduced to 1-acyl-sn-glycerol 3-phosphate by the cofactor, NADPH. The acyltransferases that catalyze the synthesis of LPA from G-3-P and fatty acyl carnitine or coenzyme A derivatives have been shown to be present in both mitochondria and microsomes (6–9). Based on the differences of substrate utilization, products formed, divalent cation requirements, and molecular weights, the mitochondrial and microsomal acyltransferases appeared to be different enzymes (10). Further, both mitochondria and microsomes have a capacity to acylate G-3-P and dihydroyacetone phosphate to LPA and acylglycerol 3-phosphate and subsequently to PA (11).

There are two possible LPA-hydrolyzing pathways, one via LPA phospholipase A and the other via LPA phosphatase. Since LPA is a biologically active lipid, its elimination by these enzymes is important for terminating the signal. LPA phospholipase A was purified from rat brain (12). The enzyme has a molecular mass of 80 kDa, is membrane-bound, and hydrolyzes LPA but not other lysophospholipids. Concerning LPA phosphatase, the existence of an ecto-type LPA phosphatase that also hydrolyzed PA was reported in PAM212 cells (13). To date, membrane-bound PA phosphatases have been purified from porcine thymus; these enzymes are relatively PA-specific with weak activity for LPA (14, 15), and from rat liver, this enzyme also hydrolyzes LPA, ceramide-1-phosphate, and sphingosine-1-phosphate (16, 17), while a LPA-specific phosphatase had not yet been purified.

Previously, we purified the LPA specific phosphatase (LPAP) from the cytosol fraction of bovine brain and characterized it (18). In the present study, we isolated a cDNA encoding LPAP from a human brain library and showed that it is homologous to acid phosphatases, including a prostatic acid phosphatase. Further, we examined the intracellular localization and biochemical activity of LPAP. These results demonstrate that LPAP is localized to mitochondria by signal peptides and regulates the biosynthesis of mitochondrial lipids by hydrolyzing LPA to monoacylglycerol.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibody against human prostatic acid phosphatase was obtained from Zymed Laboratories Inc. [2H]Orthophosphate, [γ-32P]ATP, and [α-32P]CTP were from NEN Life Science Products, Inc. Mitotracker Red CxMxR0s and rhodamine- wheat germ agglutinin were from Molecular Probes, Inc. (Eugene, OR). Fluorescein isothiocyanate- concanavalin A was from Biogenesis Ltd. Monoclonal anti-Bip antibody was from Stressgen Biotech Corp.

The abbreviations used are: LPA, lysophosphatidic acid; LPAP, LPA phosphatase; PA, phosphatic acid; PG, phosphatidylglycerol; G-3-P, sn-glycerol-3-phosphate; FISH, fluorescence in situ hybridization; Bip, GRP78 (glucose-regulated protein of 78-kDa); DAPI, 4',6-diamidino-2-phenylindole; MDCK, Madin-Darby canine kidney; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.
LPA Phosphatase

Cell Culture and Transfections—PC-3 is a human prostate cancer cell line derived from bone metastases, which were obtained from the Japan Health Sciences Foundation. PC-3 cells were maintained in Ham’s F-12K (Sigma) containing 10% fetal bovine serum. COS-7 monkey kidney cells, kidney MDCK epithelial cells, and NIH 3T3 cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. C2C12 cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 3.5% (w/v) glucose, and antibiotics, and the differentiation was initiated by medium exchange to Dulbeco’s modified Eagle’s medium supplemented with 1% horse serum and 3.5% (w/v) glucose.

To obtain stable clones expressing LPAF, 10 μg of pNeoSRα II plasmid was transfected into NIH 3T3 cells by using the calcium phosphate precipitation method (19). The transfected cells were cultured in complete medium for 3 days and then for 14 days in the same medium with geneticin (G418) at 400 μg/ml. Then stable transformant colonies were isolated with cloning cups. The expression of LPAF was verified by immunoblotting. Twenty-four clones showing different levels of expression were obtained.

Purification of LPAF—LPAF was purified as described (18) from the cytosolic fraction of bovine brain. The enzyme was finally purified by heparin column chromatography, reproducibly resulting in a product more than 3,300 times purer than that obtained from the cytosol, with a specific enzyme activity of 37.99 units/mg of protein as described (18).

Partial Amino Acid Sequencing of 44-kDa LPAF—LPAF (20 μg) was subjected to 7.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane using a Bio-Rad apparatus. The sample on polyvinylidene fluoride membrane was digested with lysylendopeptidase (Wako, Japan) for 24 h at 37 °C. The digested sample was subjected to a reverse phase high performance liquid chromatography using a Zorbax C-18 column (1–150-mm inner diameter), and the collected peptides were analyzed with a protein sequencer (PPSQ-10 protein sequencer; Shimazu).

Amplification and Screening of Human cDNA Encoding LPAF—Since the partial sequences of LPAF are very similar to the internal sequence encoded by human prostatic acid phosphatase, we designed two degenerate primers for PCR amplification: 5′-ATGGTICA(A/G)GT-3′ and 9′-ATGGTGACCA(T/C)GG-3′. The amplification reactions were done on a PCR thermal cycler (TaKaRa) at 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min for 40 cycles. A 987-base pair fragment based upon the partial sequences of LPAF cDNA was used as a probe to screen human cDNA library made from human brain (Stratagene) at 96 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min for 40 cycles. The digestion sample was subjected to a reverse phase high performance liquid chromatography using a Zorbax C-18 column (1–150-mm inner diameter), and the collected peptides were analyzed with a protein sequencer (PPSQ-10 protein sequencer; Shimazu).

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Expression Plasmids—The LPAP cDNAs were subcloned into pEF-BOS (20) at the BamHI site and into pNeoSRα II plasmid at the XhoI and BamHI sites.

Human Chromosome Map for LPAP by Fluorescence in Situ Hybridization (FISH)—Lymphocytes isolated from human blood were cultured in α-minimal essential medium supplemented with 10% fetal calf serum and phytohemagglutinin at 37 °C for 48–72 h. The lymphocyte culture was treated with bromodeoxyuridine (0.18 mg/ml; Sigma) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured at 37 °C for 6 h in α-minimal essential medium with thymidine (2.5 μg/ml; Sigma). Cells were harvested, and slides were made by using standard procedures including hypotonic treatment, fix, and air-dry. A 1.7-kilobase pair cDNA probe was biotinylated with dATP using the Life Technologies, Inc. BioNick labeling kit (15 °C, 1 h) (21). The procedure for FISH detection was performed according to Heng et al. (21) and Heng and Tsui (22). FISH signals and the DAPI banding pattern were recorded separately by taking photographs, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI banded chromosomes (22).

Site-directed Mutagenesis—To construct the mutant (H52A) in which histidine (amino acid 52) is replaced with alanine at the putative active site, we designed two primers: 5′-GGTGCAAGGTGTTGCTGGCGG-3′ and 5′-GGAGACCTCAGGCCCGG-3′ (the mutation site is underlined). To confirm the desired mutation, the mutant DNA in pBluescript II KS− was sequenced by the method described above. The complete sequence corresponding to those obtained from the amino acid sequence are underlined.
of the mutated LPAP insert was subcloned into pNeoSro II plasmid at the XhoI and BamHI sites.

Construction and Preparation of LPAP Fragment as Antigen Protein—To express LPAP with a cDNA plasmid, two primers were synthesized for PCR amplification. In the case of LPAP, the sense primer, 5'-CGCGGATCCTGTCGGGGGCAGTGGACA-3' (amino acids 165–174) was designed to generate a BamHI site (underlined). The antisense primer, 5'-CGCGGATCCTGTCGGGGGCAGTGGACA-3' (amino acids 312–317) was designed to introduce a BamHI site. The amplified fragment was digested with BamHI and subcloned into the plasmid pQE30 at the corresponding site. The recombinant plasmid containing the LPAP sequence was transformed into Escherichia coli and induced with isopropyl-1-thio-D-galactoside to produce a histidine-tagged protein. The protein—

Figure 2. A, comparison of LPAP with human prostatic acid phosphatase. Amino acids encoded by LPAP and prostatic acid phosphatase are aligned, and identical residues are boxed. Dashes indicate gaps inserted to maximize alignment. B, highly conserved peptide sequence in acid phosphatases and two other proteins. The proteins are as follows (in descending order): human LPA phosphatase, human prostatic acid phosphatase, human lysosomal acid phosphatase, rat lysosomal acid phosphatase, E. coli acid phosphatase, and three yeast acid phosphatases as well as the rat sodium channel protein and E. coli penicillin-binding protein.

Preparation and Affinity Purification of LPAP Antibody—Two rabbits were immunized with the histidine-tagged LPAP fragment (1 mg) coupled to keyhole limpet hemocyanin (23) in complete Freund’s adjuvant. The serum was collected 10 days after each booster injection. For the affinity purification of the antibody, the antigen protein (2 mg) was coupled to 0.15 g of BrCN-activated Sepharose 4B (23) in complete Freund’s adjuvant. The serum was collected 10 days after each booster injection. Using half the amount of the conjugated protein emulsified in incomplete Freund’s adjuvant (Difco). Booster injections were repeated every 2 weeks thereafter to the column and affinity-purified by elution at pH 2.5. Immune IgG was applied to the column and affinity-purified by elution at pH 2.5.

Amplification of Human cDNA Encoding Prostatic Acid Phosphatase—Total RNA was prepared from PC-3 cells by a single step guanidine isothiocyanate/phenol chloroform method (24, 25). We designed two primers for PCR amplification of the prostatic acid phosphatase based on the published cDNA sequence: 5'-CGCGGATCCTGTCGGGGGCAGTGGACA-3' or 5'-CGCGGATCCTGTCGGGGGCAGTGGACA-3' (nucleotides 1–33 in prostatic acid phosphatase; Ref. 26); 5'-CGCGGATCCTGTCGGGGGCAGTGGACA-3' (nucleotides 1155–1175 in prostatic acid phosphatase). The first strand cDNA was synthesized from total RNA of PC-3 cells using the random primer. The reaction mixture (25 μl) containing the total RNA (10 μg), random primer, and 200 units of reverse transcriptase (Life Technologies), was incubated at 37 °C for 1 h. One-fourth of the resulting DNA preparation was used as a template for the subsequent PCR amplification.

Substrate Specificity of LPAP and Human Prostatic Acid Phosphatase—COS-7 cells were transfected with LPAP or human prostatic acid phosphatase in PEF-BOS plasmid. After 48 h, cells were harvested and homogenized. The cell lysate was centrifuged at 100,000 × g for 60 min, and then the supernatant was applied onto a column of DEAE-Sephrose equilibrated with 20 mM Tris-HCl (pH 7.0). The column was washed with the same buffer, and the enzyme eluted at 0.5 ml/min with a linear NaCl gradient (0–0.5 M). The substrate specificity of enzymes was examined by the modified method of Hiroyama et al. (16). In brief, after the reaction with various lipids, chloroform/methanol (1:2; 200 μl) was added to the reaction mixture (50 μl), and then chloroform (80 μl) and 1 n HCl (80 μl) were further added. The mixture was vigorously mixed and separated to two phases by centrifugation. A part (125 μl) of the upper phase was transferred to another tube, and perchloric acid (25 μl), 10% ammonium molybdate (25 μl), and 10% ascorbic acid (50 μl) were added and boiled at 95 °C for 5 min. The absorbance of the mixture was measured at 796 nm.

Cell Stain—The cells expressing LPAP were incubated with 50 nM MitoTracker Red CMXRs for mitochondrial staining in growing medium for 30 min and then rinsed with PBS three times. They were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, permeabilized by 0.2% Triton X-100 in PBS for 5 min, and then rinsed with PBS three times. Permeabilized cells were incubated with anti-LPAP polyclonal antibody and anti-Bip monoclonal antibody for 1 h. After being rinsed with PBS, cells were then incubated with fluorescein-labeled second antibodies and fluorescein isothiocyanate-concanavalin A or rhodamine–wheat germ agglutinin for 30 min. The subcellular localization of LPAP was visualized by confocal microscopy.

Analysis of Lipids in LPAP-expressing NIH 3T3 Cells—NIH 3T3 cells and LPAP-transfected NIH 3T3 cells were labeled for 4 h with [32P]orthophosphate and then harvested with a rubber policeman after being washed in PBS. Phospholipids were extracted by the method of Bligh and Dyer (27), and 150,000 cpm of lipids were separated by two-dimensional thin layer chromatography (TLC) using chloroform/
methanol/25% ammonia/water (15:11:2:2, v/v/v/v) as the first dimensional solvent and n-butyl alcohol/acetic acid/water (30:5:5, v/v/v) as the second on oxalate-treated TLC plates, or chloroform/methanol/acetic acid (65:25:10, v/v/v) as the first and chloroform/methanol/formic acid (65:25:10, v/v/v) as the second dimensional solvent (28). After development, the spots were visualized by autoradiography. To quantitate the radioactivity in each spot, the spots were scraped off, and the radioactivity was measured by scintillation counter.

Assay of LPAP Activity Using Crude 32P-labeled Lipids in Vitro—To ascertain which phospholipid is dephosphorylated most effectively by LPAP, a crude extract of lipids was used as substrate for LPAP. The catalytic activity for the extracted lipids was measured in vitro. In brief, the 150,000 cpm of lipids were dried under nitrogen and resuspended in a reaction buffer containing 50 mM Tris-maleate (pH 7.5) and 2 mM Triton X-100. LPAP purified from bovine brain was added and then reacted at 37 °C for 15 min. The reaction mixture was extracted as described above and spotted on a TLC plate. After two-dimensional separation, each lipid was scraped off, and the radioactivity was measured.

Western Blot Analysis—Protein concentrations were determined using a Bio-Rad protein assay kit. Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with anti-LPAP antibody, followed by anti-rabbit IgG conjugated peroxidase, and the protein bands were visualized with benzamide (Sigma) and H2O2 in PBS.

RESULTS

Isolation of LPAP cDNA Clones from the Human Brain Library—We determined the amino acid sequences of six polypeptides formed by digestion of a 44-kDa protein with l-lysylendopeptidase. Next, we attempted to isolate the cDNA encoding the enzyme based on the information obtained from partial amino acid sequencing. Since two (MVQVFHRGARSPPL and EWFVQLYYRGK) in six polypeptides were similar to the sequence of prostatic acid phosphatase, two degenerate primers were designed based on them and used in PCR amplification. We obtained a single 987-base pair amplification product. After screening 1,000,000 phages from the human brain cDNA library using the 987-base pair fragment as probe, 25 putative clones were isolated. Of these, 20 clones were positive on secondary screening and further analyzed by DNA sequencing. All clones encoded the same protein. The amino acid deduced sequence, which was found to contain six polypeptides, coded a novel protein of 421 amino acid residues including the putative signal sequence, which was hydrophobic (Fig. 1). There are two putative initiation codons, ATG (positions 21 to 19) and ATG (positions 1–3) in human LPAP cDNA. To determine which is the initiation codon, we further investigated the N-terminal sequence of mouse LPAP. It was found that ATG (positions 21 to 19) upstream of ATG (positions 1–3) was not present in mouse LPAP (data not shown). Thus, we presumed that ATG (positions 1–3) is the real initiation codon. Further, we attempted to study the LPA phosphatase activity and intracellular localization of the constructs contain-
ing either ATG (positions -21 to -19) or ATG (positions 1–3). Both had LPA phosphatase activity and a similar intracellular localization (data not shown), indicating that ATG (positions 1–3) was the initiation codon of LPAP. Next, we searched for homologous proteins in a data bank with a computer. As a result, we found that human prostatic acid phosphatase was most homologous to LPAP (Fig. 2A). 28.5% of the amino acids of LPAP were identical to prostatic acid phosphatase. In addition, LPAP had a consensus sequence (LXXVXXVXRHGXRXP) with a group of acid phosphatases (Fig. 2B) at the N terminus.

**Northern Blot Analysis**—We first studied the tissue distribution of LPAP by Northern blot analysis, demonstrating an expression of a 1.75-kilobase LPAP mRNA (Fig. 3A). The LPAP mRNA was detected in all tissues examined, but the expression was marked in kidney, heart, small intestine, muscle, and liver.

**Human Chromosome Map for LPAP by FISH**—To examine the possibility that LPAP is involved in genetic diseases, we attempted to analyze the localization of the gene on chromosomes by FISH. Under the conditions used, the hybridization efficiency was approximately 79% for this probe (among 100 mitotic figures checked, 79 of which showed signals on one pair of chromosomes). DAPI banding was used to identify the specific chromosome, and an assignment between the signal from the probe and the long arm of chromosome 1 was obtained. The position was further determined based on 10 photos (Fig. 3, B (a)). No additional locus was detected by FISH under the conditions used; therefore, the LPAP gene is located at human...
antibody that was prepared using the proteins produced in E. coli also reacted with the native LPAP from bovine brain.

Immunoblotting with LPAP antibody revealed that all transfected plasmids were expressed as proteins in the COS-7 cells (Fig. 4B), although control COS-7 cells also contained the endogenous LPAP. In the wild type LPAP(+) cell lysate, a 45-kDa protein and smaller proteins including one of 37 kDa were stained by anti-LPAP antibody. To determine whether the smaller proteins were degradative products of the 45-kDa protein, we tagged LPAP with GFP at the C terminus. Immunostaining of lysates from C-terminal tagged LPAP expressing cells with tag antibody produced the same staining pattern as that for polyclonal anti-LPAP antibody, confirming that the smaller proteins are degradative proteins (data not shown). Using lysates from these protein-expressing cells, we measured LPAP phosphatase activity. As shown in Fig. 4C, LPAP phosphatase activities in mutant (H52A) and LPAP(-) expressing cells were the same as that of the negative control (vector only). The wild type LPAP(+) and LPAP(H52A) were colocalized with a mitochondrial marker, MitoTracker Red CMXRos, but LPAP(-) was cytoplasmic (Fig. 4D). We further examined the possibility that LPAP localized in other organelles, such as endoplasmic reticulum and Golgi apparatus. But LPAP was not colocalized with those organelles (Fig. 4E). The results indicate that LPAP is localized to mitochondria by the signal peptide and functions in mitochondria.

Next, we examined the intracellular localization of the endogenous LPAP in MDCK cells and differentiated C2C12 cells. Both of the cells expressed the LPAP in abundance (Fig. 3A). These endogenous LPAP also colocalized with MitoTracker Red CMXRs in MDCK cells and differentiated C2C12 cells (Fig. 5A), but in undifferentiated C2C12 cells, LPAP was not detectable (data not shown), suggesting that the endogenous LPAP localized in mitochondria and was induced with the differentiation of C2C12 cells to myotubes. To confirm whether LPAP is induced in differentiated C2C12 cells, we examined the change in LPAP content during differentiation (0, 1, 2, 3.5, and 5 days) after starvation (Fig. 5B). There were two positive proteins with anti-LPAP antibody, 37 and 44 kDa. Both of them increased with differentiation (Fig. 5B). However, in the differentiated C2C12 cells, 37-kDa protein was contained at a much higher level, while it was at a lower level in MDCK cells than the 44-kDa protein (data not shown). Since the 37-kDa protein was detected both in LPAP(+) expressing COS-7 cells and LPAP(-) expressing COS-7 cells (Fig. 4B), we thought it was a degradative or processed LPAP. Thus, LPAP was found to be induced with the differentiation from myoblast to myotube.

Substrate Specificity of LPAP and Human Prostatic Acid Phosphatase—To investigate the substrate specificity of LPAP and human prostatic acid phosphatase, two expression vectors containing cDNA encoding LPAP and human prostatic acid phosphatase were constructed. These expression vectors and pEF-BOS as a control vector were introduced into COS-7 cells, and the cell lysates were separated by a DEAE-Sepharose column chromatography. Partially purified human prostatic acid phosphatase and LPAP were used to study the substrate specificity. The presence of the enzymes was confirmed by immunoblot analysis. In this experiment, we used phosphate-containing compounds including LPA analogs for substrates. LPAP hydrolyzed LPA specifically as described previously (18), but human prostatic acid phosphatase markedly hydrolyzed p-nitrophenyl phosphate and weakly hydrolyzed PA and glycercophosphate in addition to LPA (Fig. 6).

Change in Phospholipid Composition of LPAP-expressing NIH 3T3 cells—To study the alteration in phospholipid composition brought about by LPAP transfection, we isolated NIH
c chromosome 1, region q21. An example of the mapping results is presented in Fig. 3, B (b).

**Fig. 5.** The intracellular localization and induced expression of endogenous LPAP. A, MDCK cells were stained with anti-LPAP antibody (a), antigen-absorbed anti-LPAP antibody (d), and MitoTracker Red CMXRs (b, e). C2C12 cells were differentiated in starvation medium for 3.5 days (D3.5d). Then the differentiated cells were stained with anti-LPAP antibody (j), antigen-absorbed anti-LPAP antibody (j), and MitoTracker Red CMXRs (h, k) and observed by confocal microscopy. B, C2C12 cells were induced to differentiate for 0, 1, 2, 3.5, and 5 days by starvation. The cells were harvested and sonicated in 10 mM Tris-HCl buffer (pH 7.5). The lysates (20 μg) were applied to an SDS-polyacrylamide gel and immunoblotted with anti-LPAP antibody.
3T3 cells stably expressing LPAP. The expression of LPAP was verified by immunoblotting, and 24 clones showing different levels of expression were obtained. Control NIH 3T3 cells and LPAP-stable clones were labeled with "[^32P]orthophosphate, and analyzed for phospholipids. Total counts of incorporated "[^32P] into phospholipids were almost the same between control NIH 3T3 cells and LPAP-stable clones. Visual inspection of the autoradiograms and the measurement of radioactivity of each lipid indicated that cardiolipin, phosphatidyl glycerol (PG), and PA were decreased in the LPAP-stable clone cells (Fig. 7, A and B). In contrast, phosphatidylethanolamine and phosphatidylinositol increased. Similar results were obtained using other clones.

To examine which phospholipid is most effectively hydrolyzed by LPAP in vitro, we assayed with the crude extracts of "[^32P]-labeled lipids from NIH 3T3 cells as substrate. LPAP did not hydrolyze PG, cardiolipin, or other lipids (Fig. 8, A and B), only LPA. It is known that lung surfactant contains a highly active phosphomonoesterase. This phosphatase is quite specific for the hydrolysis of PA and 1-acetyl-2-lysophosphatidic acid. In addition, this enzyme converts phosphatidylglycerol phosphate to PG and P(l, 30). Therefore, we investigated the possibility that LPAP hydrolyzed phosphatidylglycerol phosphate. However, it had no catalytic activity for phosphatidylglycerol phosphate (data not shown). These results suggest that LPAP hydrolyzes LPA synthesized by acyltransferase in mitochondria and regulates mitochondrial lipid biosynthesis, thereby regulating mitochondrial functions.

**DISCUSSION**

In a previous study, we purified an LPA-specific phosphatase from bovine brain. The partial amino acid sequencing of the bovine enzyme revealed a similarity of sequence to prostatic acid phosphatase. The amino acid sequence deduced from the cDNA showed LPAP to be a high molecular weight acid phosphatase. It is known that acid phosphatases catalyze the hydrolysis of phosphate monoesters and, in some cases, phospho-ester transfer between phosphoesters and alcohols (31-33). However, the specific substrate of acid phosphatase was not known. Many studies have demonstrated that prostatic acid phosphatase also can function as a protein-tyrosine phosphatase in cells (34, 35). In addition, it has been made clear that prostatic acid phosphatase specifically dephosphorylates tyrosine phosphates in c-ErbB2 rather than tyrosine phosphates in a wide variety of tyrosine-phosphorylated proteins (36). Interestingly, we found that prostatic acid phosphatase also hydrolyzed LPA to monoacylglycerol, and an LPAP point mutant (H52A) of the active site in prostatic acid phosphatase lost its activity (Fig. 4A). Thus, we examined whether LPAP also has protein-tyrosine phosphatase activity using a phosphorylated EGF receptor in vitro. LPAP hydrolyzed tyrosine phosphates in the EGF receptor whose activity was inhibited by Triton X-100 (data not shown), suggesting that LPAP has tyrosine phosphatase activity besides LPA phosphatase activity, although its physiological meaning is not clear. In addition, prostatic acid phosphatase was shown to resemble PA phosphatase 2a (17) in that both have the activity to hydrolyze PA and LPA, and those expressions are high in prostate and regulated by androgen (31, 37). But prostatic acid phosphatase was found not to have the activity to hydrolyze ceramide-1-phosphate and sphingo-sine-1-phosphate (Fig. 6). Further, both of prostatic acid phosphatase and LPAP are not homologous to PA phosphatase 2a in amino acid sequences, showing that these proteins are a different category of phosphatase from PA phosphatase.

Phospholipid analysis in the cells where LPAP is expressed showed the reduction of PG and cardiolipin. Cardiolipin represents 0.2-15% of all lipid phosphorous in various animal tissues and is located primarily in the mitochondrial inner membrane (38). Biochemical analysis demonstrated that cardiolipin is required for many enzymatic activities, such as cytochrome c oxidase (39) and carnitine acylcarnitine translocase (40), and is involved in cellular functions, such as protein import into mitochondria (41-44) and binding of matrix Ca^{2+} (45). PG comprises approximately 1% of all the lipid phosphorous in mammalian tissues except in lung and is found in many intracellular locations, such as mitochondrial, nuclear, and microsomal membranes. In lung, PG accounts for approximately 5% of phospholipid content; it is localized predominantly at the lamella body of membrane (41) and is also one of the main components of lung surfactant (47). A recent biochemical analysis also indicated that PG is a potential activator of the protein kinase C family, including protein kinase C0 (47)and nuclear protein kinase Co II (48). Cardiolipin and PG are synthesized mainly in mitochondria from LPA. Furthermore, we found that ectopically expressed LPAP and endogenous LPAP both localize at mitochondria. Therefore, the expression of LPAP caused the reduction of these lipids through the removal of precursor lipid, LPA, from mitochondria. However, expression of LPAP also caused an increase in phosphatidylethanolamine and phosphatidylinositol synthesis. This increase may be caused by a disturbance of the phospholipid metabolism through lack of LPA in mitochondria.

LPA and PA are key intermediates for the biosynthesis of glycerolipids (49, 50). These two simple phospholipids are formed in both the microsomes and mitochondria by sequential acylation of G-3-P catalyzed by glycerophosphate acyltrans-
ferase (EC 2.3.1.15) and monoacylglycerophosphate acyltransferase (EC 2.3.1.51) (51–55).

However, since LPAP localized at mitochondria, LPA in mitochondria is depleted, resulting in reduction of PG and cardiolipin. The expression of LPAP increased in a time-dependent manner when C2C12 cells were stimulated to differentiate to myotubes by starvation. This may in part reflect the increase in mitochondria, because myotubes contain a lot of mitochondria. Indeed, LPAP is expressed highly in mitochondria-developed tissues such as heart, kidney, liver, smooth muscle, and skeletal muscle. However, the LPAP in mitochondria is expected to degrade LPA, which is an essential precursor for cardiolipin and PG synthesis, resulting in the negative regulation of synthesis of these lipids. This would hamper mitochondrial functions. It is not clear why high levels of LPAP are expressed in mitochondria, but LPAP may regulate mitochondrial phospholipid levels as glycerol-3-phosphate acyltransferase does. Its expression is positively regulated when fasted animals are refeed a high carbohydrate diet, and it is negatively regulated by starvation, diabetes, and glucagon (56, 57). Through the regulation of LPAP expression by environmental conditions, LPA levels may be controlled, and then mitochondrial functions are

Fig. 7. Phospholipid compositional change. A, the extracted $^{32}$P-labeled phospholipids (150,000 cpm) were dried under a stream of nitrogen and spotted on TLC plates. The plates were separated by two-dimensional TLC using chloroform/methanol/25% ammonia/water (15:11:2:2, v/v/v/v) as the first solvent and n-butyl alcohol/acetic acid/water (30:5:5, v/v/v) as the second on oxalate-treated TLC plates (a, b) or chloroform/methanol/formic acid (65:25:10, v/v/v) as the second (c, d) and exposed to x-ray films for 1 or 3 h. PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine; CL, cardiolipin; PIP, phosphatidylinositol monophosphate; PIP2, phosphatidylinositol bisphosphate. B, the spots visualized were scraped off, and the radioactivities were measured.

Fig. 8. In vitro assay of LPAP activity using a crude mixture of phospholipids. The extracted $^{32}$P-labeled phospholipids (150,000 cpm) from $^{32}$P-labeled NIH 3T3 cells were dried under nitrogen and reacted with the purified LPAP at 37°C for 15 min in the reaction buffer containing 50 mM Tris-maleate and 2 mM Triton X-100. The reaction mixtures were extracted and separated by two-dimensional TLC using chloroform/methanol/25% ammonia/water (15:11:2:2, v/v/v/v) as the first solvent and n-butyl alcohol/acetic acid/water (30:5:5, v/v/v) as the second on oxalate-treated TLC plates (a, b) and chloroform/methanol/acetic acid (65:25:10, v/v/v) as the first solvent and chloroform/methanol/formic acid (65:25:10, v/v/v) as the second (c, d) (A). The spots visualized were scraped off, and the radioactivities were measured (B).
modified. However, the functions of LPAP in mitochondria remain to be elucidated.

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REFERENCES
