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Identification of membrane *O*-acyltransferase family motifs

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ABSTRACT

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

Introduction

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

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

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

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

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

Materials and methods

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

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

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

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

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

In vitro LPLAT assays. Acyltransferase activity was measured by the transfer of [^{14}C]18:1-CoA to lysophospholipids to form phospholipids. Reaction mixtures contained 100 mM Tris–HCl (pH 7.4), 1 mM EDTA, 25 μM [^{14}C]18:1-CoA (0.22 GBq/mmol), 50 μM lysophospholipid, and protein (100,000 *g* pellets) in a total volume of 0.1 ml. The amount of total protein for each assay is described in the corresponding Figure legends. After incubation at 37 °C for 10 min, reactions were stopped by the addition of 0.3 ml of chloroform:methanol (1:2, v/v). Total lipids were extracted using a slightly modified Bligh–Dyer method [33], and subsequently analyzed by TLC in chloroform:methanol:acetic acid:water (50:25:8:4, v/v/v/v). Bands at positions corresponding to the expected products were visualized by I_2 vapor, cut off the plate, placed in Microscinti-O (Perkin-Elmer Life Sciences), and analyzed in a liquid scintillation counter LS6500 (Beckman).

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

Results

Alignment of LPLATs in the MBOAT family

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

Conserved sequences among three LPLATs

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

Conserved sequences among four LPLATs

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