TABLE 3. Accession number of "motif 3-cysteine" acyltransferases

Species	Enzyme	Nucleotide	Protein
Homo sapiens	LPCAT1	AB244719	BAE94688
Homo sapiens	LPCAT2	AB244718	BAF47696
Homo sapiens	LPEAT2	NM_153613	NP_705841
Homo sapiens	GPAT3	NM_032717	NP_116106
Homo sapiens	GPAT4	NM_178819	NP_848934
Mus musculus	LPCAT1	AB244717	BAE94687
Mus musculus	LPCAT2	AB244716	BAF47695
Mus musculus	ATlike1B	NM_027599	NP_081875
Mus musculus	LPEAT2	NM_207206	NP_997089
Mus musculus	GPAT3	NM_172715	NP_766303
Mus musculus	GPAT4	NM_018743	NP_061213
Danio rerio	zgc:158232	NM_001044341	NP_001037806
Danio rerio	zgc:112165	NM_001020656	NP_001018492
Danio rerio	zgc:77292	NM_205559	NP_991122
Danio rerio	zgc:136875	NM_001040249	NP_001035339
Danio rerio	AGPAT9	NM_001002685	NP_001002685
Danio rerio	AGPAT9like	NM_001099450	NP_001092920
Ciona intestinalis	cicl007p14	AK173535	
Ciona intestinalis	cieg018n20	AK114815	
Caenorhabditis elegans	acl-4	NM_075978	NP_508379
Caenorhabditis elegans	acl-5	NM_001047817	NP_001041282

was crucial for the activity of LPCAT1. This cysteine may be responsible for the difference between the NEM sensitivity of various acyltransferases. Further experiments are needed to directly certificate this hypothesis. At this position, GPAT1 and lysophosphatidic acyltransferase 1 (LPAAT1; also called AGPAT1) contain arginines that have been shown to be crucial for their activity (28, 29). Thus, the amino acid at the end of motif 3 seems to be essential for the activity of these acyltransferases. Although GPAT2 does not possess the described motif 3-cysteine, a cysteine at motif 2 was proposed to be responsible for its NEM sensitivity (25).

The importance of the amino acid at the end of motif 3 suggests that this residue can be utilized to classify acyltransferases. Therefore, we performed an extensive BLAST search to investigate the conservation of the LPCAT1 motif 3-cysteine. The results showed that the cysteine at the end of motif 3 is highly conserved among many enzymes of many species. We termed these enzymes "motif 3-cysteine" acyltransferases in this work. The establishment of this classification allowed us to investigate the evolution of LPCAT1. We compared the motif 3-cysteine enzymes to determine when LPCAT1 first appeared. The phylogenetic tree suggested that the evolution of LPCAT1 occurred before the appearance of teleosts. It is said that the ancestor of the lung also appeared before the evolution of teleosts as a "respiratory pharynx", which became the air bladder in teleosts and the lung in other vertebrates (26). Thus, the evolution of LPCAT1 appears to coincide with that of the lung. Because both LPCAT1 and LPCAT2 possess lyso-PAF AT activity, the ancestor enzyme of LPCAT1 might be a PAF biosynthetic enzyme. Additionally, LPCAT1 might have evolved as the biosynthetic enzyme of pulmonary surfactant phospholipids. The high expression level of hLPCAT1 mRNA in the human lung underscores its involvement in pulmonary function.

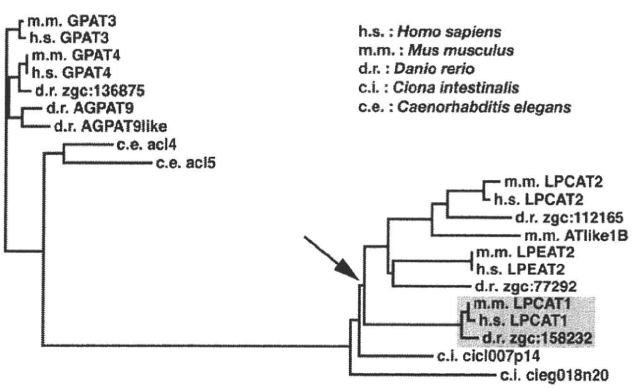

Fig. 5. Phylogenetic tree of motif 3-cysteine acyltransferases. Multiple alignment of putative lysophospholipid acyltransferases containing a cysteine at the end of motif 3 was performed. Based on the result, a phylogenetic tree was drawn. Arrow: the time of bifurcation of LPCAT1 and other proteins.

The properties of hLPCAT1 that we have described here implicate it in the biosynthesis of pulmonary surfactant lipids. First, it synthesizes DPPC and DSPG in vitro, the major lipid components of pulmonary surfactant. Second, the evolution of LPCAT1 appears to coincide with the evolution of the lung. Third, hLPCAT1 is highly expressed in the human lung. In addition to these findings, hLPCAT1 mRNA (which was called AYTL2 in the report) was shown to be upregulated during in vitro differentiation of alveolar type II cells (30). Our preliminary data suggest that hLPCAT1 exhibits substrate specificity for palmitoyl-CoA (T. Harayama, H. Shindou, and T. Shimizu, unpublished observations). Based on the above observations by us and others, we propose that hLPCAT1 is the biosynthetic enzyme of pulmonary surfactant lipids. Further studies are needed to elucidate the roles of hLPCAT1 in surfactant production in vivo. Given the fact that mutations in numerous genes involved in pulmonary surfactant function lead to various pulmonary diseases (for example, ATP-binding cassette A3 and surfactant protein B) (3, 31),

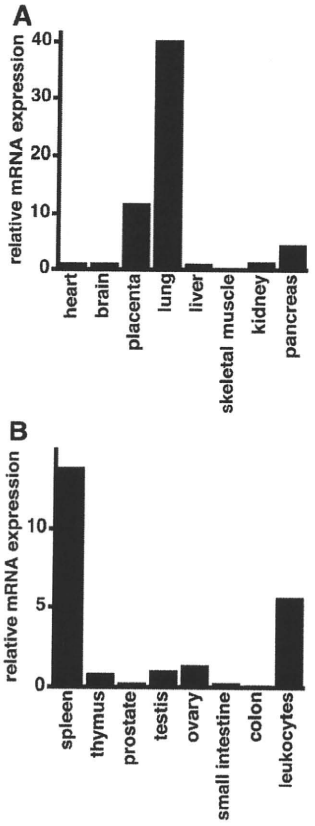

Fig. 6. Tissue distribution of hLPCAT1 mRNA. A: Quantitative PCR was performed using cDNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas as templates. Values are illustrated as the relative expression level of hLPCAT1 divided by that of glyceraldehyde-3-phosphate dehydrogenase. The value for human heart was set as 1. B: Quantitative PCR was performed using cDNA from human spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocytes as templates. Values are illustrated as the relative expression level of hLPCAT1 divided by that of glyceraldehyde-3-phosphate dehydrogenase. The value for human thymus was set as 1.

alterations in hLPCAT1 function may also be responsible for some lung dysfunctions.

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Identification of membrane *O*-acyltransferase family motifs

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ABSTRACT

Cellular membranes contain several classes of glycerophospholipids, which have numerous structural and functional roles in cells. Membrane diversity and asymmetry are important for membrane fluidity, curvature, and storage of lipid mediator precursors. Using acyl-CoAs, glycerophospholipids are first formed in the *de novo* pathway (Kennedy pathway), and then modified in the remodeling pathway (Lands' cycle) to generate mature membrane. Recently, several lysophospholipid acyltransferases (LPLATs) from two families, the 1-acylglycerol-3-phosphate *O*-acyltransferase (AGPAT) family and the membrane bound *O*-acyltransferase (MBOAT) family, were shown to function in the remodeling pathway. The MBOAT family possesses either LPLAT activity or protein *O*-acyltransferase activity. While the motifs of the AGPAT family have been well characterized, the MBOAT motifs remain unclear. In this study, we identified four MBOAT motifs essential for LPLAT activities by extensive site-directed mutagenesis. These findings further our understanding of the enzyme reaction mechanisms and will contribute to structure predictions for the MBOAT family enzymes.

Introduction

All organisms are comprised of cells that are encapsulated by a cell membrane, which contains glycerophospholipids, cholesterol, and proteins [1]. Glycerophospholipids are important not only as structural and functional components of biological membranes, but also as constituents of serum lipoproteins and pulmonary surfactant. Additionally, glycerophospholipids play important roles as precursors of lipid mediators such as platelet-activating factor (PAF) and eicosanoids [2]. Tissues maintain distinct contents and compositions of various glycerophospholipids such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL) [1]. Using acyl-CoAs, glycerophospholipids are formed by the *de novo* pathway (Kennedy pathway), and modified to their mature form by the remodeling pathway (Lands' cycle) [3,4]. Saturated and monounsaturated fatty acids are usually esterified at the *sn*-1 position, whereas polyunsaturated acyl groups are located at the *sn*-2 position. The combinations of fatty acids at the *sn*-1 and *sn*-2 positions vary among different classes of glycerophospholipids [5,6]. The rapid turnover of the *sn*-2 acyl moiety is attributed to the concerted activation of phospholipase A_2s (PLA_2s) and lysophospholipid acyltransferases (LPLATs) [2,6–8]. These asymmetry and high diversity of glycerophospholipids are established by the remodeling pathway. Although these metabolic processes are carried out in several tissues, only limited information on the enzymes involved in glycerophospholipid remodeling has been available for the more than 50 years since the discovery of the Lands' cycle.

Recently, several groups including us, identified mammalian LPLATs functioning in the remodeling pathways from the 1-acylglycerol-3-phosphate *O*-acyltransferase (AGPAT) and the membrane bound *O*-acyltransferase (MBOAT) families [9–18]. Lyso-PC acyltransferase (LPCAT)1 and 2, lyso-PE acyltransferase (LPEAT)2, lyso-PA acyltransferase (LPAAT)3, lyso-PG acyltransferase (LPGAT)1, and lyso-CL acyltransferase 1 (LCLAT1) are members of the AGPAT family [9–13,17,18]. On the other hand, LPCAT3, LPCAT4, LPEAT1, and lyso-PI acyltransferase (LPIAT)1 are members of the MBOAT family [14–16]. An yeast homolog (ALE1, LPT1, and SLC4) was also identified as an MBOAT family enzyme (MBOAT2) [19–22]. The AGPAT family also contains several acyltransferases working in the *de novo* pathway. Until now, "AGPAT motifs" important for their acyltransferase activities have been well characterized [23–25]. However, "MBOAT motifs" for LPLAT activities were not described and information on the consensus sequences are limited [26], since the enzymes were originally considered to be

Abbreviations: PAF, platelet-activating factor; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PLA_2, phospholipase A_2; LPLAT, lysophospholipid acyltransferase; AGPAT, 1-acylglycerol-3-phosphate *O*-acyltransferase; MBOAT, membrane bound *O*-acyltransferase; LPCAT, lyso-PC acyltransferase; LPEAT, lyso-PE acyltransferase; LPAAT, lyso-PA acyltransferase; LPGAT, lyso-PG acyltransferase; LCLAT1, lyso-CL acyltransferase 1; LPIAT, LPI acyltransferase; LPSAT, lyso-PS acyltransferase; CHO, Chinese hamster ovary.

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protein-*O*-acyltransferases such as ghrelin acyltransferase [27] and acyltransferases for sonic hedgehog [28]. Only the His residue at position 350 (His^{350}) in LPIAT1 has been reported to be important for LPIAT activity [16].

Thus, we focused on LPLATs of the MBOAT family to determine the motifs essential for enzyme activities. The four known LPLATs exhibit multiple activities (i) LPCAT3 (MBOAT5) has LPCAT, LPEAT, and lyso-PS acyltransferase (LPSAT) activities with 18:1-CoA, 18:2-CoA, and 20:4-CoA, (ii) LPCAT4 (MBOAT2) has LPCAT, LPEAT, and LPAAT activities with preference for 18:1-CoA, (iii) LPEAT1 (MBOAT1) has LPEAT and LPSAT activities with preference for 18:1-CoA, and (iv) LPIAT1 (MBOAT7 or mboa-7) has LPIAT activity with 20:4-CoA [14–16,29,30]. Using site-directed mutagenesis, we identified four MBOAT motifs essential for LPLAT activities, but not for protein acylation activities (except for the Motif B). To our knowledge, this is the first finding and the detailed analysis of the MBOAT motifs. This study will help understand the mechanism of the enzyme reaction of the MBOAT family enzymes.

Materials and methods

All lysophospholipids and acyl-CoAs were obtained from Avanti Polar Lipids (Alabaster, AL). [1-^{14}C]oleoyl-CoA (1.924 GBq/mmol) was purchased from Moravec Biochemicals (Mercury Lane, CA). TLC silica gel plates (type 5721) were purchased from Merck (Darmstadt, Germany).

Cloning of mouse LPLATs. We have previously described the cloning of mouse LPCAT3 (mLPCAT3, DNA Data Bank of Japan (DDBJ) accession number AB294194), mLPCAT4 (AB297383), and mLPEAT1 (AB297382) [14]. Similarly, a 1.4 kb cDNA clone encoding the full-length of mLPIAT1 (NM_029934) was obtained by PCR amplification using a forward primer, 5′-CTAGCTAGCCACC ATGGATTACAAGGAT GACGATGACAAGACACCCGAAGAATGGACATA TCTAATGGTCC-3′, with attached FLAG epitope (DYKDDDDK), and a reverse primer, 5′-CCGCTCGAGTCACTCTTCCCGGAGCTTTTCC-3′. Mouse brain cDNA was used as a template. Amplified PCR products were cloned into the pCXN2.1 vector [31] and sequenced.

Mutagenesis of mouse LPLATs. Each mutant was constructed by overlap extension PCR. The primer sets utilized are listed in Supplementary Table 1. Amplified PCR products were cloned into the pCXN2.1 vector and sequenced.

Expression of FLAG-mutants in Chinese hamster ovary (CHO)-K1 cells. CHO cells were transfected with each FLAG-tagged LPLAT cDNA or FLAG-tagged mutant cDNA using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, cells from 10-cm dishes were scraped into 1 ml of ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 300 mM sucrose, and a protease inhibitor mixture, Complete (Roche Applied Science), and then sonicated three times on ice for 30 s each time. After centrifugation for 10 min at 800 *g*, each supernatant was collected and centrifuged at 100,000 *g* for 1 h. The resulting pellets were resuspended in buffer containing 20 mM Tris–HCl (pH 7.4), 300 mM sucrose, and 1 mM EDTA. Protein concentration was measured by the method of Bradford [32], using a commercially prepared protein assay solution (Bio-Rad) and bovine serum albumin (BSA, fraction V, fatty acid-free; Sigma) as a standard.

Western blot analysis. Western blot analyses were performed using an anti-FLAG M2 mAb (IBI/Kodak) and a horseradish peroxidase-labeled anti-mouse IgG (GE Healthcare UK Ltd.) The membrane was exposed to ECL reagents (GE Healthcare UK Ltd.) and signals were detected using a Luminescent image analyzer, LAS-4000 mini (Fujifilm Corporation, Tokyo, Japan).

In vitro LPLAT assays. Acyltransferase activity was measured by the transfer of [^{14}C]18:1-CoA to lysophospholipids to form phospholipids. Reaction mixtures contained 100 mM Tris–HCl (pH 7.4), 1 mM EDTA, 25 μM [^{14}C]18:1-CoA (0.22 GBq/mmol), 50 μM lysophospholipid, and protein (100,000 *g* pellets) in a total volume of 0.1 ml. The amount of total protein for each assay is described in the corresponding Figure legends. After incubation at 37 °C for 10 min, reactions were stopped by the addition of 0.3 ml of chloroform:methanol (1:2, v/v). Total lipids were extracted using a slightly modified Bligh–Dyer method [33], 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 by I_2 vapor, cut off the plate, placed in Microscinti-O (Perkin-Elmer Life Sciences), and analyzed in a liquid scintillation counter LS6500 (Beckman).

Software. Data are presented as mean + SD. All statistical calculations were performed using Prism 4 (GraphPad Software). Sequence alignments were made using GENETYX-MAC Version 13.0.6 (GENETYX Corporation). For the alignments, the sequences of the LPCAT3 orthologues, *Homo sapiens* (NP_005759), *Gallus gallus* (XP_416516), *Danio rerio* (XP_001341285), *Caenorhabditis elegans* (NP_001022735) and mouse ghrelin *O*-acyltransferase (GOAT, NP_001119786) were obtained from the National Center for Biotechnology Information (NCBI) database.

Results

Alignment of LPLATs in the MBOAT family

There are four known MBOAT family LPLATs; mLPCAT3, mLPCAT4, mLPEAT1, and mLPIAT1. The amino acid sequences of the enzymes were aligned to identify conserved sequences (Fig. 1). We examined the sequences conserved among three (Focus 1 and 2) or four (Focus 3, 4, and 5) of the LPLATs, as indicated (Fig. 1). These 21 amino acids were substituted with Ala residues. To assess each activity of the mutants, 18:1-CoA was used as a donor, because mLPCAT3, mLPCAT4, and mLPEAT1 possessed the LPLAT activities with 18:1-CoA [14], and mLPIAT1 also recognized 18:1-CoA (data not shown).

Conserved sequences among three LPLATs

First, we focused on the conserved amino acid sequences that are conserved among the three LPLATs, mLPCAT3, mLPCAT4, and mLPEAT1, but not mLPIAT1, as indicated by Focus 1 and 2 (Fig. 1). Single amino acid mutants were generated from mLPCAT3 by Ala substitution as follows; P182A, S183A, L184A, L185A, and E186A in the Focus 1, and R312A, W313A, and D314A in the Focus 2. The LPCAT, LPEAT, and LPSAT activities of each mutant were reduced compared to mLPCAT3-wild-type (WT), with the exception of L185A. However, the expression level of each mutant was lower than that of WT (Fig. 2A). The R312A mutant had similar acyltransferase activities to WT, but the W313A and D314A mutants did not show any activity (Fig. 2B). Although the expression level of each mutant was slightly lower than that of WT, the activities of the W313A and D314A mutants were completely abolished. Thus, Trp^{313} and Asp^{314} in mLPCAT3 are essential for LPLAT activities (Fig. 2B). WT (mLPCAT3) and mutants attached with a Flag-tag at the N terminus had an apparent molecular weight of approximately 37 kDa, slightly less than the predicted molecular weight of 56 kDa. Discrepancies in molecular weight have been observed previously in other acyltransferases possessing multiple membrane spanning domains [14,18,30].

Conserved sequences among four LPLATs

Next, we focused on the amino acid sequences that are conserved among all four LPLATs, as indicated by Focus 3, 4, and 5 in Fig. 1. Single amino acid mutants of mLPCAT3 were generated by

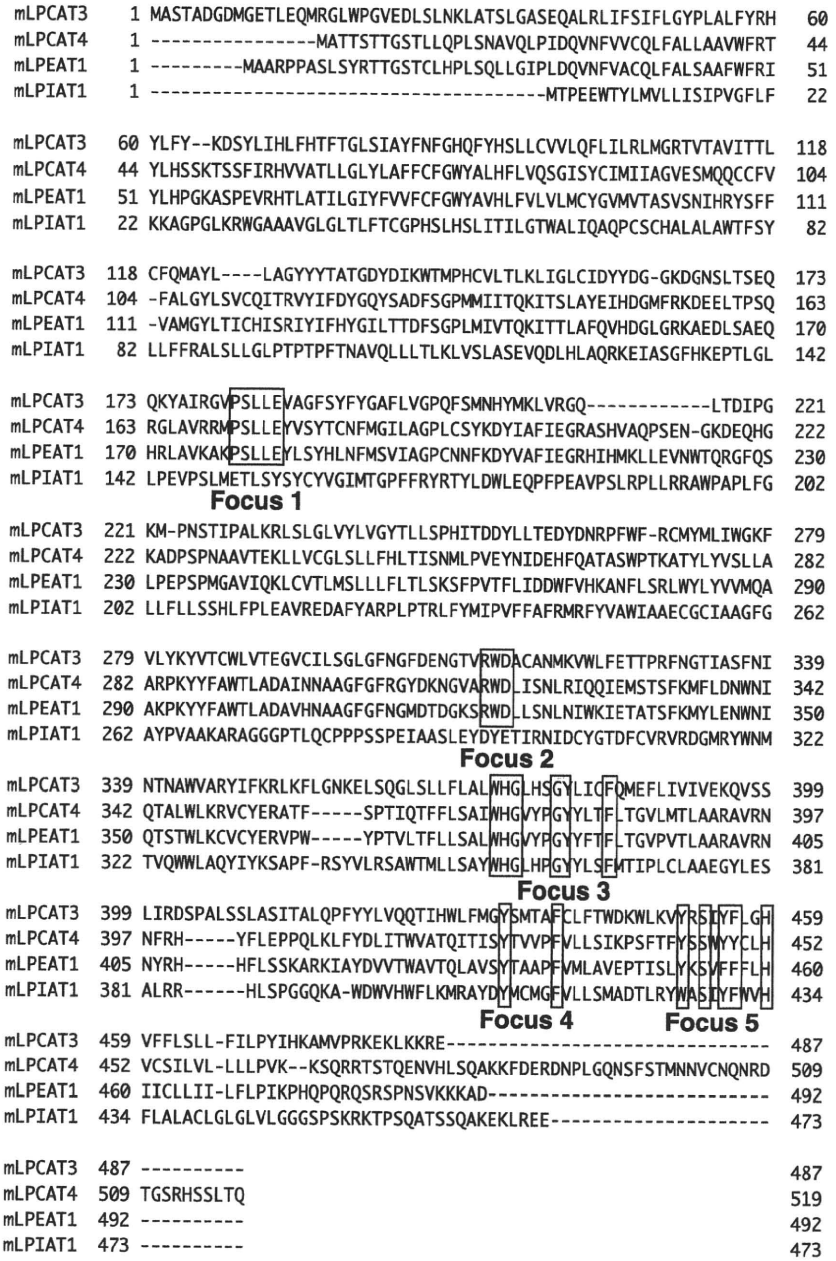

Fig. 1. Alignment of LPLATs in the MBOAT family. Alignment of mLPCAT3 (BAG12120), mLPCAT4 (BAG12122), mLPEAT1 (BAG12121), and mLPIAT1 (NP_084210) was performed using GENETYX-MAC. We focused on the five regions indicated by squares, Focus 1, 2, 3, 4, and 5.

substitution with Ala as follows; W373A, H374A, G375A, G379A, Y380A, and F384A in Focus 3, Y434A and F439A in Focus 4, and Y451A, S453A, Y455A, F456A, and H459A in Focus 5. The LPCAT, LPEAT, and LPSAT activities of each Focus 3 mutant were completely abolished by mutagenesis, even though the expression level of each mutant was similar to that of WT (Fig. 3A). The mutants, Y434A and F439A in Focus 4 and Y451A, Y455A, F456A, and H459A in Focus 5 also showed activities similar to the vector control (V), although the expression levels of Y434A and H459A were slightly lower than that of WT (Fig. 3B). We did not detect any reduction in LPLAT activities in the S453A mutant. Thus, all amino acids, in Focus 3, 4, and 5, with the exception of Ser^{453}, are key amino acids for LPLAT activities (Fig. 3B).

Identification of MBOAT motifs

We found the amino acid sequences essential for LPLAT activities in Focus 2, 3, 4, and 5 (Fig. 2 and 3). These mLPCAT3 amino acids were aligned with other LPCAT3 orthologues, *H. sapiens* (h), *G. gallus* (g), *D. rerio* (d), *C. elegans* (c). The conserved Focuses among LPLAT paralogues of the MBOAT family were very similar among the LPCAT3 orthologues (Fig. 4A). Accordingly, these results indicate that the conserved Focus 2, 3, 4, and 5 sequences should be considered the MBOAT motifs, designated the MBOAT Motifs A, B, C, and D, respectively. Thus, we determined the Motif A (^{313}WD^{314}), B (^{373}WHGxxxGYxxxF^{384}), C (^{434}YxxxxF^{439}), and D (^{451}YxxxYFxxH^{459}) in mLPCAT3 (Fig. 4B). The x means any amino acids. Although the Trp^{312} and Asp^{313} in the Motifs A and Tyr^{451}, Tyr^{455}, and Phe^{456} in the Motif D of mLPCAT3 are not identical among all the LPLAT paralogues of the family, only conservative amino acid substitutions at the indicated positions were found, for example Tyr^{295} and Glu^{296} in mLPIAT1 (Fig. 1).

Protein *O*-acyltransferases, such as GOAT are also members of the MBOAT family [27]. Mouse GOAT possesses the Motif B, but not the Motif A, C, or D (Fig. 4C). This observation may indicate that the Motifs A, C, and D are important for the recognition of lysophospholipids.

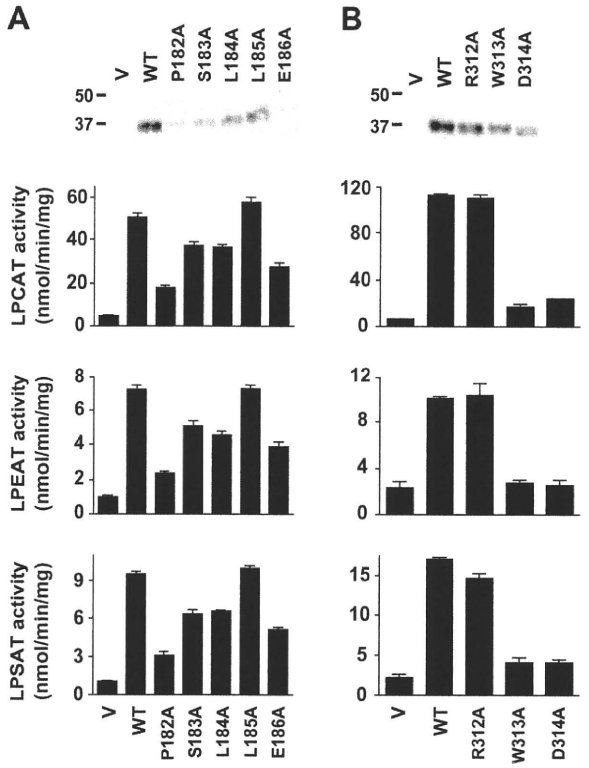

Fig. 2. Conserved sequences among three LPLATs. We focused on the amino acid sequences that are conserved among three LPLATs (mLPCAT3, mLPCAT4, and mLPEAT1) indicated by Focus 1 and 2 in Fig. 1. We performed Western blots using 3 μg microsomal protein and measured LPCAT, LPEAT, and LPSAT activities with 18:1-CoA using Focus 1 mutants; P182A, S183A, L184A, L185A, and E186A (A) and Focus 2 mutants; R312A, W313A, and D314A (B). The assays were performed with 0.2 μg microsomal protein for LPCAT activity, and 1 μg microsomal protein for LPEAT and LPSAT activities. The LPLAT activities of each mutant were decreased compared to WT, with the exception of L185A, but the expression levels of the mutants were reduced and may have affected the enzyme activities (A). The activities of W313A and D314A were similar to the vector control, V (B). No statistical analysis was performed, because the expression level of the mutants was not equal to that of the WT. V and WT indicate the vector control and mLPCAT3-wild-type, respectively. Values are means + SD. Three independent experiments were performed with similar results.

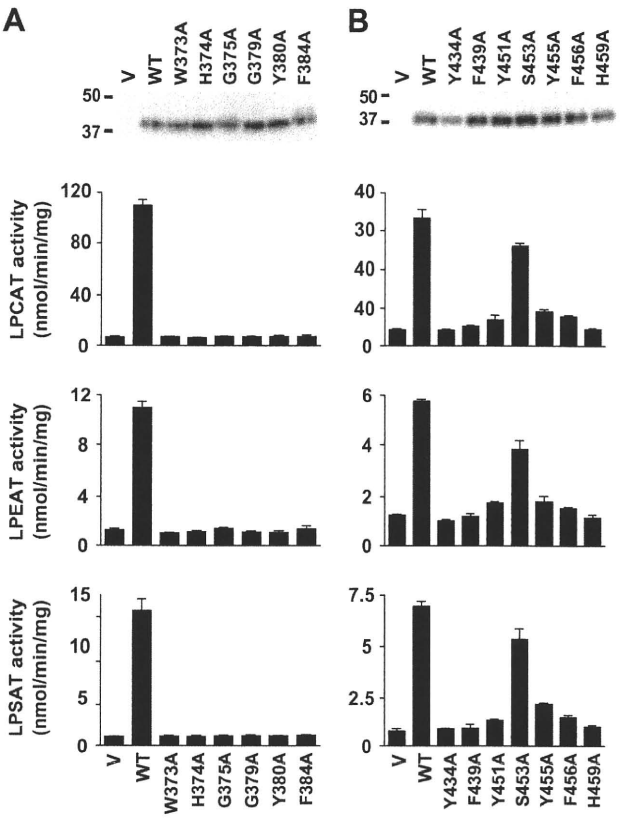

Fig. 3. Conserved sequences among all four LPLATs. We focused on the amino acid sequences that are conserved among all four LPLATs (mLPCAT3, mLPCAT4, mLPEAT1, and mLPlAT1) indicated by Focus 3, 4, and 5 in Fig. 1. We performed Western blots using 3 μg microsomal protein and measured LPCAT, LPEAT, and LPSAT activities with 18:1-CoA using Focus 3 mutants; W373A, H374A, G375A, G379A, Y380A, and F384A (A) and Focus 4 and 5 mutants; Y434A, F439A, Y451A, S453A, Y455A, F456A, and H459A (B). The enzyme assays were performed with 0.2 μg microsomal protein for LPCAT activity, and 1 μg microsomal protein for LPEAT and LPSAT activities. The LPLAT activities of all the Focus 3 mutants were abolished (A). Although we did not detect any reduction in the LPLAT activities in the S453A mutant, the activities of the other Focus 4/5 mutants decreased similar levels to V (B). No statistical analysis was performed, because the expression levels of the mutants were not equal to that of the WT. V and WT indicate the vector control and mLPCAT3-wild-type, respectively. Values are means + SD. Three independent experiments were performed with similar results.

Discussion

Here we have presented the first identification of MBOAT motifs essential for LPLAT activities. From our detailed mutagenesis studies, we identified the Motif A (^{313}WD^{314}), B (^{373}WHGxxxGYxxxF^{384}), C (^{434}YxxxxF^{439}), and D (^{451}YxxxYFxxH^{459}) in mLPCAT3 (Fig. 4B). Mouse GOAT, a protein *O*-acyltransferase, which is a member of the MBOAT family, possesses only Motif B, but not Motif A, C, nor D, suggesting that lysophospholipids may be recognized by Motif A, C, and/or D.

Over the last four years, many LPLATs involved in the remodeling pathway have been identified, resulting in the greatest advance in the LPLAT field since the discovery of the Kennedy pathway and the Lands' cycle 50 years ago. Two families of LPLATs have been discovered, namely the AGPAT and the MBOAT families. The AGPAT family also includes acyltransferases working in the *de novo* pathway. The AGPAT motifs have been well characterized using glycerol-3-phosphate acyltransferase 1, LPAAT1, and LPCAT1 [23–25]. According to these reports, there are at least four AGPAT motifs, and motif 2 of the AGPAT family interacts with acyl-CoA. However, little is known about the MBOAT motifs. The His^{350} in human LPIAT1 was only reported to be important for LPIAT activity [16]. To determine MBOAT motifs, we constructed 21 mutants targeting the conserved amino acids among LPLATs in the MBOAT family. Consequently, we identified the four MBOAT Motifs (A–D), which were highly conserved among LPCAT3 orthologues. Furthermore, the Motifs A, C, and D were not conserved in GOAT which may suggest that the Motif A, C, and D play a role in the recognition of lysophospholipids. In this study, Motifs for protein *O*-acylation activities were not investigated. Until now, since the purification of LPLATs is extremely difficult due to their multiple transmembrane domains, all the biochemical characterization of LPLATs was performed using over-expression systems or siRNA experiments. In the future, solution of the three dimensional structures of LPLATs will finally clarify the motifs and the mechanisms of interaction with substrates more precisely.

In conclusion, this is the first identification of the "MBOAT motifs", which are required for the LPLAT activities. Further studies are needed to elucidate the biochemical and biological characteristics of the MBOAT enzymes and to determine their potential as novel therapeutic targets for various diseases. To date, the study of membrane biogenesis in the remodeling pathway has been delayed due to the lack of information on key molecules involved

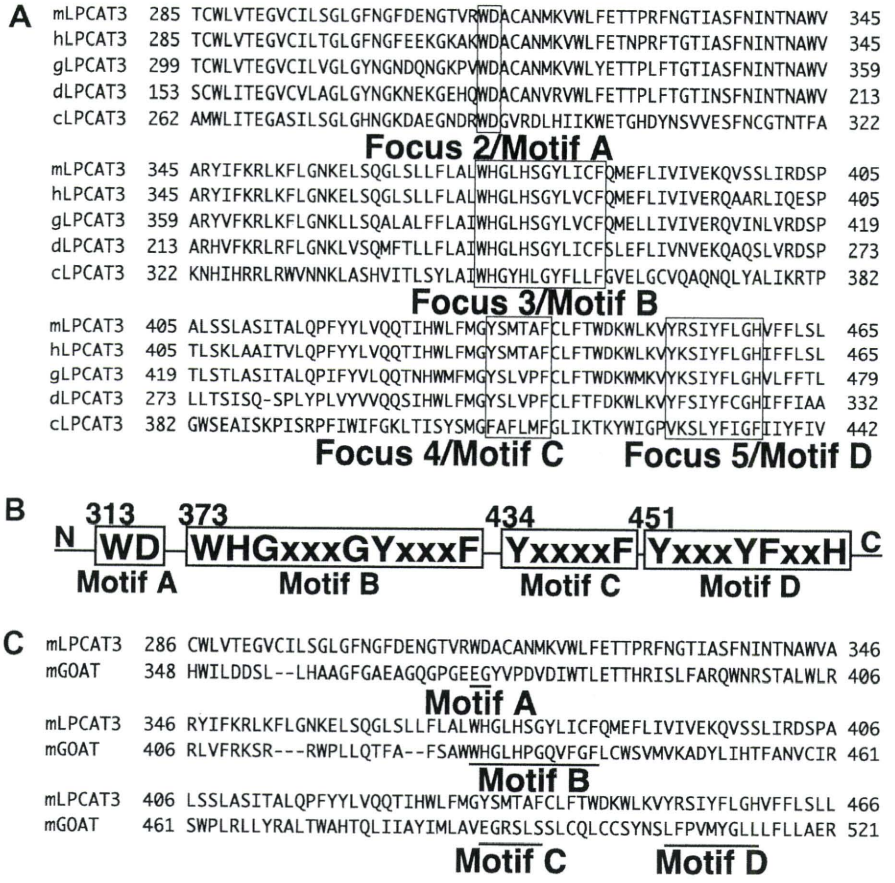

Fig. 4. The proposed MBOAT motifs essential for LPLAT activities. (A) For sequence alignment, the sequences of the LPCAT3 orthologues *Homo sapiens* (h, NP_005759), *Gallus gallus* (g, XP_416516), *Danio rerio* (d, XP_001341285), and *Caenorhabditis elegans* (c, NP_001022735) were obtained from HomoloGene in the NCBI database. Open boxes indicate the Focuses and Motifs. (B) The proposed MBOAT motifs are presented. The numbers indicate the position of amino acids in mLPCAT3. The x indicates any amino acids. (C) The alignment of mLPCAT3 and mGOAT is shown. mGOAT also contains the Motif B, but does not possess the Motifs A, C, or D.

in the process. The recent identification of LPLATs, together with the present study, expand the LPLAT field and paves the way for discovering new enzymes with the MBOAT motifs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.04.013.

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Phosphorylation of lysophosphatidylcholine acyltransferase 2 at Ser34 enhances platelet-activating factor production in endotoxin-stimulated macrophages*

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Running title: Phosphorylation of LPCAT2 for PAF biosynthesis

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Platelet-activating factor (PAF) is a potent proinflammatory phospholipid mediator that elicits various cellular functions under physiological and pathological conditions. We have recently identified two enzymes involved in PAF production; lysophosphatidylcholine acyltransferase-1 (LPCAT1) and LPCAT2. We found that LPCAT2 is highly expressed in inflammatory cells and is activated by lipopolysaccharide (LPS) treatment through Toll-like receptor 4. However, the molecular mechanism for the activation remains elusive. In this study, phos-tag SDS-PAGE revealed the LPS-induced phosphorylation of LPCAT2. Furthermore, mass spectrometry and mutagenesis analyses identified Ser34 of LPCAT2 as the phosphorylation site to enhance the catalytic activities. The experiments using inhibitors and siRNA against mitogen-activated protein kinase (MAPK) cascades demonstrated that LPCAT2 phosphorylation through LPS-TLR4 signaling may directly depend on MAPK-activated protein kinase 2 (MAPKAP kinase 2 or MK2). These findings develop a further understanding of both PAF production and phospholipid remodeling triggered by inflammatory stimuli. Specific inhibition of the PAF biosynthetic activity by phosphorylated LPCAT2 will provide a novel target for the regulation of inflammatory disorders.

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent proinflammatory lipid mediator that triggers various cellular functions through its G protein-coupled receptor (PAF[5] receptor; PAFR) (1,2). It is proposed that PAF is synthesized in various cells and tissues via two distinct pathways, the *de novo* and remodeling pathways. Through the remodeling pathway, PAF is rapidly synthesized in response to extracellular stimuli. Under such conditions, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF), the precursor of PAF, is

synthesized from 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phos phocholine (1-alkyl phosphatidylcholine, PC) by the action of phospholipase A_2 (PLA_2). Lyso-PAF is subsequently converted to PAF by acetyl-CoA:lyso-PAF acetyltransferase (lyso-PAFAT).

Endogenous lyso-PAFAT activity was initially demonstrated in 1980 (3) and partially characterized (4-7). Recently, we identified two molecular entities of lyso-PAFATs; a constitutively expressed lyso-PAFAT, lysophosphatidylcholine acyltransferase (LPCAT) 1 (8), and an inducible lyso-PAFAT, LPCAT2 (9). In these previous reports, endogenous lyso-PAFAT in inflammatory cells was activated by prophlogistic stimuli (6) and LPCAT2 in mouse peritoneal macrophages was indeed activated by lipopolysaccharide (LPS)-stimulation (9). However, the exact mechanisms for LPCAT2 activation remain unknown.

LPS activates Toll-like receptor (TLR) 4, which plays a central role in the activation of the innate immune system. Through its association with different combinations of four adaptors, the TLR4 signaling pathway leads to the phosphorylation of MAPKs; p38, ERK, and JNK. Subsequently, activated p38 phosphorylates MAPK-activated protein kinase 2 (MK2) (10,11), which can induce inflammatory cytokines (12,13) and lipid mediators (14,15).

LPCAT2 also possesses LPCAT activity to produce the major membrane phospholipid, PC, which mainly contains polyunsaturated fatty acids (PUFAs) at the *sn*-2 position. This biosynthetic pathway of phospholipids, known as Lands' cycle or remodeling pathway, is responsible for generating the membrane diversity (16). PUFAs in phospholipids may affect membrane curvature and fluidity, and store lipid mediator precursors that are converted to eicosanoids, such as prostaglandins, leukotrienes, and lipoxins (1). PC plays an important role as a precursor of both eicosanoids and PAF.

By mass spectrometry and mutagenesis studies, we demonstrated that LPCAT2 is activated by Ser34 phosphorylation in mouse peritoneal

macrophages and RAW264.7 cells with LPS-treatment. Consensus sequence and experiments with an MK2 inhibitor, and siRNA suggested that MK2 might directly phosphorylate and activate LPCAT2. These findings contribute to a better understanding of the regulatory mechanisms of PAF biosynthesis in inflammatory cells.

EXPERIMENTAL PROCEDURES

Materials

PC from frozen egg yolk, LPS from *Salmonella minnesota*, and anti-FLAG M2 antibody were from Sigma (St. Louis, MO). Lyso-PAF was from Cayman Chemical Co. (Ann Arbor, MI). Arachidonoyl-CoA was from Avanti Polar Lipids (Alabaster, AL). [^{3}H] Acetyl-CoA (129.5 GBq/mmol), horseradish peroxidase-linked anti-rabbit IgG, and horseradish peroxidase-linked anti-mouse IgG were from GE Healthcare (Buckinghamshire, UK). [1-^{14}C] Arachidonoyl-CoA (2.22 GBq/mmol) was from Moravec Biochemicals (Brea, CA). Thin layer chromatography (TLC) silica gel plates (type 5721) were from Merck (Darmstadt, Germany). Cell line Nucleofector kit V was from LONZA (Basel, Switzerland). Acetyl-CoA, DMSO and acrylamide bis 29:1 were from WAKO (Osaka, Japan). Phos-tag acrylamide was from NARD Institute, Ltd. (Hyogo, Japan). (5*Z*)-7-Oxozeanol was from TOCRIS Bioscience (Ellisville, MO). SB202474, SB203580, and MK2 inhibitor III were from Calbiochem (San Diego, CA). The siRNAs (ON-TARGETplus Non-targeting Pool D-001810-10-20 and ON-TARGETplus SMARTpool L-040135-00-0005) were from Thermo Scientific (Dharmacon) (Waltham, MA). Anti-MK2, anti-phospho-MK2, anti-p38 MAPK, and anti-phospho-p38 MAPK antibodies were from Cell Signaling Technology (Beverly, MA). The proteinase inhibitor mixture, EDTA-free Complete, was from Roche (Mannheim, Germany).

Mice

C57BL/6J mice were obtained from Clea Japan, Inc. (Tokyo, Japan). Mice were maintained in a light-dark cycle with lights on from 0800-2000 h at 22°C. 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.

Isolation of mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated as previously described (6). Cells were cultured for 16 h before stimulation.

Preparation of cell lysates

Cells were pretreated with or without 20 μM MK2 inhibitor III, 20 μM SB203580 (p38 MAPK inhibitor) or 1 μM (5*Z*)-7-oxozeaenol (TAK1 inhibitor) for 1 h, and then stimulated with 100 ng/ml LPS for 30 min. After stimulation, cells (peritoneal macrophages or RAW264.7 cells) were washed with ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 0.3 M sucrose, and 1 mM sodium orthovanadate, and collected in buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM 2-mercaptoethanol, and 1× EDTA-free Complete. Subsequently, cells were sonicated twice on ice for 30 s each time and centrifuged at 9,000 × *g* for 10 min at 4°C to remove cellular debris, intact cells, and mitochondria.

For primary cultured mouse peritoneal macrophages, the resultant supernatant at 9,000 × *g* was centrifuged at 100,000 × *g* for 1 h at 4°C. The resultant pellet was re-suspended with ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM 2-mercaptoethanol, 1× EDTA-free Complete. The concentration of each protein was measured by the Bradford method (17), using protein assay solution (Bio-Rad). Bovine serum albumin (fraction V, fatty acid-free; Sigma) served as a standard.

Site-directed mutagenesis of LPCAT2

Mouse LPCAT2 mutants (S34A and S34D) were constructed by overlap extension PCR. The amplified PCR products were cloned into the pCXN2.1 vector and the sequence was confirmed. The primer sets utilized are described were S34A (Forward: CGC CAG GCG GCC TTC TTC CCG CCG C, Reverse: GCG GCG GGA AGA AGG

CCG CCT GGC G); and S34D (Forward: CGC CAG GCG GAC TTC TTC CCG CCG C, Reverse: GCG GCG GGA AGA AGT CCG CCT GGC G).

Transfection into RAW264.7 cells

RAW264.7 cells (5×10^6 cells), 100 μl of Nucleofector solution V, and 5 μg of each DNA of vector, FLAG-mLPCAT2, S34A, or S34D were mixed. The mixture in the cuvette was set onto the amaxa Nucleofector, and electroporated with program D-032. Then, cells were seeded onto 6 cm dishes. Twenty-four hours after transfection, cells were stimulated with 100 ng/ml LPS for 30 min. The siRNA transfection was performed similarly. The mixture in the cuvette contained 120 pmol siRNA.

Production of anti-LPCAT2 and anti-phospho-LPCAT2 antibodies

Anti-LPCAT2 antiserum was generated at Immuno-Biological Laboratories (Gunma, Japan). The C-terminal peptide, SNKVSPESQEEGTSDKKVD was used to immunize rabbits. Anti-LPCAT2 antibody was purified from the anti-LPCAT2 antiserum using activated thiol Sepharose 4B binding to the LPCAT2 epitope. Anti-phospho-LPCAT2 antibody was generated by SCRUM (Tokyo, Japan) using a phospho-peptide, RQA(pS)FFPPP, at the N-terminus of LPCAT2.

Western blot analysis

Western blot analyses were performed as previously described (18). To detect the band shift, which represents phosphorylated protein, an SDS-PAGE gel containing 50 μM Phos-tag acrylamide with 100 μM Mn^{2+} was used.

Assay of lyso-PAF acetyltransferase and LPCAT

Lyso-PAF acetyltransferase and LPCAT assays were performed as previously described (8,9).

Quantitative real-time PCR

Total RNAs were prepared using the RNeasy Mini Kit (QIAGEN), and first strand cDNA was subsequently synthesized using Superscript III (Invitrogen). The PCRs

were performed using Fast Start DNA Master SYBR Green I (Roche Applied Science). The primers for MK2 designed to amplify a 185-bp fragment were (Forward: GGA TCT TCG ACA AGA GAA CCC AG. Reverse: GAG ACA CTC CAT GAC AAT CAG CA).

Software

All statistical calculations were performed using Prism 4 (GraphPad Software). Alignment of mammal LPCAT2 was performed using GENETYX-MAC Version 13.0.6 (GENETYX Corporation). Sequences of mouse (BAF47695), human (BAF47696), bovine (XP_592529), dog (XP_854080), and rat (XP_001064713) LPCAT2 are available in the DDBJ/EMBL/GenBank databases.

RESULTS

Phosphorylation of LPCAT2 by LPS-stimulation

To examine the different characteristics of the two lyso-PAFATs (LPCAT1 and LPCAT2), FLAG-tagged LPCAT1 and LPCAT2 were transiently transfected into the mouse macrophage cell line RAW264.7 using amaxa Nucleofector transfection kit V. Since RAW264.7 cells express TLR4 signaling molecules, cells were stimulated with LPS for 30 min and the lyso-PAFAT activity was examined using the supernatant at 9,000 × *g* for 10 min. The lyso-PAFAT activities of LPCAT1 and LPCAT2 were measured by radioisotope assays. While the LPCAT1 activity was unchanged after LPS-stimulation, the LPCAT2 activity was enhanced 4-fold compared to non-stimulated LPCAT2 (Fig. 1A). Lyso-PAFAT activity in the vector-transfected cells was slightly increased by LPS-stimulation, possibly due to the presence of endogenously expressed LPCAT2 in RAW264.7 cells.

The mechanism of LPCAT2 activation was investigated using Phos-tag acrylamide gel electrophoresis. Phos-tag makes a complex with two Mn^{2+} ions and acts as a phosphate-binding molecule (19). The complex is used for phosphate affinity SDS-PAGE, which results in the mobility shift of the phosphorylated proteins. A shifted band of FLAG-LPCAT2, but not FLAG-LPCAT1, was observed after

LPS-stimulation (Fig. 1B). The upper band may represent the phosphorylated form of LPCAT2. This result suggests that LPCAT2 is phosphorylated and activated by extracellular stimuli.

To identify the phosphorylated amino acid residue(s) of LPCAT2, RAW264.7 cells stably overexpressesing FLAG-LPCAT2 were established using Fugene HD in the presence of geneticin. The cells were stimulated with LPS for 30 min and the pellet at 100,000 × *g* for 1 h was analyzed by Phos-tag SDS-PAGE. The position corresponding to the shifted band in the Phos-tag Western blot was cut and subjected to in-gel trypsin digestion (20). After immobilized metal affinity chromatography (IMAC) enrichment of phosphopeptides (21), only one phospho-LPCAT2 peptide candidate (^{32}QApSFFPPPVPNPFVQQTTISASR^{54}) was detected by liquid chromatography-mass spectrometry (LTQ, Thermo Electron, San Jose, CA) (data not shown). Peptides containing unphosphorylated Ser34 were not detected in the phosphopeptide- enriched fraction. The flow through fraction of IMAC contained several other unphosphorylated peptides derived from LPCAT2. Although the Mascot score was 38, which is not significant, these results suggest that Ser34 of LPCAT2 is a candidate residue of the phosphorylation induced by LPS-stimulation. Ser34 of mouse LPCAT2 is well-conserved among mammals, such as human, bovine, dog, and rat (Fig. 1C).

Site-directed mutagenesis of LPCAT2

To confirm Ser34 as the target of phosphorylation, site-directed mutagenesis of LPCAT2 was performed. Ser34 was substituted for alanine (S34A) and aspartate (S34D). These constructs were transiently transfected into RAW264.7 cells using amaxa, and the cells were stimulated with LPS for 30 min. In the Phos-tag Western blot analysis using the M2 anti-FLAG antibody, a mobility shift was detected in wild-type (WT) LPCAT2, but not in the S34A or S34D mutant (Fig. 2A).

Next, we examined the effect of phosphorylation on the dual activities of LPCAT2 (lyso-PAFAT and LPCAT). Both activities of mutants were measured by

radioisotope assays. Lyso-PAFAT and LPCAT activities were enhanced in WT LPCAT2 with LPS-stimulation (Fig. 2B and C). The enzyme activity of S34A was similar to WT, but was not increased by LPS-stimulation. In contrast, S34D exhibited a higher enzyme activity than WT, but no further stimulation was observed (Fig. 2B). The expression level of each mutant was similar to that of WT (Fig. 2A). These results indicate that both lyso-PAFAT and LPCAT activities were enhanced by the Ser34 phosphorylation of LPCAT2.

Signaling pathway for LPCAT2 phosphorylation

To investigate the time course of LPCAT2 phosphorylation, thioglycolate-induced murine peritoneal macrophages were stimulated with LPS for varying times (0-120 min). Each microsomal protein (pellet at 100,000 × *g* for 1 h) was analyzed by Western blot using anti-LPCAT2 and anti-phospho-LPCAT2 antibodies. The amount of total LPCAT2 was nearly equal among the samples. The most intense phospho-LPCAT2 signal was detected at 15-30 min, and decreased as the incubation continued until 120 min (Fig. 3). This is consistent with lyso-PAFAT activation in our previous study (6). Similarly, MK2 phosphorylation reached a peak at 15-30 min. The consensus phosphorylation sequence (Hyd-X-R-X-X-S) of MK2 substrates (22) is conserved around Ser34 (VPRQAS) in LPCAT2 (Fig. 1C), where Hyd represents a hydrophobic residue and X represents any amino acid. These results suggest that LPCAT2 is one of the protein substrates of MK2. Murine MK2 has two splice variant proteins (23), and thus MK2 appeared at the positions of 45 and 55 kDa by the Western blot.

The signal transduction pathway for LPCAT2 phosphorylation was studied using several inhibitors of TLR4 signaling molecules: tumor growth factor-β activated kinase-1 (TAK1), p38 MAPK, and MK2 (see Fig. 6). RAW264.7 cells overexpressing FLAG-LPCAT2 were pretreated with each inhibitor for 1 h and stimulated with LPS for 30 min. Treatment with (5*Z*)-7-oxozeaenol (a TAK1 inhibitor) abolished the phosphorylation of p38 MAPK, MK2, and

LPCAT2 (Fig. 4A). SB203580 (a p38 MAPK inhibitor) also inhibited the phosphorylation of MK2 and LPCAT2, while the inactive analogue, SB202474, did not affect their phosphorylation (Fig. 4B). Pyrrolopiridine (MK2 inhibitor III) (24) treatment diminished the phosphorylation of LPCAT2 (Fig. 4C). Combined with the consensus sequence of the MK2 substrates, these data strongly suggested that LPS-induced phosphorylation of LPCAT2 is dependent on MK2, a downstream kinase of TAK1 and p38 (see Fig. 6).

Suppression of LPCAT2 phosphorylation by MK2-siRNA

The involvement of MK2 in LPCAT2 phosphorylation was further examined using the MK2 knockdown (KD) of RAW264.7 cells stably expressing LPCAT2. MK2-siRNA was transiently transfected into RAW264.7 cells by amaxa Nucleofector transfection kit V. After 48 h, the cells were treated with LPS for 30 min. The level of MK2-mRNA expression was decreased by 70-80% in MK2-siRNA-transfected cells (MK2-KD) compared to cells transfected with negative control (NC)-siRNA (Fig. 5A). The supernatant at 9,000 × *g* for 10 min was analyzed by Western blot using anti-MK2, anti-phospho-MK2, anti-LPCAT2, and anti-phospho-LPCAT2 antibodies. The amounts of total MK2 and phosphorylated MK2 were decreased in MK2-KD cells (Fig. 5B), consistent with their MK2-mRNA levels. Although the amount of total LPCAT2 was nearly equal in both MK2-KD and NC-cells, that of phospho-LPCAT2 was significantly diminished in MK2-KD cells.

We also performed enzymatic assays and examined the effect of MK2-siRNA on LPCAT2 activation. Both lyso-PAFAT and LPCAT activities were enhanced by LPS-stimulation in the NC-cells; however, both activations were abolished in MK2-KD cells (Fig. 5C and D). These results are consistent with the effect of the MK2-inhibitor on LPCAT2 phosphorylation (Fig. 4C), and thus indicate the MK2-dependent phosphorylation of LPCAT2.

DISCUSSION

Here, we present the activation mechanism of PAF biosynthetic enzyme by endotoxin-stimulation. In response to inflammatory stimuli, LPCAT2 was phosphorylated and activated in mouse peritoneal macrophages and RAW264.7 cells. Mass spectrometry and mutagenesis analyses identified Ser34 of LPCAT2 as the phosphorylation site to enhance the enzymatic activities. MK2 inhibitor and siRNA suppressed LPCAT2 phosphorylation, suggesting that LPCAT2 might be directly phosphorylated by MK2 to promote PAF and PC biosynthesis (Fig. 6).

In 1980, the lyso-PAFAT activity as the PAF biosynthetic enzyme was reported (3). Since then, several groups have attempted to characterize the enzyme. Lyso-PAFAT is rapidly activated in response to extracellular stimuli, such as calcium ionophore (4), acid stress (7), and LPS (16). However, neither the lyso-PAFAT cDNA sequence nor the mechanism of lyso-PAFAT activation had been elucidated. Recently, we identified two types of lyso-PAFATs: LPCAT2 that is an inducible lyso-PAFAT (9), and LPCAT1 that has constitutive lyso-PAFAT activity (8). LPCAT2 mRNA in macrophages is also upregulated by LPS-treatment for 16 h (9). The difference between LPCAT1 and LPCAT2 resembles that of cyclooxygenase-1 and -2 to produce prostaglandins (25,26). In mouse peritoneal macrophages, LPCAT2 is activated within 30 min by LPS-stimulation (Fig.3), consistent with the characteristics of endogenous lyso-PAFAT (6,9).

In this study, phosphorylated LPCAT2 was detected with the Phos-tag Western blot, by mobility shift (Fig. 1B and 2A). Through mass spectrometric analysis of the phosphorylated enzyme, Ser34 was identified as a phosphorylation site. Both the band shift and the activation were observed in WT LPCAT2, while the S34A mutant displayed neither characteristic (Fig. 2). Since mutagenesis at Ser34 did not abolish the basal activities, it is proposed that Ser34 is located in a regulatory region of LPCAT2. Moreover, the mutagenesis study indicated that Ser34 was the only target of the phosphorylation that led to the enzymatic activation of LPCAT2. Furthermore, Ser34 phosphorylation enhanced both the