Identification of a Novel Noninflammatory Biosynthetic Pathway of Platelet-activating Factor*§

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Platelet-activating factor (PAF) is a potent lipid mediator playing various inflammatory and physiological roles. PAF is biosynthesized through two independent pathways called the de novo and remodeling pathways. Lyso-PAF acetyltransferase (lyso-PAF AT) was believed to biosynthesize PAF under inflammatory conditions, through the remodeling pathway. The first isolated lyso-PAF AT (LysoPAFAT/LPCAT2) had consistent properties. However, we show in this study the finding of a second lyso-PAF AT working under noninflammatory conditions. We partially purified a Ca2+-independent lyso-PAF AT from mouse lung. Immunoreactivity for lysophosphatidylcholine acetyltransferase 1 (LPCAT1) was detected in the active fraction. Lpcat1-transfected Chinese hamster ovary cells exhibited both LPCAT and lyso-PAF AT activities. We confirmed that LPCAT1 transfers acetate from acetyl-CoA to lyso-PAF by the identification of an acetyl-CoA (and other acyl-CoAs) interacting site in LPCAT1. We further showed that LPCAT1 activity and expression are independent of inflammatory signals. Therefore, these results suggest the molecular diversity of lyso-PAF ATs is as follows: one (LysoPAFAT/LPCAT2) is inducible and activated by inflammatory stimulation, and the other (LPCAT1) is constitutively expressed. Each lyso-PAF AT biosynthesizes inflammatory and physiological amounts of PAF, depending on the cell type. These findings provide important knowledge for the understanding of the diverse pathological and physiological roles of PAF.

Phosphatidylcholine (PC)§ is the most abundant glycerophospholipid in eukaryotes and exerts various biological functions. PC is biosynthesized through a de novo pathway (1), and the sn-2 acyl group is subsequently modified through a remodeling pathway (2). In the remodeling pathway, PC is cleaved at its sn-2 position by phospholipase A2 (PLA2) (EC 3.1.1.4), generating lysophosphatidylcholine (LPC). Next, an acyl group is transferred to LPC by LPC acyltransferase (LPCAT) (EC 2.3.1.123), generating PC with various acyl compositions. Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is an ether analogue of PC and contains an acetyl group at its sn-2 position. PAF is a potent lipid mediator that has many inflammatory and noninflammatory roles (3–5). PAF has been implicated in many inflammatory diseases, such as thrombosis (6), multiple sclerosis (7), acute lung injury (8), asthma (9), and anaphylaxis (10). Under noninflammatory circumstances, PAF plays roles in several physiological processes such as glycogen degradation of fetal lung (11), fertility (12), and long term potentiation of neurons (13). Similarly to PC, PAF is biosynthesized by the de novo and remodeling pathways. In the remodeling pathway, alkyl-PC is cleaved at its sn-2 position by PLA2, generating lyso-PAF (alkyl-LPC). Among PLAs, cytosolic PLAs (cPLA2s) plays a major role in PAF production of inflammatory cells (14). PAF is then biosynthesized from lyso-PAF by lyso-PAF acetyltransferase (lyso-PAF AT) (EC 2.3.1.67) (15). It was long thought that PAF production occurs through the remodeling pathway under inflammatory conditions, whereas the de novo pathway produces physiological (noninflammatory) PAF (as summarized in Fig. 5E, upper panel) (4, 16).

It has been demonstrated that endogenous lyso-PAF AT activity is enhanced by several inflammatory stimuli, probably both by post-translational modifications and by mRNA induction (17). This activity was shown to be Ca2+-dependent (18, 19). We recently reported the cDNA cloning of a lyso-PAF AT, LysoPAFAT/LPCAT2. This enzyme has both lyso-PAF AT and LPCAT activities in the presence of Ca2+ (20). The lyso-PAF AT activity and the mRNA level of LysoPAFAT/LPCAT2 are enhanced by various stimuli, consistently with the endogenous lyso-PAF AT activity in inflammatory cells. However, it was unknown whether additional lyso-PAF AT(s) exist.

The gene with the highest homology to Lysopafat/LPCat2 is Lpcat1 (21). LPCAT1 is thought to be involved in the biosynthesis of PAF in eukaryotes. Therefore, the finding of a lyso-PAF AT working under noninflammatory conditions is significant for the understanding of the diverse biological and physiological roles of PAF.

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** The abbreviations used are: PC, phosphatidylcholine; PLA2, phospholipase A2; LPC, lysophosphatidylcholine; LPCAT, LPC acyltransferase; PAF, platelet-activating factor; cPLA2, cytosolic phospholipase A2; lyso-PAF AT, lyso-PAF acetyltransferase; COX, cyclooxygenase; LPS, lipopolysaccharides; APMFS, 4-aminophenylmethanesulfonyl fluoride; ODN, CpG oligonucleotide; poly(I:C), polyinosine-polycytidylic acid; CHO, Chinese hamster ovary; WT, wild type; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; ANOVA, analysis of variance.
thesis of pulmonary surfactant lipids based on several experimental observations as follows: substrate preference for medium-chain saturated acyl-CoAs to produce disaturated PC, high expression in alveolar type II cells, and a robust induction at the perinatal period (21, 22). Both LPCAT1 and LysoPAFAT/LPCAT2 are members of the lysosphospholipid acyltransferase family. The members of this family contain four conserved acyltransferase motifs (motifs 1–4) (23). Although several studies were performed based on site-directed mutagenesis using other lysosphospholipid acyltransferases, the precise role of each motif is not yet clear (23–25).

Here we show the finding of a novel Ca2+-independent lyso-PAF AT activity in the murine lung, mediated by LPCAT1. Depending on the substrate concentration, LPCAT1 recognizes a wide range of acyl-CoAs as substrates, ranging from acetyl-CoA to palmitoyl-CoA. We further characterized the nature of this substrate specificity by site-directed mutagenesis and identified several amino acid residues responsible for acyl-CoA (including acetyl-CoA) binding.

We investigated the role of LPCAT1 in PAF biosynthesis and showed that LPCAT1 is neither activated nor up-regulated at the mRNA level by inflammatory stimuli, in contrast to LysoPAFAT/LPCAT2. Thus, LPCAT1 may be involved in the noninflammatory PAF production. This relationship is similar to that of the two cyclooxygenases (COXs). COX-1 is constitutive and exerts protective functions, whereas COX-2 is induced in inflammation and plays many pathological roles (26). Our findings show the existence of a noninflammatory remodeling pathway involved in PAF biosynthesis. The enzymes involved in each remodeling pathway were expressed differently depending on the cell type. We propose that PAF is mainly biosynthesized by cPLA2 and LysoPAFAT/LPCAT2 in the inflammatory remodeling pathway, whereas PLA2s and LPCAT1 produce PAF in the noninflammatory remodeling pathway. Thus, the classical hypothesis needs some revision, and physiological levels of PAF may be produced not only through the de novo pathway but also through the noninflammatory remodeling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Acetyl-CoA, 1-[1-14C]palmitoyl-LPC, [1-14C]palmitoyl-CoA, a HiTrap DEAE-Sepharose Fast Flow column (5:50) (DEAE-Sepharose column), and ÄKTAExplorer 105 were obtained from GE Healthcare. Lyso-PAF was purchased from Cayman Chemical Co. (Ann Arbor, MI). 1-O-Alkenyl-LPC was from Doosan (Toronto, Canada). All species of 1-acyl-LPC and acyl-CoA ranging from butanoyl-CoA to arachidoyl-CoA were obtained from Avanti Polar Lipids (Alabaster, AL). Phosphatidylcholine (PC) from frozen egg yolk and lipopolysaccharides (LPS) from *Salmonella minnesota* were purchased from Sigma. BIGCHAP was from DOJINDO Laboratories (Kumamoto, Japan). QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA).

**Animals**—C57BL/6j mice and Sprague-Dawley rats were obtained from Clea Japan, Inc. (Tokyo, Japan). Mice were maintained in a light-dark cycle with light from 8:00 to 20:00 at 21 °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.

**Preparation of Microsomes from Mouse Tissues**—Mouse lungs, spleen, brain, liver, and kidney were homogenized with a Polytron homogenizer in 3× volume of cold buffer containing 100 mM Tris-HCl (pH 7.4), 300 mM sucrose, 5 mM 2-mercaptoethanol, 20 μM 4-amidinophenylmethanesulfonyl fluoride (APMSF), and a protease inhibitor mixture Complete (1×). After centrifugation for 10 min at 9,000 × g, the supernatant was collected and centrifuged for 1 h at 100,000 × g. The pellet was resuspended in a buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM sucrose, 5 mM 2-mercaptoethanol, 20 μM APMSF and 1× Complete.

**Partial Purification of Murine Lung Ca2+-independent Lyso-PAF AT**—The microsomal fraction of lung was solubilized with 0.5% BIGCHAP (w/v) for 40 min and centrifuged at 100,000 × g for 1 h. The supernatant of this centrifugation was designated solubilized microsomes. Solubilized microsomes were applied on a DEAE-Sepharose column equilibrated with Buffer A containing 20 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol, 20 μM APMSF, and 0.1% BIGCHAP using ÄKTAExplorer 105. Proteins were eluted by a 160–500 mM linear gradient of NaCl.

**Measurement of Lyso-PAF AT and LPCAT Activities**—Lyso-PAF AT and LPCAT activities were measured as described previously, using microplate chromatography or thin layer chromatography (20, 21, 27). For the measurement of lyso-PAF AT activity, the protein was incubated with 0–200 μM [3H]acetyl-CoA (1.11 GBq/mmol) and 20 μM lyso-PAF in a buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol, 2 mM CaCl2, 20 μM APMSF, 1× Complete, and 1 mg/ml PC. Eventually, EDTA, CoCl2, CuCl2, FeCl2, MgCl2, or MnCl2 was used instead of CaCl2 in some experiments. For the measurement of LPCAT activity, protein was incubated with 0–200 μM acyl-CoA and 50 μM LPC in a buffer containing 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mg/ml PC. As radiolabeled substrate, [14C]palmitoyl-CoA or [14C]palmitoyl-LPC was utilized.

**Production of Anti-LysoPAFAT/LPCAT2 and Anti-LPCAT1 Antiserum**—Antiserum were generated at Immuno-Biological Laboratories (Gunma, Japan). C-terminal peptides were used for immunization of rabbits (Lyso-PAFAT/LPCAT2, SNKVSPESEQEQTSDKKVD, and LPCAT1, EMYPDYAE-DYLPDQTHFDS). Specificity of the antiserum was examined by Western blot using microsomes from vector-, LysoPAFAT/Lpcat2-, or Lpcat1-transfected cells.

**Western Blot Analysis**—Western blot analyses were performed as described previously (21). Antiserum were used in a dilution factor of 1:1000.

**Site-directed Mutagenesis of LPCAT1**—Mutants of LPCAT1 were constructed using QuikChange site-directed mutagenesis kit or by overlap extension PCR (28). The primer sets utilized are listed in the supplemental material.

**Identification of PAF by PAF Receptor Binding Assay and Mass Spectrometry**—PAF receptor binding assay was done as described previously (14). Mass spectrometry was performed as described previously (20, 29) and as precisely described in supplemental Fig. 5.
Isolation and Stimulation of Mouse Peritoneal Cells—Mouse peritoneal macrophages induced by thioglycolate were prepared as described previously (17). The cells were treated with 100 ng/ml LPS, 0.8 μg/ml CpG oligonucleotide (ODN) 1826, or 1 μg/ml polyinosine-polycytidylic acid (poly(I:C)) for 16 h. Cells were then washed with ice-cold buffer containing 20 mM Tris-HCl (pH 7.4) and 300 mM sucrose. Total RNAs were then extracted and subsequently used for the synthesis of first-strand cDNA.

Isolation of Rat Alveolar Type II Cells and Macrophages—Alveolar type II cells were isolated by the method of Dobbs and Mason (30), as described previously (21). Alveolar macrophages for RNA extraction were recovered from bronchoalveolar lavage fluid of 7-week-old Sprague-Dawley rats. Rats were anesthetized and euthanized, and bronchoalveolar lavage fluid was obtained by four times lavage using phosphate-buffered saline. A part of the cells was cytocentrifuged with Cytospin 3 at 200 rpm for 2 min and stained with Diff-Quick confirming that >90% of the cells were macrophages.

Quantitative PCR—Quantitative PCR experiments were performed as described previously (21) using LightCycler 1.5 (Roche Diagnostics). The primers used are indicated in the supplemental material.

Software—All statistical calculations were performed using Prism 4 (GraphPad Software). Edmundson wheel analysis was performed using GENETYX-MAC version 13.0.6 (Genetyx Corp.).

RESULTS

Identification of a Ca2+-independent Lyso-PAF AT Activity—To characterize PAF biosynthesis in mice, we measured lyso-PAF acetyltransferase activities in lung, spleen, brain, liver, and kidney microsomes in the presence of EDTA or Ca2+. Consistently with the expression pattern of Lysopafat/Lpcat2 (20), high lyso-PAF AT activity was observed in spleen microsomes in the presence of Ca2+, which was inhibited by EDTA (Ca2+-dependent lyso-PAF AT activity) (Fig. 1A). The Ca2+-dependent lyso-PAF AT activity was weaker in brain and liver microsomes and not detectable in kidney microsomes. Surprisingly, EDTA did not inhibit lyso-PAF AT activity in lung microsomes, indicating the presence of a Ca2+-independent lyso-PAF AT (Fig. 1A).

Partial Purification of LPCAT1 as a Lyso-PAF AT—We next tried to identify the Ca2+-independent lyso-PAF AT by partial purification. After solubilization (with 0.5% (w/v) BIGCHAP), lung microsomes retained the Ca2+-independent lyso-PAF AT activity (Fig. 1C). Solubilized microsomes were applied on a DEAE-Sepharose column and fractionated (Fig. 1B). For each fraction, Ca2+-independent lyso-PAF AT activity was measured. The activity was detected from fractions 10 to 13 (Fig. 1C).
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We performed Western blot using anti-LysoPAFAT/LPCAT2 antiserum. Two major bands appeared in the spleen microsomes and the solubilized lung microsomes, the lower one having the size expected from the primary sequence of LysoPAFAT/LPCAT2 (60 kDa). However, the 60-kDa band was barely detected after fractionation (Fig. 1D). Thus, the proteins exhibiting immunoreactivity against LysoPAFAT/LPCAT2 were different from that with Ca\(^{2+}\)-independent lyso-PAF AT activity (Fig. 1, C and D). We performed similar experiments using anti-LPCAT1 antiserum, because LPCAT1 possesses the highest homology (48.2%) to LysoPAFAT/LPCAT2. The immunoreactivity was present in fractions 10–14, with similarity to the Ca\(^{2+}\)-independent lyso-PAF AT activity (Fig. 1, C and E), suggesting that LPCAT1 might be the Ca\(^{2+}\)-independent lyso-PAF AT in lung.

To obtain more direct evidence for the lyso-PAF AT activity in LPCAT1, microsomes were prepared from Lpcat1- or vector-transfected Chinese hamster ovary (CHO)-K1 cells, and lyso-PAF AT activity was measured. Lpcat1-transfected cells had a higher lyso-PAF AT activity than the control cells, indicating that LPCAT1 is the Ca\(^{2+}\)-independent lyso-PAF AT partially purified from murine lung (Fig. 2A).

Characterization of LPCAT1 as a Lyso-PAF AT—We characterized kinetic properties of LPCAT1. The apparent \(K_m\) values for acetyl-CoA and lyso-PAF were 82.4–128.2 and 7.9–18.4 \(\mu\)M, respectively (supplemental Fig. 1, A and B). Lyso-PAF AT activity of LPCAT1 was similar with or without Ca\(^{2+}\) (Fig. 2A). On the other hand, the activity of LysoPAFAT/LPCAT2 was detected in the presence of Ca\(^{2+}\) but not EDTA (Fig. 2A). The activity of LPCAT1 was not influenced by the presence of Co\(^{2+}\), Mg\(^{2+}\), or Mn\(^{2+}\), whereas Cu\(^{2+}\) and Fe\(^{3+}\) inhibited it. The activity of LysoPAFAT/LPCAT2 was detected in the presence of Co\(^{2+}\), Mg\(^{2+}\), Fe\(^{3+}\), or Mn\(^{2+}\) but not Cu\(^{2+}\) (Fig. 2A). The activity of LPCAT1 was unaltered by the presence of dithiothreitol, but the activity of LysoPAFAT/LPCAT2 was 2-fold increased (supplemental Fig. 1C). The pH optimum for LPCAT1 activity was around pH 7.5 (supplemental Fig. 1D).

We next investigated the lyso-phospholipid preference of LPCAT1. LPCAT1 showed high activity toward alkyl-LPC (C16), acyl-LPC (C16), and alkenyl-LPC (mixture). The activity was lower for acyl-LPC (C18) and not significant for alkyl-LPC (C18) (Fig. 2B). Thus, LPCAT1 can synthesize PAF (C16) much more efficiently than PAF (C18).

Acetyltransferase activity of LPCAT1 had not been reported previously, probably because of the concentration of acetyl-CoA used (21, 22). Thus, we measured acetyltransferase activities of LPCAT1 with two different concentrations (10 and 200 \(\mu\)M) of various acyl-CoAs (C2 to C20) as acetyl donors. At 10 \(\mu\)M acetyl-CoA, LPCAT1 utilized all saturated acyl-CoAs ranging from hexanoyl-CoA (C6) to palmitoyl-CoA (C16) as substrates, and the maximal activity was observed for decanoyl-CoA (C10) (Fig. 2C). On the other hand, at 200 \(\mu\)M acetyl-CoA, the substrate preference of LPCAT1 shifted to shorter chain acyl-CoAs (Fig. 2D). Similarly to our previous report, LPCAT1 showed no acetyltransferase activity at the substrate concentration of 10 \(\mu\)M (Fig. 2C). However, we could detect the activity for acetyl-CoA (C2) at 200 \(\mu\)M, as expected from the result shown in supplemental Fig. 1A. Activity for butanoyl-CoA (C4) was also seen, but it was not significantly different from that in vector-transfected cells. Thus, LPCAT1 recognizes a wide range of acyl-CoA.
of acyl-CoAs (from C2 to C16) and exerts acyltransferase activity at a high concentration of acetyl-CoA.

Analysis of Conserved Motifs in LPCAT1—To prove that the wide range of acyltransferase activity seen in Lpcat1-transfected cells is directly exerted by LPCAT1, we tried to characterize the recognition sites of acyl-CoAs in LPCAT1. Because acyl-CoA recognition may be a common characteristic of acyltransferases, we hypothesized that some of the acyltransferase motifs may play roles in acyl-CoA recognition. We compared the sequences of the following lysophospholipid acyltransferases: glycerol-3-phosphate acyltransferase 1, lysophosphatidic acid acyltransferase α/β (LPAATα and LPAATβ), lysophosphatidylglycerol acyltransferase 1 (LPGAT1), and lysocardiolipin acyltransferase 1 (ALCAT1) were compared for searching consensus sequences of motifs 1–4. The localization of putative motifs in LPCAT1 is illustrated in the lower panel. B–E, mutants of each motif were constructed, and lyso-PAF AT (white bars) and LPCAT (black bars) activities were measured. The results are illustrated as the relative activity compared with WT (remaining activity). Background activity was measured from vector-transfected cells and subtracted. C, mutants G164A, F174A, and R177A show different remaining lyso-PAF AT and LPCAT activities. ND, not detectable. Error bars are S.E. of three independent experiments, each performed in triplicate.

We next investigated the effects of the deletion of each motif on the activity of LPCAT1. Each motif region (from His135 to Asp140, from Gly164 to Arg177, from Ile203 to Gly209, and from Pro227 to Pro233) was deleted from LPCAT1, generating mutants del1, del2, del3, and del4. Each mutant was transfected into CHO-K1 cells, and the expression was examined by Western blot. Each mutant was expressed but was weaker than wild type LPCAT1 (WT) (supplemental Fig. 2A). Lyso-PAF AT and LPCAT activities were then measured by thin layer chromatography analyses. Lyso-PAF AT and LPCAT activities were abolished in these deletion mutants (supplemental Fig. 2B).

We then examined the effect of point mutations in each motif. We generated mutants H135A and D140A in motif 1; G164A, R168A, R171A, F174A, and R177A in motif 2; E208A and G209A in motif 3; and P227A, P230A, and P233A in motif 4. Each mutant was transfected into CHO-K1 cells, and the expression was detected by Western blot. The expression levels of D140A, R171A, F174A, E208A, and P233A decreased, whereas those of other mutants were similar to that of WT (supplemental Fig. 2, C–F). For each mutant, we measured lyso-PAF AT (1-hexadecyl-lyso-PAF and acetyl-CoA as substrates) and LPCAT (1-myristoyl-LPC and palmitoyl-CoA as substrates) activities relative to WT (hereafter, we will refer these relative activities as “remaining activity”). H135A, D140A, and E208A had no remaining activity, whereas R168A, R171A, G209A, P227A, P230A, and P233A had partially remaining lyso-PAF AT and LPCAT activities (Fig. 3, B–E). Notably, in G164A, lyso-PAF AT activity was slightly increased (~150%), but LPCAT activity was similar to WT (~100%). F174A and R177A had no detectable lyso-PAF AT activity (<10%), whereas LPCAT activity was only partially diminished (~40%) (Fig. 3C).

Analysis of Motif 2 in LPCAT1—We investigated further lyso-PAF AT and LPCAT activities in F174A and R177A. The relative lyso-PAF AT and LPCAT activities were not affected by reaction buffers, lysophospholipid species (lyso-PAF and LPC), or acyl-chain length at the sn-1 position of LPC (C14 and C16) (data not shown). However, the relative
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FIGURE 4. Analysis of motif 2 in LPCAT1. A, remaining LPCAT activities of mutants F174A (black circles) and R177A (white circles) were measured, using various acyl-COAs as acyl donors. Remaining activities were higher when using longer acyl-COAs as substrates. Results of two independent experiments were plotted. B, amino acids around motif 2 are highly hydrophobic. When illustrated in an Edmundson wheel representation, hydrophobic residues are located on one side. The primary structure of residues 160–177 is illustrated with hydrophobic residues in boldface. The underlined region was used for Edmundson wheel analysis. C, mutants of each hydrophobic residue were constructed, and remaining LPCAT activities using acetyl-CoA (white bars) and palmitoyl-CoA (black bars) as acyl donors were measured. Mutant I160A had LPCAT activity for acetyl-CoA similar to WT, but that for palmitoyl-CoA was decreased. Error bars are S.E. of three independent experiments, each performed in triplicate. D, remaining LPCAT activity of mutant I160A was measured, using various acyl-COAs (C2–C16) as acyl donors. Remaining LPCAT activity was decreased for acyl-COAs ranging from butanoyl-CoA (C4) to palmitoyl-CoA (C16), but it was less affected for longer acyl-CoAs. Results of two independent experiments were plotted. E, proposed model explaining interaction between LPCAT1 and acyl-CoA. Ile160 interacts with the third or fourth carbon of acyl-CoA. Other hydrophobic residues of motif 2 are in an α-helix configuration and interact with carbons near CoA. Phe174 and Val175 interact with the first or second carbon of acyl-CoA, and Arg177 may interact with CoA (43).

activity for acetyl-CoA was different from that for palmitoyl-CoA (Fig. 3C and Fig. 4A).

We measured LPCAT activities of F174A and R177A for various chain length acyl-CoAs (C2 to C16). The substrate concentrations were 200 μM for short acyl-CoAs (C2 to C8), and 10 μM for medium-chain acyl-CoAs (C10 to C16). The remaining LPCAT activities in F174A and R177A were higher for longer acyl-CoAs as substrates (Fig. 4A). We confirmed that such relationships were found in several concentrations of acyl-CoAs (supplemental Fig. 3, A–C). Thus, the preference of F174A and R177A for longer chain acyl-CoA was not because of substrate concentration but because of acyl chain length. These results suggest that mutations in motif 2 modulate acyl-CoA selectivity.

Motif 2 of LPCAT1 contains many hydrophobic amino acids conserved among species, thus these residues may interact with acyl-CoA (Fig. 4B and supplemental Fig. 4A). The hydrophobic region (from Trp162 to Tyr169) was predicted to form an α-helix using the PredictProtein Server (31). When this hydrophobic region (in this example, Ile160 to Arg177) is illustrated by an Edmundson wheel representation (32), all hydrophobic residues are located at one side (Fig. 4B). This suggests that this region forms an α-helix, and the hydrophobic residues are located favorably to interact with the acyl chain. To investigate their roles in acyl-CoA interaction, we constructed mutants of each hydrophobic residue (I160A, I162A, W163A, L166A, I167A, Y169A, I170A, V173A, and V175A) and transfected them into CHO-K1 cells. The expression of each mutant was detected by Western blot. The expression levels of I162A, V173A, and F174A (as described previously) were reduced compared with WT, but those of other mutants were not changed (supplemental Fig. 4B). We compared the remaining LPCAT activity in these mutants using acetyl-CoA or palmitoyl-CoA as acyl donors. The remaining activities in I162A, W163A, L166A, and V173A were similarly changed compared with WT for both substrates. Both activities were also slightly but reproducibly decreased in I167A, Y169A, and I170A (Fig. 4C). In particular, I160A retained fully its activity for acetyl-CoA, but the activity for palmitoyl-CoA prominently decreased (Fig. 4C). In contrast, V175A had no activity for acetyl-CoA but partially remaining activity for palmitoyl-CoA (Fig. 4C), similarly to F174A (Fig. 4, A and C) and R177A (Fig. 4A). We further investigated the substrate specificity of I160A by measuring LPCAT activity for various chain length acyl-CoAs (C2 to C16). The activity for acetyl-CoA was similar to WT, but those for longer acyl-CoAs than butanoyl-CoA decreased. The mutation mostly affected the activity for butanoyl-CoA (C4) and hexanoyl-CoA (C6) and less for longer chain acyl-CoAs (Fig. 4D). These results led us to conclude that some of the above described amino acids play roles in the interaction with acyl-CoA (including acetyl-CoA), as will be explained further under “Discussion.”

LPCAT1 Is a Cell Type-specific, Noninflammatory PAF Biosynthetic Enzyme— The identification of an acetyl-CoA interacting site confirmed direct PAF biosynthesis by LPCAT1. Additionally, the product synthesized from lyso-PAF and acetyl-CoA by LPCAT1 was indeed PAF, as determined by PAF receptor binding assay and by mass spectrometry (supplemental Fig. 5, A–C).

PAF is a potent inducer of inflammation, and lyso-PAF AT activity is increased by various inflammatory stimuli, through mRNA up-regulation and post-translational modification (17, 20). Therefore, we first examined whether
LPCAT1 is activated by LPS. LPS is an agonist of Toll-like receptor (TLR) 4, which is expressed in RAW264.7 cells (33, 34). RAW264.7 cells were transiently transfected with Lpecat1 and stimulated by LPS. However, LPS stimulation had no effect on lyso-PAF AT activity associated with LPCAT1 (supplemental Fig. 6A), in contrast to LysoPAFAT/LPCAT2, which was activated under the same conditions. We next examined whether agonists of TLRs up-regulate mRNA levels of Lpcat1 in thioglycolate-elicited peritoneal macrophages. The macrophages were stimulated by poly(i:C), LPS, or ODN1826. Poly(i:C) and ODN1826 are agonists of TLR3 and TLR9, respectively (33), and LPS and ODN1826 are known to up-regulate Lysopafat/Lpcat2 mRNA (20). However, neither agonist up-regulated Lpecat1 mRNA (supplemental Fig. 6, B and C). Thus, LPCAT1 is a lyso-PAF AT but biosynthesizes PAF under noninflammatory conditions, showing a sharp contrast with LysoPAFAT/LPCAT2 (20).

Our results show the presence of inflammatory and noninflammatory remodeling pathways involved in PAF biosynthesis, which can be distinguished by Ca\(^{2+}\) dependence. We hypothesized that Ca\(^{2+}\)-dependent and -independent lyso-PAF AT activities are cell type-specific and have different biological roles. We examined lyso-PAF AT activity in lung, alveolar type II cells, and alveolar macrophages of rat. Lyso-PAF AT activity was mainly Ca\(^{2+}\)-independent in alveolar type II cells, whereas it was Ca\(^{2+}\)-dependent in alveolar macrophages (Fig. 5A). We performed Western blot analysis using anti-LPCAT1 antiserum, and consistently with the Ca\(^{2+}\)-independent lyso-PAF AT activity, immunoreactivity for LPCAT1 was high in alveolar type II cells and not detected in alveolar macrophages (Fig. 5B). We performed quantitative PCR to investigate the genes involved in cell type-specific remodeling pathways for PAF biosynthesis, using cDNAs prepared from lung, alveolar type II cells, and alveolar macrophages. Lpecat1 mRNA was strongly expressed in alveolar type II cells, whereas Lysopafat/Lpcat2 mRNA was found in alveolar macrophages (Fig. 5C), consistently with the Ca\(^{2+}\) dependence of lyso-PAF AT activities. Because cPLA\(_2\) is involved in inflammatory PAF production (14), we investigated whether it also provides lyso-PAF in noninflammatory PAF biosynthesis. The relative mRNA level of cplas\(_{2\alpha}\) in alveolar macrophages compared with lung was high, at a similar extent to Lysopafat/Lpcat2. The enrichment of cplas\(_{2\alpha}\) in alveolar type II cells was modest compared with that of Lpecat1 (Fig. 5D). Thus, although cPLA\(_2\) is the major phospholipase involved in the inflammatory PAF remodeling pathway, other phospholipases may also contribute to the noninflammatory one.

**DISCUSSION**

Identification and Characterization of LPCAT1 as a Ca\(^{2+}\)-independent Lyso-PAF AT—In the literature, lyso-PAF AT activity has long been characterized as Ca\(^{2+}\)-dependent, because EDTA inhibits this activity (18, 19). We recently reported the cloning of a lyso-PAF AT, LysoPAFAT/LPCAT2 (20). Its activity was Ca\(^{2+}\)-dependent and inhibited by the addition of EDTA, consistently with previous studies of intrinsic lyso-PAF AT activity. In this study, we showed a Ca\(^{2+}\)-independent lyso-PAF AT activity in murine lung.

We solubilized and partially purified the Ca\(^{2+}\)-independent lyso-PAF AT. By Western blot analyses, the activity seemed to be derived from LPCAT1 present in the lung (Fig. 1, A–E). Indeed, Lpecat1-transfected cells showed higher Ca\(^{2+}\)-independent lyso-PAF AT activity than control cells. High concentration of acetyl-CoA was needed to detect lyso-PAF AT activity of LPCAT1. As it is reported that the concentration of acetyl-CoA can exceed 100 \(\mu\)m in tissues (35), LPCAT1 might produce enough amount of PAF under physiological conditions.

LPCAT1 activity was Ca\(^{2+}\)-independent and not increased by dithiothreitol, in contrast to that of LysoPAFAT/LPCAT2 (Fig. 2A and supplemental Fig. 1C). Both LPCAT1 and LysoPAFAT/LPCAT2 possess putative EF-hand motifs, similarly to many Ca\(^{2+}\)-binding proteins (20, 21, 36). Further studies are needed to analyze the differences in the structure and function of the “EF-hand motifs” in these two lyso-PAF ATs to explain the different Ca\(^{2+}\) dependence.

Motif 2 Is an Acyl-CoA-binding Site in LPCAT1—The idea that a single enzyme synthesizes both PAF and PC is controversial, because many enzymes have more restricted substrate specificity. Thus, before arguing the biological roles of LPCAT1, we had to exclude the possibility that LPCAT1 indirectly stimulates the acyltransferase activity. Therefore, we tried to obtain evidence for the acyl-CoA (including acetyl-CoA) binding of LPCAT1 by site-directed mutagenesis based on two hypotheses. First, because acyl-CoA binding is a common feature of acyltransferases, some of the conserved motifs might be involved in acyl-CoA binding. Second, mutations in the acyl-CoA-binding site should modulate the substrate specificity for acyl-CoA.

Mutants F174A and R177A showed no lyso-PAF AT activity and partially reduced LPCAT activity (Fig. 3C). We found that both mutants had higher remaining activity for longer chain acyl-CoAs (Fig. 4A). We explain this by the following model: Phe\(^{174}\) and Arg\(^{177}\) of motif 2 are localized in the acyl-CoA binding site, in a position that can interact with both acetyl-CoA and longer chain acyl-CoAs. The acyl-CoA-binding site is a deep pocket with many residues that interact with acyl-CoA. A longer acyl-CoA has more interacting residues, because it has more hydrocarbons. The mutation in Phe\(^{174}\) or Arg\(^{177}\) leads to a decreased interaction with acyl-CoA, but because longer acyl-CoA has more interacting partners, the loss of one interacting residue has less effect on its activity. As a consequence, F174A and R177A retain relatively high activity for longer acyl-CoAs (Fig. 4E).

We also constructed mutants of each hydrophobic residue in motif 2 and measured their acyltransferase activities. 1160A had acyltransferase activity similar to WT, whereas acyltransferase activity for butanoyl-CoA and longer chain acyl-CoAs decreased (Fig. 4, C and D). The activities were better retained for longer chain acyl-CoA, except for acetyl-CoA. The reason for the increased activity for longer acyl-CoA can be explained similarly to F174A and R177A. Because acyltransferase activity was not altered, we conclude that Ile\(^{160}\) interacts with the third or fourth carbon of acyl-CoA.

Noninflammatory PAF Biosynthesis by LPCAT1
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A

Lys-PAF Acetyltransferase Activity (pmol/min/mg)

E

"Classical" Hypothesis

non-inflammatory
PAF biosynthesis

inflammatory
PAF biosynthesis

E

Reviewed Hypothesis

non-inflammatory
PAF biosynthesis

inflammatory
PAF biosynthesis

E

Lung
Alveolar Macrophages

E

Relative mRNA Level

E

Lung
Alveolar Type II Cells

E

Relative mRNA Level

E

Lung
Alveolar Type II Cells
the acyl chain. V175A had similar activity to F174A and R177A, and thus we think that Val175 is located between Phe174 and Arg177 in the substrate-binding pocket and also plays a role in acyl-CoA binding.

We summarized these observations as a model shown in Fig. 4E. We identified important residues for acyl-CoA binding, although more residues interacting with long-chain acyl-CoAs seem to exist (illustrated as X in the model shown in Fig. 4E). As far as we know, this is the first study showing direct evidence that motif 2 interacts with acyl-CoA. This is also the first identification of acetyl-CoA-binding site in a lyso-PAF AT. The three-dimensional structure of LPCAT1 will clarify the mechanism of interaction more precisely in the future.

**LPCAT1 Is a Noninflammatory Lyso-PAF AT**—The finding of a region interacting with acetyl-CoA and longer acetyl-CoAs confirmed a direct role of LPCAT1 in the biosynthesis of both PAF and PC. Therefore, we next examined the biological roles of LPCAT1 through PAF production.

PAF is well characterized as an inflammatory mediator and is thought to be biosynthesized through the remodeling pathway by lyso-PAF AT during inflammation (4, 16). Indeed, LysoPAFAT/LPCAT2 first isolated as a lyso-PAF AT was both activated and up-regulated by inflammatory stimuli (20). However, in similar experiments, LPCAT1 was neither activated nor up-regulated (supplemental Fig. 6, A and B). Thus LPCAT1 is a constitutive lyso-PAF AT, providing a novel pathway involved in PAF biosynthesis under the physiological state: the Ca2+-independent, noninflammatory PAF remodeling pathway (Fig. 5E).

We investigated whether both remodeling pathways exist in the same or in different cells. In alveolar type II cells and alveolar macrophages, lyso-PAF AT activities were Ca2+-independent and Ca2+-dependent, respectively, consistently with mRNA distribution of Lpcat1 and Lysopafat/Lpcat2 (Fig. 5, A–C). Furthermore, the relative mRNA level of cplα,α, which has been implicated in inflammatory PAF production, was high in alveolar macrophages but existed at a basal level in alveolar type II cells, compared with whole lung (Fig. 5, B and C). Thus, the inflammatory PAF remodeling pathway consists of cplα,α and LysoPAFAT/LPCAT2 in macrophages, and the noninflammatory PAF remodeling pathway consists of other PLA2s (in addition to cplα,α) and LPCAT1 in alveolar type II cells. Thus, these pathways are cell type-specific and have different roles (inflammatory or physiological). We summarized this hypothesis in Fig. 5E.

**FIGURE 5. Different PAF biosynthetic pathways in different cells.** A, lyso-PAF AT activities of lung, alveolar type II cells, and alveolar macrophages were measured in the presence of EDTA (white bars) or Ca2+ (black bars). Lyso-PAF AT activities were Ca2+-independent and Ca2+-dependent in alveolar type II cells and alveolar macrophages, respectively. Error bars are S.D. The figure shows one representative result of two independent experiments with similar results. B, Western blot analysis was performed using anti-LPCAT1 antiseraum. The expression of LPCAT1 was detected in lung and highly in alveolar type II cells. No signal was detected in alveolar macrophages. Each lane contains 4.5 μg of microsomal proteins. C and D, expression levels of lpcat1, Lysopafat/lpcat2, cplα,α, and β-actin were measured by quantitative PCR in lung (circles), C, alveolar macrophages (squares), and D, alveolar type II cells (triangles). Relative values compared with lung are illustrated. The results of two independent experiments are shown, and values under the detection range are illustrated as open symbols. C, relative amounts compared with lung of Lysopafat/lpcat2 and cplα,α were similar in alveolar macrophages but not Lpcat1. D, Lpcat1 is highly expressed in alveolar type II cells but not Lysopafat/lpcat2. The relative amount compared with lung of cplα,α was not as high as Lpcat1. E, upper panel, the classical hypothesis was that PAF is produced through the de novo pathway under noninflammatory conditions, whereas it is biosynthesized through the remodeling pathway under inflammatory conditions. Lower panel, we found a novel cell type-specific PAF synthetic pathway, which is mediated by some PLA2s and LPCAT1. Although this pathway is a remodeling pathway, it is noninflammatory. Thus we named it the noninflammatory remodeling pathway (indicated by an arrowhead).
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enable us to better understand the roles of PAF in physiological conditions.

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