View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Biochemical and Biophysical Research Communications 443 (2014) 718–724

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel lysophosphatidic acid acyltransferase enzyme (LPAAT4) with a possible role for incorporating docosahexaenoic acid into brain glycerophospholipids☆☆

Miki Eto [a,b], Hideo Shindou [a,c,*], Takao Shimizu [a,b]

[a] Department of Lipid Signaling, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan
[b] Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

ARTICLE INFO

Article history:
Received 3 December 2013
Available online 12 December 2013

Keywords:
Phosphatidic acid
Glycerophospholipid
Brain
Lysophosphatidic acyltransferase
Docosahexaenoic acid

ABSTRACT

Glycerophospholipids are important components of cellular membranes, required for constructing structural barriers, and for providing precursors of bioactive lipid mediators. Lysophosphatidic acid acyltransferases (LPAATs) are enzymes known to function in the *de novo* glycerophospholipid biosynthetic pathway (Kennedy pathway), using lysophosphatidic acid (LPA) and acyl-CoA to form phosphatidic acid (PA). Until now, three LPAATs (LPAAT1, 2, and 3) have been reported from the 1-acyl-glycerol-3-phosphate *O*-acyltransferase (AGPAT) family. In this study, we identified a fourth LPAAT enzyme, LPAAT4, previously known as an uncharacterized enzyme AGPAT4 (LPAATδ), from the AGPAT family. Although LPAAT4 was known to contain AGPAT motifs essential for acyltransferase activities, detailed biochemical properties were unknown. Here, we found that mouse LPAAT4 (mLPAAT4) possesses LPAAT activity with high acyl-CoA specificity for polyunsaturated fatty acyl-CoA, especially docosahexaenoyl-CoA (22:6-CoA, DHA-CoA). mLPAAT4 was distributed in many tissues, with relatively high expression in the brain, rich in docosahexaenoic acid (DHA, 22:6). mLPAAT4 siRNA in a neuronal cell line, Neuro 2A, caused a decrease in LPAAT activity with 22:6-CoA, suggesting that mLPAAT4 functions endogenously. siRNA in Neuro 2A cells caused a decrease in 18:0–22:6 PC, whereas mLPAAT4 overexpression in Chinese hamster ovary (CHO)-K1 cells caused an increase in this species. Although DHA is considered to have many important functions for the brain, the mechanism of its incorporation into glycerophospholipids is unknown. LPAAT4 might have a significant role for maintaining DHA in neural membranes. Identification of LPAAT4 will possibly contribute to understanding the regulation and the biological roles of DHA-containing glycerophospholipids in the brain.

© 2013 The Authors. Published by Elsevier Inc. Open access under CC BY-NC-ND license.

1. Introduction

Cellular membranes are comprised of glycerophospholipids, which have many important structural and functional roles for cells, for example maintaining cellular barriers and acting as precursors of lipid signaling molecules [1,2]. Tissues contain distinct contents of glycerophospholipids, such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL), each with different composition of fatty acids [1,3].

Glycerophospholipids are first formed from glycerol-3-phosphate (G3P) through the *de novo* pathway (Kennedy pathway) [4]. G3P is converted to lysoPA (LPA) by glycerol-3-phosphate acyltransferases (GPATs), and are subsequently transformed to PA by LPA acyltransferases (LPAATs) [5]. Once different classes of glycerophospholipids are synthesized from the common intermediate

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; AGPAT, 1-acyl-glycerol-3-phosphate *O*-acyltransferase; DHA-CoA, docosahexaenoyl-CoA; DHA, docosahexaenoic acid.

☆☆ This work was supported by a Grant-in-Aid for Scientific Research (S) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (TS) and The Grant for National Center for Global Health and Medicine (24-001, 25-201; TS). HS was supported by a CREST, Japan Science and Technology Agency, a Gushinkai, and The Cell Science Research Foundation.

* Corresponding author. Address: National Center for Global Health and Medicine, Tokyo 162-8655, Japan. Fax: +81 3 3202 7364.
E-mail address: hshindou-tky@umin.net (H. Shindou).

0006-291X © 2013 The Authors. Published by Elsevier Inc. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.bbrc.2013.12.043

PA, glycerophospholipids are reconstituted through the remodeling pathway (Lands' cycle) [6]. In the Lands' cycle, glycerophospholipids are degraded to lysophospholipids by phospholipase A_2s (PLA_2s), and are formed back to glycerophospholipids by lysophospholipid acyltransferases (LPLATs) [2,3,7]. Mammalian LPAATs were identified from the 1-acyl-glycerol-3-phosphate *O*-acyltransferase (AGPAT) family, and LPLATs of the remodeling pathway were found from both the AGPAT and the membrane bound *O*-acyltransferase (MBOAT) family [2,7].

The AGPAT family members possess highly conserved motifs [5], and many have been already characterized. LPAAT1, LPAAT2 and LPAAT3 function as LPAATs in the Kennedy pathway [5,8,9]. Other members are reported to function as GPATs, or as LPLATs of the Lands' cycle [5,7].

AGPAT4 (LPAATδ) is a member of the AGPAT family, and has highly homology with LPAAT3 (60%). Although structural and tissue distribution of AGPAT4 is studied in the past [10], little biochemical information has been reported. We newly found that AGPAT4 functions as an LPAAT with high selectivity for polyunsaturated fatty acyl-CoA, especially docosahexaenoyl-CoA (DHA-CoA, 22:6-CoA). From biochemical characteristics identified in this manuscript, we will rename this enzyme as LPAAT4 according to the proposed LPLAT nomenclature [7]. Mouse LPAAT4 (mLPAAT4) was expressed in many tissues, with highest mRNA expression in the brain, particularly rich in docosahexaenoic acid (DHA, 22:6)-containing glycerophospholipids [11]. Although DHA is known to be important for brain functions [11], the mechanism of DHA incorporation into cellular membrane glycerophospholipids is still unknown. LPAAT4 might have an important role for maintaining adequate DHA levels of brain glycerophospholipids.

2. Materials and methods

Please see details in the Supplementary text.

2.1. Materials

Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA). Ham's F-12 nutrient mixture, minimal essential medium (MEM) and geneticin (G418 Disulfate Aqueous Solution) were obtained from Nacalai Tesque (Kyoto, Japan). 14:0/14:0 PC was purchased from NOF corporation (Tokyo, Japan). G3P and DHA were purchased from Sigma (St. Louis, MO). All other glycerophospholipids, lysophospholipids and acyl-CoAs were purchased from Avanti Polar Lipids (Alabaster, AL). Methanol, chloroform, acetonitrile and ammonium bicarbonate were purchased from Wako (Osaka, Japan).

2.2. Plasmids and vectors

mLPAAT4 (NCBI accession number NM_026644, registered as AGPAT4) cDNA was amplified by PCR with forward 5′-CTAGCTAGCCACCATGGATTACAAGGATGACGATGACAAGGACCTCATCGGGCTGCTGAAGTCCC-3′, and reverse 3′-CCGCTCGAGTCAGTCCGTTTGTTTCCGTTTGTTGTCG-5′ primers using mouse brain as a template. mLPAAT4 H96A with a mutation in AGPAT motif I (NHX_4D motif, histidine was converted to alanine) was constructed by overlap extension PCR with forward 5′-GGTCCTCAATGCCAAGTTTG-3′, and reverse 5′-CAAACTTGGCATTGAGGACC-3′ primers. Mouse GPAT1 (mGPAT1) (NCBI accession number NM_0008149) cDNA was amplified by PCR with forward 5′-CTAGCTAGCCACCATGGATTACAAGGATGACGATGACAAGGAGGAGTCTTCAGTGACAGTTGGC-3′, and reverse 3′-CCGCTTAAGCTACAGCACCACAAAACTC-5′ primers using mouse liver as a template. FLAG epitopes (DYKDDDDK) were attached to the N-terminus. PCR products were ligated into the pCXN2.1 vector [12] and sequenced.

2.3. Transfection and siRNA

Chinese hamster ovary (CHO)-K1 cells (provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan) were transfected with cDNAs using Lipofectamine 2000 (Life Technologies). Microsomal proteins were extracted 48 h post-transfection. For stable overexpression, transfected CHO-K1 cells were selected with 3 mg/ml geneticin for four days, and were maintained in 0.3 mg/ml geneticin. siRNAs of mLPAAT4 (ON-TARGETplus SMARTpoolsiRNA, L-051038-01) and a negative control (D-001810-10) were obtained from Dharmacon. 5 nM siRNA was transfected using Lipofectamine RNAiMAX reagent (Life Technologies) into Neuro 2A cells (ATCC, Manassas, VA).

2.4. LPLAT and GPAT activity measurement

0.5 μg microsomal protein was incubated for 10 min at 37 °C with reaction mixtures containing lysophospholipid and acyl-CoAs (LPLAT activities) or G3P and acyl-CoAs (GPAT activities). Lipids were extracted by Bligh and Dyer method, and were measured using liquid chromatography–mass spectrometry (LC–MS).

2.5. Glycerophospholipid composition analysis

CHO-K1 cells were transfected with pCXN2.1 vector, mLPAAT4 cDNA or mLPAAT4 H96A cDNA, and Neuro 2A cells were transfected with control or mLPAAT4 siRNA. For both cell types, cells were treated with 50 μM DHA in 2% FBS medium 48 h post-transfection, and lipids were extracted at various time points by directly adding methanol to the dishes. Fatty acid composition of PC, PE and PS were analyzed using LC–MS.

2.6. Reversed phase LC–MS

Glycerophospholipids were separated with Acquity Ultraperformance LC (UPLC) system (Waters, Milford, MA), and were detected by TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Waltham, MA) with a HESI-II electrospray ionization source.

2.7. Quantitative PCR analysis

Quantitative PCRs (LightCycler System; Roche Applied Science, Mannheim, Germany) were performed using FastStart DNA Master SYBR Green I (Roche Applied Science). mRNA levels were normalized by 36B4, a housekeeping gene. Primers used were: mLPAAT4 forward, CAAGATCAATGCCAGACTCTGCT; mLPAAT4 reverse, AAACTTGTGATTGAGGACCACGA; 36B4 forward, CTGAGATTCGGGATATGCTGTTG; and 36B4 reverse, AAAGCCTGGAAGAAGGAGGTCTT.

2.8. Confocal microscopy

CHO-K1 cells stably overexpressing FLAG-tagged mLPAAT4 were used to study subcellular localization. mLPAAT4 was detected using anti-FLAG M2 antibody. ER, mitochondria and Golgi were visualized by anti-PDI antibody (Cell Signaling Technology, Danvers, MA), MitoTracker Red CMXRos (Life Technologies) and anti-GM130 antibody (Abcam, Cambridge, UK), respectively. Confocal microscopy was performed with LSM510 Laser Scanning Microscope (Carl Zeiss, Germany).

2.9. Statistics and softwares

Statistical evaluations and analysis of kinetics were performed with PRISM 5 software (Graphpad Software Inc., La Jolla, CA). Statistics were calculated by using Student's *t*-test, or one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Kinetics were analyzed by fitting the data to the Michaelis–Menten equation.

2.10. Mice

C57BL6 N mice were obtained from Clea Japan, Inc. (Tokyo, Japan). 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.

3. Results

3.1. Cloning and tissue distribution of mLPAAT4

Until now, three LPAATs (LPAAT1, LPAAT2 and LPAAT3) have been reported. We newly identified a fourth LPAAT enzyme, previously known as AGPAT4 (NM_026644). From the biochemical characteristics described later, we renamed AGPAT4 to LPAAT4. mLPAAT4 encodes a 378 amino acid protein of 43.8 kDa with 6 transmembrane domains predicted by HMMTOP (http://www.enzim.hu/hmmtop) [13,14]. Tissue distribution of mLPAAT4 was analyzed using quantitative PCR, and normalized by 36B4, a housekeeping gene. mLPAAT4 mRNA was expressed highest in the brain, followed by lung, stomach and colon (Fig. 1A). Next, Western blot analysis was performed to study mLPAAT4 protein expression in various tissues, using anti-mLPAAT4 antibody (Scrum Inc.) against a C-terminal peptide (IDNKRKQTD). The inconsistency between the apparent molecular mass (~37 kDa) and the calculated molecular weight (43.8 kDa), also observed in other acyltransferases, may be caused by multiple membrane spanning domains [8,15]. mLPAAT4 was expressed in most tissues, besides heart, liver and kidney (Fig. 1B). Inconsistent with mRNA expression levels, highest protein expression was observed in the testis.

3.2. Subcellular localization of mLPAAT4

To study subcellular localization of mLPAAT4, CHO-K1 cells stably overexpressing FLAG-tagged mLPAAT4 were examined by confocal microscopy. Cells were stained with anti-FLAG M2 antibody (Sigma) to detect mLPAAT4, and with organelle markers: anti-PDI antibody for ER, MitoTracker Red CMXRos for mitochondria and anti-GM130 antibody for Golgi. The expression pattern of mLPAAT4 was similar to that of the ER marker, but not the mitochondria and Golgi markers (Fig. 1C). These results suggest that mLPAAT4 is mainly localized to the ER when overexpressed in CHO-K1 cells.

3.3. Acyltransferase activities of mLPAAT4

To study the enzymatic activities of mLPAAT4, pCXN2.1 vector, FLAG-tagged mLPAAT4 or mLPAAT4 H96A were overexpressed in CHO-K1 cells. Microsomal fractions (100,000*g* pellet) were used

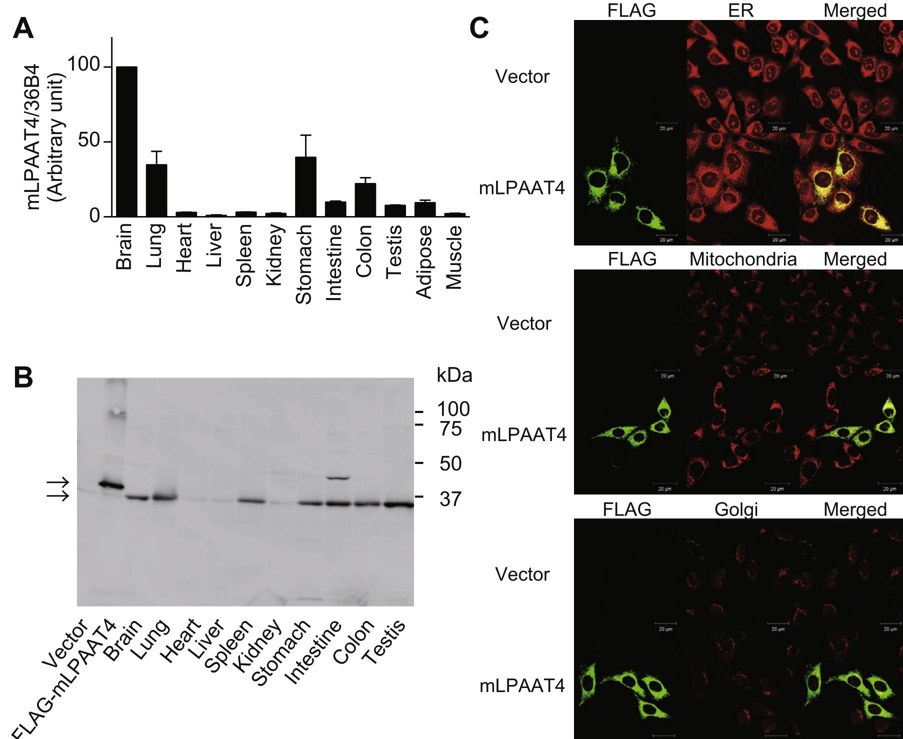

Fig. 1. Tissue distribution and subcellular localization of mLPAAT4. (A) mLPAAT4 mRNA expression levels in various tissues of mice (8 weeks) were analyzed by quantitative PCR, and normalized by 36B4. Data are shown as the mean + S. E. of three independent experiments. (B) Western blot analysis was performed using anti-mLPAAT4 antibody. Microsomal protein from tissues (10 μg/lane), or CHO-K1 cells transfected with pCXN2.1 vector or FLAG-tagged mLPAAT4 (0.5 μg/lane), were loaded. Arrowheads indicate FLAG-tagged mLPAAT4 (upper) and mLPAAT4 (lower). (C) Immunocytochemical studies were performed using CHO-K1 cells stably overexpressing pCXN2.1 vector or FLAG-tagged mLPAAT4. mLPAAT4 was detected using anti-FLAG M2 antibody (green). ER, mitochondria and Golgi were visualized by anti-PDI antibody (red), MitoTracker Red (red) and anti-GM130 antibody (red), respectively. *Scale bars* correspond to 20 μm. Three independent experiments were performed with similar results (B, C).

for the measurement of enzyme activities. Expression levels of mLPAAT4 and mLPAAT4 H96A were similar, confirmed by Western blot analysis using anti-M2 antibody (Fig. 2A). First, LPLAT activities were measured using 25 μM 16:0 lysophospholipid and a mixture of 5 different acyl-CoAs: 1 μM each of 16:0-, 18:1-, 18:2-, 20:4 and 22:6-CoA. mLPAAT4 possessed significant LPAAT activity with high preference for long chain polyunsaturated fatty acyl-CoA, especially 22:6-CoA (Fig. 2B); however, mLPAAT4 did not show other LPLAT activities (Fig. S1). mLPAAT4 also did not possess GPAT activity (Fig. S2A). Activity of mLPAAT4 H96A was decreased to similar levels to the vector control (Fig. 2B), indicating that the motif is essential for its activity. These results demonstrated that LPAAT activities in this assay were caused by mLPAAT4 itself. Activities of mLPAAT4 were also confirmed with 18:0 LPA and 18:1 LPA as substrates (Fig. S2B).

3.4. Kinetics of mLPAAT4 as an LPAAT

To evaluate substrate binding affinities of mLPAAT4, we examined LPAAT activities with different concentrations of 22:6-CoA and LPA. First, LPAAT assays were performed with 25 μM 16:0 LPA and 0–10 μM 22:6-CoA (Fig. 2C). Next, using 10 μM 22:6-CoA, activities were measured with 0–40 μM 16:0 LPA (Fig. 2D). Furthermore, we studied LPAAT activities with 10 μM 22:6-CoA and 0–30 μM 18:0 LPA (Fig. 2E). LPAAT activities were increased concentration dependently of substrates. At higher concentrations of 22:6-CoA (>10 μM), 16:0 LPA (>40 μM) or 18:0 LPA (>30 μM), LPAAT activities were inhibited (data not shown). The maximum velocities and km values calculated from three independent experiments are shown in the figures.

3.5. DHA incorporation into mLPAAT4 overexpressing cells

We next investigated how mLPAAT4 influences DHA-incorporation into glycerophospholipids, because mLPAAT4 possessed high activity with 22:6-CoA (DHA-CoA) (Fig. 2B). pCXN2.1 vector, mLPAAT4 or mLPAAT4 H96A were overexpressed in CHO-K1 cells. 48 h after transfection, cells were treated with 50 μM DHA in 2% FBS medium, and lipids were extracted at the indicated time points (0, 1, 3, 6, 9, 12 and 24 h). Glycerophospholipid composition of PC, PE and PS were analyzed, since PA synthesized through the *de novo* pathway is mainly converted to these species. Using LC–MS, all major species of PC, PE and PS (16:0, 18:0 and 18:1 at the *sn*-1 position, 16:0, 18:1, 18:2, 20:4 and 22:6 at the *sn*-2 position) were analyzed. Signal intensities for each species were summed, and the percentage of each signal was calculated. DHA addition increased all analyzed DHA-containing glycerophospholipid species. Interestingly, mLPAAT4 overexpression caused an increase in the percentage of 18:0–22:6 PC compared to vector and mutant transfected cells (Fig. 2G); however, the percentage of 16:0–22:6 PC (Fig. 2F) and 18:1–22:6 PC (Fig. 2H) did not change. These results are inconsistent with the *in vitro* activities, which will be discussed later. DHA-containing PE and PS species (16:0–22:6, 18:0–22:6 and 18:1–22:6) were also analyzed; however, none of these were altered by mLPAAT4 overexpression (data not shown).

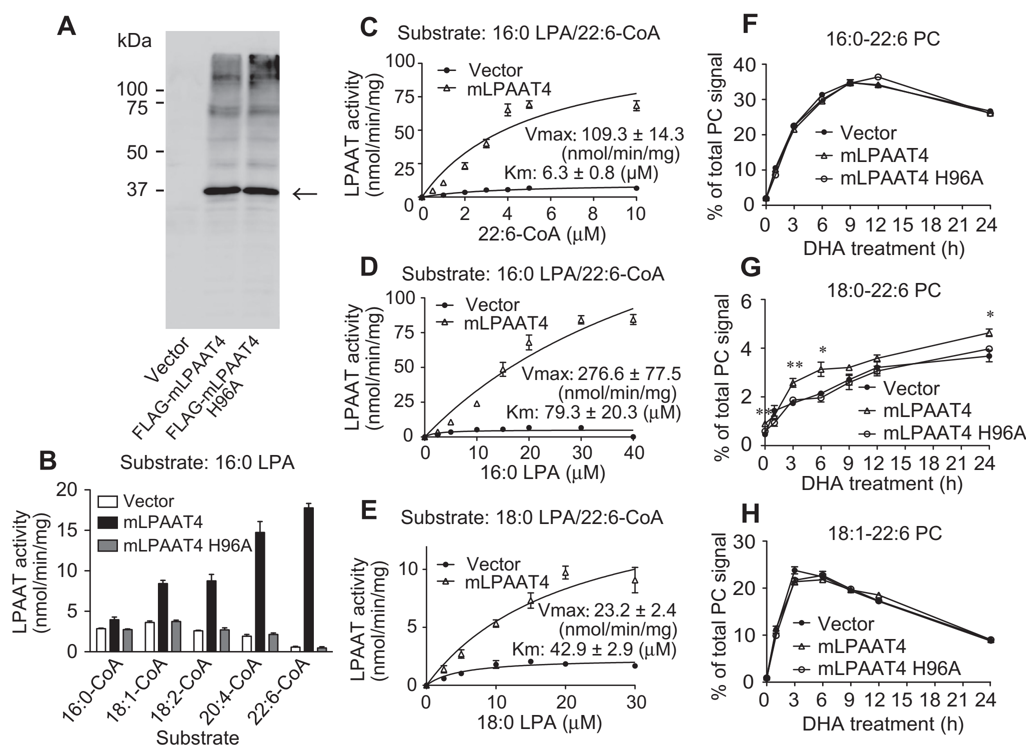

Fig. 2. Substrate specificity of mLPAAT4. (A) Western blot analysis was performed with anti-FLAG M2 antibody, to check transfection efficiencies of proteins used for LPLAT assays (5 μg/lane). Arrowheads indicate FLAG-tagged mLPAAT4 or FLAG-tagged mLPAAT4 H96A. (B) LPAAT activities were measured with 25 μM 16:0 LPA and a mixture of 5 different acyl-CoAs (1 μM of 16:0-, 18:1-, 18:2-, 20:4- and 22:6-CoA), using 0.5 μg microsomal protein of CHO-K1 cells expressing pCXN2.1 vector (*white bars*), FLAG-tagged mLPAAT4 (*black bars*) or FLAG-tagged mLPAAT4 H96A mutant (*gray bars*). Data are shown by the mean + S. D. of triplicate measurements. (C) LPAAT activities were measured using 25 μM 16:0 LPA and 0–10 μM 22:6-CoA. (D) LPAAT activities were measured with 10 μM 22:6-CoA and 0–40 μM 16:0 LPA. (E) LPAAT activities were measured with 10 μM 22:6-CoA and 0–30 μM 18:0 LPA. The maximum velocities (V_{max}) and km values calculated from three independent experiments are shown as the mean ± S. E. in the insets (C–E). (F–H) CHO-K1 cells transfected with pCXN2.1 vector, mLPAAT4 or mLPAAT4 H96A were treated with 50 μM DHA, and fatty acid composition of PC was analyzed at various time points. The signal intensities of 16:0–22:6 PC (F), 18:0–22:6 PC (G) and 18:1–22:6 PC (H) are shown as the percentage of total PC signal. Data are shown by the mean ± S. E. of triplicate measurements. Three independent experiments were performed with similar results (A-H). Statistics were calculated with one-way analysis of variance (ANOVA) followed by post hoc Tukey's test; $^{*}p < 0.05$, $^{**}p < 0.01$.

3.6. siRNA-mediated knockdown of mLPAAT4

To confirm endogenous activity of mLPAAT4, siRNA-mediated knockdown was performed using Neuro 2A cells. siRNA transfection caused an approximately 90% decrease in mLPAAT4 mRNA level (Fig. 3A). Western blot analysis showed that endogenous mLPAAT4 protein was undetectable in mLPAAT4 siRNA transfected cells (Fig. 3B). siRNA of mLPAAT4 caused an approximately 40% decrease in LPAAT activity with 22:6-CoA, suggesting that mLPAAT4 indeed has endogenous activity (Fig. 3C).

Next, we studied the effect of mLPAAT4 siRNA on DHA incorporation into glycerophospholipids. Neuro 2A cells were transfected with mLPAAT4 siRNA, and DHA incorporation into PC, PE and PS were analyzed in the same procedure as in overexpression experiments using CHO-K1 cells. All DHA-containing species showed an increase by addition of DHA. Intriguingly, the percentage of 18:0–22:6 PC (Fig. 3E) and 18:1–22:6 PC (Fig. 3F) were decreased by mLPAAT4 knockdown; however, no changes were observed in the percentage of 16:0–22:6 PC (Fig. 3D). All analyzed PE and PS species (16:0–22:6, 18:0–22:6 and 18:1–22:6) showed no significant differences (data not shown). These results are consistent with the fatty acid composition changes in mLPAAT4 overexpressing cells, but contradict the *in vitro* LPAAT activities of mLPAAT4 with 16:0 LPA. This will be discussed later.

4. Discussion

In this study, we identified LPAAT4, previously known as AGPAT4, as a novel type of LPAAT. Although LPAAT4 was already recognized structurally as a member of the AGPAT family [10], the LPAAT activity shown in the previous study using 18:1-CoA was very weak, and the actual substrate of this enzyme was unknown. Here, we newly demonstrated that mLPAAT4 has LPAAT activity with high acyl-CoA selectivity for long chain polyunsaturated fatty acyl-CoA, especially 22:6-CoA (Figs. 2B and 4). mLPAAT3 which has high homology with mLPAAT4 also has LPAAT

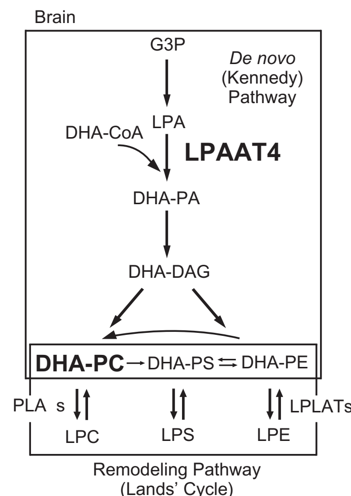

Fig. 4. A proposed scheme for the function of mLPAAT4 in the brain. mLPAAT4 is highly expressed in the brain, and showed LPAAT activity with high preference for 22:6-CoA. DHA-containing PA synthesized by mLPAAT4 is changed to DHA-containing diacylglycerol (DAG), and is further converted to DHA-containing PC, PE and PS. mLPAAT4 might be important for synthesizing DHA-containing glycerophospholipids in the brain.

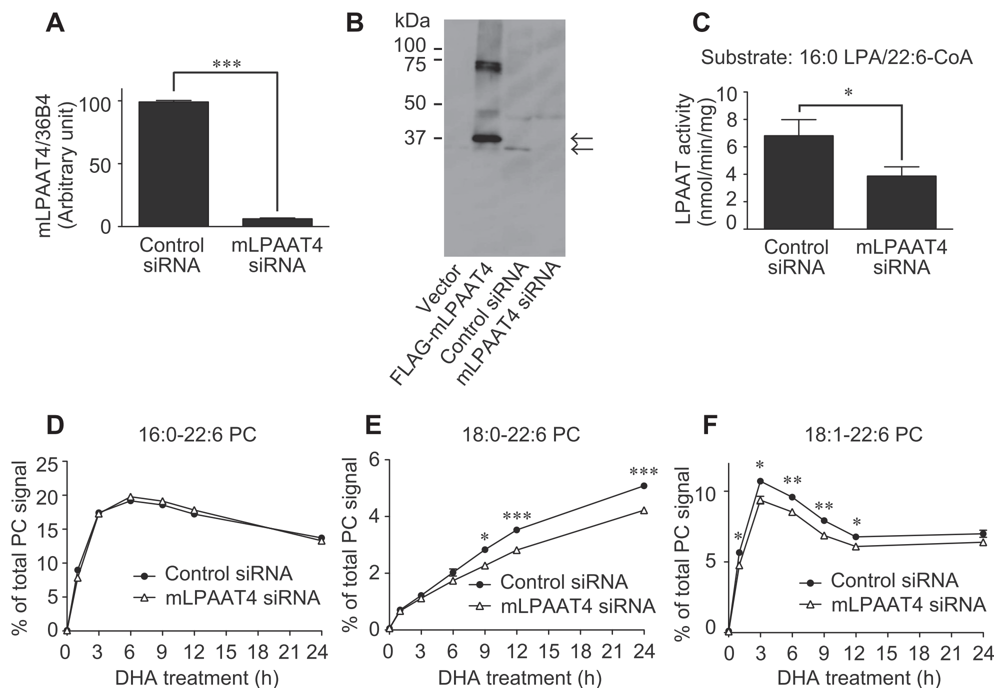

Fig. 3. siRNA-mediated knockdown of mLPAAT4 in Neuro 2A cells. (A) Neuro 2A cells were transfected with control or mLPAAT4 siRNA, and mRNA expression levels of mLPAAT4 were analyzed using quantitative PCR analysis, and normalized by 36B4. (B) Western blot analysis was performed with 10 μg microsomal protein from Neuro 2A cells transfected with control or mLPAAT4 siRNA, using anti-mLPAAT4 antibody. mLPAAT4 overexpressed in CHO-K1 cells was used as a positive control (0.5 μg/lane). Arrowheads indicate FLAG-tagged mLPAAT4 (upper) and mLPAAT4 (lower). (C) LPAAT activities were measured using 0.5 μg microsomal protein of Neuro 2A cells transfected with control or mLPAAT4 siRNA, with 25 μM 16:0 LPA and 10 μM 22:6-CoA as substrates. (D–F) Control or mLPAAT4 siRNA transfected cells were treated with 50 μM DHA, and fatty acid composition of PC was analyzed with LC–MS at various time points. The signal intensities of 16:0–22:6 PC (D), 18:0–22:6 PC (E) and 18:1–22:6 PC (F) are shown as the percentage of total PC signal. The percentage of 18:0–22:6 PC and 18:1–22:6 PC were decreased by inhibition of mLPAAT4. Data are shown by the mean ± S. E. of triplicate measurements. Three independent experiments were performed with similar results (A–F). Statistics were calculated with Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

activity with preference for polyunsaturated fatty acyl-CoA [8,9]. mLPAAT3 is reported to possess LPAAT and LPIAT activity, but we could only observe LPAAT activity of mLPAAT4. Although mLPAAT4 might show other LPLAT activities by changing the experimental conditions, our data suggest that mLPAAT4 mainly functions as an LPAAT. mLPAAT4 was distributed in many tissues including the brain, a tissue abundant in DHA-containing glycerophospholipids.siRNA-mediated knockdown of mLPAAT4 in Neuro 2A cells decreased LPAAT activity with 22:6-CoA (Fig. 3C), indicating that mLPAAT4 functions as an LPAAT endogenously. Compared to the dramatic decrease in mLPAAT4 mRNA and protein expression (Fig. 3A and B), the decrease in LPAAT activity was approximately 40%, suggesting the existence of other LPAATs including unknown enzymes, with activity for 22:6-CoA in this cell line.

Overexpression of mLPAAT4 in CHO-K1 cells caused an increase in the percentage of 18:0–22:6 PC (Fig. 2G). 16:0–22:6 PC, 18:1–22:6 PC, and all DHA-containing PE and PS species were unchanged (Fig. 2F and H). On the other hand, siRNA of mLPAAT4 in Neuro 2A cells lead to a decrease in the early incorporation rate of 18:1–22:6 PC, and a decrease in 18:0–22:6 PC (Fig. 3E and F); however, 16:0–22:6 PC and all DHA-containing PE and PS species were not altered (Fig. 3D). The reason for these results is obscure, since mLPAAT4 showed clear activity towards 16:0 LPA. There are several possibilities to explain the contradictory results. One is that other LPAAT enzymes are involved in maintaining 16:0–22:6 PC, so the change in mLPAAT4 expression was not strong enough to influence its content. A second is that 16:0–22:6 PC is mainly produced through the Lands' cycle. A third is that the level of 16:0–22:6 PC is regulated by the degradation rate. A lipid profiling of the spinal cord of calcium-independent phospholipase $A_2\beta$ (iPLA$_2\beta$) knockout mice showed a prominent increase in 16:0–22:6 PC, suggesting the importance of iPLA2β for degrading these species [16]. Although further studies are needed to clarify the reason for the partial change in DHA-containing glycerophospholipids, the change in 18:0–22:6 PC suggests that mLPAAT4 produces DHA-containing glycerophospholipids in cellular levels. In neural membranes, DHA is enriched in PE and PS, mainly in the form of 18:0–22:6 [17]. Microsomes from the cerebral cortex synthesizes PS from 18:0–22:6 PC most favorably [18]. PS decarboxylation, a process which synthesizes PE from PS in the mitochondria, is also reported to prefer 18:0–22:6 species [19]. Although we could not see changes in 18:0–22:6 PE and PS in our experiments using cell lines, the *de novo* synthesis of 18:0–22:6 glycerophospholipids by mLPAAT4 might be important for biosynthesis of 18:0–22:6 PE and PS in the nervous system, where enzymes required for synthesizing these PE and PS species are present.

DHA is believed to have many important roles for the nervous system. Many animal studies show that an n-3 fatty acid deficient diet for two generations, which decreases brain and retinal DHA, impairs visual performance and causes defects in neural functions [20]. DHA is reported to increase neurite outgrowth in hippocampal neurons [21], and reduced DHA levels are observed in neurodegenerative diseases, such as Alzheimer's Disease [11]. It is controversial whether DHA treatment is clinically effective as a treatment for Alzheimer's Disease [22,23], however, recent reports suggest that DHA might be effective for some patients, those with the accumulation of intracellular amyloid β oligomers [24]. Future investigations are needed to confirm if DHA is truly effective, but these reports suggest a possibility that DHA might lead to treatments for dementia or neurodegenerative diseases.

Although many studies show the importance of DHA for neural functions, hypothesized to show beneficial effects through the form of glycerophospholipids, the mechanism of how DHA incorporates into brain glycerophospholipids is still unknown. Until now, the only LPLAT reported to have high preference for 22:6-CoA was LPAAT3, which has low expression in the brain [9]. LPAAT4, which is expressed in the brain, and has high substrate specificity for 22:6-CoA, might be one of the key enzymes for synthesizing DHA-containing glycerophospholipids in the nervous system (Fig. 4). Although further investigations are needed to confirm whether this enzyme actually contributes to synthesis of DHA-containing glycerophospholipids *in vivo*, this study might lead to a new understanding of the molecular mechanism of DHA incorporation into brain glycerophospholipids, and possibly contribute to new treatments for diseases such as cognitive disorders or neurodegenerative diseases.

Acknowledgments

We are grateful to Dr. K. Waku (Teikyo University) for valuable suggestions, and Dr. J.-i. Miyazaki (Osaka University) for supplying pCXN2. We also thank Drs. Y. Kita and F. Hamano (The University of Tokyo), and D. Hishikawa, K. Yanagida, T. Harayama, R. Morimoto, and all members of our laboratories for valuable suggestions, and Y. Takahashi for technical assistance (National Center for Global Health and Medicine).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.043.

References

[1] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat. Rev. Mol. Cell Biol. 9 (2008) 112–124.
[2] T. Shimizu, Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation, Annu. Rev. Pharmacol. Toxicol. 49 (2009) 123–150.
[3] A. Yamashita, Y. Hayashi, Y. Nemoto-Sasaki, et al., Acyltransferases and transacylases that determine the fatty acid composition of glycerolipids and the metabolism of bioactive lipid mediators in mammalian cells and model organisms, Prog. Lipid Res. 53 (2013) 18–81.
[4] E.P. Kennedy, S.B. Weiss, The function of cytidine coenzymes in the biosynthesis of phospholipides, J. Biol. Chem. 222 (1956) 193–214.
[5] R.A. Coleman, D.G. Mashek, Mammalian triacylglycerol metabolism: synthesis, lipolysis, and signaling, Chem. Rev. 111 (2011) 6359–6386.
[6] W.E. Lands, Metabolism of glycerolipides; a comparison of lecithin and triglyceride synthesis, J. Biol. Chem. 231 (1958) 883–888.
[7] H. Shindou, T. Shimizu, Acyl-CoA:lysophospholipid acyltransferases, J. Biol. Chem. 284 (2009) 1–5.
[8] K. Yuki, H. Shindou, D. Hishikawa, T. Shimizu, Characterization of mouse lysophosphatidic acid acyltransferase 3: an enzyme with dual functions in the testis, J. Lipid Res. 50 (2009) 860–869.
[9] A. Koeberle, H. Shindou, T. Harayama, T. Shimizu, Role of lysophosphatidic acid acyltransferase 3 for the supply of highly polyunsaturated fatty acids in TM4 Sertoli cells, FASEB J. 24 (2010) 4929–4938.
[10] B. Lu, Y.J. Jiang, Y. Zhou, et al., Cloning and characterization of murine 1-acyl-sn-glycerol 3-phosphate acyltransferases and their regulation by PPARα in murine heart, Biochem. J. 385 (2005) 469–477.
[11] J.R. Marszalek, H.F. Lodish, Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you, Annu. Rev. Cell Dev. Biol. 21 (2005) 633–657.
[12] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.
[13] G.E. Tusnady, I. Simon, Principles governing amino acid composition of integral membrane proteins: application to topology prediction, J. Mol. Biol. 283 (1998) 489–506.
[14] G.E. Tusnady, I. Simon, The HMMTOP transmembrane topology prediction server, Bioinformatics 17 (2001) 849–850.
[15] D. Hishikawa, H. Shindou, S. Kobayashi, et al., Discovery of a lysophospholipid acyltransferase family essential for membrane asymmetry and diversity, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 2830–2835.
[16] G. Beck, Y. Sugiura, K. Shinzawa, et al., Neuroaxonal dystrophy in calcium-independent phospholipase A2β deficiency results from insufficient remodeling and degeneration of mitochondrial and presynaptic membranes, J. Neurosci. 31 (2011) 11411–11420.
[17] H.Y. Kim, Novel metabolism of docosahexaenoic acid in neural cells, J. Biol. Chem. 282 (2007) 18661–18665.
[18] H.Y. Kim, J. Bigelow, J.H. Kevala, Substrate preference in phosphatidylserine biosynthesis for docosahexaenoic acid containing species, Biochemistry 43 (2004) 1030–1036.

[19] J.H. Kevala, H.Y. Kim, Determination of substrate preference in phosphatidylserine decarboxylation by liquid chromatography-electrospray ionization mass spectrometry, Anal. Biochem. 292 (2001) 130–138.
[20] N. Salem Jr., B. Litman, H.Y. Kim, K. Gawrisch, Mechanisms of action of docosahexaenoic acid in the nervous system, Lipids 36 (2001) 945–959.
[21] F. Calderon, H.Y. Kim, Docosahexaenoic acid promotes neurite growth in hippocampal neurons, J. Neurochem. 90 (2004) 979–988.
[22] Y. Freund-Levi, M. Eriksdotter-Jonhagen, T. Cederholm, et al., Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial, Arch. Neurol. 63 (2006) 1402–1408.
[23] J.F. Quinn, R. Raman, R.G. Thomas, et al., Docosahexaenoic acid supplementation and cognitive decline in Alzheimer disease: a randomized trial, JAMA 304 (2010) 1903–1911.
[24] T. Kondo, M. Asai, K. Tsukita, et al., Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Aβ and differential drug responsiveness, Cell Stem Cell 12 (2013) 487–496.