学位論文

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Comparative analyses of isoforms of the calcium-independent phosphatidylethanolamine *N*-acyltransferase PLAAT-1 in humans and mice

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Abstract ***N*-Acylphosphatidylethanolamines (NAPEs) are a class of glycerophospholipids, which are known as precursors for different bioactive *N*-acylethanolamines. We previously reported that phospholipase A/acyltransferase-1 (PLAAT-1), which was originally found in mammals as a tumor suppressor, catalyzes *N*-acylation of phosphatidylethanolamines to form NAPEs. However, recent online database suggested the presence of an uncharacterized isoform of PLAAT-1 with an extra sequence at the N terminus. In the present study, we examined the occurrence, intracellular localization, and catalytic properties of this longer isoform, as well as the original shorter isoform from humans and mice. Our results showed that human tissues express the longer isoform but not the short isoform at all. In contrast, mice expressed both isoforms with different tissue distribution. Unlike the cytoplasmic localization of the shorter isoform, the long isoform was found in both cytoplasm and nucleus, inferring that the extra sequence harbors a nuclear localization signal. As assayed with purified proteins, neither isoform required calcium for full activity. Moreover, the overexpression of each isoform remarkably increased cellular NAPE levels. These results conclude that the new long isoform of PLAAT-1 is a calcium-independent *N*-acyltransferase existing in both cytoplasm and nucleus and suggest a possible formation of NAPEs in various membrane structures including nuclear membrane.**—Hussain, Z., T. Uyama, K. Kawai, I. A. S. Rahman, K. Tsuboi, N. Araki, and N. Ueda. **Comparative analyses of isoforms of the calcium-independent phosphatidylethanolamine *N*-acyltransferase PLAAT-1 in humans and mice.** ***J. Lipid Res.*** **2016.** 57: **2051–2060.**

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Fatty acyl ethanolamides [*N*-acylethanolamines (NAEs)] are a class of lipid mediators. Among them, arachidonoylethanolamide (anandamide) is known to be an endogenous ligand of cannabinoid receptors (namely, an endocannabinoid) (1). In addition, palmitoylethanolamide and oleoylethanolamide show biological activities such as anti-inflammation, analgesia, and appetite suppression via different receptors including PPAR-α (2–5). These NAEs are biosynthesized principally through a two-step pathway from membrane glycerophospholipids via *N*-acylphosphatidylethanolamines (NAPEs) (6). The first reaction is the transfer of an acyl chain from a glycerophospholipid molecule such as phosphatidylcholine (PC) to the amino group of phosphatidylethanolamine (PE), resulting in the formation of NAPE, and the enzyme responsible is known as PE *N*-acyltransferase. Very recently, cPLA$_2$ε, a member of the cytosolic phospholipase A$_2$ (PLA$_2$) family (PLA2G4), has been identified as the calcium-stimulated *N*-acyltransferase capable of catalyzing this step (7). However, a series of our recent studies revealed that five members of the HRAS-like suppressor (HRASLS) family, which were originally discovered as tumor suppressors, possess calcium-independent phospholipid-metabolizing activities including NAPE-forming *N*-acyltransferase and PLA$_{1/2}$ activities (8–12), and we proposed to give HRASLS-1–5 the names phospholipase A/acyltransferase-1–5 (PLAAT-1–5), respectively (11). Among the five members, PLAAT-1 particularly received our attention because of its relatively high PE *N*-acyltransferase activity over PLA$_{1/2}$ activity in vitro and predominant expression in testis, skeletal muscle, brain, and heart of humans, mice, and rats, where NAPEs accumulate in response to ischemia and inflammation (11, 13).

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Abbreviations: HRASLS, HRAS-like suppressor; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NLS, nuclear localization signal; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA$_2$, phospholipase A$_2$; PLAAT-1, phospholipase A/acyltransferase-1; UTR, untranslated region.

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cDNAs of PLAAT-1 (originally referred to as A-C1) were cloned from a mouse chondrogenic cell line (ATDC5) (accession number, AF163095) and human renal cell carcinoma-derived cells (RCC-K1) by Akiyama et al. (14) and Ito et al. (15), respectively. Based on their nucleotide sequences, the primary structures of human and mouse PLAAT-1 proteins were deduced to be 168 and 167 amino acids long, respectively (14, 15). We reported that the recombinant PLAAT-1 proteins with these primary structures show the aforementioned enzyme activity (11, 13). However, recently the NCBI database exhibited the existence of a longer isoform with an extra amino acid sequence at the N terminus in various mammals, including humans (NM_020386) and mice (XM_006522203). The deduced amino acid sequence of this longer isoform comprised 273 (humans) or 277 (mice) amino acids, respectively. In this article, we will tentatively refer to the original shorter isoform and later reported longer isoform of humans (h) and mice (m) as hPLAAT-1S and hPLAAT-1L or mPLAAT-1S and mPLAAT-1L, respectively (**Fig. 1**). Moreover, the database suggested that mice have two different transcripts encoding mPLAAT-1S (NM_013751 and XM_006522204). We will refer to NM_013751 and XM_006522204 as mPLAAT-1SA and mPLAAT-1SB (**Fig. 2**), respectively.

So far, endogenous PLAAT-1 has been examined without discriminating between PLAAT-1S and PLAAT-1L. Thus, the first aim of the present study was to clarify whether both isoforms are endogenously expressed in human and mouse tissues. Second, because we noticed that the N-terminal extra domain of PLAAT-1L is a kind of polybasic domain (see Results for details), we investigated the intracellular localization. Moreover, because only recombinant PLAAT-1S protein has been characterized, our third aim was to examine the catalytic properties of recombinant PLAAT-1L.

The results suggest that PLAAT-1L is endogenously expressed in both human and mouse tissues, whereas PLAAT-1S exists only in mice. We also report that PLAAT-1L localizes in both nucleus and cytoplasm and functions as an NAPE-forming *N*-acyltransferase in a Ca^{2+}-independent manner.

EXPERIMENTAL PROCEDURES

Materials

1,2-[1′-^{14}C]dipalmitoyl-PC was purchased from PerkinElmer Life Science (Boston, MA). [1,2-^{14}C]ethanolamine HCl was from Moravek Biochemicals (Brea, CA). Horseradish peroxidase-linked anti-mouse and anti-rabbit IgGs were from GE Healthcare (Piscataway, NJ). 1,2-Dipalmitoyl-PC, 1,2-dioleoyl-PE, mouse anti-FLAG monoclonal antibody M2, anti-FLAG M2-conjugated agarose affinity gel, and FLAG peptide were from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies against lamin A/C and syntaxin 6 and rabbit anti-FLAG (DYKDDDDK) polyclonal antibody were from Cell Signaling Technology (Danvers, MA). Dulbecco's modified Eagle's medium, fetal bovine serum, Lipofectamine 2000, TRIzol, pEF1/Myc-His vector, pEF6/Myc-His vector, and Alexa Fluor® 488-conjugated goat anti-mouse IgG were from Invitrogen/Thermo Fisher Scientific (Carlsbad, CA). Triton X-100, Nonidet P-40, and Proteinase K were from Nacalai Tesque (Kyoto,

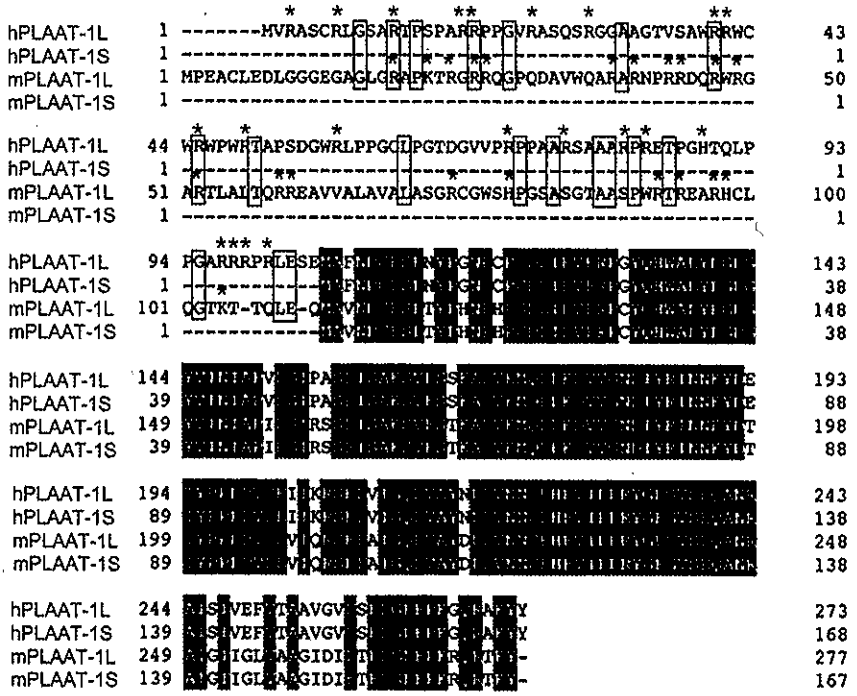

Fig. 1. Alignment of the amino acid sequences of PLAAT-1 isoforms. All these sequences were aligned using the program GENETYX-MAC (version 15). Closed shaded and clear boxes indicate identity among isoforms and identity between the polybasic domains of hPLAAT-1L and mPLAAT-1L, respectively. Asterisks indicate basic amino acids (Arg, Lys, and His) in the polybasic domains.

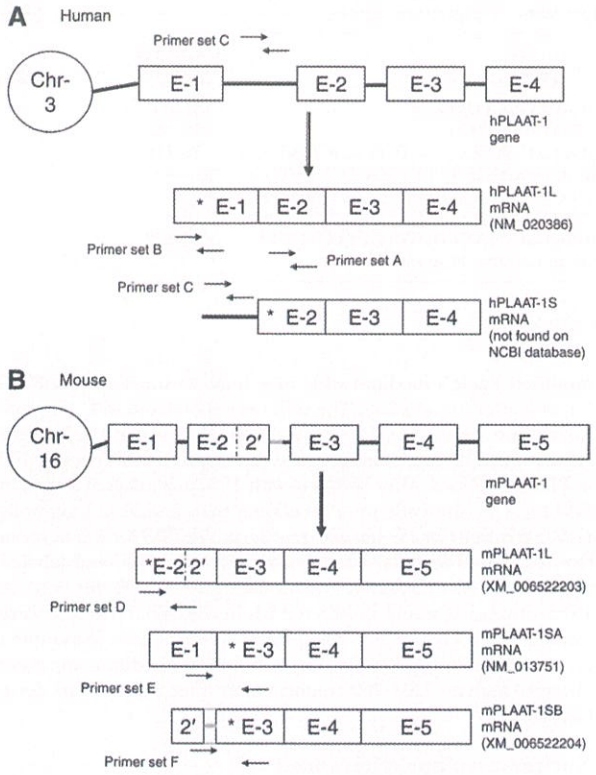

Fig. 2. The structures of mRNAs encoding PLAAT-1 isoforms. The schematic organization of mRNAs of human (A) and mouse (B) PLAAT-1 isoforms is shown. "E" stands for an exon, and the lines between exons indicate introns. Asterisks and arrows indicate the positions of initiation codons and primer sets A–F, respectively. In B, 2′ and a gray line indicate part of exon 2 and part of the intron between exons 2 and 3, respectively, which are contained in mPLAAT-1SB. Accession numbers are shown in parentheses. Chr, chromosome.

Japan). KOD-Plus-Neo and KOD FX DNA polymerases were from Toyobo (Osaka, Japan). Protein assay dye reagent concentrate was from Bio-Rad (Hercules, CA). Protease inhibitor cocktail set III, Immobilon-P, and precoated silica gel 60 F_{254} aluminum sheets (20 × 20 cm, 0.2 mm thick) for TLC were from Merck (Darmstadt, Germany). Human MTC™ Panels I and II were purchased from Clontech (Mountain View, CA). An NE-PER Nuclear and Cytoplasmic Extraction Reagents kit and Pierce® Western Blotting Substrate Plus were purchased from Thermo Fisher Scientific (Rockford, IL). Paraformaldehyde fixative solution was from Muto Pure Chemicals (Tokyo, Japan). Normal goat serum was from Vector Laboratories (Burlingame, CA). Permafluor was from Immunotech (Marseille, France). Silver Staining MS Kit was from Wako Pure Chemical Industries (Osaka, Japan). *Ex* Taq DNA polymerase, Prime-STAR DNA polymerase and a PrimeScript RT reagent kit were from TaKaRa Bio (Ohtsu, Japan). Human prostate cancer cell line (Du-145) was obtained from American Type Culture Collection (Rockville, MD).

Construction of expression vectors

pEF6/Myc-His expression vectors harboring the cDNAs of the C-terminally FLAG-tagged PLAAT-1S from humans (hPLAAT-1S-FL) and mice (mPLAAT-1S-FL) were constructed as described previously (11). The cDNA encoding the C-terminally FLAG-tagged hPLAAT-1L (hPLAAT-1L-FL) was obtained from human skeletal muscle cDNA in MTC™ Panel I by PCR amplification using two pairs of nested primers. The first PCR using primers F1 and R1 (**Table 1**) was carried out with KOD-Plus-Neo DNA polymerase for 30 cycles at 98°C for 10 s, 55°C for 30 s, and 68°C for 60 s, and the second PCR using primers F2 and R2 was for 25 cycles under the same conditions. The obtained DNA fragment was inserted between *Spe*I and *Not*I sites of the pEF1/Myc-His vector. The cDNA encoding the C-terminally FLAG-tagged mPLAAT-1L (mPLAAT-1L-FL) was constructed in two steps of PCR using primers F3 and R3. The first step PCR amplification was carried out with Prime-STAR DNA polymerase for 35 cycles at 98°C for 10 s, 61°C for 30 s, and 72°C for 60 s. cDNA used as a template was prepared from mouse heart as described in the next section. The second step PCR was carried out using the first PCR product as a template for 15 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 60 s. The obtained DNA fragment was inserted between *Spe*I and *Not*I sites of the pEF6/Myc-His vector. All constructs were sequenced in both directions using an Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific).

RT-PCR

To see the tissue distribution of hPLAAT-1 isoforms, cDNAs from human MTC™ Panels I and II were used as templates and subjected to PCR amplification by *Ex* Taq DNA polymerase. As shown in **Table 2** and Fig. 2, the primers were designed on the basis of a common sequence to hPLAAT-1S and hPLAAT-1L [primer set A, which was used previously (11)], a unique sequence for hPLAAT-1L (primer set B), and 5′-untranslated region (5′-UTR) of hPLAAT-1S (primer set C). The PCR conditions used were as follows: 30 cycles with denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s for primer set A; 35 cycles with denaturation at 98°C for 10 s, annealing and extension at 68°C for 60 s for primer set B; 30 cycles with denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 68°C for 60 s for primer set C; 25 cycles with denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 68°C for 60 s for human GAPDH (hGAPDH) as a control. Du-145 cell line was chosen arbitrarily to prepare human genomic DNA. The cells were homogenized in 100 mM Tris-HCl (pH 8.5) containing 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and Proteinase K. After centrifugation for 5 min at 20,400 *g*, the genomic DNA was precipitated by adding isopropanol, washed with 70% ethanol, and resuspended in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

To see the tissue distribution of mPLAAT-1 isoforms, C57BL/6 mice (male, 8 weeks old; Japan SLC Inc.) were anesthetized and euthanized by decapitation according to the guidelines for care and use of animals established by Kagawa University (Kagawa, Japan). Total RNAs were then isolated from different mouse tissues using TRIzol. First strand cDNAs were prepared from 5 μg of total RNA using a PrimeScript RT reagent kit and subjected to PCR amplification by KOD FX DNA polymerase. Unique sequences for mPLAAT-1L (primer set D), mPLAAT-1SA (primer set E) and mPLAAT-1SB (primer set F) were used to design primers (Table 2 and Fig. 2). The PCR conditions used were as follows: 35 cycles with denaturation at 98°C for 10 s, annealing and extension at 72°C for 60 s for primer sets D–F; 28 cycles with denaturation at 98°C for 10 s, annealing at 58°C for 30 s, and extension at 68°C for 60 s for mouse GAPDH (mGAPDH) as a control.

Expression and purification of recombinant proteins

COS-7 cells were grown at 37°C to 90% confluency in 100 mm plastic dishes containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified 5% CO_2 and 95% air incubator. The expression vectors harboring cDNAs for FLAG-tagged PLAAT-1 isoforms were introduced into COS-7

TABLE 1. Primers used for the construction of expression vectors

cDNA (Accession Number)	Primer[a]	Sequence[b]	Location of Nucleotides
hPLAAT-1L (NM_020386)	F1	5′-CCAAGCGAGGTCTAGCCGGAGCGACTGTGC-3′	31–60
	R1	5′-TCCTCCCAAATTCCTTCAATATCAATATC-3′	956–928
	F2	5′-cgcactagtccaagATGGTCAGAGCCTCGTGCCGGCTCGGC-3′ (*Spe*I site)	92–118
	R2	5′-cgcgcggccgcctacttatcgtcgtcatccttgtaatcATAGTATTTTGCTCTTTGTCC-TTTTGGAAAC-3′ (*Not*I site followed by an in-frame FLAG sequence)	910–880
mPLAAT-1L (XM_006522203)	F3	5′-cgcactagtccaagATGCCTGAGGCGTGCTTGGAGGATTTG-3′ (*Spe*I site)	16–42
	R3	5′-cgcgcggccgcctacttatcgtcgtcatccttgtaatcATATTTCGTTCTTTGTCTTT TGGG-3′ (*Not*I site followed by an in-frame FLAG sequence)	846–823

[a]F, forward primer; R, reverse primer.
[b]Restriction sites and FLAG tag sequences are indicated as small letters.

cells using Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested and sonicated twice each for 5 s in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl.

As described previously for the purification of PLAAT-1S (11), the cell homogenates overexpressing human PLAAT-1 isoforms were subjected to ultracentrifugation at 105,000 *g* for 30 min, and PLAAT-1 proteins were purified from the resultant supernatant (cytosol) by anti-FLAG antibody affinity chromatography. The eluted fractions were analyzed by silver staining. The protein concentration was determined by the method of Bradford with BSA as a standard (16).

Western blotting

COS-7 cells transiently expressing FLAG-tagged PLAAT-1 isoforms were harvested after 48 h of transfection, suspended in 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, and then homogenized by sonic disruption twice for 5 s each. Each sample (20–30 μg of protein) was separated by SDS-PAGE on 14% gel and electrotransferred to a hydrophobic polyvinylidene difluoride membrane (Immobilon-P). The membrane was blocked with PBS containing 5% dried skimmed milk and 0.1% Tween 20 (buffer A) and then incubated with anti-FLAG antibody (1:2,000 dilution) in buffer A at room temperature for 1 h, followed by incubation with horseradish peroxidase-labeled anti-rabbit IgG antibody (1:4,000 dilution) in buffer A at room temperature for 1 h. The membrane was finally treated with Pierce® Western Blotting Substrate Plus and visualized with the aid of a LAS1000plus lumino-imaging analyzer (FUJIX Ltd., Japan).

Immunocytochemistry

COS-7 cells overexpressing FLAG-tagged PLAAT-1 isoforms were cultured on 18 mm glass coverslips containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C for 24 h after transfection. The cells were then fixed with 4% paraformaldehyde in 0.1 M PBS for 15 min. The fixed cells were rinsed with PBS twice and permeabilized with 0.2% Triton X-100 in PBS for 15 min. After blocking with 10% normal goat serum in PBS for 1 h, the cells were incubated with anti-FLAG antibody (1:500 dilution) in 1% normal goat serum in PBS for 1 h at room temperature. The cells were washed with PBS twice and labeled with Alexa 488-conjugated anti-mouse IgG (1:1,000 dilution) in 1% normal goat serum in PBS for 1 h in the dark. The cells were washed with PBS twice, and the specimen coverslips were mounted on glass slides using Permafluor, a mounting medium, and were observed with an LSM 700 confocal laser microscope (Carl Zeiss, Germany).

Nuclear-cytoplasmic fractionation

FLAG-tagged PLAAT-1 isoforms were expressed in COS-7 cells as described in Expression and Purification of Recombinant Proteins. After 48 h of transfection, the nuclear-cytoplasmic fractionation was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit according to the manufacturer's protocol (17). The obtained nuclear and postnuclear supernatant fractions were then subjected to Western blotting as described above, using antibodies against FLAG (1:2,000 dilution), lamin A/C (1:2,000 dilution), and syntaxin 6 (1:2,000 dilution) as primary antibodies and horseradish peroxidase-labeled anti-mouse and anti-rabbit IgGs (1:4,000 dilution) as secondary antibodies.

Enzyme assay

Purified human PLAAT-1 isoforms (0.2 μg of protein) were incubated with 40 μM 1,2-[1′-^{14}C]dipalmitoyl-PC (45,000 cpm) and 80 μM 1,2-dioleoyl-PE in 100 μl of 100 mM glycine-NaOH buffer

TABLE 2. Primers used for PCR

cDNA (Accession Number)	Primer Set	Direction	Sequence	Location of Nucleotides
hPLAAT-1L (NM_020386) and hPLAAT-1S	A	Forward	5′-CCCTGTGGAAGAAATCATAAAGCGGTC-3′	685–711
		Reverse	5′-CCCAGGAATGAGAAGACACCAACAGC-3′	876–851
hPLAAT-1L (NM_020386)	B	Forward	5′-TGCCTCCCGGTGCGAGAAGAAGACC-3′	366–390
		Reverse	5′-ACAGGGCCCAGTGCTGATAGCCAGG-3′	506–482
5′-UTR of hPLAAT-1S [reported by Ito et al. (15)]	C	Forward	5′-CCCCTTGTGCACACATCAGTGTTGG-3′	220–244
		Reverse	5′-AAGAAGAGCTAGCTAATACAACTTGCC-3′	384–358
hGAPDH (NM_002046)	—	Forward	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	199–224
		Reverse	5′-CATGTGGGCCATGAGGTCCACCAC-3′	1,181–1,158
mPLAAT-1L (XM_006522203)	D	Forward	5′-TGCCTGAGGCGTGCTTGGAGGATTTG-3′	17–42
		Reverse	5′-CGCCGCGCCACCTCTGATCCCTCCGC-3′	166–141
mPLAAT-1SA (NM_013751)	E	Forward	5′-CTGAGCTGTGAGCAGGCGATTTGTGTG-3′	39–65
		Reverse	5′-AGCCATCACCCAAGTACAGTGCCCAG-3′	230–205
mPLAAT-1SB (XM_006522204)	F	Forward	5′-TAAGACAACCCAGCTTGAGCAGGGAG-3′	177–202
		Reverse	5′-AGCCATCACCCAAGTACAGTGCCCAG-3′	317–292
mGAPDH (NM_008084)	—	Forward	5′-AACTCCCACTCTTCCACCTTCGATG-3′	1,099–1,123
		Reverse	5′-CCTGTTGCTGTAGCCGTATTCATTG-3′	1,203–1,179

(pH 8.2), 2 mM dithiothreitol, and 0.1% Nonidet P-40 at 37°C for 30 min. In some assays, 1 mM EDTA or 1 mM $CaCl_2$ was also added. Reactions were terminated by the addition of 320 μl of a mixture of chloroform and methanol (2:1, v/v) containing 5 mM 3(2)-*t*-butyl-4-hydroxyanisole. After centrifugation, 100 μl of the organic phase was spotted on a silica gel thin-layer plate (10 cm height) with a calibrated capillary glass pipet connected to a rubber aspirator tube (Drummond Scientific Co., Broomall, PA) and was dried under the airflow of a hair dryer. Later on, the TLC plate was developed at 4°C for 25 min in a mixture of chloroform/methanol/28% ammonium hydroxide (80:20:2, v/v) (solvent A). The distribution of radioactivity on the plate was visualized and quantified using an image reader FLA-7000 (Fujifilm, Tokyo, Japan).

Metabolic labeling

COS-7 cells transiently expressing PLAAT-1 isoforms were grown at 37°C to 80% confluency in 6-well plates containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum and were labeled with [^{14}C]ethanolamine (0.32 μCi/well) for 18 h. Cells were then harvested and washed twice with PBS. Total lipids were extracted by the method of Bligh and Dyer (18), spotted on a silica gel thin-layer plate (20 cm height), and developed at 4°C for 90 min in solvent A. The distribution of radioactivity on the plate was visualized and quantified using an image reader FLA-7000.

RESULTS

cDNA cloning of human and mouse PLAAT-1L

We previously cloned cDNAs of PLAAT-1S from human testis and mouse brain (11). In the present study, we cloned cDNAs of PLAAT-1L from human skeletal muscle and mouse heart based on the reported nucleotide sequences (accession number NM_020386 and XM_006522203, respectively). The sequences of cDNAs that we cloned were fully coincident with the reported ones. These results showed that human skeletal muscle and mouse heart express mRNA of PLAAT-1L.

Comparing with PLAAT-1S, the deduced amino acid sequences of human and mouse PLAAT-1L had an N-terminal extra sequence comprising 105 and 110 amino acids, respectively (Fig. 1). The sequences of PLAAT-1L except the extra sequences were exactly identical with those of PLAAT-1S in both humans and mice. Though mPLAAT-1S showed 83.9% amino acid identity with hPLAAT-1S, the identity between the extra sequences of hPLAAT-1L and mPLAAT-1L was as low as 18.1%. However, as pointed by asterisks in Fig. 1, both the extra sequences were abundant in basic amino acids (20 Arg and 1 His in hPLAAT-1L and 17 Arg, 2 Lys, and 2 His in mPLAAT-1L), and their isoelectric points were 12.09 and 11.50, respectively. Thus, the extra sequence of PLAAT-1L appeared to form a polybasic domain.

Detection of PLAAT-1 mRNA in human tissues

As shown in Fig. 2A, hPLAAT-1L mRNA comprises four exons (exons 1–4) (NM_020386), while hPLAAT-1S mRNA lacks exon 1 (15). We found that most of the 5′-UTR sequence of hPLAAT-1S mRNA [36–401 in (15)] is identical to the 3′ sequence of the intron between exon 1 and 2 and ended by AG, the established 3′-consensus sequence of an intron. In order to examine whether both isoforms of hPLAAT-1 are endogenously expressed in human tissues, we prepared three sets of PCR primers (primer sets A–C in Table 2 and Fig. 2A). Primer set A was used to detect hPLAAT-1 mRNA isoforms without discriminating between hPLAAT-1L and hPLAAT-1S mRNAs, while primer set B was to specifically recognize hPLAAT-1L mRNA. To selectively detect hPLAAT-1S mRNA, we used primer set C, both forward and reverse primers of which were contained in the 5′-UTR sequence of hPLAAT-1S mRNA (15). By using primer set A, hPLAAT-1 (hPLAAT-1S + hPLAAT-1L) mRNA levels were shown to be high in testis, skeletal muscle, brain, heart, and thyroid, followed by many other tissues at low levels (**Fig. 3A**). A similar distribution profile of

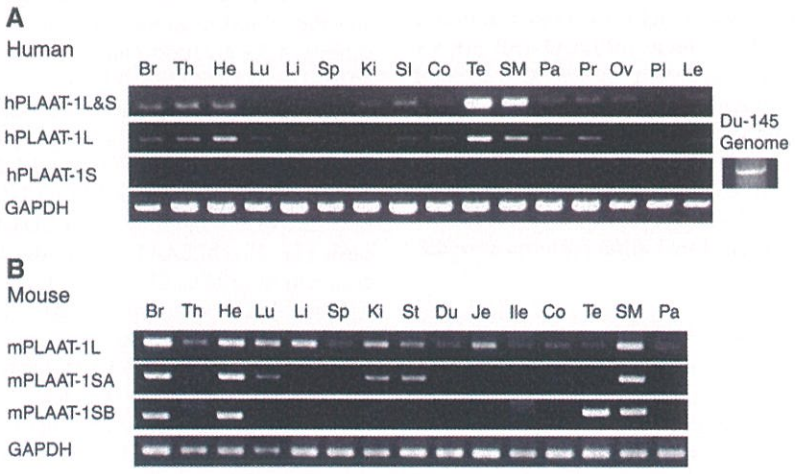

Fig. 3. The expression profile of endogenous PLAAT-1 isoforms. The expression of PLAAT-1 isoforms in human (A) and mouse (B) tissues was examined by PCR using primers as shown in Table 2. In A, human genomic DNA extracted from Du-145 cells was also amplified with primer set C. Br, brain; Co, colon; Du, duodenum; He, heart; Ile, ileum; Je, jejunum; Ki, kidney; Le, leukocyte; Li, liver; Lu, lung; Ov, ovary; Pa, pancreas; Pl, placenta; Pr, prostate; SI, small intestine; SM, skeletal muscle; Sp, spleen; St, stomach; Te, testis; Th, thymus.

PLAAT-1L mRNA was also obtained with primer set B. In contrast, PLAAT-1S mRNA was not detected in any human tissue with primer set C. We also analyzed cDNAs of human prostate cancer cells (Du-145) and human embryonic kidney 293 cells (HEK293) with PCR and found that both the cell lines expressed the long isoform, but not the short isoform (data not shown). To see whether primer set C can functionally amplify a DNA fragment, we performed PCR using human genomic DNA prepared from Du-145 cells as a template and could amplify a DNA band with the expected size (right panel of Fig. 3A). These results strongly suggested that hPLAAT-1L, rather than hPLAAT-1S, is an endogenous PLAAT-1 in human tissues.

Detection of PLAAT-1 mRNA in mouse tissues

We next analyzed RNA derived from mouse tissues. The mPLAAT-1 gene had five exons (exons 1–5), which gave rise to three different mRNAs: mPLAAT-1L, mPLAAT-1SA, and mPLAAT-1SB (Fig. 2B). Exons 3–5 were common in all these transcripts, whereas exon 1 or 2 was present in mPLAAT-1SA and mPLAAT-1L, respectively. mPLAAT-1SB mRNA started with a portion in exon 2 (designated as 2′ in Fig. 2B) and contained four nucleotides of the intron between exons 2 and 3 (indicated as a gray line). Thus mPLAAT-1SA and mPLAAT-1SB mRNAs differed only in their 5′-UTR sequences but encoded the identical mPLAAT-1S protein. We designed three sets of PCR primers (D–F) to specifically detect mPLAAT-1L, mPLAAT-1SA, and mPLAAT-1SB mRNAs, respectively (Fig. 2B and Table 2). mPLAAT-1SA and mPLAAT-1SB mRNAs were distinguishable by using different forward primers in primer sets E and F, although a common reverse primer was used in both primer sets.

As shown in Fig. 3B, unlike humans, mice expressed all three transcripts of PLAAT-1. Additionally, their expression profiles were clearly different from one another. mPLAAT-1L mRNA was abundantly expressed in brain, heart, and skeletal muscle, followed by many other tissues at low levels. mPLAAT-1SA mRNA was highly expressed in brain, heart, and skeletal muscle, whereas mPLAAT-1SB mRNA was highly expressed in brain, heart, testis, and skeletal muscle. Thus, as for mPLAAT-1S transcripts, brain, heart, and skeletal muscle abundantly expressed both transcripts A and B, while testis predominantly expressed transcript B. These results suggested that mice expressed both of PLAAT-1L and PLAAT-1S (mPLAAT-1SA and mPLAAT-1SB) mRNAs, although their distribution patterns were different among tissues.

Intracellular localization of PLAAT-1L

It was previously reported that recombinant PLAAT-1S protein, transiently expressed in COS-7 cells, is localized in cytoplasm and perinuclear region, but not within the nucleus (14). To compare the subcellular localization between PLAAT-1S and PLAAT-1L, we transiently expressed FLAG-tagged PLAAT-1 isoforms of humans and mice in COS-7 cells. Western blot analysis of the cell homogenates with anti-FLAG antibody confirmed successful expression of these recombinant proteins (**Fig. 4A**). We next observed the cells by immunocytochemistry. As shown in Fig. 4B, both hPLAAT-1S and mPLAAT-1S were mostly localized throughout cytoplasm (right panels). In contrast, both hPLAAT-1L and mPLAAT-1L were distributed throughout cytoplasm and nucleus (left panels) or mostly localized to nucleus (middle panels). Nucleoli were not stained in any of the panels. Moreover, we separated the nuclear fraction from postnuclear supernatant with the aid of Nuclear and Cytoplasmic Reagents kit. As expected, lamin A/C (a marker protein for nuclei) and syntaxin 6 (a marker protein for Golgi apparatus) were almost exclusively localized in the nuclear fraction and the postnuclear supernatant, respectively (Fig. 4C). In this latter assay, PLAAT-1L was consistently detected in both the fractions, while most of PLAAT-1S was seen in the postnuclear supernatant. Taken together, these results showed that PLAAT-1L is localized in both nuclei and cytoplasm in contrast to PLAAT-1S, and a certain sequence in the N-terminal polybasic domain of PLAAT-1L was presumed to function as a nuclear localization signal (NLS), assisting its partial translocation to the nucleus.

Catalytic properties of PLAAT-1L

FLAG-tagged hPLAAT-1L and hPLAAT-1S were purified from the soluble fractions of the COS-7 cells overexpressing each enzyme by anti-FLAG antibody affinity chromatography. The purified proteins were then allowed to react with 1,2-[1′-^{14}C]dipalmitoyl-PC in the presence of nonradiolabeled PE, and the products were separated by TLC (**Fig. 5A**). In agreement with our previous results with COS-7 cell homogenates overexpressing PLAAT-1S (13), purified hPLAAT-1S produced radioactive bands corresponding to *N*-palmitoyl-PE and free palmitic acid. A similar TLC image was also obtained with purified hPLAAT-1L (Fig. 5A). Because the formations of *N*-[^{14}C]palmitoyl-PE and [^{14}C]palmitic acid are attributed to *N*-acyltransferase and $PLA_{1/2}$ activities, respectively, the results showed that hPLAAT-1L and hPLAAT-1S had these dual activities (Fig. 5B). Their specific *N*-acyltransferase activities were similar to each other (14.5 ± 1.2 nmol/min/mg of protein for hPLAAT-1L and 12.9 ± 0.3 for hPLAAT-1S). The ratio of *N*-acyltransferase activity to $PLA_{1/2}$ activity (2.0–2.5 for hPLAAT-1L and 1.9–2.4 for hPLAAT-1S) was not significantly different between hPLAAT-1L and hPLAAT-1S. Neither *N*-acyltransferase nor $PLA_{1/2}$ activity of hPLAAT-1S was stimulated by 1 mM Ca^{2+} or inhibited by 1 mM EDTA as we reported previously (11, 13). hPLAAT-1L was also insensitive to the same concentration of $CaCl_2$ and EDTA (Fig. 5B).

Moreover, to examine whether PLAAT-1L exerts the *N*-acyltransferase activity in intact cells, we metabolically labeled PLAAT-1S- or PLAAT-1L-overexpressing COS-7 cells with [^{14}C]ethanolamine (**Fig. 6A**). As analyzed by TLC, the radioactive bands corresponding to NAPEs and NAEs were seen in both cells, and their radioactivities were much higher than those in control cells (Fig. 6B and C, respectively). These results showed that PLAAT-1L as well as PLAAT-1S has Ca^{2+}-independent *N*-acyltransferase activity and that the NAPEs produced by both isoforms are further metabolized to NAEs.

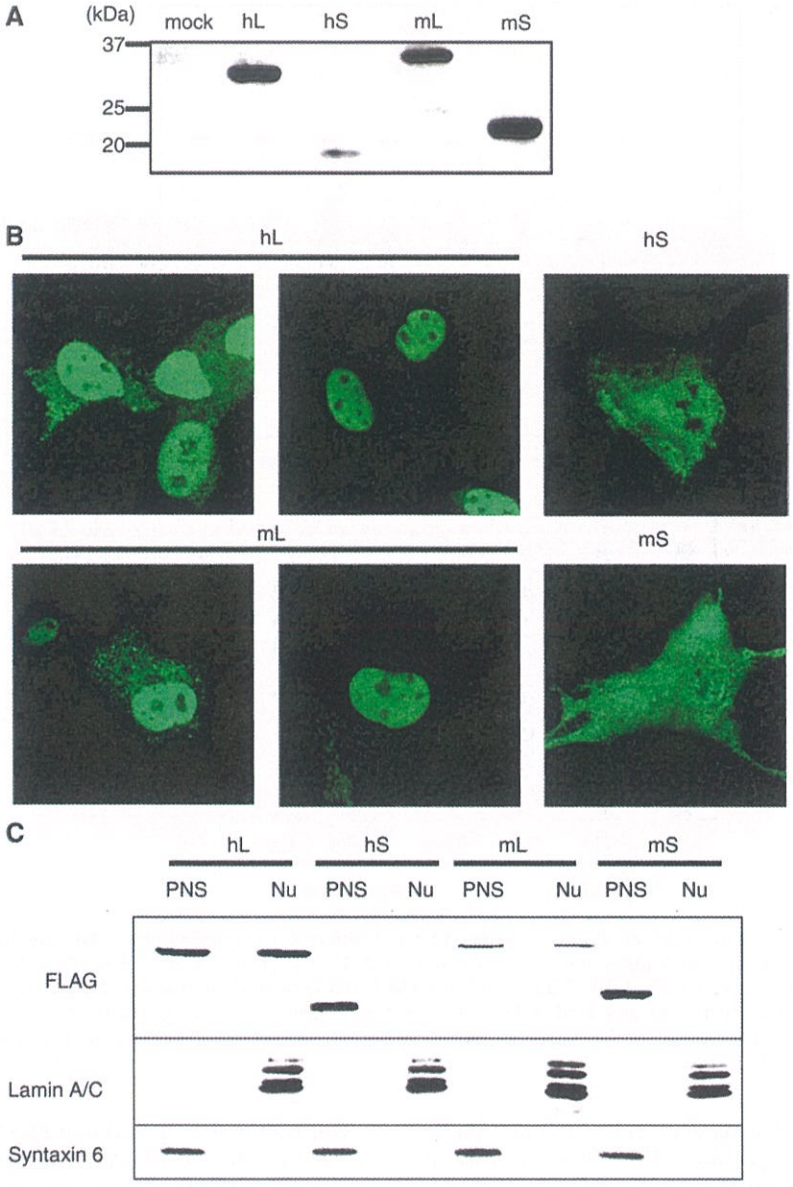

Fig. 4. Intracellular localization of recombinant PLAAT-1 isoforms. The FLAG-tagged PLAAT-1 isoforms were transiently expressed in COS-7 cells. A: The cells were homogenized by sonic disruption, and the obtained homogenate samples (20–30 μg of protein) were analyzed by Western blotting with anti-FLAG antibody. B: The cells were immunocytochemically analyzed with anti-FLAG antibody. C: The cells were separated into nuclear (Nu) and postnuclear supernatant (PNS) fractions as described in Experimental Procedures. Each fraction (16, 10, and 4 μl of PNS and 8, 5, and 2 μl of nuclear fraction) was subjected to Western blotting using anti-FLAG, anti-lamin A/C and anti-syntaxin 6 antibodies, respectively. hL, hPLAAT-1L; hS, hPLAAT-1S; mL, mPLAAT-1L; mS, mPLAAT-1S.

DISCUSSION

So far, PLAAT-1 proteins (referred to as PLAAT-1S in this study) of humans, mice, and rats (NP_001099341) have been characterized as tumor suppressors (14, 15) or phospholipid-metabolizing enzymes (11–13). According to the NCBI database, PLAAT-1 is also present in various other mammals including chimpanzees (XP_516953), cows (NP_001300994), pigs (XP_003132643), dogs (XP_535846), rabbits (XP_008264890), and guinea pigs (XP_003477141). We found that among mammals, at least primates (including humans and chimpanzees), mice, and guinea pigs have PLAAT-1L isoforms with an N-terminal polybasic domain, pI values of which are around 12.

One of our aims was to detect which isoform of PLAAT-1 was endogenously expressed in human and mouse tissues. We could detect mRNA of PLAAT-1L in many human tissues. However, referring to the previously reported 5′-UTR

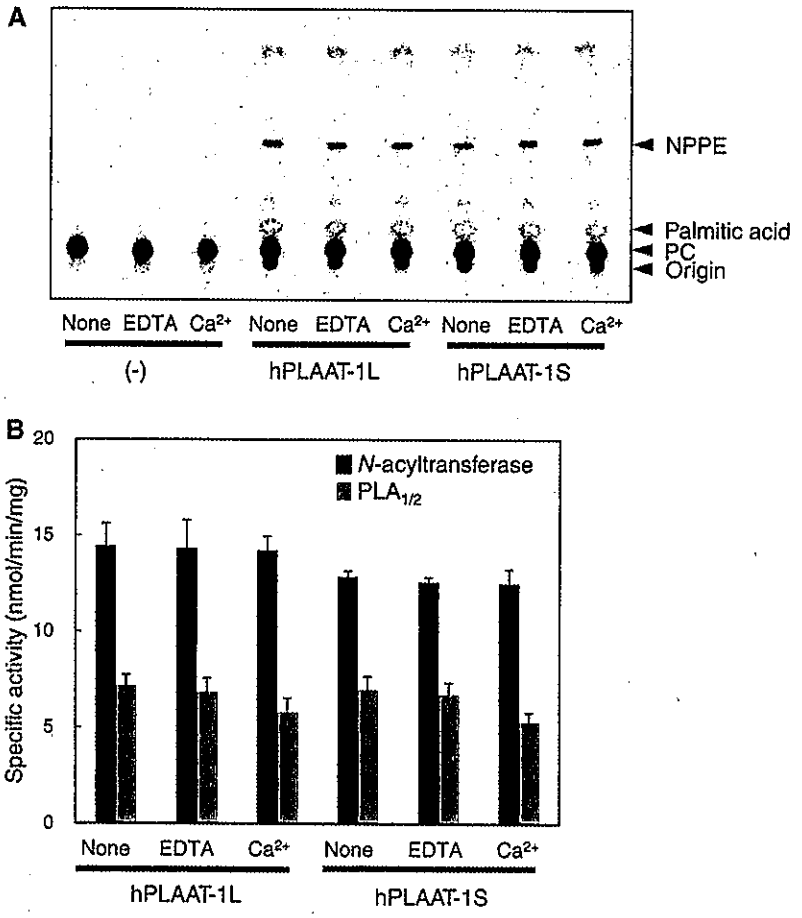

Fig. 5. Enzymatic activities of purified human PLAAT-1 isoforms. The purified hPLAAT-1L and hPLAAT-1S (0.2 μg of protein) were allowed to react with 40 μM 1,2-[1′-^{14}C]dipalmitoyl-PC and 80 μM 1,2-dioleoyl-PE in the presence of 1 mM EDTA (EDTA) or 1 mM $CaCl_2$ (Ca^{2+}) or in their absence (None). The resultant radioactive products were separated by TLC (A), and *N*-acyltransferase and $PLA_{1/2}$ activities were calculated by quantifying the produced *N*-[^{14}C]palmitoyl-PE (NPPE) and [^{14}C]palmitic acid, respectively (mean values ± SD, n = 3) (B).

sequence of A-C1 by Akiyama et al. (15), we could not detect the endogenous expression of the short form in humans. In contrast, we found that mice endogenously express both the short and long isoforms of this protein. In addition, mice had two transcripts differing in the 5′-UTR region to generate the same short isoform of PLAAT-1. As for tissue distribution, both hPLAAT-1L and previously reported human A-C1 (15) were shown to be highly expressed in testis, skeletal muscle, brain, heart, and thyroid, followed by many other tissues at low levels, which suggested that both data represent the same protein. However, in mice the short isoform was found to be highly expressed in brain, heart, skeletal muscle (from both transcripts A and B), and testis (mostly from transcript B) as opposed to the almost ubiquitous expression of the long isoform, supporting the dual presence of PLAAT-1 isoforms. This difference between humans and mice is unclear but may partly be explained by the absence of other family members, PLAAT-2 and -4 in mice (10, 19, 20), which may be compensated by multiple PLAAT-1 isoforms.

Our results also showed that PLAAT-1S was localized to cytoplasm, whereas PLAAT-1L existed in both cytoplasm and nucleus. The localization of a protein to the nucleus is typically controlled by the presence of NLS sequences, and a number of carrier proteins such as importin-α and importin-β are involved in the transportation process to the nucleus (21). According to cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), which claims to accurately predict NLSs specific to the importin αβ pathway (22), amino acids 12–44 (score 3.7) and 95–124 (score 3.2) of hPLAAT-1L and amino acids 19–48 of mPLAAT-1L (score 3.5) were predicted to be putative consensus sequences for bipartite NLS, defined as (K/R)(K/R)$X_{10\text{-}12}$(K/R)$_{3/5}$ where X indicates any amino acid and (K/R)$_{3/5}$ represents at least three of either lysine or arginine out of five consecutive amino acids. Proteins with a score of 3, 4, or 5 were predicted to be localized to both the nucleus and the cytoplasm. These results suggest that nuclear import of PLAAT-1L occurs at least partly through the importin αβ pathway. In addition, the C-terminal

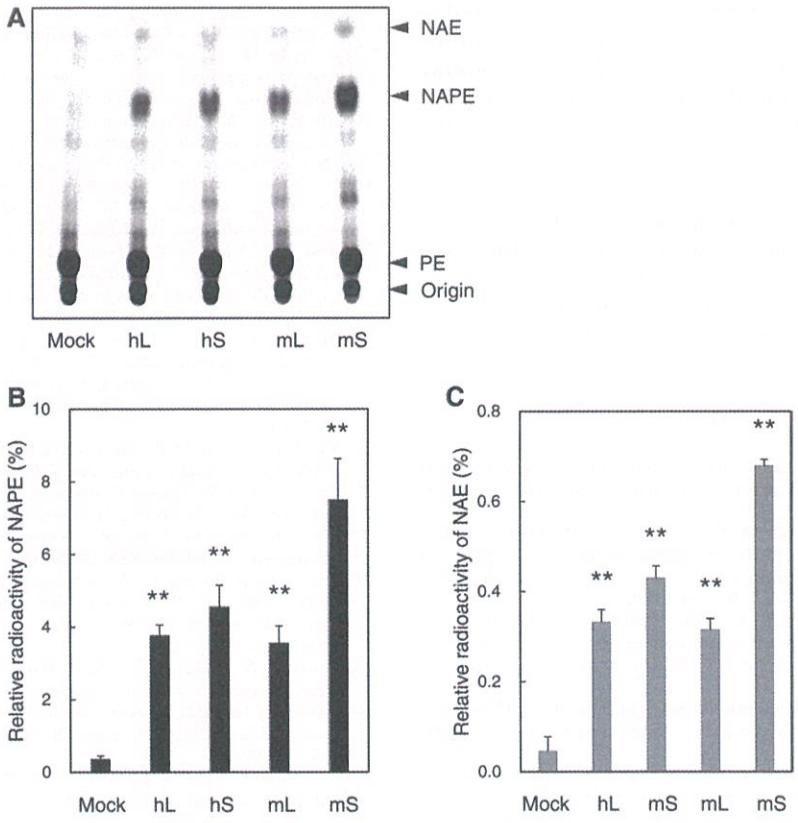

Fig. 6. Production of NAPEs and NAEs in COS-7 cells expressing the recombinant PLAAT-1 isozymes. COS-7 cells were transiently transfected with the insert-free vector (Mock) or expression vectors harboring hPLAAT-1L (hL), hPLAAT-1S (hS), mPLAAT-1L (mL), or mPLAAT-1S (mS) and were incubated with [^{14}C] ethanolamine. The total lipids were then extracted and separated by TLC (A), and relative radioactivities of NAPEs (B) and NAEs (C) were calculated (mean values ± SD, n = 3). ** $P < 0.01$, compared with mock cells by two-sided *t*-test.

region (amino acids 266–277) of mPLAAT-1L was predicted to be a class 2 monopartite NLS (score 3.0) because this region contains K(K/R)X(K/R) where X indicates any amino acid. Alternatively, NucPred analyses (https://www.sbc.su.se/~maccallr/nucpred/) (23) also predicted that PLAAT-1L molecules spend more time in the nucleus than PLAAT-1S molecules. Polybasic domains have also been found to constitute NLSs, as in SV40 large T antigen (24) and the nuclear lamin proteins (25). It is here worth reminding that a polybasic motif was reported to target the protein to phosphatidylinositol 4,5-bisphosphate-rich membranes (26) and increases the extent and avidity of membrane association probably through a charge interaction as shown in $p21^{K\text{-}ras(B)}$ (27). Thus, it turns out that some portions of the polybasic domain of PLAAT-1L may facilitate the protein to adhere to the membrane structures such as plasma and nuclear membranes so that the enzymatic reactions take place in their close proximity.

At present, we do not know the biochemical significance of this nuclear localization, but a possible role of nuclear PLAAT-1L is to generate NAPEs in the nuclear membrane. NAPEs serve as membrane stabilizers (28, 29) and are well known to function as precursors for various bioactive NAEs (1–4). Concomitantly, other signaling lipid molecules like phosphatidic acids (30) and lysophosphatidic acids (31) can be formed from NAPEs by NAPE-hydrolyzing phospholipase D (NAPE-PLD) (32) or through the NAPE-PLD-independent pathway (33), respectively. However, further studies are required to clarify whether endogenous PLAAT-1 produces NAPEs in the nuclear membrane of living cells and whether NAPEs play these roles on the nuclear membrane.

As far as catalytic property is concerned, the long isoform did not show any significant differences from the short isoform; namely, the ratio of *N*-acyltransferase activity to $PLA_{1/2}$ activity was similar, and Ca^{2+} dependency was not seen. Therefore, the polybasic domain of the long isoform was suggested to merely affect the intracellular localization of this protein without altering its catalytic properties. However, we cannot rule out a possibility that the domain serves as an enzymatic complement, which might be responsible in vivo to regulate the enzyme activity of PLAAT-1.

In summary, this study revealed that PLAAT-1 long isoform exists in both cytoplasm and nucleus, thus showing the first evidence of a possible NAPE generation within the nucleus. Additionally, in terms of the endogenous expression

of PLAAT-1, this long isoform seemed to have replaced its shorter predecessor (originally reported as A-C1) in humans while mice invariably expressed the both isoforms. Future studies are expected to uncover the mechanism of nuclear shuttling and the physiological significance of this nuclear PLAAT-1.

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