LPCAT2

We identified LPCAT2 (also called LysoPAFAT/LPCAT2 or AT like 1), which catalyzes both PAF and PC synthesis mainly in inflammatory cells (Fig. 2) (24). Thus, two types of glycerophospholipids (PAF and PC) are synthesized from the same precursor (lyso-PAF) using a single enzyme (LPCAT2) (24). LPCAT2 mRNA is upregulated in mouse thioglycollate-induced peritoneal macrophages by treatment with Toll-like receptor 4 agonists, lipopolysaccharide, but the upregulation is suppressed by dexamethasone treatment. Upon acute inflammatory stimulation with lipopolysaccharide, the lyso-PAF acetyltransferase activity of LPCAT2 was activated in the p38 mitogen-activated protein kinase dependent pathway, consistent with the previous report of endogenous lyso-PAF acetyltransferase activity (29). In contrast, the LPCAT activity of LPCAT2 was not enhanced (24). Thus, a single enzyme catalyzes membrane biogenesis (LPC acyltransferase activity) of inflammatory cells, while producing PAF (lyso-PAF acetyltransferase activity) in response to external stimuli. How the two activities are differentially regulated remains to be elucidated. Furthermore, specific inhibitors of LPCAT2 may be better anti-inflammatory drugs than PAF receptor antagonists, since such inhibitors could potentially inhibit proliferation of inflammatory cells by disturbing membrane biogenesis, in addition to inhibiting the inflammatory signaling by PAF.

We recently reported that LPCAT1 also possesses a lyso-PAF acetyltransferase activity (Fig. 2) (15). In addition, the amino acid residues of LPCAT1 that are essential for each activity (LPCAT or lyso-PAF acetyltransferase activity) were identified by site-directed mutagenesis. In contrast to LPCAT2, LPCAT1 was neither activated nor upregulated by lipopolysaccharide stimulation in the mouse macrophages. While LPCAT2 activity is Ca^{2+}-dependent, LPCAT1 activity is Ca^{2+}-independent. These findings indicate two distinct remodeling pathways for PAF synthesis; the inflammatory/inducible (LPCAT2) and the non-inflammatory/constitutive (LPCAT1) remodeling pathway (15). The relationship between LPCAT1 and LPCAT2 is similar to that of cyclooxygenase 1 and 2, which are constitutively expressed and inducible enzymes, respectively (30). It is still possible that other constitutive lyso-PAF acetyltransferase is present, as LPCAT1 is enriched only in alveolar type II cells.

LPEAT2 also shows LPCAT activity, but an siRNA specific for LPEAT2 did not decrease the LPCAT activity (see PE section for detail) (Fig. 2) (23). In addition, LPEAT2 (AGPAT7) was reported to exhibit LPCAT activity in red blood cells, although the activity was relatively weak (31).

LPCAT3 and LPCAT4

We and Zhao et al. independently identified LPCAT3 in the MBOAT family, which contains DAG acyltransferase 1 (DGAT1), cholesterol acyltransferases 1 and 2 (ACAT1 and 2), protein acyltransferases, such as ghrelin acyltransferase (2, 7, 32). LPCAT3 (also called MBOAT5), exhibits LPCAT, LPEAT, and lyso-PS acyltransferase (LPSAT) activities (Fig. 2) (25-27). Thus, the MBOAT family is a novel LPLAT family. Mouse LPCAT3 mRNA was detected ubiquitously and showed higher acyltransferase activity toward polyunsaturated fatty acyl-CoAs, 20:4-CoA and 18:2-CoA, than saturated fatty acyl-CoAs. Transfection with LPCAT3-siRNA into B16 melanoma cells reduced endogenous LPCAT, LPEAT, and LPSAT activities with 20:4-CoA and the amount of PC, PE, and PS containing arachidonic acid at the sn-2 position.

LPCAT4 (originally called MBOAT2) possesses LPCAT and LPEAT activities with a clear preference for 18:1-CoA (Fig. 2) (25). Mouse LPCAT4 mRNA is highly expressed in the epididymis, brain, testis, and ovary. Since LPCAT3 and LPCAT4 had higher LPCAT activity with 1-acyl-LPC than 1-O-alkyl-LPC or 1-O-alkenyl-LPC as acceptors, these enzymes may recognize the difference between the ester and ether bond at the sn-1 position of LPC (25).

(iii) PE and PS synthesis in the remodeling pathway

LPEAT1 (also called MBOAT1) is a member of the MBOAT family and possesses LPEAT and LPSAT activities with a clear preference for 18:1-CoA (Fig. 2) (25). Mouse LPEAT1 mRNA is highly expressed in the stomach, epididymis, and colon. LPCAT3 and LPCAT4 also have LPEAT activities toward polyunsaturated acyl-CoAs and 18:1-CoA, respectively. In contrast to the LPCAT activities of LPCAT3 and LPCAT4, no clear differences were observed between the 1-acyl-LPEAT and 1-O-alkenyl-LPEAT activities of each enzyme. LPCAT3 and LPEAT1 catalyze PS synthesis in the remodeling pathway (Fig. 2). LPCAT3 recognizes polyunsaturated acyl-CoAs and LPEAT1 prefers 18:1-CoA in the presence of LPS. Thus, LPCAT3 synthesizes PC, PE, and PS containing polyunsaturated fatty acids at the sn-2 position. In contrast, LPCAT4 (PC and PE formation) and LPEAT1 (PE and PS formation) esterify oleic acid (18:1) to the sn-2 position of lysophospholipids (25).

Recently, LPEAT2 (previously called AGPAT7, LPAATη, or AT like 3) was identified from the AGPAT family (23). The enzyme is similar to LPCAT1 and LPCAT2, and show LPEAT, LPGAT, LPSAT, and LPCAT activities toward 18:1-CoA or 20:4-CoA (Fig.

2). However, an siRNA specific for LPEAT2 decreased only the LPEAT activity, but not the LPGAT, LPSAT, or LPCAT activities in HEK293T cells (23). Since mouse LPEAT2 was expressed primarily in the brain, LPEAT2 may play crucial roles in the biogenesis of brain PE.

(iv) PI synthesis in the remodeling pathway

LPIAT1 (also called MBOA-7, MBOAT7, and LRC4) is the first PI synthetic enzyme in the remodeling pathway to be identified (33) (Fig. 2). LPIAT1 exhibits LPIAT activity toward 20:4-CoA and the site-directed mutagenesis of human LPIAT1 revealed a predicted active site residue, His350 within a long hydrophobic region. An LPIAT1 mutant of *C. elegans* exhibits larval arrest and egg-laying defects. Mammals may possess more than one LPIAT.

IV. Conclusion

Phospholipids are the major constituents of biological membranes, playing important roles in the maintenance of the cellular boundary and the regulation of cellular signaling. Kennedy and Lands reported the synthesis of phospholipids in the *de novo* pathway (Kennedy pathway), and the maturation of phospholipids in the remodeling pathway (Lands' cycle), respectively, to produce membrane diversity (Fig. 1). Membrane diversity is important for membrane fluidity and curvature and is produced by the concerted and overlapping reactions of multiple LPLATs that recognize both the polar head groups of lyso-glycerophospholipids and various acyl-CoAs in the remodeling pathway. In the last 4 years, many LPLATs functioning in the remodeling pathway have been identified, resulting in the most spectacular advance in the LPLAT field since the discovery of the Kennedy pathway and the Lands' cycle 50 years ago (2, 7). As each enzyme has several names and each name refers to several enzymes, we have proposed renaming LPLATs in order to clarify and standardize the nomenclature (see Fig. 2) (7). Identification of additional LPLATs may contribute to further elucidation of membrane diversity and asymmetry. It will be intriguing to determine how many enzymes are present and sufficient to produce over 800 different molecular species of glycerophospholipids.

Since acyl-CoAs and lysophospholipids are the substrates of LPLATs, clarification of which acyl-CoA synthetases (an alternative name; acyl-CoA ligase) and PLA$_2$s are functionally coupled to individual LPLATs will be important. How phospholipids synthesized in the ER are transported to target organelles, including lamellae bodies in alveolar type II cells, remains to be determined. Additionally, analysis of enzyme activities with mixtures of

acyl-CoA or lysophospholipid substrates is important. Although enzyme purification is not an easy task because of the multitransmembrane spanning nature of the LPLATs, determination of the 3D structure by x-ray crystallography is of interest. Further studies is needed to elucidate the biological roles of these enzymes *in vivo*, such as analyzing LPLAT knockout mice or *in vivo* siRNA experiments. The recent findings reviewed here constitute a critical milestone for greater understanding of how membrane diversity and asymmetry are established and the biological significance of these phenomena.

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FOOTNOTES

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Fig. 1; The biogenesis of membrane diversity

Kennedy and Lands reported the first synthesis of phospholipids in the *de novo* pathway

(Kennedy pathway), and subsequently maturation of phospholipids in the remodeling pathway (Lands' cycle) to produce membrane diversity. The remodeling pathway is attributed to the concerted and coordinated actions of PLA_2s and LPLATs.

Fig. 2; A proposal for LPLAT nomenclature

Proposed names are shown in red. AGPAT and MBOAT family members are indicated by yellow and blue, respectively. Representative former names are shown.

Fig. 1

de novo biosynthesis

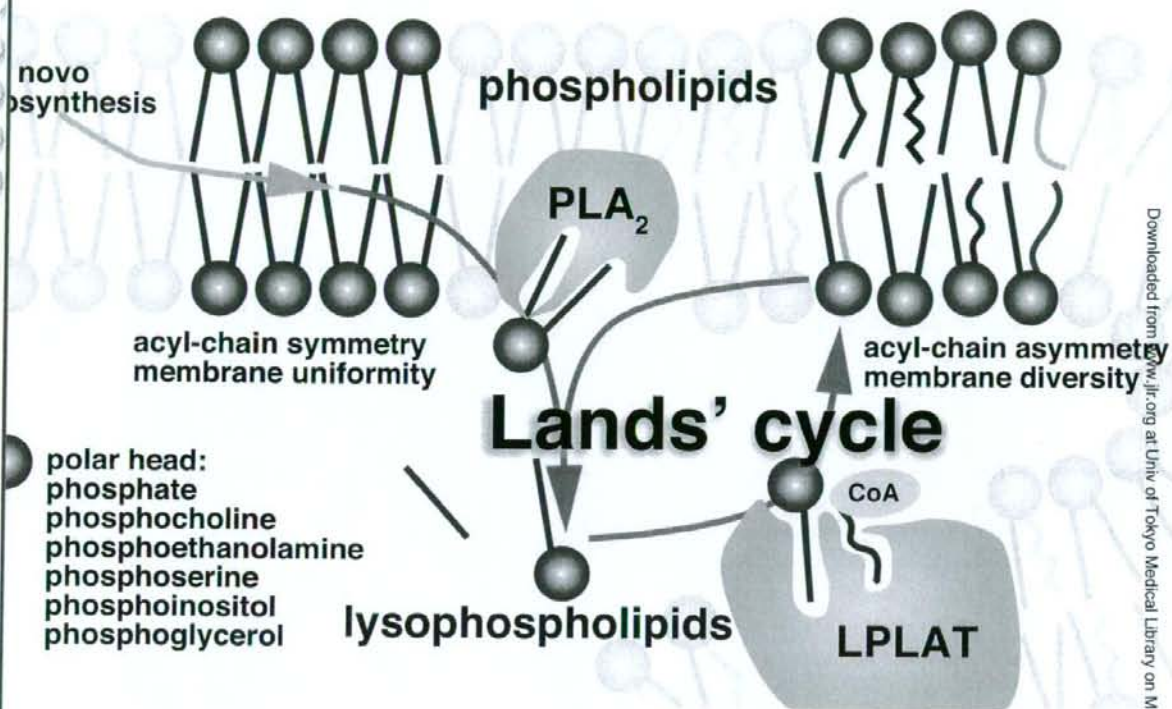phospholipids

PLA₂

acyl-chain symmetry
membrane uniformity

acyl-chain asymmetry
membrane diversity

Lands' cycle

polar head:
phosphate
phosphocholine
phosphoethanolamine
phosphoserine
phosphoinositol
phosphoglycerol

lysophospholipids

CoA

LPLAT

Fig. 2

Product	Proposed Name	Former Name			
LPA	**GPAT1**	GPAT1			
	GPAT2	GPAT2	xGPAT1		
	GPAT3	GPAT3	AGPAT8	AGPAT9	LPAATθ
	GPAT4		AGPAT6		LPAATζ
PA	**LPAAT1**		AGPAT1		LPAATα
	LPAAT2		AGPAT2		LPAATβ
	LPCAT1	LPCAT1	AGPAT9	AT like 2	
PC	**LPCAT1**	LPCAT1	AGPAT9	AT like 2	
	LPCAT2	LysoPAFAT/LPCAT2		AT like 1	
	LPCAT3	MBOAT5			
	LPCAT4	MBOAT2			
	LPEAT2	LPEAT2	AGPAT7	AT like 3	LPAATη
PE	**LPCAT3**	MBOAT5			
	LPCAT4	MBOAT2			
	LPEAT1	MBOAT1			
	LPEAT2	LPEAT2	AGPAT7	AT like 3	LPAATη
PS	**LPCAT3**	MBOAT5			
	LPEAT1	MBOAT1			
	LPEAT2	LPEAT2	AGPAT7	AT like 3	LPAATη
PG	**LPCAT1**	LPCAT1	AGPAT9	AT like 2	
	LPGAT1	LPGAT1			
	LPEAT2	LPEAT2	AGPAT7	AT like 3	LPAATη
PI	**LPIAT1**	MBOA-7	MBOAT7	LRC4	
CL	**LCLAT1**	ALCAT	AGPAT8		
PAF	**LPCAT1**	LPCAT1	AGPAT9	AT like 2	
	LPCAT2	LysoPAFAT/LPCAT2		AT like 1	
Unknown			AGPAT3		LPAATγ
			AGPAT4		LPAATδ
			AGPAT5		LPAATε
				AT Like 1B	

de novo pathway — LPA, PA

Remodeling pathway pathway — PC, PE, PS, PG, PI, CL, PAF

Characterization of mouse lysophosphatidic acid acyltransferase 3: An enzyme with dual functions in the testis*

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Running title: An acyltransferase with dual enzyme activities in the testis

Abbreviations:

PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phophatidylserine; LPLAT, lysophospholipid acyltransferases; AGPAT, 1-acylglycerol-3-phosphate O-acyltransferase; LPAAT, lyso-PA acyltransferase; LPIAT, lysoPI acyltransferase; ER, endoplasmic reticulum; CDP-diacylglycerol, cytidine diphosphodiacylglycerol; DDBJ, DNA Data Bank of Japan; NCBI, National Center for Biotechnology Information; CHO, Chinese hamster ovary; and DHT, dihydrotestosterone

Abstract

Glycerophospholipids are structural and functional components of cellular membranes as well as precursors of various lipid mediators. Using acyl-CoAs as donors, glycerophospholipids are formed by the *de novo* pathway (Kennedy pathway) and modified in the remodeling pathway (Lands' cycle). Various acyltransferases, including two lysophosphatidic acid acyltransferases (LPAATs), have been discovered from a 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) family. Proteins of this family contain putative acyltransferase motifs, but their biochemical properties and physiological roles are not completely understood. Here, we demonstrated that mouse LPAAT3, previously known as mouse AGPAT3, possesses strong LPAAT activity and modest lysophosphatidylinositol acyltransferase activity with a clear preference for arachidonoyl-CoA as a donor. This enzyme is highly expressed in the testis, where CDP-diacylglycerol synthase 1 preferring 1-stearoyl-2-arachidonoyl-phosphatidic acid as a substrate is also highly expressed. Since 1-stearoyl-2-arachidonoyl-species are the main components of phosphatidylinositol, mouse LPAAT3 may function in both the *de novo* and remodeling pathways and contributes to effective biogenesis of 1-stearoyl-2-arachidonoyl-phosphatidylinositol in the testis. Additionally, the expression of this enzyme in the testis increases significantly in an age-dependent manner, and β-estradiol may be an important regulator of this enzyme's induction. Our findings identify this acyltransferase as an alternative important enzyme to produce phosphatidylinositol in the testis.

Supplementary key words

1-acylglycerol-3-phosphate O-acyltransferase, phosphatidic acid, phosphatidylinositol, testis, β-estradiol

Introduction

Tissues maintain distinct content and composition of various glycerophospholipids such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (1-4). They are formed by two pathways using acyl-CoAs as donors. One is the *de novo* pathway (Kennedy pathway) in which glycerophospholipids are formed from glycerol 3-phosphate (5,6). The other is the remodeling pathway (Lands' cycle), where the concerted activation of phospholipase A_2s and lysophospholipid acyltransferases (LPLATs) occurs (6-10). These pathways are the basis of membrane asymmetry and diversity. In general, saturated and monounsaturated fatty acids are esterified at the *sn*-1 position, whereas polyunsaturated fatty acids are at the *sn*-2 position. The combinations of fatty acids at the *sn*-1 and *sn*-2 positions vary among different classes of phospholipids. In the rat liver and brain, for example, PA possesses a low arachidonic acid content (11-13), whereas arachidonic acid is a major component of PI (4,13,14).

Extensive studies of acyltransferases have been conducted over the last decade, mostly using homology searches (6,7,15-35). Several acyltransferase families have been proposed, including 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) family. At least seven AGPAT family members have been identified in mouse (21, 36, 37) and each of them contains a highly conserved putative catalytic motif (NHX_4D) and putative substrate binding motif (EGTR) (38-40) (Fig. 1A). Some of AGPAT family members are relatively well characterized. LysoPA acyltransferase (LPAAT) activity of mouse LPAAT1 (previously known as mouse AGPAT1) and mouse LPAAT2 (or mouse AGPAT2) is well documented (21, 26) and mutations in human LPAAT2 cause congenital generalized lipodystrophy (41). Recently, mouse AGPAT6 was shown to have glycerol-3-phosphate acyltransferase activity (33). Mouse AGPAT3 has been investigated in the past, but the characterization was far from being done (37). The existence of at least seven members in AGPAT family raises questions as to the specific role of each member. Therefore, investigation of their tissue distributions or biochemical properties will be important in understanding their biological roles.

We present here, to our knowledge, the first detailed biochemical and biological characterization of mouse AGPAT3 (mAGPAT3). Surprisingly, mAGPAT3 possesses both LPAAT and lysoPI acyltransferase (LPIAT) activities and prefers arachidonoyl-CoA as a donor, indicating its dual roles in the *de novo* and remodeling pathways. Point mutations in highly conserved motifs NHX_4D or EGTR completely suppressed both LPAAT and LPIAT activities.

31

The enzyme was localized in endoplasmic reticulum (ER) and expressed in the liver, kidney, and testis. In the testis, cytidine diphosphodiacylglycerol (CDP-diacylglycerol) synthase 1 is highly expressed and particularly converts 1-stearoyl-2-arachidonoyl-PA to CDP-diacylglycerol, a phospholipid precursor (42, 43). This might suggest that mLPAAT3 produces PI effectively. Additionally, mAGPAT3 expression in the testis increases significantly in an age-dependent manner. Since β-estradiol induced this enzyme in testicular cell line, mAGPAT3 may play an important role in the testis coupled with sex hormone. We renamed this enzyme as LPAAT3 according to a proposal for the standardization of LPLAT nomenclature by Shindou et al. (6).

MATERIALS AND METHODS

Materials

DMEM, 12F-HAM, and RPMI1640 are obtained from Sigma. TLC silica gel plates (type 5721) were purchased from Merck (Darmstadt, Germany). Various lysophospholipids and acyl-CoAs were from Avanti Polar Lipids (Alabaster, AL). [1-^{14}C]Oleoyl-CoA (1.924 GBq/mmol), [1-^{14}C]Linoleoyl-CoA (2.035 GBq/mmol), and [1-^{14}C]Arachidonoyl-CoA (2.035 GBq/mmol) were purchased from Moravec Biochemicals (Mercury Lane, CA). [1-^{14}C]Palmitoyl-CoA (2.22 GBq/mmol) and [^{3}H]Acetyl-CoA (185 GBq/mmol) were obtained from GE Healthcare UK Ltd. (Buckinghamshire, England).

Cloning of mLPAAT1 and mLPAAT3—

The entire coding region of mLPAAT3 (DNA Data Bank of Japan (DDBJ) accession number AB377215) was identified in the National Center for Biotechnology Information (NCBI) database. A 1.1-kb cDNA clone encoding the full-length mLPAAT3 was obtained by PCR amplification using a forward primer designed to encode FLAG epitope (DYKDDDDK) in frame with the start codon of target DNA coding region (5'-CTAGCTAGCCACCATGGATTACAAGGATGACGATGACAAGGGCCTGCTTGCCTA CCTGAAGACCC), and a reverse primer (CCGCTCGAGTTATTCCTT TTTCTTAAGCTCTTGGTTGCC-3'). Mouse heart cDNA was used as a template. Amplified PCR products were cloned into the pCXN2.1 vector (32) and sequenced. Similarly, mLPAAT1 (DDBJ accession number NM_018862) was identified in NCBI, and full-length mLPAAT1 was obtained by PCR amplification using a forward primer designed to encode FLAG epitope in frame (CTAGCTAGCCACCATGGATTACAAGGATGACGATGACAAGGAGCTGTGGC CCGGGGCCTGG), and a reverse primer (CCGCTCGAGTCAGAGCCGGGCTTCGCCCGCT CCCCC).

Mutagenesis of mLPAAT3

mLPAAT3 constructs with single mutations in the highly conserved motif NHX$_4$D (His→Ala, or Asp→Ala) or EGTR (Glu→Ala) were made using cloned mLPAAT3 cDNA as a template. Amplified PCR products were cloned into the pCXN2.1 vector and sequenced. Details of the method have been previously described (39).

Expression of FLAG-mLPAAT3 in Chinese hamster ovary (CHO)-K1 cells—

33

CHO-K1 cells were seeded onto 10 cm dishes, at a density of 3×10^6 cells /dish 1 day before transfection. 12 µg each of pCXN2.1 vector or FLAG-mLPAAT3-pCXN2.1 was transfected using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, transfected cells were scraped into 1 ml of ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM sucrose, and a proteinase inhibitor cocktail Complete (Roche Applied Science) and sonicated three times on ice for 30 s each time. Cell lysates were centrifuged at 9,000 x g for 15 min. Supernatants were then centrifuged at 100,000 x g for 1 h. Pellets were suspended in buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM sucrose, and 1 mM EDTA. The protein concentration was measured by the method of Bradford (45), using a commercially prepared protein assay solution (Bio-Rad) and BSA (fraction V, fatty acid-free; Sigma) as a standard.

Production of Anti-mLPAAT3 Antiserum—

Antiserum was generated at SCRUM (Tokyo, Japan). C-terminal peptides were used for immunization of rabbits (EKGSSYGNQELK and FPGEQFKPARRPWT). Specificity of the aniserum was examined by Western blot using mircosomes from vector-, mLPAAT3-transfected cells. Microsomal fractions from mAGPAT4-, or AGPAT5-transfected cells were also used as negative controls.

Western Blot Analysis—

Cell lysates were centrifuged at 800 x g for 10 min. Supernatants were centrifuged at 9,000 x g for 15 min. Supernatants were then centrifuged at 100,000 x g for 1 h. Initial 9000 x g pellets were homogenized again and centrifuged at 9000 x g. The pellets were used as 9000 x g pellets. 2 µg each of 9,000 x g pellets, 100,000 x g pellets, and 100,000 x g supernatants were resolved by 10% SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare UK Ltd.). The membrane was blocked with 5% skim milk, incubated with anti-FLAG M2 mAb (IBI/Kodak) or anti-cytochrome c oxidase antibody (Invitrogen), washed and incubated with horseradish peroxidase-labeled anti-mouse IgG (GE Healthcare UK Ltd.). After washing, the membrane was exposed to ECL reagents (GE Healthcare UK Ltd.) and x-ray film (GE Healthcare UK Ltd.) to visualize immunoreactive proteins. Expression of the FLAG-tagged target protein was confirmed. For the examination of endogenous mLPAAT3 subcellular localization, rabbit anti-mLPAAT3 antiserum and horseradish peroxidase-labeled anti-rabbit IgG (GE Healthcare UK Ltd.) were used as primary and secondary antibodies, respectively.

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Confocal microscopy—

CHO-K1 cells transfected with pCXN2.1 vector or FLAG - mLPAAT3 - pCXN2.1 cells were fixed with 4% paraformaldehyde and permeabilized with methanol/acetone solution (1:1, v/v). Cells were incubated with 10 μg/ml primary antibody for 30 min. As a marker, M5 anti-FLAG mouse mAb or anti-FLAG rabbit antibody (Sigma) for FLAG epitope, anti-calnexin antibody (BD Biosciences) for ER, anti-cytochrome c oxidase antibody (Invitrogen) for mitochondria, and anti-GM130 (Invitrogen) for Golgi were used. Cells were blocked with 1% BSA and then incubated with 10 μg/ml Alexa Fluor 546 goat anti-rabbit IgG (Eugene, OR) and Alexa Fluor 488 goat anti-mouse IgG (Eugene, OR). Confocal microscopy was performed using an LSM510 Laser Scanning Microscope (Carl Zeiss, Germany) equipped with a 63 x water immersion objective lens (NA=1.2). Cells were observed by excitation at 543 nm with a He/Ne laser, and emission through a 585 nm long pass filter for the detection of red fluorescence. For the detection of green fluorescence, the excitation was at 488 nm with an argon laser, and emissions were taken collected using a 505-550 nm band pass filter.

Assay of LPLAT activity—

Acyltransferase activity was measured by the transfer of $[1-^{14}C]$acyl-CoAs or $[^3H]$acetyl-CoA to lysophospholipids to form phospholipids. Reaction mixtures contained 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, and indicated concentrations of acyl-CoA, lysophospholipids and enzyme (100,000 x g pellets) in a total volume of 100 μl. The amount of total protein, concentrations of acceptors and donors are described in corresponding figure legends. After incubation at 37°C for 10 min, reactions were stopped by the addition of 300 μl of chloroform:methanol (1:2, v/v). The reaction progressed linearly at least for 10 min. Total lipids were extracted using the Bligh-Dyer method (46) and subsequently analyzed by TLC in chloroform: methanol: acetic acid: water (50:25:8:4, v/v/v/v). Bands at positions corresponding to the expected products were visualized with I_2 vapor, cut off the plate, placed in Microscinti-O (PerkinElmer Life Sciences), and analyzed in a liquid scintillation counter LS6500 (Beckman).

Quantitative Real-time RT-PCR—

Total RNA was prepared using RNeasy Mini Kit (QIAGEN). First strand cDNAs were synthesized using Superscript II (Invitrogen). PCR was conducted in microcapillary tubes, in 20 μl reaction volumes consisting of 2 μl of cDNA solution, 1 x FastStart DNA Master SYBR Green I (Roche Applied Science), and 0.5 μM each of the forward and reverse primers.

Sequences of primers used in RT-PCR are shown in Supplemental Table 1.

Stimulation of testicular cell line TM4 cells with various sex hormones—

Testicular cell line TM4 cells were cultured in F12-HAM:DMEM (1:1,v/v) containing 5% horse serum (Gibco) and 2.5% FBS. 1×10^5 cells were incubated with either mock, 100 nM β-estradiol (Sigma), dihydrotestosterone (DHT; Sigma), or testosterone (Sigma) for 24 h and collected.

Animals—

C57BL/6J mice were obtained from Clea Japan, Inc. (Tokyo, Japan). Mice were maintained at 21°C in a light-dark cycle with light from 08:00 to 20:00. Mice were fed with a standard laboratory diet and water ad libitum. All animal studies were conducted in accordance with the guidelines for Animal Research at The University of Tokyo and were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Statistics—

Data are presented as mean + SD. P values less than 0.05 were considered statistically significant. All statistical calculations were performed using Prism 4 (GraphPad Software).

RESULTS

Cloning of mLPAAT3

To identify novel LPLATs, we focused on AGPAT family proteins. A phylogenetic tree was drawn by pairwise comparisons of the amino acid sequences of LPAAT family members by using ClustalW, DDBJ (http://www.clustalw.ddbj.nig.ac.jp/top-j.html) (Fig. 1A) (49). The amino acid sequences of mLPAAT1, mLPAAT2 and mLPAAT3 are shown in Fig. 1B. A 1.1-kb cDNA clone encoding the full-length mouse LPAAT3 enzyme was obtained by PCR amplification. mLPAAT3 encodes a 376-amino acid protein of 43.3 kDa, containing four transmembrane domains, predicted by ConPred II (48), and the conserved motifs (NHX_4D and EGTR) found in members of AGPAT family (Fig. 1B). The protein also possesses the C-terminal sequence motif KKXX (49), suggesting the mLPAAT3 localizes to the ER, similar to LPCAT1 and LysoPAFAT/LPCAT2 (15, 16).

Tissue distribution of mLPAAT3 and mMBOA7

The tissue distribution of mLPAAT3 mRNA was analyzed by quantitative real-time PCR analysis. mLPAAT3 was predominantly expressed in the testis (Fig. 2A), whereas mMBOA7 was ubiquitously expressed (Fig. 2C). We examined mLPAAT3 expression profile in protein level by Western blot using rabbit anti-mLPAAT3 antiserum. mLPAAT3 was highly expressed in the testis (Fig. 2B). mLPAAT3 was also expressed in the liver and kidney (Fig. 2B). Discrepancy between mRNA and protein expression level of mLPAAT3 may possibly be due to the difference in translational efficiency from mRNA into protein and/or half-life of this enzyme within tissues.

Subcellular localization of FLAG-mLPAAT3 and endogenous mLPAAT3

To facilitate immunocytochemical and Western blot analyses of mLPAAT3, we constructed an mLPAAT3 fusion protein that contains the FLAG epitope fused in-frame to the N terminus of mLPAAT3. FLAG-mLPAAT3 was transfected into CHO-K1 cells, and the subcellular localization was examined by confocal microscopy after 48 h. Cells were stained for ER (anti-calnexin N-terminal), Golgi (anti-GM130) or mitochondrial (anti cytochrome c oxidase) markers. The subcellular distribution pattern of FLAG-mLPAAT3 was similar to that of calnexin N terminal (Supplemental Fig. 1). Neither Golgi nor mitochondrial marker protein distributions overlapped with mLPAAT3 (data not shown). To confirm these observations, CHO-K1 cells were transiently transfected with FLAG-mLPAAT3, homogenized, and

differential subcellular fractions were collected using an ER extraction kit (Sigma). When the subcellular fractions were analyzed by Western blot using anti-FLAG M2 antibody, the enzyme was found in ER fraction (Fig. 3A), consistent with the data obtained by confocal microscopy (Supplemental Fig. 1). FLAG -mLPAAT3 had an apparent molecular weight of approximately 37 kDa, slightly less than the predicted molecular weight of 43.3 kDa. The discrepancy in molecular weight was observed in other acyltransferases possessing multiple membrane spanning domains as previously described (17). To examine subcellular localization of endogenous mLPAAT3, subcellular fractions of testicular cell line TM4 cells were analyzed by Western blot using rabbit anti-mLPAAT3 antiserum. Consistent with the finding in FLAG-mLPAAT3 over-expression system, endogenous mLPAAT3 was predominantly localized to the ER fraction (Fig. 3B).

Substrate selectivity of mLPAAT3

Using $[1-^{14}C]$arachidonoyl-CoA as an acyl donor, we analyzed the substrate specificity of mLPAAT3 using a variety of lysophospholipid acceptors (Fig. 4A). The microsomal fraction from CHO-K1 cells transfected with mLPAAT3 had detectable LPLAT activity for LPA, LPC and LPI (Fig. 4A). However, its LPC acyltransferase activity was not pursued further because of its very limited activity. We also checked substrate selectivity using $[1-^{14}C]$oleoyl-CoA as an acyl donor, and did not observe any significant LPLAT activity of mLPAAT3 (data not shown).

Next, we examined the preference of mLPAAT3 for various LPA acceptor, using $[1-^{14}C]$oleoyl-CoA or $[1-^{14}C]$arachidonoyl-CoA as acyl donors. As seen in Fig. 4B, mLPAAT3 exhibited strong LPAAT activity using arachidonoyl-CoA as a donor with palmitoyl-LPA, stearoyl-LPA, oleoyl-LPA and octadecenyl-LPA as acceptors (Fig. 4B). We then examined the acyl-CoA selectivity of mLPAAT3 using palmitoyl-LPA as an acceptor. mLPAAT3 demonstrated a clear preference for arachidonoyl-CoA as a donor (Fig. 4C). We also examined the acyl-CoA selectivity of mLPAAT3 using 1-acyl-LPI as an acceptor. mLPAAT3 showed LPIAT activity using arachidonoyl-CoA as a donor (Fig. 4D). Other LPI acyltransferases such as MBOA7 would explain the high endogenous activity with vector-transfected cells.

Kinetics of mLPAAT3 expressed in CHO-K1 cells

We examined the acyltransferase activity of mLPAAT3 using palmitoyl-LPA and