The fatty acid transport protein (FATP1) is a very long chain acyl-CoA synthetase.

The primary sequence of the murine fatty acid transport protein (FATP1) is very similar to the multigene family of very long chain (C20-C26) acyl-CoA synthetases. To determine if FATP1 is a long chain acyl coenzyme A synthetase, FATP1-Myc/His fusion protein was expressed in COS1 cells, and its enzymatic activity was analyzed. In addition, mutations were generated in two domains conserved in acyl-CoA synthetases: a 6-amino acid substitution into the putative active site (amino acids 249–254) generating mutant M1 and a 59-amino acid deletion into a conserved C-terminal domain (amino acids 464–523) generating mutant M2. Immunolocalization revealed that the FATP1-Myc/His forms were distributed between the COS1 cell plasma membrane and intracellular membranes. COS1 cells expressing wild type FATP1-Myc/His exhibited a 3-fold increase in the ratio of lignoceryl-CoA synthetase activity (C24:0) to palmitoyl-CoA synthetase activity (C16:0), characteristic of very long chain acyl-CoA synthetases, whereas both mutant M1 and M2 were catalytically inactive. Detergent-solubilized FATP1-Myc/His was partially purified using nickel-based affinity chromatography and demonstrated a 10-fold increase in very long chain acyl-CoA specific activity (C24:0/C16:0). These results indicate that FATP1 is a very long chain acyl-CoA synthetase and suggest that a potential mechanism for facilitating mammalian fatty acid uptake is via esterification-coupled influx.

The murine fatty acid transport protein (FATP1) was identified and cloned by Schaffer and Lodish (1) from a 3T3-L1 adipocyte cDNA expression library and is localized to the plasma and other membranes of adipocytes and other target tissues such as brain, skeletal muscle, heart, and kidney (1). The FATP1 gene is conserved widely in biology from bacteria to mammals and is one of several putative transporters of fatty acids (1–4). Factors that control FATP1 gene expression have been investigated by a number of laboratories and reveal regulation by several effector systems: up-regulation during preadipocyte differentiation (1, 5) by peroxisome proliferator-activated receptors (6–8) and by fasting (5) and down-regulation by insulin (9). Despite a growing body of knowledge relating to the control of FATP1 gene expression, studies on the FATP1 protein and its mechanistic role in fatty acid uptake have been limited (10).

FATP1 exhibits broad-based amino acid similarity to a family of very long chain acyl-CoA synthetases but exhibits only limited sequence similarity to the multigene family of long chain acyl-CoA synthetases. Disruption of Saccharomyces cerevisiae FAT1, the yeast homologue to mammalian FATP1 (2), results in decreased fatty acid uptake, a substantial reduction in very long chain fatty acyl-CoA synthetase activity, and the accumulation of very long chain fatty acids (11, 12). Moreover, in animal cells, fatty acid uptake is diminished in cell lines overexpressing FATP1 if either endogenous ATP levels are depleted or if FATP1 is mutated (S250A) at a putative covalent AMP binding site (10). Since the mechanism of fatty acid activation with coenzyme A requires the formation of an enzyme-adenylate intermediate and uptake is linked to the presence of the AMP binding site, we hypothesized that FATP1 may be a plasma membrane very long chain acyl-CoA synthetase.

To test the hypothesis that FATP1 is a plasma membrane very long chain acyl-CoA synthetase, COS1 cells were transfected with an epitope-tagged FATP1 cDNA construct. In addition, two mutant forms of FATP1 were also epitope-tagged and expressed: a 6-amino acid substitution mutation from amino acids 249–254, which encompasses the putative catalytic site, and a 59-amino acid deletion within the carboxyl region of the protein in a domain highly conserved in acyl-CoA synthetases. Expression was verified by Western analysis, and localization of FATP1 to the plasma membrane and intracellular membranes was confirmed by immunofluorescence. Enrichment of FATP1 via affinity chromatography coincided with an increase in very long chain acyl-CoA specific activity. These results indicate that FATP1 is a very long chain acyl-CoA synthetase and suggest that fatty acid uptake in mammalian cells is mediated by esterification-coupled influx, similar to the mechanism used by bacteria (13, 14).

### EXPERIMENTAL PROCEDURES

**Construction of FATP1 cDNA Expression Plasmid**—The FATP1 cDNA was a generous gift of Jean Schaffer, Washington University, St. Louis, MO. A KpnI restriction site was engineered at the C terminus of FATP1 and used to subclone the coding region into pcDNA3.1-Myc/His (Invitrogen) to create pFATP1-Myc/His encoding a FATP1-Myc/His translational fusion protein under control of the cytomegalovirus promoter. To develop the M1 substitution mutation at amino acids 249–254 (TSGGTG), oligonucleotide-directed mutagenesis (5′-AGGCTTGGAGGGGAGGGGCGGGGCGGGCGCCCTCCAGATGATGTTAAGAAGCAGGATC-3′) was used to introduce a XhoI site and convert the sequence to LEAAAA. To develop the deletion mutant M2 at amino acids...
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464–523, two polymerase chain reaction products corresponding to the N-terminal and C-terminal sectors were generated using the following four primers:

N-5': GCCATACATGGGGCTCCTGGAGCAGGAAACA,
N-3': CCGTACTCGAGATCGAAACGCCGCAGAGGGTC,
C-5': CGGCGCTGAGGTCGACCTTGAGCCGC,
C-3': GTAGGATCCAGCAGCTAGTGAGGATC.

The resulting polymerase chain reaction products were ligated at the engineered XhoI site (found at the N-3' end and the C-5' end) and captured by polymerase chain reaction using the N-5' and C-3' primers. The amplified fragment was subsequently cloned into pcDNA3.1-Myc/His. Following transfection, R2G substitution found embedded within N-5' was corrected to the original wild type arginine at position 2. All cloning and polymerase chain reaction reactions were verified by DNA sequencing.

Viral-mediated DNA Transfection in COS1 Cells—Transfection of COS1 cells for either immunofluorescence or acyl-CoA synthetase activity measurements was performed using polylysine-coated adenovirus-mediated transfection (15). Replication-deficient adenovirus was polylysine-coated as described by Allgood et al. (15) and stored at -70 °C until use. For transfection, COS1 cells were plated in 10-cm plates (1.3 x 10⁶ cells) and grown overnight at 37 °C. Adenovirus (typically ~200 plaque-forming units/cell) was incubated with pFATP1-Myc/His (10 μg) at 25 °C for 20 min, and the virus/DNA mixture incubated with additional polylysine (125-fold molar to DNA) for an additional 30 min. The virus/DNA/polylysine mixture was delivered to the COS1 cells in 4-mL medium, and 2 h post-transfection, additional medium (4.0 mL) supplemented with fetal bovine serum (20%) was added. Forty-eight h post-transfection, cells were harvested for analysis.

Indirect Immunofluorescence of FATP-Myc/His in Transfected COS1 Cells—COS1 cells were plated on 13-mm cover slips (1.6 x 10⁶ cells/coverslip). Twenty-four h post-plating, cells were transfected with 0.5 μg/coverslip of either pFATP1-Myc/His or pcDNA3.1 β-galactosidase-Myc/His (Invitrogen) complexed to polylysine-coated adenovirus and allowed to grow for an additional 24 h. Cells were then fixed with formaldehyde and glutaraldehyde in the presence or absence of 0.01% Triton X-100 at 37 °C. After fixation, the cells were incubated with anti-Myc monoclonal antibodies (Invitrogen) for 2 h at 37 °C, washed with phosphate-buffered saline, and then incubated with fluorescein-labeled secondary antibody (Organon Teknika) for 1 h at 25 °C. Preparations were viewed using a Nikon Eclipse E800 photomicroscope equipped with brightfield phase and fluorescence optics including a 100-W mercury lamp epi-fluorescence illumination with standard fluorescence filter sets. Digital images were collected using a CoolCam liquid-cooled, three-chip color CCD camera (Cool Camera Company, Decatur, GA) and captured to a 486DX2 personal computer using Image Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD).

Western Blot Analysis—Protein concentrations (samples with Triton X-100) were determined by using bicinchoninic/copper sulfate protein assay (Pierce). SDS-PAGE analysis and normalization with reference to a bovine serum albumin standard. SDS-polyacrylamide gel electrophoresis of samples was followed by transfer of proteins to polyvinylidifluoride membranes (Millipore). Membranes were blocked in phosphate-buffered saline containing 0.05% Tween 20 and 10 mg/mL bovine serum albumin and probed with monoclonal anti-Myc horseradish peroxidase antibodies (Invitrogen). Blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech).

Affinity Column Enrichment of FATP1—COS1 cells were transfected using polylysine-coated adenovirus as described, and 48 h post-transfection, cells were harvested by centrifugation and immediately frozen in liquid nitrogen. The cell pellet was thawed in Buffer A (25 mM sucrose, 100 mM Tris-HCl, pH 8.0) and subjected to 4 sequential freeze/thaw cycles. Triton X-100 treatment (1% for 10 min at 4 °C, washed extensively in Buffer B). The bound protein was eluted with Buffer A supplemented with imidazole. Fractions were concentrated by centrifugation (Amicon 10), immediately frozen, and stored at -70 °C until further analyzed.

Acyl-CoA Synthetase Activity Assay—Samples were assayed for palmitoyl-CoA and lignoceryl-CoA synthetase activity by conversion of 14C-labeled palmitic acid (Amersham Pharmacia Biotech) or 3H-labeled lignoceric acid (American Radiochemicals) into their CoA derivatives as described previously (11). For solubilization of long chain and very long chain fatty acids, palmitic and lignoceric acids were dried under nitrogen and solubilized in 50 μL of α-cyclodextrin (10 mg/mL) before use.

RESULTS

pFATP1-Myc/His was transfected into COS1 cells using virus-mediated transfection, and protein expression was verified by Western analysis utilizing anti-Myc antibodies. FATP1-Myc/His exhibited a relative molecular mass of 73 kDa (Fig. 1), consistent with its predicted mass of ~70 kDa plus the Myc tag and polyhistidine tract. As expected, nontransfected cells showed no corresponding immune-reactive protein. There is no detectable endogenous FATP1 in COS1 cells as determined by Northern blotting of COS1 DNA using a FATP1 probe or by immunoblotting using a monoclonal anti-β-galactosidase antibody directed toward amino acids 192–215 of FATP1 (results not shown).

To determine the intracellular location of FATP1-Myc/His, immune localization was performed using anti-Myc monoclonal antibodies. Immunohistochemical analysis of COS1 cells transfected with either pFATP1-Myc/His or a pcDNA3.1 β-galactosidase-Myc/His control vector was carried out in the presence and absence of detergent (Fig. 2), as described under “Experimental Procedures.” The outer membrane of COS1 cells transfected with pFATP1-Myc/His was labeled (Fig. 2, panel A), indicating the presence of antigen on the cell surface. Subsequent dispersion of this immunofluorescent signal by detergent, as shown in the Triton X-100-treated cells (Fig. 2, panel B), coupled with the labeling in nondisrupted transfected COS1 cells (Fig. 2, panel A) substantiate the presence of some FATP1-Myc/His in the COS1 plasma membrane. However, as seen in Fig. 2, Triton X-100 treatment also revealed the presence of additional FATP1-Myc/His within internal membranes. This point demonstrates that FATP1-Myc/His is distributed broadly within cellular membranes, being present on both the plasma membrane and intracellular membranes. Cytoplasmically localized β-galactosidase-Myc/His was not immune-reactive unless the immunostaining was disrupted with Triton X-100, verifying the integrity of the plasma membrane in the absence of detergents (Fig. 2, panels C and D).

To test the hypothesis that FATP1 is a very long chain CoA synthetase, pFATP1-Myc/His-transfected COS1 cells were assayed for both palmitoyl-CoA and lignoceryl-CoA synthetase activities. As shown in Table I, a 3- to 4-fold increase in ligno-
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Very long chain acyl-CoA synthetase activity of FATP1-Myc/His COS1 cells were treated with virus alone or transfected with pcDNA 3.1 FATP1-Myc/His and assayed for lignoceroyl-CoA synthetase (C24:0) and palmitoyl-CoA synthetase (C16:0) specific activity (Sp. Act.) as described under “Experimental Procedures.” Data are representative of at least three separate trials ± S.E.

| Treatment               | C16:0 Sp. Act. | C24:0 Sp. Act. | C24:0/C16:0 Activity Ratio |
|-------------------------|----------------|----------------|---------------------------|
| Virus-treated           | 47.3 ± 8.5     | 1.0 ± 0.2      | 2.1 ± 0.1                 |
| pFATP1-Myc/His          | 57.5 ± 6.1     | 3.9 ± 0.8      | 6.8 ± 0.8                 |

A positive correlation between FATP1 expression and fatty acid uptake has been previously demonstrated (1, 8, 10, 17). Such internalized fatty acids may act as transcriptional regulators, potentially activating gene expression of a COS1 cell very long chain acyl-CoA synthetase. To ensure that the increase in lignoceroyl-CoA synthetase activity measured in pFATP1-Myc/His-transformed cells was directly due to the enzymatic activity of FATP1-Myc/His and not indirectly due to the fatty acid transport activity of FATP1, the experiment was performed using medium containing delipidated serum. Transfection of COS1 cells with pFATP1-Myc/His in delipidated medium followed by assay of acyl-CoA synthetase activity resulted in a C24:0/C16:0 specific activity ratio (×100) of 7.3 (results not shown for serine at position 250 in FATP1 inhibits ATP binding and reduces fatty acid transport activity. These results suggested that the domain surrounding amino acid 250, which contains the signature motif TSGTTG critical for ATP-dependent ligase reactions, may be required for synthetase activity. Therefore, we generated mutant M1, which contains a 6-amino acid substitution at those positions introducing the sequence LEAAAA. Whereas the motif 1 signature is not diagnostic for acyl-CoA synthetases, a domain within the C-terminal half of the protein is highly conserved in the enzymes capable of esterifying either long chain or very long chain fatty acids (11). We introduced a deletion of this second motif (M2) into FATP1 (amino acids 464 to 523), producing a protein truncated by 59 amino acids. Mutants M1 and M2 were introduced into COS1 cells via polylysine-coated adenovirus-mediated transfection, and the level of heterologous protein expression was assayed by blotting with anti-Myc antibodies. As shown in Fig. 3, although all FATP1 forms were similarly expressed, only the wild type FATP1 yielded a substantial increase in C24:0/C16:0 activity; both mutant M1 and M2 were devoid of any very long chain acyl-CoA synthetase activity. This finding is consistent with that of Stuhlsatz-Krouper et al. (10), who demonstrate that mutation at serine 250 abolished ATP binding and transport activity. Moreover, analysis of mutant M2 demonstrates that this highly conserved domain, whose function is not known, is also required for catalytic activity.

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FATP1 is a Very Long Chain Acyl-CoA Synthetase

FIG. 4. Chromatography of FATP1-Myc/His on nickel-chelate resin. COS1 cells were transformed with pFATP1-Myc/His as described under “Experimental Procedures,” and membrane proteins were solubilized in Triton X-100-containing buffer. The membrane extract was separated by chromatography on nickel-chelate resin, and the long chain acyl-CoA and very long chain acyl-CoA synthetase was assayed in all fractions. Column fractions were separated by SDS-10% polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes, and probed with monoclonal anti-myc antibodies. FATP1-Myc/His was detected using enhanced chemiluminescence. The intensity of the immune image was scanned using a Molecular Dynamics densitometer with IPLab Gel software and is presented as solid bars. The ratio of very long chain (C24:0) to long chain (C16:0) acyl-CoA synthetase activity is presented in the gray bars. Results are shown for one experiment, representative of two separate trials.

shown). These results indicate that exogenous fatty acids internalized due to the cell-surface expression of FATP1-Myc/His are not likely up-regulating the expression of an endogenous very long chain acyl-CoA synthetase gene.

To further demonstrate that the lignoceroyl-CoA synthetase activity associated with pFATP1-Myc/His transformed COS1 cells was directly attributable to an enzymatic activity intrinsic to FATP1-Myc/His, the protein was partially purified using chelating Ni$^{2+}$-affinity chromatography. Total COS1 cell membranes, solubilized membrane proteins, the wash, and elution column fractions were assayed for lignoceroyl-CoA and palmitoyl-CoA synthetase activity (Fig. 4). The ratio of the acyl-CoA synthetase activities (C24:0/C16:0) increased 10-fold in the FATP1-enriched elution column fraction when compared with the starting solubilized COS1 cell membrane material (Fig. 4). Western blotting of the various fractions indicated that an increase in FATP1 immunoreactivity paralleled the increase in C24:0/C16:0 specific activity.

**DISCUSSION**

Our results characterize the FATP1 gene product as a very long chain acyl-CoA synthetase. This finding is supported by the C24:0/C16:0 activity measurements in transfected cells and in the activity of partially purified FATP1-Myc/His protein. The substantial enrichment of FATP1-Myc/His by nickel-based affinity chromatography resulted in a 10-fold increase in very long chain acyl-CoA synthetase specific activity (Fig. 4), supporting the conclusion that FATP1 itself is responsible for the activity measured in transfected COS1 cells (Table 1). The amino acid sequences of very long chain acyl-CoA synthetases are overall very similar but are generally characterized by two domains of very high identity, shown in Fig. 5 as motif 1 and motif 2 (11). Motif 1 is a covalent AMP binding site common to all acyl-CoA synthetases (long chain and very long chain) and necessary for formation of the acyl-adenylate reaction intermediate. Mutagenesis of serine 250 to alanine within motif 1 results in an FATP1 form with markedly diminished fatty acid transport activity (10). Our results also demonstrated that mutation at motif 1 renders the protein catalytically inactive (Fig. 3). Motif 2, whose function is not known, is unique to acyl-CoA synthetases and is somewhat diagnostic in distinguishing very long chain acyl-CoA sequences from long chain acyl-CoA synthetases in data base searches (11). Fig. 3 shows that motif 2 is also necessary for catalytic activity. This is interesting, for a number of folding algorithms used to predict the topology of membrane proteins such as FATP1 place a transmembrane domain between motif 1 and motif 2 from amino acids 294–313. If such topology predictions were proven to be true, it would suggest that domains on either side of the membrane are necessary for catalytic activity. It is important to note that the fatty acid substrate specificity of long chain versus very long chain acyl-CoA synthetases is not absolute. That is, there are overlapping substrate specificities for fatty acids of varying carbon lengths. The use of the ratios of two end points, C24:0 and C16:0, provides a convenient and technically manageable method for assessing and describing an enzyme as a long chain or a very long chain acyl-CoA synthetase. It is likely that the BODIPY 3823-labeled fatty acid analog used by Schaffer and Lodish (1) is a substrate for both FATP1 and the long chain acyl-CoA synthetase, which led to their concurrent identification in the functional screening assay. Experiments are under way to evaluate this possibility.

Several proteins localized to the fat cell plasma membrane have been implicated in fatty acid uptake. Many of these have been characterized by their ability to bind fatty acids or fatty acid derivatives (reviewed in Refs. 3 and 4). In contrast, FATP1 was cloned from a 3T3-L1 adipocyte cDNA library as the product of a functional screen utilizing a BODIPY 3823-labeled fatty acid and a fluorescence-activated cell sorting assay. This screen yielded two different clones; one was FATP1, and the other was the murine long chain acyl-CoA synthetase 1 (1). Transfection of either clone into COS7 cells resulted in a 3-4-fold increase in oleic acid uptake when compared with non-transfected cells. In light of our finding that FATP1 is a very long chain acyl-CoA synthetase, it seems reasonable to suggest that the screening method employed by Schaffer and Lodish (1) identified two metabolic enzymes whose functions in sum are linked to fatty acid uptake for molecules of 12–26 carbons or for fatty acids containing branched chains. Our results do not preclude a role for FATP1 as a fatty acid transporter. However, its amino acid sequence similarity to the very long chain acyl-CoA synthetase multigene family make it more likely that the protein functions in metabolic activation of very long chain fatty acids and not in fatty acid transport per se. Interestingly, recent work by Gargiulo et al. (31) shows that the adipocyte long chain acyl-CoA synthetase is also expressed in part on the plasma membrane, similar to the distribution of FATP1.

Our results do not address the possibility of a fatty acid permease capable of working in conjunction with either the long chain or very long chain acyl-CoA synthetases to mediate fatty acid uptake. Fatty acid uptake may occur by a diffusional (18–22) or protein-mediated (23–25) event followed by esterification of the internalized fatty acid. In general, we refer to this process as esterification-coupled influx. That is, the uptake of fatty acids at the plasma membrane, either via a diffusional...
or protein-mediated event, is coupled to the ATP-dependent esterification of the lipid, producing the corresponding acyl-CoA. Indeed, Schaffer and co-workers (10) show that in cells expressing the S250A mutant of FATP1 or in cells depleted of ATP, fatty acid uptake is severely compromised, presumably due to an inability to form the acyl-adenylate intermediate. Consistent with this, in *Escherichia coli*, fatty acids traverse the inner membrane in an ATP-dependent event coupled to esterification catalyzed by the *FadD* gene product, an acyl-CoA synthetase (14, 26–29). The advantage of the esterification-coupled influx mechanism is that the acyl-CoAs are not able to diffuse back across the plasma membrane, effectively locking the fatty acid in the cell. This process would be considered functionally analogous to phosphorylation of glucose by either hexokinase or glucokinase producing glucose 6-phosphate, thereby preventing its back diffusion from the cell.

In conclusion, we have identified the FATP1 gene product as a very long chain acyl-CoA synthetase and proposed that fatty acid uptake occurs via esterification-coupled influx. The available clones and cell models allow for the testing of this model. In light of these findings, recent data base analysis of genes predicted to encode fatty acid transporters (30) in a wide variety of genera may need to be expanded to include an evolutionarily-conserved family of acyl-CoA synthetases capable of esterifying very long chain or branched chain fatty acids.

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