An accurate and versatile method for determining the acyl group-introducing position of lysophospholipid acyltransferases

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Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Foetal bovine serum (FBS) and Opti-MEM were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Honey bee venom PLA2, lipase from *Rhizomucor miehei* which has intrinsic PLA1 activity and palmitoyl- [$^{13}C_{16}$] coenzyme A were obtained from Sigma-Aldrich (St. Louis, MO). All glycerophospholipids and lysophospholipids including palmitoyl-d62 PC (1,2-dipalmitoyl-d62-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids (Alabaster, AL). LC-MS grade methanol and acetonitrile were purchased from Kanto Chemical (Tokyo, Japan). Chloroform, formic acid and ammonium formate were purchased from Fujifilm-Wako (Osaka, Japan).

Preparation of sn-1-acyl dominant LPLs and sn-2-acyl dominant LPLs

To obtain palmitoyl lysophosphatidylcholine (LPC) with palmitic acid at the sn-2 position of the glycerol backbone (sn-2-palmitoyl LPC), dipalmitoyl PC (DPPC) was subjected to an in vitro PLA1 reaction using the method by M. Okudaira et al. with some modifications. Non-labelled or deuterium-labelled (d62) DPPC (1,2-dipalmitoyl-d62-sn-glycero-3-phosphocholine) (1 µmol each) was incubated with reaction mixture containing 78 mM phosphate, 10% diethyl ether, 2 mM CaCl2, 0.05% TritonX-100 and 4.5 µg/ml *Rhizomucor miehei* lipase (pH 7.4). The mixture was incubated at 37 °C for 80 min. Then the reaction was stopped by adding 9 volumes of acidic methanol (pH 4.0). After centrifugation (21,500 ×g, 5 min), the supernatant was applied to a Bond Elut C18 solid phase cartridge column (Agilent Technologies) to remove the remaining DPPC and free palmitic acid, the by-product of the PLA1 reaction. The extract was dried by evaporation and dissolved in acidic methanol. The sn-1/sn-2 ratio and the concentration of sn-2-palmitoyl LPC preparation were determined by LC-MS/MS. The sn-2-palmitoyl LPC preparation predominantly contained LPC with palmitic acid at the sn-2 position (>95%) and the level of remaining PC was very low (<0.001% of LPC). The preparation, which we called “stock sn-2-palmitoyl dominant LPC”, was stored at −80 °C. The sn-1 LPC isomer was prepared from the sn-2 LPC isomer by using the spontaneous acyl-migration reaction. Since the acyl-migration reaction is dependent on the pH of the solution and the incubation time, the “stock sn-2-palmitoyl dominant LPC” was dissolved in weak alkaline solution (100 mM Tis-HCl pH 8.9). To prepare the sn-2 LPC isomer, the reaction was stopped immediately by adding acidic methanol. To prepare the sn-1 LPC isomer, the reaction mixture was incubated for 2 h at 37 °C before stopping the reaction. The new preparations were called “sn-2-palmitoyl dominant LPC” and “sn-1-palmitoyl dominant LPC”, respectively. We confirmed that the prepared “sn-1-palmitoyl dominant LPC” and “sn-2-palmitoyl dominant LPC” predominantly contained LPC with palmitic acid at the sn-1 position (>90%) and sn-2 position (>90%), respectively (**Fig. 1**). We also prepared sn-1and sn-2-oleoyl dominant LPG and sn-1and sn-2-stearoyl dominant LPC using the same procedure as stated above, except that dioleoyl PG (DOPG) and distearoyl PC (DSPC) were used instead of DPPC.

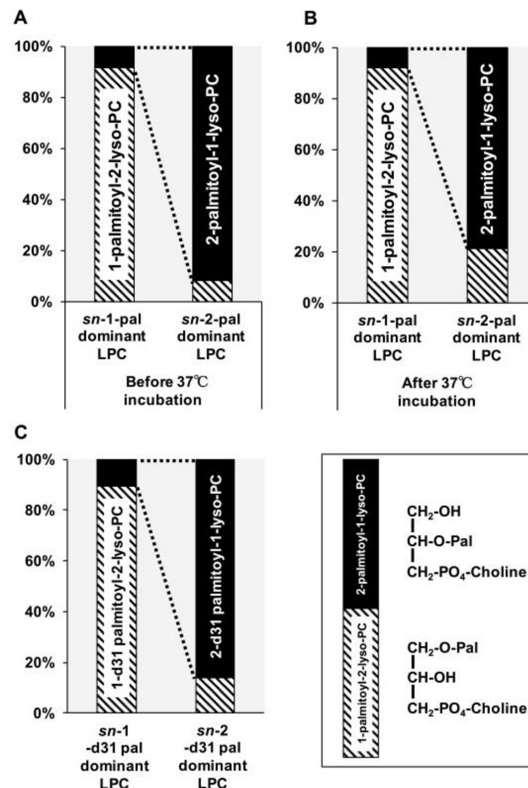

Fig. 1. Preparation of sn-1- and sn-2-palmitoyl dominant LPCs. (A–C) Ratio of 2-palmitoyl-1-lyso-PC (sn-2-palmitoyl LPC: filled) and 1-palmitoyl-2-lyso-PC (sn-1-palmitoyl LPC: dashed) in the present LPC preparations used for LPCAT1 assay. "sn-2-pal (palmitoyl) dominant LPC" was prepared from DPPC by *Rhizomucor miehei* lipase (PLA1) and "sn-1-pal (palmitoyl) dominant LPC" was prepared from "sn-2-pal (palmitoyl) dominant LPC" by using a spontaneous acyl-migration reaction. (A and B) Ratio of sn-1 and sn-2-palmitoyl LPC before (A) and after (B) 37 °C 10 min incubation. (C) Ratio of sn-1 and sn-2-palmitoyl LPC preparations in the deuterium-labelled palmitoyl LPC. The data are representative of three independent experiments with similar results.

LC-MS/MS analyses

The LC-MS/MS system consisted of an UltiMate 3000 HPLC system and TSQ Quantiva Triple-Stage Quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with a heated-electrospray ionization-II (HESI-II) source. For HPLC, we used a reverse-phase column (Capcell Pak C18 ACR (250 mm × 1.5 mm,3 µm particle size; Osaka Soda) with a gradient elution of solvent A (5 mM ammonium formate in water, pH 4.0) and solvent B (5 mM ammonium formate in 95% (v/v) acetonitrile, pH 4.0) at 200 µl/min. This method enabled us to separate 1-acyl-2-lyso-PL (sn-1-acyl LPL) and 2-acyl-1-lyso-PL (sn-2-acyl LPL).

Diacyl-phospholipids (PLs) including PC and PG were quantified by a similar method with slight modifications. The LC-MS/MS system consisted of a NANOSPACE SI-II HPLC system (Osaka Soda) and TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with a heated-electrospray ionization-II (HESI-II) source. LC separation was performed using a reverse-phase column (Capcell Pak C8 UG120, 150 mm × 1.5 mm, 5 µm particle size; Osaka soda) with a gradient elution of solvent A and solvent B (described above) at 400 µl/min.

LPC and PC were detected by selected reaction monitoring (SRM) in positive ion mode. At MS1, the m/z values of [M + H]+ ions for non-labelled, [^{13}C]-labelled, deuterium-labelled palmitoyl LPC and stearoyl LPC are 496.3, 512.3, 526.3 and 524.4 respectively. The m/z values for non-labelled DPPC, DPPC with a non-labelled palmitoyl and a [^{13}C]-labelled palmitoyl ([$^{13}C_{16}$] DPPC), and DPPC with a [^{13}C]-labelled palmitoyl and deuterium-labelled palmitoyl ([$^{13}C_{16}$]-[d31] DPPC), and 38:4 (stearoyl-arachidonoyl) PC are 734.6, 750.6, 780.6 and 810.6 respectively. At MS3, phosphocholine fragments (m/z = 184.1) were detected. To measure phosphatidylglycerol (PG), at MS1 the m/z values of [M + NH4]+ ion for 36:1 (stearoyl-oleoyl) PG is 794.6. At MS3, corresponding diacylglycerol fragment (m/z = 605.5) was detected in positive ion mode. For measurement of

lysophosphatidylglycerol (LPG), at MS1 the m/z values of [M-H]− ion for oleoyl LPG is 509.3. At MS3, corresponding fatty acid fragment (m/z = 281.2) was detected in negative ion mode.

To confirm incorporated fatty acid composition of the assay reactants, the product ions were scanned in negative mode to detect [M + HCOO]− ions at MS1.

Plasmids and vectors

cDNA for human LPCAT1 (hLPCAT1; NCBI accession number NM_024830) was amplified by PCR using PrimeSTAR HS Polymerase (TAKARA BIO Inc.) and HEK293A cell cDNA as a template. The PCR primers used were as follows.

Forward 5′-CGCCACCATGAGGCTGCGGGGATGCGG-3′

Reverse 5′-GAGCTCGAGCTAATCCAGCTTCTTGCGAAC-3′

The amplified cDNA fragments were inserted into the pCAGGS vector (a gift from J. Miyazaki, Osaka University) with or without an N-terminal FLAG-tag. The nucleotide sequences of all the plasmids prepared were checked by DNA sequencing. Mouse LPCAT1, LYCAT and LPCAT3 clone in pCXN2.1 vector were previously described by Okudaira et al.

Cell culture and transfection of plasmids

HEK293A cells (Thermo Fisher Scientific) were maintained in DMEM supplemented with 10% FBS, 2 mM l-Glutamine (Thermo Fisher Scientific), 100 U/ml penicillin (Sigma-Aldrich), and 100 µg/ml streptomycin (Thermo Fisher Scientific) at 37 °C in the presence of 5% CO2 gas. HEK293A cells were transfected with cDNAs encoding several LPLATs using Opti-MEM and Lipofectamine 2000 (Thermo Fisher Scientific).

Preparation of membrane fractions

HEK293A cells were harvested and transferred to ice-cold TSC buffer (20 mM Tris-HCl, pH 7.4, 300 mM sucrose and cOmplete™ Protease Inhibitor Cocktail (Roche, Mannheim, Germany)), and were sonicated ten times for 10 s using a probe-type sonicator (MICROTEC). The cell homogenates were centrifuged at 800 ×g for 10 min, and the supernatants were further centrifuged at 100,000 ×g for 1 h. The resultant pellets were re-suspended in TSE buffer (20 mM Tris-HCl, pH 7.4, 300 mM sucrose and 1 mM EDTA). Protein concentrations were determined with a BCA protein assay kit (Thermo Fisher Scientific). The membrane fractions were used as an enzyme source.

LPL acyltransferase assays

LPC acyltransferase (LPCAT) activities that introduce palmitic acid into palmitoyl LPC were determined by measuring the product of the LPCAT reaction, i.e. DPPC with a slight modification of T. Harayama et al. In the LPCAT reaction, membrane fractions (containing LPCAT1 recombinant protein), LPC ("sn-1-palmitoyl dominant LPC" or "sn-2-palmitoyl dominant LPC") and [$^{13}C_{16}$] palmitoyl-CoA were mixed and incubated at 37 °C for 10 min. The final reaction mixture contained 110 mM Tris-HCl (pH 7.4), 150 mM sucrose, 0.5 mM EDTA, 10 µM LPC, 2 µM Acyl-CoA, 0.015% Tween-20 and 0.05 µg of membrane protein. The reaction was stopped by adding chloroform/methanol (1:2). Dilauryl-PC (DLPC) or dilauryl-PG (DLPG) was added as an internal standard and lipids were extracted by the Bligh & Dyer method. The organic (lower) layer was evaporated, dissolved in methanol, and the amounts of DPPC containing [$^{13}C_{16}$]-labelled palmitic acid were quantified by LC–MS/MS. To quantify the amount of labelled-DPPC, commercially available non-labelled DPPC was used as a calibration standard. For kinetics analysis, various concentrations of LPC ("sn-1-palmitoyl dominant LPC" or "sn-2-palmitoyl dominant LPC") and [$^{13}C_{16}$] palmitoyl-CoA were subjected to the LPCAT reaction using fixed concentrations of [$^{13}C_{16}$] palmitoyl-CoA (2 µM) and LPC (10 µM), respectively. Similar assay conditions were employed for LPG acyltransferase (LPGAT) activities for LYCAT and LPC acyltransferase (LPCAT) activities for LPCAT3. LPGAT activities for LYCAT were determined in the presence of LYCAT-overexpressed membrane fraction (0.05 µg) and stearoyl-CoA using sn-2 or sn-1-oleoyl dominant LPG. LPCAT activities for

LPCAT3 were determined in the presence of LPCAT3-overexpressed membrane fraction (0.005 µg) and arachidonoyl-CoA using sn-2 or sn-1-stearoyl dominant LPC.

Confirmation of the position of incorporated fatty acid

To determine position of incorporated palmitic acid, LPCAT1 assay products were subjected to the PLA2 reaction and the position of the incorporated palmitic acid in the resulting palmitoyl-LPC was determined. Since palmitoyl-LPC can be produced from palmitoyl-containing PC in the membrane fraction that was used as an enzyme source, which may disturb measurement of the palmitoyl LPC concentration, a deuterium-labelled [d31]palmitoyl LPC (both "sn-1-palmitoyl dominant LPC" and "sn-2-palmitoyl dominant LPC") was prepared from [d62] DPPC (possessing two [d31]-labelled palmitic acids) and used instead of non-labelled palmitoyl LPC as an acceptor. [$^{13}C_{16}$]-[d31] DPPC produced by the LPCAT1 assay was reacted with PLA2. The position of [$^{13}C_{16}$] palmitic acid and [d31] palmitic acid in the resulting palmitoyl LPC was then determined. To separate [$^{13}C_{16}$]-[d31] DPPC from [d31] palmitoyl LPC (because it was used an acceptor, the reaction mixture had excess [d31] palmitoyl LPC), the assay products were applied to a Bond Elut C8 solid phase cartridge column (Agilent Technologies) and eluted with 5 mM ammonium formate in 99.5% (v/v) methanol. The eluted fraction containing [$^{13}C_{16}$]-[d31] DPPC was evaporated and dissolved in 100 mM Tris-HCl buffer containing 0.1% Triton X-100. The PLA2 reaction was started by adding 0.1unit bee venom PLA2, allowed to continue at 37 °C for 10 min and then stopped by acidic methanol containing 1 µM 17:0 LPC and 100 nM 17:0 LPA as an internal standard. After centrifugation (21,500 ×g, 5 min), the supernatant was subjected to LC-MS/MS analysis to determine the concentrations of sn-1 [$^{13}C_{16}$] palmitoyl LPC and sn-1 [d31] palmitoyl LPC. The LYCAT and LPCAT3 assay products were also digested with PLA2, and the resulting sn-1-acyl-LPG and sn-1-acyl-LPC were analysed by LC-MS/MS.

Preparation of LPC with palmitic acid predominantly at the sn-1 or sn-2 position

To determine at which position of the LPC glycerol backbone LPCAT1 introduces palmitic acid, we first prepared two types of palmitoyl-LPC that have palmitic acid at either the sn-2 or sn-1 position from dipalmitoyl PC (DPPC). As described in Material and methods section, the two LPC isomers were separated and quantified by LC-MS/MS to determine their purity. The analysis revealed that the present sn-2 LPC isomer preparation was composed of >90% of 2-palmitoyl-1-lyso-PC and < 10% of 1-palmitoyl-2-lyso-PC, while the sn-1 LPC isomer preparation was composed of >90% of 1-palmitoyl-2-lyso-PC and < 10% of 2-palmitoyl-1-lyso-PC (**Fig. 1A**). After incubating the lipids for 10 min in enzymatic assay buffer, the assay condition for the pre-set LPCAT1 assay, most of the 2-palmitoyl-1-lyso-PC remained intact (**Fig. 1B**). In the following studies, we call the sn-1- and sn-2-palmitoyl LPC isomer preparations as the sn-1- and sn-2-palmitoyl dominant LPCs, respectively. We also produced deuterium labelled sn-1-palmitoyl dominant LPC and sn-2-palmitoyl dominant LPC from deuterium labelled DPPC (**Fig. 1C**).

LPCAT1 introduces palmitic acid at both the sn-1 and sn-2 positions of LPC

In order to determine the position of the LPC glycerol backbone at which LPCAT1 introduces palmitic acid, we performed an LPCAT assay using sn-1-palmitoyl dominant LPC or sn-2-palmitoyl dominant LPC as the acceptor, [$^{13}C_{16}$] palmitoyl-CoA as the acyl donor, and the membrane fraction of LPCAT1-overexpressed cells as the enzyme source. We evaluated the LPCAT1 activity by measuring the [$^{13}C_{16}$] DPPC formed. Using the membrane fraction from HEK293A cells expressing mouse LPCAT1, significant amounts of [$^{13}C_{16}$] palmitic acid were incorporated in both sn-1-palmitoyl dominant LPC and sn-2-palmitoyl dominant LPC although the amount incorporated in the latter was almost twice the amount incorporated in the former (**Fig. 2A**). Interestingly, the membrane fraction prepared from mock-transfected HEK293A cells also had similar [$^{13}C_{16}$] palmitic acid-incorporating activity (**Fig. 2A**), indicating that LPCAT1 is endogenously expressed in HEK293 cells. A similar result was obtained when human LPCAT1 expressed in HEK293 cells (**Fig. 2B**). The difference in activity could not have been caused by the substrate preparation method because the activity for sn-1-palmitoyl dominant LPC prepared from the sn-2-palmitoyl dominant LPC was almost the same as the activity for commercially available palmitoyl-LPC which has a similar content of 1-palmitoyl-2-lyso-PC (data not shown).

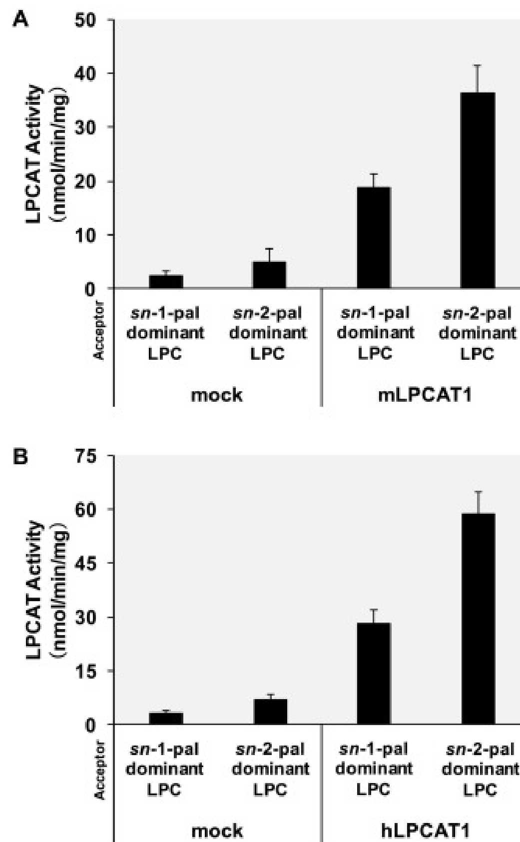

Fig. 2. LPCAT1 activities toward sn-1 and sn-2-pal dominant LPCs.

LPCAT assays were performed using "sn-1-pal dominant LPC" or "sn-2-pal dominant LPC" as an acceptor, and a membrane fraction of LPCAT1-overexpressing HEK293A cells as an enzyme source. The results using recombinant mouse LPCAT1 (A) and human LPCAT1 (B) are shown on the right and the results using control membrane fraction (mock) were also shown on the left. Data are shown as the mean + SD of three data points. Three experiments using independently-prepared membrane fractions were performed with similar results.

[$^{13}C_{16}$] DPPC formed by the LPCAT1 reaction would be either 1-[$^{13}C_{16}$] palmitoyl-2-palmitoyl-PC or 1-palmitoyl-2-[$^{13}C_{16}$] palmitoyl -PC. To confirm which isomers were produced, we subjected the reactants to MS/MS analysis. In negative mode, formate adducts ([M-HCOO]-) of reactants PC were fragmented by collision induced dissociation (CID). The resulting fragments obtained from both the sn-2-palmitoyl dominant LPC and the sn-1-palmitoyl dominant LPC reactants were 255.2 and 271.2, which correspond to unlabelled and [$^{13}C_{16}$]-labelled palmitic acid, respectively. Interestingly, the intensities of the two fragments were different among the two reactants (**Fig. 3A**). Since the fatty acid moiety is more easily liberated from the sn-2 position than from the sn-1 position, 1-palmitoyl-2-[$^{13}C_{16}$] palmitoyl-PC was abundant in the reactants produced from sn-1-palmitoyl dominant LPC, while 1-[$^{13}C_{16}$] palmitoyl-2-palmitoyl-PC was abundant in the reactants produced from sn-2-palmitoyl dominant LPC.

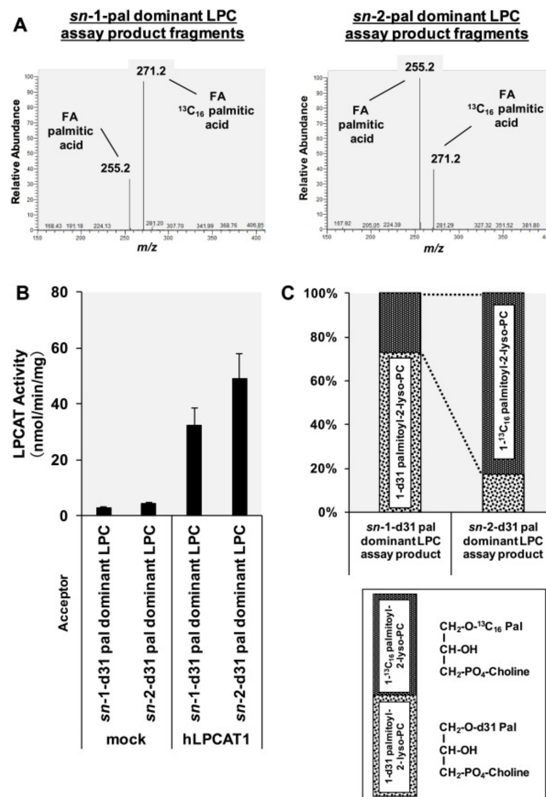

Fig. 3. Confirmation of the position of incorporated palmitic acid.

(A) Fragment spectrum of [$^{13}C_{16}$] palmitoyl-palmitoyl-PC produced by LPCAT1 reaction. LPCAT1 assay products containing [$^{13}C_{16}$] palmitoyl-palmitoyl-PC were subjected to mass spectrometry (MS) analysis. In the negative ion mode, PC [M + HCOO]− ions were fragmented using a collision energy of 45 eV and two fatty acid (FA) fragments (palmitic acid m/z = 255.2 and [$^{13}C_{16}$]palmitic acid m/z = 271.2) were detected. Left and right panels show representative fragment patterns of assay products using “sn-1-pal dominant LPC” and “sn-2-pal dominant LPC” as an acceptor, respectively. (B and C) Determination of the position of incorporated [$^{13}C_{16}$] palmitic acid by using PLA2 reaction and LC-MS/MS. (B) LPCAT1 activities using sn-1- or sn-2-[d31]pal dominant LPC as an acceptor and recombinant human LPCAT1. Similar results were obtained when non-labelled sn-1 or sn-2 pal dominant LPC was used (**Fig. 2**). Data are shown as the mean + SD of three data points. Three experiments using independently-prepared membrane fractions were performed with similar results. (C) Ratio of sn-1-[$^{13}C_{16}$] palmitoyl-LPC (dot) and sn-1-[d31] palmitoyl-LPC (shadowed) in PLA2-digested assay mixtures. LPCAT1 assay products [$^{13}C_{16}$] palmitoyl-[d31] palmitoyl-PC were subjected to bee venom PLA2 treatment. The resulting LPC species were analysed by LC-MS/MS. The data are representative of three independent experiments with similar results.

To perform more quantitative analysis, we used sn-2-[d31] palmitoyl dominant LPC and sn-1-[d31] palmitoyl dominant LPC as the acceptors in the LPCAT1 reaction. A similar result was obtained when unlabelled palmitoyl LPC was used as the acceptor (**Fig. 3B**). Double-labelled [$^{13}C_{16}$]-[d31] DPPC formed by the LPCAT1 reaction would be either 1-[$^{13}C_{16}$] palmitoyl-2-[d31] palmitoyl-PC or 1-[d31] palmitoyl-2-[$^{13}C_{16}$] palmitoyl-PC. The reactants were digested with PLA2 and analysed by LC-MS/MS. When sn-2-[d31] palmitoyl dominant LPC was used as the acceptor, the major LPC detected in PLA2 reactant was 1-[$^{13}C_{16}$] palmitoyl-LPC, while when sn-1-[d31] palmitoyl dominant LPC was used as the acceptor, the major LPC detected was 1-[d31] palmitoyl-LPC (**Fig. 3C**). The above two results clearly show that LPCAT1 incorporated palmitic acid at both the sn-1 position and sn-2 position of LPC.

Kinetic parameters of sn-2 and sn-1-palmitoyl dominant LPCs

To determine the kinetic parameters, we used human LPCAT1 and either [1] an LPC concentration of 10 µM with varying concentrations of [$^{13}C_{16}$] palmitoyl-CoA (**Fig. 4A**) or [2] a [$^{13}C_{16}$] palmitoyl-CoA concentration of 2 µM and a varying the concentration of LPC (**Fig. 4B**). The apparent Km value toward [$^{13}C_{16}$] palmitoyl-CoA was

0.23 ± 0.12 μM for sn-1-palmitoyl dominant LPC and 0.64 ± 0.16 μM for sn-2-palmitoyl dominant LPC. The Km value toward LPC was 2.08 ± 0.12 μM for sn-1-palmitoyl dominant LPC and 3.67 ± 0.08 μM for sn-2-palmitoyl dominant LPC. In both cases, the Vmax values toward sn-2-palmitoyl dominant LPC were found to be about 2 times higher than those toward sn-1-palmitoyl dominant LPC. Therefore, sn-1-palmitoyl LPC appears to have a slightly higher affinity for LPCAT1 than sn-2-palmitoyl LPC, while sn-2-palmitoyl LPC has a higher turnover rate.

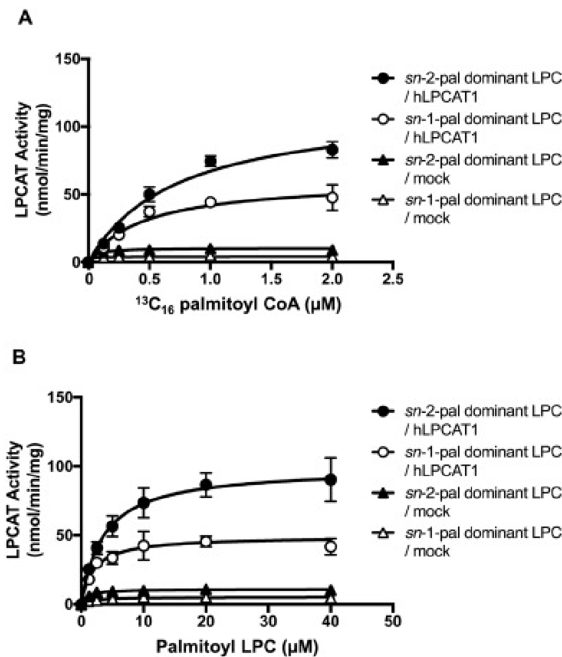

Fig. 4. Kinetic analyses of LPCAT1 activities toward sn-1- and sn-2-pal dominant LPCs.

(A and B) The kinetic analyses to determine the Km and Vmax values for human LPCAT1 were performed using varying concentration of [$^{13}C_{16}$] palmitoyl-CoA (A) and palmitoyl-LPC (B). Closed circles, sn-2-pal dominant LPC; open circles, sn-1-pal dominant LPC. Results obtained using mock-transfected membrane fraction as an enzyme source were also shown. Closed triangles, sn-2-pal dominant LPC; open triangles, sn-1-pal dominant LPC. Data are shown as the mean ± SD of three data points. The results are representative of three independent experiments with similar results. The Km and Vmax values calculated from three independent experiments are shown as the mean ± SD in the text.

LYCAT and LPCAT3 introduce stearic acid at the sn-1 position of LPG and arachidonic acid at the sn-2 positions of LPC, respectively

We next applied the method to determine the acyl position of other LPLATs. We chose LYCAT and LPCAT3. Preceding studies suggested that LYCAT introduced stearic acid (18:0) selectively at the sn-1 position of lysophosphatidylinositol (LPI), while LPCAT3 was critical for introduction of polyunsaturated fatty acids (PUFAs), especially arachidonic acid (20:4) at the sn-2 position of various LPLs, generating various types of arachidonoyl phospholipids. To examine the activity of LYCAT, we used stearoyl-CoA as a donor and lysophosphatidylglycerol (LPG) as an acceptor instead of LPI, since LYCAT was able to introduce stearic acid into LPG with a similar manner to LPI. We prepared an sn-2-oleoyl dominant LPG substrate from di-oleoyl phosphatidylglycerol (PG) by PLA1 reaction and then converted the sn-2-oleoyl dominant LPG to an sn-1-oleoyl dominant LPG in alkaline condition as we did for palmitoyl LPC. We confirmed the present sn-2 LPG isomer preparation (sn-2-acyl dominant LPG) was composed of >90% of 2-oleoyl-1-lyso-PG and <10% of 1-oleoyl-2-lyso-PG, while the sn-1 LPG isomer preparation (sn-1-acyl dominant LPG) was composed of >90% of 1-oleoyl-2-lyso-PG and <10% of 2-oleoyl-1-lyso-PG (**Fig. 5A left**). Unlike LPCAT1 which introduced palmitic acid both at the sn-1 and sn-2 position of LPC, LYCAT introduced stearic acid predominantly at the sn-1 position of LPG (**Fig. 5B left and Fig. 5C left**). Similarly, we prepared an sn-2-stearoyl dominant LPC and an sn-1-stearoyl dominant LPC substrates from di-stearoyl phosphatidylcholine (PC) and used them for evaluation of LPCAT3 activity using arachidonoyl-CoA as an acyl donor. In contrast to LPCAT1 and LYCAT, LPCAT3 was found to

introduce arachidonic acid predominantly at the sn-2 position of LPC (**Fig. 5A right, Fig. 5B right and Fig. 5C right**).

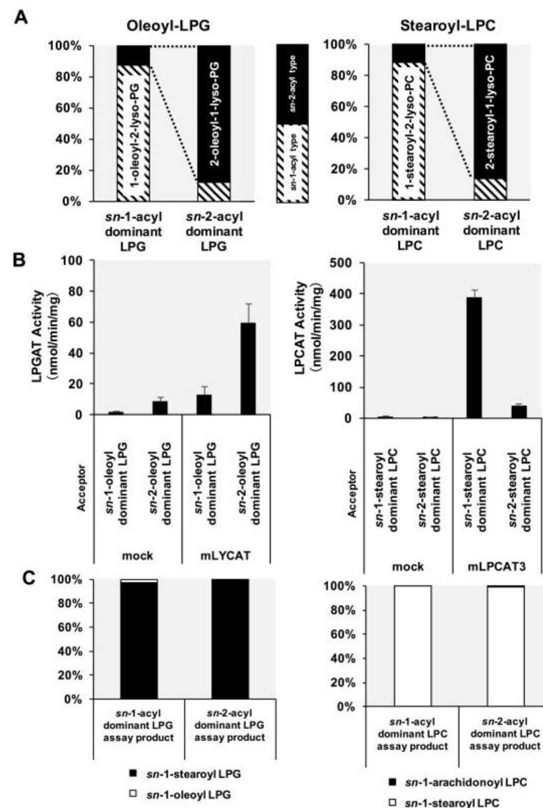

Fig. 5. Positions of glycerol backbone at which LYCAT and LPCAT3 introduce a fatty acid.

(A) Ratio of 2-oleoyl-1-lyso-PG (sn-2-oleoyl LPG: filled) and 1-oleoyl-2-lyso-PG (sn-1-oleoyl LPG: dashed) in the present LPG preparations used for LYCAT assay (left). Ratio of 2-stearoyl-1-lyso-PC (sn-2-stearoyl LPC: filled) and 1-stearoyl-2-lyso-PC (sn-1-stearoyl LPC: dashed) in the present LPC preparations used for LPCAT3 assay (right). (B) LYCAT (left) and LPCAT3 (right) activities toward "sn-1-acyl dominant LPLs" and "sn-2-acyl dominant LPLs". LYCAT activity was determined using "sn-1-oleoyl dominant LPG" or "sn-2-oleoyl dominant LPG" as an acceptor, stearoyl-CoA as a donor and a membrane fraction of mouse LYCAT-overexpressing HEK293A cells as an enzyme source (left). LPCAT3 activity was determined using "sn-1-stearoyl dominant LPC" or "sn-2-stearoyl dominant LPC" as an acceptor, arachidonoyl-CoA as a donor and a membrane fraction of mouse LPCAT3-overexpressing HEK293A cells as an enzyme source (right). The results using control membrane fraction (mock) were also shown. Data are shown as the mean + SD of three independent experiments. (C) Ratio of sn-1-acyl LPLs in PLA2-digested assay products. LYCAT assay products (36:1 (stearoyl-oleoyl) PG) or LPCAT3 assay products (38:4 (stearoyl-arachidonoyl) PC) were subjected to bee venom PLA2 digestion. The resulting LPL species were analysed by LC-MS/MS. Ratio of sn-1-stearoyl LPG (closed) and sn-1-oleoyl LPG (open) in PLA2-digested LYCAT products (left) and ratio of sn-1-arachidonoyl LPC (closed) and sn-1-stearoyl LPC (open) in PLA2-digested LPCAT3 products (right) were shown. The data are representative of three independent experiments with similar results.