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Membrane topology of murine glycerol-3-phosphate acyltransferase 2

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ABSTRACT

Glycerol-3-phosphate acyltransferase (GPAT) is a rate-limiting enzyme in mammalian triacylglycerol biosynthesis. GPAT is a target for the treatment of metabolic disorders associated with high lipid accumulation. Although the molecular basis for GPAT1 activation has been investigated extensively, the activation of other isoforms, such as GPAT2, is less well understood. Here the membrane topology of the GPAT2 protein was examined using an epitope-tag-based method. Exogenously expressed GPAT2 protein was present in the membrane fraction of transformed HEK293 cells even in the presence of Na_2CO_3 (100 mM), indicating that GPAT2 is a membrane-bound protein. Trypsin treatment of the membrane fraction degraded the N-terminal (FLAG) and C-terminal (*myc*-epitope) protein tags of the GPAT2 protein. Bioinformatic analysis of the GPAT2 protein sequence indicated four hydrophobic sequences as potential membrane-spanning regions (TM1–TM4). Immunoblotting of the *myc*-epitope tag, which was inserted between each TM region of the GPAT2 protein, showed that the amino acid sequence between TM3 and TM4 was protected from trypsin digestion. These results suggest that the GPAT2 protein has two transmembrane segments and that the N-terminal and C-terminal regions of this protein face the cytoplasm. These results also suggest that the enzymatically active motifs I–III of the GPAT2 protein face the cytosol, while motif IV is within the membrane. It is expected that the use of this topological model of GPAT2 will be essential in efforts to elucidate the molecular mechanisms of GPAT2 activity in mammalian cells.

1. Introduction

Glycerol-3-phosphate acyltransferase (GPAT), a rate-limiting enzyme in triacylglycerol (TAG) biosynthesis, catalyzes the esterification of glycerol-3-phosphate with a fatty acyl-CoA to form 1-acylglycerol-3-phosphate (lysophosphatidic acid; LPA). Four GPAT isoforms (GPAT1, GPAT2, GPAT3 and GPAT4) have been identified in mammals [1,2]. Previous studies showed that exogenous over expression of a GPAT gene resulted in TAG accumulation [3], whereas knockout or knockdown of a GPAT isoform decreased lipid levels in both cultured cells and experimental animals [4–6]. A recent study clearly demonstrated that pharmacological inhibition of GPAT with a novel inhibitor, FSG67, reduced body weight and food intake, and also ameliorated hepatic steatosis and insulin resistance in diet-induced obese mice [7]. Thus, GPAT is an attractive target for the treatment of metabolic disorders resulting in high lipid accumulation. However, the molecular basis for activation of each GPAT isoform in mammalian cells is still unknown.

GPAT1 and GPAT2 are considered to be localized in the outer mitochondrial membrane, while GPAT3 and GPAT4 are in the endoplasmic reticulum (ER) [1,2]. All GPAT isoforms have four enzymatically active motifs (motifs I–IV) encoded by their amino acid sequences [1,2]. These motifs are considered to face the cytosol in order to bind the substrates, i.e. fatty acyl-CoA and glycerol-3-phosphate [1,2]. Consistent with these hypothetical considerations, an elegant series of experiments has confirmed the cytosolic location of the active motifs within the GPAT1 protein [8]. However, the topography (topology) of other GPATs on the organelle membrane has not yet been clarified experimentally.

GPAT2 (also called xGPAT1) was cloned by our laboratory and by Wang et al. in 2007 [9,10]. GPAT2 is expressed in the heart, liver, spleen, kidney, adipose tissue and testis [9,10], and it has 72% amino acid sequence identity with GPAT1. GPAT2 can utilize both a saturated acyl-CoA and an unsaturated acyl-CoA as synthetic substrates, in contrast to GPAT1, which selectively utilizes saturated acyl-CoAs [1,2]. Although it has been believed that all four active motifs (I–IV) of GPATs are exposed to the cytosol [1,2], recent

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studies showed that several active motifs of the 1-acylglycerol-3-phosphate acyltransferases (AGPATs), which are also members of the GPAT family, are located in the ER membrane or on the luminal side of the ER, where they can bind hydrophobic substrates including LPA, which has a long-chain fatty acid [11]. In addition, it has been reported that the catalytically active sites of 1,2-diacylglycerol acyltransferases-1 (DGAT1) are located on the luminal side of the ER, where they catalyze formation of TAG, resulting in the excretion of very low density lipoprotein from the liver [12,13]. A previous study from our laboratory reported that a portion of the GPAT2 protein population was detected in microsomal membrane fractions without mitochondria [9]. Thus, analysis of the membrane topology of GPAT2 would facilitate studies to elucidate the role of this protein in lipid metabolism. Here, we show that the GPAT2 protein contains two transmembrane segments. In addition, three active motifs (I–III) of this protein are exposed to the cytosol, while the remaining motif (IV) is within the membrane. These facts might help to distinguish the molecular basis of GPAT2 activity from that of other GPAT isoforms.

2. Materials and methods

2.1. Cell lines

Human embryonic kidney (HEK) 293 cells were used for this study. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.1% gentamicin.

2.2. Bioinformatics analysis

Bioinformatic on-line programs: SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) [14], PSORT II (http://psort.ims.u-tokyo.ac.jp/form2.html) [15], HMMTOP (http://www.enzim.hu/hmmtop/) [16], and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) [17] were used to predict transmembrane regions of the murine GPAT2 protein (GenBank Accession No. BAF03614).

2.3. Plasmid constructs

Construction of expression plasmids encoding murine GPAT1 or GPAT2 protein is described in the Supplementary methods.

2.4. Isolation of the membrane fraction from GPAT-transfected cells

HEK293 cells were transiently transfected with one of the GPAT expression plasmids (Fig. 1) or with an empty vector (pcDNA3.1/myc-His vector) using PolyFect transfection reagent (Qiagen, Valencia, CA). After 24 h, cells were washed with phosphate-buffered saline (including 137 mM NaCl, 2.7 mM KCl, and 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4) and scraped using a homogenizing buffer (called medium I) that included 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and 1 mM EDTA. The cells were homogenized in a Dounce homogenizer by 13 strokes of an A-type pestle, followed by centrifugation at 600*g* for 10 min to remove large debris and nuclei. The postnuclear supernatant (PNS) was further centrifuged at 100,000*g* for 60 min to separate the total membrane fraction. The membrane pellet was resuspended in medium I and used for the studies thereafter.

2.5. Carbonate extraction assay

The membrane sample was diluted 50-fold with either 1 M NaCl or 100 mM sodium carbonate buffer (pH 11.5) and incubated for 30 min at 0 °C as described by Fujiki et al. [18]. The suspensions were centrifuged at 4 °C for 60 min at 105,000*g*. The supernatants were concentrated to 5 μl by centrifugation using a MICROCON® centrifugal filter and tubes (YM-10; Millipore, Bedford, MA) at 14,000*g* for 30 min. Membrane pellets were gently rinsed twice with ice-cold medium I and dissolved in 5 μl of RIPA buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet-P40, and 2 mM EDTA). Samples (5 μl each) were mixed directly with SDS sample buffer for immunoblot analysis.

2.6. Protease protection assay

Protease protection assays of the membrane sample were performed as described previously [19] with some modifications. The total membrane sample from HEK293 cells was incubated in the presence or absence of trypsin (50 μg/ml) at 37 °C. In some cases, Triton X-100 was added (final concentration 1%) before addition of trypsin. After 60 min, trypsin inhibitor (40 mg/ml) was added to each sample to stop the reaction. Each sample was directly mixed with SDS sample buffer for immunoblot analysis.

2.7. Immunoblotting

Equal amounts of the sample proteins were denatured by boiling for 5 min in SDS sample buffer containing 1% beta-mercaptoethanol. The proteins were then electrophoresed on an SDS–polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore). The membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h and incubated with an anti-*myc* tag rabbit polyclonal antibody (anti-*myc* antibody 1, 1:500, Cell Signaling Technology, Inc., MA, USA), anti-*myc* tag mouse monoclonal antibody (anti-*myc* antibody 2, 1:1000, Santa Cruz Biotechnology, Inc., CA, USA), anti-FLAG tag mouse monoclonal antibody (1:500, Sigma, St. Louis, MO, USA), anti-calnexin rabbit polyclonal antibody (1:2000, Santa Cruz Biotechnology Inc.), or anti-Bip/GRP78 mouse monoclonal antibody (1:250, BD Transduction Laboratories, Lexington, KY). Proteins were then visualized with an anti-mouse or an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Biosource International, Camarillo, CA) using an ECL plus Western Blotting Detection System (Amersham Pharmacia Biotech, Aylesbury, UK).

3. Results

3.1. Expression of exogenous GPAT proteins in HEK 293 cells

Each of the GPAT-encoding plasmids (Fig. 1) was transfected into HEK293 cells and exogenously expressed GPAT proteins in the PNS fraction were detected by immunoblotting using an anti-FLAG or anti-*myc*-epitope tag antibody. As shown in Fig. 2A, immunoblotting with the anti-*myc*-epitope tag antibody 1 detected the C-terminal *myc*-fused GPAT proteins (GPAT1-M, GPAT1-FM, GPAT2-M, and GPAT2-FM), while the anti-FLAG tag antibody detected the N-terminal FLAG-fused GPATs (GPAT1-FM and GPAT2-FM). The anti-*myc*-epitope tag antibody 1 could also be used to detect GPATs with a *myc*-epitope tag between the (predicted) transmembrane segments (GPAT1-m1, GPAT2-m1, GPAT2-m2, and GPAT2-m3), although the detection level was relatively low for the GPAT2-m1 protein (Fig. 2B and C). Alternatively, an anti-*myc*-epitope tag antibody 2 was used for robust detection of GPAT2-m1 (Fig. 2C).

3.2. GPAT2 is a membrane-bound protein

Sodium carbonate extraction was performed to determine whether GPAT2 is a membrane-bound protein. Treatment of