Membrane Topology of the Murine Fatty Acid Transport Protein 1

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The murine fatty acid transport protein (FATP1) was identified in an expression cloning screen for proteins that facilitate transport of fatty acids across the plasma membranes of mammalian cells. Hydropathy analysis of this protein suggests a model in which FATP1 has multiple membrane-spanning domains. To test this model, we inserted a hemagglutinin epitope tag at the amino terminus or a FLAG tag at the carboxyl terminus of the FATP1 cDNA and expressed these constructs in NIH 3T3 cells. Both tagged constructs produce proteins of the expected molecular masses and are functional in fatty acid import assays. Indirect immunofluorescence studies with selective permeabilization conditions and protease protection studies of sealed membrane vesicles from cells expressing epitope-tagged FATP1 were performed. These experiments show that the extreme amino terminus of tagged FATP1 is oriented toward the extracellular space, whereas the carboxyl terminus faces the cytosol. Additionally, enhanced green fluorescent protein fusion constructs containing predicted membrane-associated or soluble portions of FATP1 were expressed in Cos7 cells and analyzed by immunofluorescence and subcellular fractionation. These experiments demonstrate that amino acids 1–51, 52–100, and 101–190 contain signals for integral association with the membrane, whereas residues 258–313 and 314–475 are only peripherally membrane-associated. Amino acid residues 191–257 and 476–646 do not direct membrane association and likely face the cytosol. Taken together, these data support a model of FATP1 as a polytopic membrane protein with at least one transmembrane and multiple membrane-associated domains. This study provides the first experimental evidence for topology of a member of the family of plasma membrane fatty acid transport proteins.

Import of free long chain fatty acids (LCFAs) into cells is a function central to the normal physiology of myocytes and adipocytes, which metabolize and store these molecules, respectively. LCFAs are taken up across the plasma membrane of these cells efficiently despite low physiologic concentrations of unbound free LCFAs in human serum (low nanomolar range) (1, 2). Developmental and pathophysiological regulation of fatty acid utilization in a variety of tissues in accordance with nutritional and hormonal signals suggests that protein-mediated mechanisms exist for regulated movement of LCFAs across the plasma membrane. Experiments demonstrating that LCFA uptake is saturable and inhibited by prior protease treatment of the cell surface are consistent with a protein-mediated mechanism of LCFA transport (3–6). Although biophysical and cellular studies show that LCFAs are capable of traversing the membranes by simple flip-flop (7–9), such a mechanism is likely to contribute significantly to fatty acid permeation primarily at high concentrations of fatty acids, such as may occur in pathophysiological states. On the other hand, protein-mediated permeation is likely to be important under conditions of low LCFA concentrations.

In previous work we identified the 63-kDa fatty acid transport protein 1 (FATP1) as a protein that facilitates the import of LCFAs when expressed in mammalian cells (10). Although CD36 and mitochondrial aspartic aminotransferase were identified on the basis of their ability to bind LCFAs and subsequently shown to participate in fatty acid transport, FATP1 was isolated in an expression cloning screen for functional fatty acid import. FATP1 is expressed at high levels in adipose and muscle tissues and is a member of a large family of related proteins that are expressed in different tissues. Thus far, six human FATPs and numerous orthologs in organisms as diverse as Caenorhabditis elegans and Saccharomyces cerevisiae have been identified on the basis of sequence conservation (11). FATPs share no significant sequence identity with other proteins that play a role in lipid transport across membranes such as CD36, mitochondrial aspartic amino transferase, and ABC transporters (e.g. Pat1p or Pat2p).

The mechanism of action of FATP1 is not well understood. FATP1 is an integral membrane protein that localizes to the plasma membrane of adipocytes, where it co-distributes with long chain acyl-CoA synthetase (10, 12). These findings are consistent with the hypothesis that transport of LCFAs into mammalian cells is coupled to esterification of imported LCFAs at the inner leaflet of the plasma membrane. This proposed mechanism is analogous to the well characterized fatty acid transport system in Escherichia coli (13), in which the outer membrane protein, FadL, binds and transports LCFAs. Imported substrates are esterified upon transport across the inner membrane by a membrane-associated acyl-CoA synthetase, FadD. The combined actions of FadL and FadD transport and metabolically trap the imported LCFAs, creating a concentration gradient favorable for LCFA import. Whether FATP1 functions as a transporter or a protein that binds fatty acids at the plasma membrane remains unresolved. FATP1 contains an AMP-binding motif that is essential for transport function (14).
and has 40% overall sequence identity with a purified peroxisomal very long chain acyl-CoA synthetase (15). A number of studies have suggested that FATP1 has very long chain acyl-CoA synthetase activity (16–18). However, knockout and overexpression experiments may be complicated by compensatory metabolic regulation. In the absence of purified FATP1, it is also not possible to rule out co-fractionation of a distinct esterification enzyme because of physical association of transport and esterification proteins. The observation that FATP1 facilitates import of long chain substrates, whereas very long chain acyl-CoA synthetase has no activity toward these shorter fatty acid sequences, has led to the proposal that FATP1 may have independent transport and esterification functions (19).

Structure-function studies of FATP1 will help elucidate the mechanism of action of this protein in LCFA import. The amino acid sequence of FATP1 contains many hydrophobic stretches, consistent with multiple (four to six) membrane spanning domains (Fig. 1). However, the topology of FATP1 is difficult to predict from the primary sequence, given that hydrophobic domains in this protein may indicate regions of FATP1 that interact with LCFAAs instead. The goal of the present study is to characterize the membrane topology of FATP1, which will assist in understanding the structure and function of this protein. Using a combination of approaches, we show that FATP1 is oriented such that the extreme amino terminus faces the extracellular space, whereas the carboxyl terminus faces the cytosol with several intervening membrane-associated domains. These results are discussed in the context of FATP1 function.

EXPERIMENTAL PROCEDURES

Materials—We obtained mouse monoclonal anti-FLAG antibody (M2) from Sigma-Aldrich Inc., mouse monoclonal anti-HA antibody (HA.11) from Covance Inc., affinity-purified rabbit polyclonal antisera against Grp78 (BiP, PA1–014) from Affinity Bioreagents Inc., rabbit polyclonal antisera against protein disulfide isomerase (PDI, SPA-890) from StressGen Biotechnologies Corp., mouse monoclonal anti-tubulin antibody (DM 1A) from Sigma-Aldrich Inc., mouse monoclonal anti-GFP antibody (8362–1) from CLONTECH Laboratories, saponin from Sigma-Aldrich Inc., paraformaldehyde from Electron Microscopy Sciences, fibronectin-coated glass coverslips from Becton Dickinson Biosciences, ALEXA 488-coupled anti-mouse and anti-rabbit IgGs and Prolong anti-fade mounting reagent from Molecular Probes, ALEXA 488-coupled anti-mouse and anti-rabbit IgGs and Prolong anti-fade mounting reagent from Molecular Probes Inc., proteinase K from Roche Molecular Biochemicals, horseradish peroxidase (HRP-coupled and gold-coupled IgGs from Jackson ImmunoResearch Laboratories, Inc., and Renaissance Western blot chemiluminescence reagents from PerkinElmer Life Sciences. Antibodies to native FATP1 sequences were generated as described previously (10, 14).

Plasmids—Polymerase chain reaction was used to insert amino-terminal HA and carboxyl-terminal FLAG tags into the ΔU3FATP1 retroviral vector (14). The Gene Editor site-directed mutagenesis kit (Promega) was used to introduce internal HA tags (at residues 54, 138, 190, 285, 313, 354, 430, 473, and 559) and glycosylation mutations (A54N, G75N, C48N, D97N, G199N, and V262N) into the cDNA of FATP1 in the vector pcDNA3.1(−/−) (Invitrogen). All polymerase chain reaction- and Gene Editor-generated sequences were confirmed by automated sequencing. Polymerase chain reaction was also used to generate fragments of FATP1 (amino acid residues 1–51, 1–646, 52–100, 101–190, 191–257, 258–313, 314–475, 476–550, 476–646, and 550–646), which were cloned into the EcoRI and BamHI sites of the vector pC2 MCS (Clontech).

Cell Culture and Expression of Full-length and Partial FATP1 Sequences—NIH 3T3 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium with 10% calf serum supplemented with 2 mM l-glutamine, 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate. Cos7 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum supplemented with 2 mM l-glutamine, 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate. Cos7 cells were transfected with plasmid DNA using LipofectAMINE Plus or LipofectAMINE 2000 reagents (Life Technologies, Inc.) according to the manufacturer’s protocols. 293GPG cells (20) were grown in Dulbecco’s modified Eagle’s medium with 10% inactivated fetal bovine serum supplemented with 2 mM l-glutamine, 50 units/ml penicillin G sodium, 50 units/ml streptomycin sulfate, 0.3 mg/ml G418, 1 μg/ml puromycin, and 1 μg/ml tetracycline. Transient transfections of 293GPG cells and harvest of viral supernatants were performed to generate high titer vesicular stomatitis virus G-glycoprotein pseudotyped retrovirus as described previously (20). 108 NIH 3T3 cells were transduced in a 35-mm well by two successive 7-h exposures to 1 ml of ΔU3FATP1 retrovirus. Parallel transduction with a virus encoding b-galactosidase indicated transduction efficiency of ∼90%. For immunofluorescence studies, the cells were grown 24–28 h in fibronectin-coated glass coverslips.

Fatty Acid Uptake Assays—Parental and retrovirally transduced NIH 3T3 cells were assayed for fatty acid uptake as described previously (14). Flow cytometric analysis of samples of 105 cells was carried out in triplicate using a Becton Dickinson FACScan.

Isolation of Sealed Membrane Vesicles—Sealed microsomal membrane vesicles were prepared as described previously (21). All manipulations were carried out at 4°C. Monolayers of fibroblasts (∼106 cells) were washed in phosphate-buffered saline (PBS), scraped and pelleted, and resuspended in Buffer B (10 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM EGTA, 1 μM protease complete (Roche Molecular Biochemicals), 1 mM PMSF, 1 μM dithiothreitol). The cells were homogenized by 20 strokes in a glass/Teflon homogenizer and centrifuged at 1000 × g for 10 min, and the nuclei and unbroken cell pellet were discarded. Membrane vesicles were pelleted by centrifugation at 356,000 × g for 30 min, washed in Buffer A (10 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM NaCl), and resuspended in buffer A. Membranes were homogenized by six passes through a 26-gauge needle. Protein was quantified by BCA (Pierce).

Proteolysis—Sealed membrane vesicles (50 μg of protein) were incubated with freshly prepared proteinase K (final concentration, 0.008–
0.2 mg/ml) for 30 min on ice. The reactions were stopped by the addition of 5 mM PMSF and 1 μM leucine, sample buffer. The samples were boiled for 5 min and immediately separated by SDS-PAGE.

Protein Isolation—For Western blot analysis of protein expression in FATPnHA- and FATPcFLAG-expressing cells, total post-nuclear membrane fractions were isolated as described (22). For subcellular fractionation, transfected cells were disrupted using a glass/Teflon homogenizer in sucrose homogenization buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 255 mM sucrose, 1 μM PMSF, 1 μM protease complete) or sucrose homogenization buffer (20 mM Na2CO3, pH 11.5, 1 mM EDTA, 255 mM sucrose, 0.6 μM PMSF, 1 μM protease complete) (10).

Membrane and soluble proteins were separated by centrifugation at 35,000 × g for 30 min. Proteins were quantified by BCA.

Western Blot Analysis—Proteins were separated by 7.5% or 10% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell; 0.2-μm pore). Primary antibodies for Western analysis were used at the following dilutions: anti-FATP 628–640 (1:2500), anti-FATP 455–470 (1:2500), anti-HA (1:2000), and anti-PDI (1:1000). Detection was performed using HRP-coupled IgG and Renaissance Western blot chemiluminescence reagents (PerkinElmer Life Sciences).

Glycosylation Analysis—Proteins were synthesized in vitro transcription/translation using the TNT Quick Coupled Transcription/Translation System (Promega) in the presence of canine pancreatic microsomes (Promega) and [35S]methionine according to the manufacturer's instructions. As a control for glycosylation, a parallel transcription/translation of CD36 was performed in all experiments. The proteins were digested overnight with N-glycosidase F (Roche Molecular Biochemicals) according to the manufacturer's recommended protocol. The proteins were separated by 7.5% SDS-PAGE, and the gels were fluorographed and analyzed by autoradiography.

Immunofluorescence—For live cell immunofluorescence, all staining was performed at 4°C. The cells were washed with PBS containing 1% fatty acid-free bovine serum albumin (PBS-BSA), blocked by incubation with normal goat IgG in PBS-BSA (200 μg/ml), and incubated with primary antibody for 1 h (20 μg/ml). The cells were washed with PBS, incubated with ALEXA-coupled secondary antibody (5 μg/ml), washed, fixed with 4% paraformaldehyde for 10 min, and mounted on glass slides using Prolong anti-fade mounting solution (Molecular Probes). For fixed cell immunofluorescence, the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, washed, and permeabilized with 0.25% Triton X-100 or 20 μg/ml saponin for 10 min. The cells were washed with PBS, blocked with PBS-BSA containing normal goat IgG (200 μg/ml), and incubated with primary antibodies (anti-HA, 1:250; anti-FLAG, 1:1000; antitubulin, 1:1000; and anti-FATP (455–470), 1:1000). The cells were washed, incubated with secondary antibody (1:250), washed, and mounted using Prolong anti-fade mounting solution. Microscopy was performed using a Zeiss Axioskop microscope, and digital images were obtained on a Spot camera (Advanced Spot software, Diagnostic Instruments).

Electron Microscopy—The cells were removed from culture dishes with 5 mM EDTA, washed in PBS, pelleted, and fixed with 2% paraformaldehyde/glutaraldehyde in PBS (pH 7.2) for 1 h at room temperature for 20 h. Cell pellet was embedded in 10% gelatin and processed for ultramicrotomy (23). Ultrathin sections were labeled with monoclonal anti-HA antibody (1:100) for 2 h, followed by 18-nm gold, goat anti-mouse IgG for 1 h. After washing, the sections were stained with uranyl acetate and embedded in methyl cellulose (23). Specimens were viewed and photographed using a Zeiss 902 electron microscope.

RESULTS

Amino- and Carboxyl-terminal Epitope-tagged FATP1 Constructs Function in LCFA Import—To define the topology of the amino and carboxyl termini of FATP1, we generated FATP1 constructs in the ΔU3 retroviral vector with either an HA tag at the amino terminus (FATPnHA) or a FLAG tag at the carboxyl terminus (FATPcFLAG) (Fig. 2A). These constructs were used to generate retroviruses and transduce NIH 3T3 cells. As a control, NIH 3T3 fibroblasts were transfected in parallel with a retroviral construct for wild-type FATP1. Each tagged construct produces a protein of the expected molecular mass (Fig. 2B). The levels of expression of these constructs are comparable with the levels of wild-type FATP1. To demonstrate that the tagged constructs are functional, we assayed parental and transfected cells for LCFA uptake. Both epitope-tagged constructs function to facilitate fatty acid import (Fig. 2C). Though FATPnHA function is comparable with wild-type FATP1 in this assay, the addition of a FLAG tag at the carboxyl terminus diminishes fatty acid import 35% compared with wild-type FATP1.

FATP1 Amino Terminus Faces the Extracellular Space, Whereas FATP1 Carboxyl Terminus Faces the Cytosol—To determine the orientation of these terminal epitope tags in the membrane, we performed indirect immunofluorescence studies of fibroblasts expressing FATPnHA or FATPcFLAG. After fixation with 4% paraformaldehyde, the cells were selectively...
permeabilized with saponin or Triton X-100 (Figs. 3 and 4). In each experiment, control staining for a cytosolic protein, tubulin, and for a resident endoplasmic reticulum (ER) luminal protein, BiP, demonstrate that treatment of cells with saponin at 20 μg/ml permeabilizes the plasma membrane but not the ER, whereas treatment with 0.25% Triton X-100 permeabilizes the plasma membrane and the ER. To evaluate the orientation of the carboxyl terminus of FATP1, FATPcFLAG-expressing cells were fixed, permeabilized, and stained with an antibody to the FLAG epitope tag (Fig. 3). FATPcFLAG-expressing cells show staining in an ER and reticular pattern after saponin or Triton X-100 permeabilization. A similar pattern of staining is observed using antibody directed against FATP1 residues 628–640 in NIH 3T3 cells expressing untagged FATP1 (data not shown). These findings are consistent with a cytosolic orientation of the FLAG epitope and the FATP1 carboxyl terminus. The prominent ER pattern observed when FATP1 is overexpressed in cultured cells likely reflects inefficient targeting of the overexpressed protein to the plasma membrane. To evaluate the orientation of the amino terminus of FATP1, FATPnHA-expressing cells were fixed, permeabilized, and stained with an antibody to the amino-terminal HA epitope tag after saponin permeabilization, whereas a comparable exposure of cells stained after Triton X-100 permeabilization shows abundant reticular staining. These findings are consistent with a luminal orientation of the HA epitope tag and the amino terminus of FATP1.

In addition, we stained live FATPnHA- and FATPcFLAG-expressing cells to further evaluate the location of the amino and carboxyl termini of FATP1 (Fig. 5). Although the FLAG-tagged FATP1 is abundantly expressed, αFLAG antibody does not stain in live cells, consistent with a cytosolic orientation of the FATP1 carboxyl terminus. By contrast, HA staining is observed on the surface of live FATPnHA cells, consistent with an orientation of the amino-terminal residues toward the extracellular space. The intensity of plasma membrane staining of live FATPnHA cells is small compared with the staining of fixed permeabilized cells, consistent with inefficient trafficking of the overexpressed protein to the plasma membrane. Localization of FATPnHA at the plasma membrane of these cells is also observed by immunoelectron microscopy (Fig. 6). Gold particles are associated with the outer aspect of the plasma membrane, consistent with localization of the HA epitope at the extracellular leaflet of the plasma membrane. Staining was also observed on intracellular membranes in FATPnHA cells (data not shown). The staining in these cells was specific because it was not observed in the parental NIH 3T3 cells.

To confirm the orientation of the termini of FATP1, we carried out protease protection studies on sealed membrane vesicles from cells expressing FATPnHA (Fig. 7). These microsomes are closed vesicles with a cytoplasmic-side-out orientation (24). Protease treatment selectively digests proteins that face the cytosol. Protein epitopes that reside within the membrane, the ER lumen (in the secretory pathway), or the extracellular space (at the plasma membrane) are protected from digestion in the absence of detergent. Upon trypsin treatment of the vesicles, full-length FATP1 is digested to yield a 37-kDa protected polypeptide. This fragment is detected by Western blot analysis using an HA antibody, but not antibodies directed against FATP1 residues 628–40 that lie close to the carboxyl terminus. Control Western blots using an antibody to the luminal PDI show that vesicles remain sealed at the trypsin concentrations used. The HA epitope and PDI are not inherently protease-resistant, because they are digested when membrane are disrupted with detergent prior to trypsin treatment. We have also observed similar patterns of protease sensitivity in 3T3L1 adipocytes expressing FATPnHA (data not shown). These results are consistent with a topology model in which the amino terminus of FATP1 is either imbedded within the mem-
brane or faces the extracellular/vesicular space, and the carboxyl terminus faces the cytosol.

**Amino Acids 455–470 of FATP1 Face the Cytosol**—To evaluate an internal region of FATP1 between the amino and carboxyl termini, we performed protease protection and immunofluorescence studies of FATPnHA-expressing cells using an antiserum raised against FATP1 amino acid residues 455–470. Western blot analysis of trypsin-treated membrane vesicles shows that this epitope is readily degraded by treatment with trypsin at concentrations that leave the lumenal PDI intact (Fig. 7). These results suggest a cytosolic location for residues 455–470. We also affinity-purified the antiserum directed against FATP1 residues 455–470 and used it to stain fixed permeabilized cells (Fig. 8). A reticular pattern of staining is observed, consistent with FATP1 membrane association. Because this antiserum stained cells permeabilized with either saponin or Triton X-100, amino acids 455–470 are likely to face the cytosol. These findings are in agreement with selective permeabilization immunofluorescence studies.

**Native and Inserted Glycosylation Sites in FATP1 Are Not Utilized**—In another approach to map the topology of FATP1, we evaluated the glycosylation status of native and inserted asparagine-linked (N-linked) glycosylation sites in FATP1. Glycosylation of such sites would indicate an orientation toward the luminal/extracellular space. The native FATP1 sequence contains potential N-linked glycosylation sites at residues 330, 393, and 518. We examined FATP1 produced by in vitro transcription/translation (in a rabbit reticulocyte lysate in the presence of [35S]methionine and dog pancreatic microsomes), FATP1 overexpressed in fibroblasts, and FATP1 natively expressed in adipocytes. When treated with N-glycosidase F and analyzed by SDS-PAGE, FATP1 shows no shift in electrophoretic mobility compared with nonglycosidase treated samples (data not shown). Thus potential N-linked glycosylation sites in the native FATP1 sequence are not utilized. Additionally, we generated seven full-length FATP1 glycosylation mutants, each of which contained one or two amino acid substitutions to create an N-linked glycosylation consensus sequence in a hydrophilic stretch of the sequence (A6N, G75N, C48N, D97N, R163S, G199N, and V262N). The constructs were sequenced to confirm fidelity of the mutated region, and each mutant was tested by in vitro transcription/translation in the presence of dog pancreatic microsomes. The synthesized proteins were treated with N-glycosidase F and analyzed by SDS-PAGE and autoradiography. For each mutant, a 63-kDa protein was produced whose mobility did not change upon deglycosylation, indicating that the mutant sites were not utilized (data not shown). Lack of glycosylation of native and inserted consensus sites suggests that these regions face the cytosol, lie within a membrane segment, or are located too close to the luminal/extracellular space.

**Fig. 5. Orientation of FATP1 termini as determined by live cell immunofluorescence.** NIH 3T3 cells expressing FATPnHA (A and B), NIH 3T3 cells expressing FATPcFLAG (E and F), and parental NIH 3T3 cells (C, D, G, and H) were stained live at 4°C (B, D, F, and H) or were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 prior to staining (A, C, E, and G). Primary anti-HA (A–D) or anti-FLAG (E–H) antibodies were used with ALEXA488-coupled secondary antibodies. Bar, 10 μm.

**Fig. 6. Immunoelectron micrographs of plasma membrane FATP1.** NIH 3T3 cells expressing FATPnHA were fixed, embedded in gelatin, infused with sucrose, and sectioned. The sections were immunolabeled using primary α-HA and secondary 18-nm gold-coupled antimouse IgG. Shown is the periphery of two representative cells with distinct labeling at the extracellular aspect of the plasma membrane (arrows). Bars, 200 nm.
to a membrane-spanning domain to support glycosylation.

**Reporter Constructs Define FATP1 Membrane-associated Domains**—Given the ability of terminally tagged FATP1 constructs to function in LCFA uptake and to serve as topology reporters, we attempted to refine our topology model by insertion of tags in internal hydrophilic segments of FATP1. This approach has also been used for topology mapping of a number of polytopic membrane proteins, and data from functional, full-length tagged proteins are considered reliable when insertion of the tag does not compromise protein function. We inserted the 9-amino acid HA epitope tag into seven hydrophilic stretches in FATP1 (at residues 54, 138, 190, 285, 313, 354, 430, 473, and 559) that are predicted to lie outside the membrane. These constructs were transfected into Cos7 cells. Western blot analysis revealed that only four of the insertion mutants (HA-191, HA-285, HA-430, and HA-473) were expressed stably in dog pancreatic microsomes produced fusion proteins of the predicted molecular masses that did not change upon treatment with N-glycosidase F (data not shown). The observation that none of the glycosylation sites is used suggests that hydrophilic regions at amino acids 90, 293, 520, and 646 face the cytosol or lie too close to membrane segments to support glycosylation.

Polytopic membrane proteins are initially targeted to the ER by a signal sequence that may form the first transmembrane domain, and subsequent transmembrane domains are thought to contain information that directs insertion and contributes to topogenesis (26). We thus sought to determine whether hydrophobic segments of FATP1 contain sufficient information to direct membrane association. We generated a series of chimeric proteins in which hydrophobic predicted membrane-associated segments (1–51, 101–190, 258–313, and 475–550) and hydrophilic predicted nonmembrane associated domains (52–100, 191–257, 314–475, and 550–646) were fused in-frame downstream of EGFP. These constructs were expressed transiently in Cos7 cells and evaluated for membrane association.

Immunofluorescence of fixed cells demonstrates that the EGFP-1–646 chimera containing full-length FATP1 is membrane-associated with an ER and reticular staining pattern (Fig. 9). A similar pattern is observed with EGFP-1–51, containing the putative FATP1 signal sequence, suggesting that these amino-terminal residues are sufficient to direct membrane association. Chimeras containing residues 52–100, 101–190, 258–313, and 314–475 are all membrane-associated and excluded from the nucleus. Residues 52–100 appear to direct association of the chimera to small perinuclear vesicular en-
These findings were confirmed by subcellular fractionation. Transfected Cos7 cells were homogenized, separated into membrane and soluble fractions by differential density centrifugation, and analyzed by Western blot using an antibody directed against GFP (Fig. 10A). When this fractionation was performed using sucrose homogenization buffer, pH 7.4, most EGFP localized to the soluble fraction (lanes 3 and 4). Similarly, chimeras containing residues 191–257 and 476–646 are largely soluble. The relatively small amount of signal in the membranes of cells expressing these constructs as well as the EGFP control likely represents incomplete separation. By contrast, chimeras containing residues 1–464 (full length), 1–51, 52–100, 101–190, 258–313, and 314–475 are predominantly membrane-associated. These findings are in agreement with immunofluorescence data and suggest that segments of FATP1 from amino acids 1–51, 52–100, 101–190 258–313, and 314–475 are each individually sufficient for membrane association.

To characterize the membrane association of these chimeric reporter constructs, fractionation was also performed in a carbonate buffer at pH 11.5. Under these conditions, peripheral membrane proteins should be solubilized and only integral membrane proteins should remain membrane-associated in the pellet fraction. After alkaline extraction, chimeras containing FATP residues 1–464 (full-length), 1–51, 52–100, and 101–190 remain predominantly membrane-associated, consistent with integral membrane association (Fig. 10B). Although chimeras containing FATP1 residues 258–313, and 314–475 are membrane-associated at neutral pH, they are partially extracted from the membrane under extreme alkaline conditions and are thus likely to represent peripherally membrane-associated domains. Taken together, these findings suggest that FATP1 contains three integral membrane domains and two peripheral membrane-associated domains. There are two large stretches of non-membrane-associated residues, one in the central region of the molecule and one containing the carboxyl terminus.

**DISCUSSION**

The data presented in this study provide the first experimental evidence for the topology of FATP1. In the absence of x-ray crystallographic data for this integral membrane protein, we have used several indirect approaches to characterize the topology of FATP1. First, we have inserted epitope tags at the termini of full-length and truncated FATP1 molecules and a crystallographic model in which no significant regions of FATP1 face the extracellular/lumenal space after the amino terminus. The amino-terminal 51 amino acids are sufficient to target FATP1 to membrane structures and thus contain a functional signal se-
sequence. This signal sequence is not cleaved. Within this region, the secondary structure of residues 1–28 are strongly predicted to assume a helical configuration (Predictprotein) (27). FATP1 also contains internal uncleaved sequences that direct targeting of membrane-associated domains that include amino acids 52–100, 101–90, 258–313, and 314–475. The region containing amino acids 1–190 directs integral membrane association, whereas the region containing residues 258–475 directs peripheral membrane association. Residues 191–257 are not membrane-associated and likely face the cytosol. This region contains IYTSGTTG\textsubscript{X}PK, which is critical for ATP binding and FATP1 function. A 170-amino acid stretch at the carboxyl terminus does not contain sequences to direct it to the membrane, and within FATP1, this region likely projects into the cytosol where it may serve as a platform for protein-protein interactions. Finally, our protease protection studies demonstrate that a 37-kDa fragment containing the amino terminus is protease-resistant in part because it lies close to the membrane. A highly compact structure might also be intolerant of even small internal epitope tag insertions.

The membrane topology of FATP1 does not resemble those of polytopic membrane transporters for hydrophilic substrates. Many classical transporters are predicted to have transmembrane domains consisting of primarily α-helical structures of 17 or more amino acids that span the phospholipid bilayer. Depending on the specific transporter, between 4 and 12 transmembrane domains are thought to form a three-dimensional channel through which substrate passes. Although FATP1 has multiple membrane-associated domains, only the amino-terminal 28 residues are strongly predicted to form an α-helical structure. Moreover, there is no evidence that regions after the amino terminus extend completely through the bilayer and are exposed to the extracellular space. The amino-terminal 190

\begin{figure}[h]
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\caption{Subcellular fractionation of EGFP-FATP1 constructs expressed in Cos\textsuperscript{7} cells. Cos\textsuperscript{7} cells were transfected with fusion constructs or EGFP alone. The cells were disrupted 48 h after transfection in sucrose homogenization buffer (A) or in carbonate buffer, pH 11.5 (B). Membrane and soluble proteins were separated by differential density centrifugation. Membrane and soluble proteins (5 μg of each) were separated by 10% SDS-PAGE and analyzed by Western blot using primary αGFP antibody. Bands were detected using HRP-coupled IgG and chemiluminescence. These results are representative of four independent experiments.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Fig11.png}
\caption{Proposed topology model for FATP1. Based on protease protection, immunofluorescence, and EGFP fusion protein studies, we predict that the amino terminus of FATP1 is oriented toward the extracellular space, whereas the carboxyl terminus is cytosolic. Amino acid residues 1–190 of FATP1 are integrally associated with the membrane. Amino acid residues 190–257 that are not membrane-associated, contain the AMP-binding motif (shaded rectangle) and are oriented toward the cytosol. Amino acid residues 258–475 are peripherally associated with the inner leaflet of the plasma membrane. Amino acid residues 476–646 are not membrane-associated and project into the cytosol. Arrowheads and numbers indicate the approximate positions of amino acid residues.}
\end{figure}
Site-directed mutagenesis of FATP1, evaluation of candidate regions for protein-protein interactions, and construction of chimeras of FATP1 with other family members will be guided by this model. Studies to characterize the structure and function of FATP1 will further our understanding of LCFA transport, which is central to normal cellular physiology. Moreover, accumulation of LCFA in nonadipose cells is thought to contribute importantly to skeletal muscle insulin resistance (34), cardiomyopathy (22, 35), and pancreatic beta cell failure (36) in non-insulin-dependent diabetes mellitus. Structure-function characterization of proteins involved in LCFA transport will help elucidate mechanisms that may play a role in human diseases in which lipid homeostasis is perturbed.

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