Substitution of Alanine for Serine 250 in the Murine Fatty Acid Transport Protein Inhibits Long Chain Fatty Acid Transport

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The murine fatty acid transport protein (FATP) was identified on the basis of its ability to facilitate uptake of long chain fatty acids (LCFAs) when expressed in mammalian cells. To delineate FATP domains important for transport function, we cloned the human heart FATP ortholog. Comparison of the human, murine, and yeast amino acid sequences identified a highly conserved motif, IYTSGTTGXPKP, also found in a number of proteins that form adenylated intermediates. We demonstrate that depletion of intracellular ATP dramatically reduces FATP-mediated LCFA uptake. Furthermore, wild-type FATP specifically binds [α-32P]azido-ATP. Introduction of a serine to alanine substitution (S250A) in the IYTSGTTGXPKP motif produces an appropriately expressed and metabolized mutant FATP that demonstrates diminished LCFA transport function and decreased [α-32P]azido-ATP binding. These results are consistent with a mechanism of action for FATP involving ATP binding that is dependent on serine 250 of the IYTSGTTGXPKP motif.

The precise mechanism of long chain fatty acid (LCFA) transport is not well understood. In mammalian cells such as myocytes and adipocytes, LCFA uptake is efficient and highly regulated. Experiments demonstrating specific, saturable LCFA uptake that is inhibited by prior protease treatment of the cell surface suggested that LCFAs are transported by a protein-mediated mechanism (1-5). CD36 and mitochondrial aspartate aminotransferase were initially proposed to facilitate LCFA transport because they are capable of binding LCFAs (6-10). More recently, we identified the fatty acid transport protein (FATP) on the basis of its function in LCFA uptake. We isolated the cDNA encoding the murine FATP using an expression cloning strategy to screen a 3T3-L1 adipocyte cDNA library for cDNAs that increase LCFA uptake when expressed in mammalian cells (11). FATP may function as an LCFA transporter that facilitates bi-directional LCFA movement across the plasma membrane (12, 13).

FATP is a 63-kDa integral plasma membrane protein. Stable overexpression of FATP confers a 4-fold increase in initial rates of LCFA uptake with a Km of 0.2 μM for oleic acid, comparable to the Km for oleic acid uptake by 3T3-L1 adipocytes (11). FATP facilitates uptake of saturated and mono-enoic LCFAs with 14–22 carbons, suggesting that it has broad specificity with respect to fatty acid chain length and degree of saturation (11).2 FATP expression in cultured adipocytes is inhibited by insulin (13). In mice, FATP expression is induced during fasting in adipose and heart tissue, suggesting that FATP may be important not only for uptake of LCFAs into tissues with a metabolic requirement for this substrate, but also for efflux of LCFAs from adipocytes during lipolysis. Disruption of the gene encoding the Saccharomyces cerevisiae (yeast) ortholog, Fat1p, results in a 2–3-fold decrease in the rate of oleate uptake and impaired growth of yeast in which de novo fatty acid synthesis is inhibited (14).

The mechanism of action of FATP is unknown. Hydropathy analysis of the amino acid sequence of FATP predicts a protein with six transmembrane domains. The primary sequence and predicted number of membrane-spanning domains for FATP are distinct from other transporters; therefore, analogies to other transport mechanisms may not be appropriate. Unlike models in which classical transporters promote movement of their substrates through a polypeptide pore in the plasma membrane, FATP might function as a flipase, facilitating transport of its amphiphatic LCFA substrate in direct contact with plasma membrane phospholipids.

In the present study, we sought to identify evolutionarily conserved residues in the primary sequence of FATP that may have functional importance. We cloned and sequenced FATP from a human heart cDNA library. Comparison with the murine and yeast orthologs delineated several conserved regions that may be essential for FATP function. We focused on the 11-amino acid motif IYTSGTTGXPKP (in which X is any amino acid) in a hydrophilic segment of the protein that is predicted to be intracellular. This motif is present in a number of proteins that form adenylated intermediates and may be involved in an interaction with ATP. In the present study, we show that FATP function is dependent on cellular ATP levels, that FATP binds azido-ATP, and that mutation of serine 250 to alanine (S250A) in the IYTSGTTGXPKP motif impairs LCFA transport function and nucleotide binding.

EXPERIMENTAL PROCEDURES

cDNA Cloning—The 1.8-kb BaAI-XbaI restriction fragment from the coding region of murine FATP was used to screen an oligo(dT) and random-primed normal adult human heart λ phage cDNA library (Stratagene). Standard molecular biology techniques were used for plaque lifts. Filters were hybridized with random-primed 32P-labeled DNA probes and washed at high stringency. Filters were exposed to x-ray film for 1 to 2 weeks.

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probe using Quikhyb Rapid Hybridization Solution (Stratagene) at 68°C. Filters were washed with 2× SSC, 0.1% SDS at 50°C. Hybridizing bacteriophage were isolated, and ExAssist Helper phage (Stratagene) was used for excision of pBluescript SK plasmids containing cDNA inserts. Double-stranded DNA sequencing was performed by the method of Sanger et al. (15). Sequence comparisons were made using DNASTAR software.

Cells—NIH 3T3 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum supplemented with 2 mM l-glutamine, 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate. 293GP cells (16) were grown in DMEM 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 293GP cells and harvest of virus supernatants were performed as described (16).

Stable Cell Lines—Murine FATP was subcloned into the pUC3 retroviral expression vector (ΔU3/SFATP) and transiently transfected into 293GP packaging cells to produce high titer VSV-G pseudotyped retrovirus as described previously (16). 106 NIH 3T3 cells were infected in a 35-mm well by two successive 7-h exposures to 1 ml of the ΔU3/SFATP retrovirus. Retrovirally transduced populations of NIH 3T3 cells were plated at limiting dilution to allow isolation of independent clonal cell lines. Individual cell lines were screened for FATP expression by Western blot analysis of microsomal proteins. Similar methods were employed by the other laboratories that overexpressed S250A FATP.

Protein Preparations—For total post-nuclear membrane (TM) preparations, confluent monolayers of cells were scraped and homogenized in 255 mM sucrose, 20 mM Tris, pH 7.4, 1 mM EDTA with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml pepstatin A, and 1 µg/ml leupeptin (SHB) using a glass-Teflon tissue grinder. Nuclei were removed by centrifugation at 1000 × g for 10 min. The remaining membranes and their associated proteins were pelleted by centrifugation at 356,000 × g for 30 min. For plasma membrane (PM) isolation, whole cell homogenates were initially centrifuged at 16,000 × g for 20 min to pellet nuclei, mitochondria, and plasma membranes. This pellet was resuspended in SHB and layered on a 1.2 M sucrose cushion. After centrifugation at 99,000 × g for 20 min, plasma membranes were isolated at the interface and pelleted at 48,000 × g for 45 min.

Western Blot Analysis—TM and PMs were resuspended in 1% Triton X-100, 50 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 µg/ml pepstatin A, and 1 µg/ml leupeptin, and proteins were quantified by BCA assay (Pierce). Equivalent amounts of protein from various cell lines were separated by 7.5% or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (0.2-µm pore). Ponceau-S staining was used to confirm even transfer. For Western analysis of FATP or mutant expression, we used a rabbit polyclonal antiserum directed against murine FATP residues 455–470 (0.25 µg/ml) or a rabbit polyclonal antiserum against murine FATP residues 628–640 at 1:100 dilution. In control immunoprecipitations, we included excess immunizing peptide against which the antiserum was raised. Immunoprecipitates were separated by 7.5% SDS-PAGE, and dried gels were submitted to autoradiography and analysis using densitometry. For calculation of the amount of azido-ATP bound per µg plasma membrane protein in the initial labeling reaction, the relevant band was cut from the gel and radioactivity counted.

RESULTS

Cloning of hFATP from a Human Heart cDNA Library—We used a 1.8-kb fragment of the murine FATP coding sequence to screen an adult human heart cDNA library under moderate stringency. We identified and sequenced several overlapping cDNA clones that contained the human heart FATP sequence (hFATP). hFATP contained a 1932 base pair open reading frame (data not shown) and encoded a 643-amino acid protein (Fig. 1). The hFATP sequence shows 61% identity with the murine FATP sequence (11) and 34% identity with the yeast FATP ortholog (14, 20). Sequence similarity is highest in regions defined by murine residues 247–257, 316–325, 335–348, 363–371, 389–391, and 503–525. Evolutionary conservation of these regions of primary sequence is consistent with an essential role of such residues in FATP function. The amino terminus is the most divergent.

The 11-amino acid sequence between murine residues 247 and 257 is completely conserved in the human, murine, yeast, and rat (21) FATP. Similar sequences are found in a number of proteins, including luciferase, long chain fatty acyl-CoA synthetases, several ligases, and several Fatty acid transporters. The conserved region may be involved in trans-membranous synthesis of cyclic peptide antibiotics (gramicidin S synthetase 2, aminoadipyl-cysteinyl-valine synthetase), suggesting that the YTSSGTGPKP motif has an important function (Fig. 2A). A common feature among these proteins is their ability to activate the carboxyl group of their respective substrates by transfer of adenosine from ATP to the substrate to form an adenylylated intermediate, liberating pyrophosphate. The adenylylated-substrate intermediate is then hydrolyzed with release of AMP and formation of an ester or amide derivative of the substrate. Hydropathy analysis of the amino acid sequence of FATP predicts there are six membrane-associated the site of mutagenesis on the coding and non-coding strands, respectively. The following internal primers were chosen for the S250A mutation: B (5’TGG CCC CGC GTT GAT GAT C-3’) and C (5’ATG CTA TAC CGG GGC CAC-3’). Initial 0.3-kb PCR products were generated with the following primer pairs: A and C, B and D. The two initial PCR products were used as template for PCR with primers A and D to generate a 0.6-kb PCR product that incorporated the S250A mutation. This product was digested with BglII and ApaLI to generate a 167-base pair fragment that was subcloned into ΔU3/SFATP. All PCR-derived sequences were confirmed by double-strandedideoxy chain termination sequencing to verify introduction of the desired mutation and fidelity of amplification.

Azido-ATP Labeling—Cross-linking experiments were performed using PM preparations from the stable cell lines described above and [α-32P]azido-ATP. This photoaffinity probe contains an azido group substitution at position 8 in the base ring that is inert until photoinactivated by short wave ultraviolet light (254 nm) to form a highly reactive nitrone. A covalent linkage is then formed between the nitrone and neighboring polypeptides. Since the nitrone group formed with irradiation is located at the purine ring, it is expected to react with adenine binding sites in proteins. This photoaffinity probe has been used to identify sequences lining the nucleotide binding sites of several proteins (17, 18).

PMs were resuspended in MES buffer (20 mM MES, pH 6.75, 150 mM NaCl, 1 mM PMSF, 1 µg/ml pepstatin A, and 1 µg/ml leupeptin), and proteins levels quantified by BCA. 25 µg of protein was incubated with [α-32P]azido-ATP on ice and exposed to ultraviolet light (254 nm) for 5 min. ATP-S was included as a competitor. The buffer was adjusted to a final 1% Triton X-100, 50 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. Wild-type and mutant FATP molecules were immunoprecipitated as described (19) using a rabbit polyclonal anti-peptide antisera directed against FATP residues 628–640 at 1:100 dilution. In control immunoprecipitations, we included excess immunizing peptide against which the antisera was raised. Immunoprecipitates were separated by 7.5% SDS-PAGE, and dried gels were submitted to autoradiography and analysis using densitometry. For calculation of the amount of azido-ATP bound per µg plasma membrane protein in the initial labeling reaction, the relevant band was cut from the gel and radioactivity counted.

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domains (Fig. 2B), and our prior immunofluorescence studies suggest the carboxyl terminus of FATP is intracellular (11). Based on this model, we predict the IYTSGTTG motif lies on an intracellular loop of the protein.

Effects of FCCP and Cyanide on FATP-mediated LCFA Uptake—Because the IYTSGTTG motif is present in proteins known or thought to form adenylated intermediates, this motif may facilitate an interaction with ATP. We postulated that FATP-mediated LCFA uptake may depend on cellular ATP levels. To test this hypothesis, we generated stable cell lines overexpressing murine FATP at high levels in NIH 3T3 fibroblasts that have low basal expression of FATP and low level LCFA uptake and metabolism (11). The murine FATP cDNA was subcloned into the DU3 retroviral expression vector (16). High titer retrovirus was generated by transient transfection of the construct into the 293GPG packaging cell line, and used to transduce NIH 3T3 cells. Clonal cell lines with stable integration of the FATP-encoding provirus were isolated by plating cells at limiting dilution. We screened for cell lines with high level expression of FATP by Western blot analysis of proteins from individual cell lines. Western analysis data from a representative pair of cell lines is shown in Fig. 3A. An anti-peptide antiserum directed against FATP residues 455–470 specifically recognizes the 63-kDa FATP in the stable FATP-overexpressing cell line, whereas parental NIH 3T3 cells have low level FATP expression (not appreciated on the exposure shown). Moreover, compared with parental NIH 3T3 cells, LCFA uptake was increased 9-fold in the FATP-overexpressing cell line, as measured using a 1-min incubation with the fluorescent LCFA analog BODIPY3823 at 37 °C, followed by flow cytometric analysis (Fig. 3, B and C). This increase in LCFA uptake is greater than that observed with previously published cell lines (11), likely due to higher levels of expression in the present cell lines (data not shown).

To examine whether FATP-mediated LCFA uptake is dependent on cellular ATP levels, we assayed LCFA uptake after cells were treated with metabolic inhibitors. Parental and FATP-overexpressing cells were incubated for 15 (cyanide) or 30 (carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)) min with increasing concentrations of inhibitor, washed and tested as above for LCFA uptake. For flow cytometric analysis, 1 μM propidium iodide was included in the media in order to gate out non-viable cells. LCFA uptake specifically attributable to FATP overexpression was calculated at each concentration of inhibitor by subtracting the LCFA uptake of parental cells from that of FATP-overexpressing cells. The inhibitors shown deplete cellular ATP by independent mechanisms. Cyanide inhibits re-oxidation of cytochrome a3 in the mitochondrial electron transport chain, whereas FCCP is a proton ionophore that dissipates proton gradients and uncou-
plexes ATP synthesis and mitochondrial electron transport.

Both inhibitors depleted cellular ATP in a dose-dependent manner, and this was correlated with a commensurate decrease in LCFA uptake in the FATP-overexpressing cells (Fig. 3, D and E). There was no significant effect of the inhibitors on the low levels of uptake by the parental NIH 3T3 cells (data not shown). In FATP-overexpressing cells, there was a significant reduction in cellular ATP concentrations at inhibitor concentrations that demonstrated an effect on LCFA transport. With FCCP we observed 50% inhibition at a concentration of 1 μM (ATP level 79% of control), and with cyanide we observed 50% inhibition at 1 mM (ATP level 80% of control). These results suggest that LCFA uptake specifically attributable to FATP expression (the difference between FATP-overexpressing and parental cell lines) is impaired in the face of cellular ATP depletion. These experiments cannot distinguish whether this effect on FATP function is due to a direct interaction of FATP with ATP (i.e., primary active transport) or to the indirect effects of ATP depletion on the general maintenance of coupled electrochemical gradients (i.e., secondary active transport in which ATP is used to establish a gradient of a driving cotransported solute or symported species).

The Effect of S250A Mutation on LCFA Uptake—To evaluate the role of the IYTSGTTGXXPK motif in FATP function, PCR was used to substitute alanine for serine at position 250 (S250A) of this motif. The mutant cDNA was cloned into the DU3 retroviral expression vector. Multiple stable cell lines overexpressing the mutant FATP were isolated and screened by Western analysis (data not shown), and a representative cell line is shown in all subsequent experiments. Pulse-chase studies were used to evaluate the expression and metabolism of S250A as compared with wild-type FATP (Fig. 4). We observed comparable levels of expression of S250A and wild-type FATP molecules, with half-lives of 10 and 8 h for S250A and FATP, respectively. Thus, S250A is expressed and metabolized in a fashion indistinguishable from wild-type FATP.

To determine whether S250A was correctly targeted to the plasma membrane, plasma membrane fractions of S250A- and wild-type FATP-overexpressing cell lines were isolated by differential density centrifugation and the use of a discontinuous sucrose gradient. For Western blot analysis of plasma membranes from S250A and FATP cells, we used a polyclonal rabbit antiserum specifically directed against FATP residues 455–470 that are identical in the mutant and wild-type proteins (Fig. 5). Total cellular membranes are shown as a positive control for proteins relatively depleted from the plasma membrane. Western blots using antibodies to the G protein β subunit (Gβ, plasma membrane), the GLUT1 glucose transporter (unglycosylated and core glycosylated forms of GLUT1 in the endoplasmic reticulum (ER)/Golgi and fully glycosylated form in the plasma membrane and Golgi), MCAD (mitochondria), and the 70-kDa peroxisomal membrane protein (PMP70) demonstrate that the plasma membrane fractions were enriched in plasma membrane proteins and relatively depleted of ER, Golgi, mitochondrial and peroxisomal proteins. This analysis ensured that the S250A mutant was appropriately targeted to the plasma membrane. The level of expression of S250A is comparable to wild-type FATP. The conservative S250A substitution is thus unlikely to have grossly altered FATP structure or targeting.

The ability of mutant and wild-type FATP molecules to promote LCFA uptake was examined using the fluorescent assay described above. Compared with wild-type FATP, overexpression of S250A resulted in a 78% reduction in LCFA uptake (Fig. 6). These results show that S250A is minimally active in LCFA transport and that serine 250 is critical for FATP function.
control studies show that this difference does not result from lower levels of expression of the S250A protein at the plasma membrane compared with wild-type FATP.

Azido-ATP Binding by Wild-type FATP and S250A—Given the conservation of the IYTSGTTGXPK motif and the evidence that a mutation of this sequence impairs FATP function, we examined the ability of wild-type FATP to bind the nucleotide analog \([\alpha^{32}P]8\)-azido-ATP. Equivalent amounts of plasma membrane protein isolated from different cell lines were incubated with \([\alpha^{32}P]8\)-azido-ATP, cross-linked by exposure to...
short wave ultraviolet light, and immunoprecipitated with a polyclonal anti-peptide antiserum directed against FATP residues 628–640, which are identical in the wild-type and mutant proteins (Fig. 7A). Immunoprecipitable FATP was labeled by \([\alpha-32P]8\)-azido-ATP in plasma membranes derived from FATP-overexpressing cells (lane 2), but not when immunoprecipitation was carried out in the presence of excess immunizing peptide (lane 3) or when plasma membrane proteins were derived from NIH 3T3 cells (lane 1). Experiments shown in Fig. 7 (B and C) were performed to assess the specificity of this labeling. Labeling of a fixed amount of plasma membrane protein with increasing concentrations of \([\alpha-32P]8\)-azido-ATP demonstrated saturation of label incorporation with half-maximal incorporation into 25 \(\mu\)g of plasma membrane protein at 130 \(\mu\)M \([\alpha-32P]8\)-azido-ATP (Fig. 7B). Approximately 3 fmol of \([\alpha-32P]8\)-azido-ATP was incorporated per \(\mu\)g of plasma membrane protein. Azido-ATP incorporation was effectively competed with a 100-fold molar excess of ATP.\(s\), a non-hydrolyzable ATP analog, with respect to FATP function (Fig. 7C). The saturability of labeling and the inhibition of labeling by a non-hydrolyzable ATP analog are features consistent with an important role of this protein in the physiology of LCFA metabolism in eukaryotic cells. The IYTSGTTPK motif common to all FATP sequences is homologous to domains in other proteins postulated or known to form adenylate cyclase. This observation suggested that FATP interacts directly with ATP, and mutation of the central serine in the IYTSGTTPK motif dramatically reduces FATP function. Our \([\alpha-32P]8\)-azido-ATP cross-linking studies suggest that FATP interacts directly with ATP, and that serine 250 is important for this interaction. This residue is critical for LCFA transport function likely due to a role in ATP binding.

Prior studies have examined the effect of uncoupling agents (2, 4-dinitrophenol, sodium arsenate), proton ionophores (carbonyl cyanide m-chlorophenylhydrazone), and electron transport inhibitors (cyanide, azide) on LCFA uptake by mammalian cells. In studies by Abumrad and co-workers, 1 \(\mu\)M dinitrophenol (2) and 25 \(\mu\)M sodium arsenate (22) did not affect basal oleate transport in adipocytes. However, insulin-mediated antagonism of epinephrine stimulation of LCFA transport was blocked by 1 \(\mu\)M dinitrophenol (23). In contrast, studies by Stremmel and co-workers (5) demonstrated inhibition of oleate uptake into isolated rat hepatocytes by 100 \(\mu\)M carbonyl cyanide m-chlorophenylhydrazone, 4 mM 2,4 dinitrophenol, and 1 \(\mu\)M sodium cyanide. Our findings of significant inhibition of LCFA uptake with decreases in intracellular ATP induced by

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**FIG. 5. Western analysis of wild-type and mutant FATP.** TMs and PMs were isolated as described under “Experimental Procedures” from NIH 3T3 and FATP- and S250A-overexpressing cell lines. For each sample, 12 \(\mu\)g of protein was separated by SDS-PAGE and analyzed by Western blot. A polyclonal rabbit antiserum directed against FATP residues 455–470 was used to detect wild-type and mutant FATP. Control blots include G-protein \(\beta\) subunit (PM), GLUT1 glucose transporter (unglycosylated and core glycosylated form in ER, glycosylated form in PM and Golgi), MCAD (mitochondrial), and the 70-kDa peroxisomal membrane protein (PMP70). Detection for each blot was performed with a horseradish peroxidase-coupled anti-rabbit antiserum and chemiluminescence.

**DISCUSSION**

FATP is highly conserved among widely divergent species, consistent with an important role of this protein in the physiology of LCFA metabolism in eukaryotic cells. The IYTSGTTPK motif common to all FATP sequences is homologous to domains in other proteins postulated or known to form adenylate cyclase. This observation suggested that FATP interacts directly with ATP, and mutation of the central serine in the IYTSGTTPK motif dramatically reduces FATP function. Our \([\alpha-32P]8\)-azido-ATP cross-linking studies suggest that FATP interacts directly with ATP, and that serine 250 is important for this interaction. This residue is critical for LCFA transport function likely due to a role in ATP binding.

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**FIG. 4. Metabolism of wild-type and mutant FATP molecules.** A. FATP- and S250A-overexpressing cell lines were labeled for 1 h with \([^{35}S]\)cysteine-methionine and chased for 0–17 h in media without radiolabeled precursor. Wild-type and mutant FATP were immunoprecipitated using a rabbit polyclonal anti-peptide antiserum directed against FATP residues 628–640. In control immunoprecipitations, excess immunizing peptide was included at a concentration of 100 \(\mu\)g/ml. Proteins were separated by SDS-PAGE and analyzed by autoradiography. B. \(^{35}S\) incorporation into a fixed amount of plasma membrane proteins (Fig. 7B) was quantified using an LKB laser densitometer. The bar graph displays wild-type or mutant FATP at each chase time point as a percentage of the amount of protein observed at 0 h of chase.

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**TABLE**

|            | TM | PM |
|------------|----|----|
|            | 3T3 | FATP | S250A | 3T3 | FATP | S250A |
| **FATP**   |     |     |       |     |     |       |
| **G\(\beta\)** |     |     |       |     |     |       |
| **GLUT 1**  |     |     |       |     |     |       |
| **MCAD**    |     |     |       |     |     |       |
| **PMP70**   |     |     |       |     |     |       |

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**REFERENCES**

1. Stremmel, W., and co-workers (5) demonstrated inhibition of oleate uptake into isolated rat hepatocytes by 100 \(\mu\)M carbonyl cyanide m-chlorophenylhydrazone, 4 mM 2,4 dinitrophenol, and 1 \(\mu\)M sodium cyanide. Our findings of significant inhibition of LCFA uptake with decreases in intracellular ATP induced by...
metabolic inhibitors are most consistent with the latter studies. These experiments, together with our observation that FATP specifically binds [α-32P]8-azido-ATP, are consistent with a model in which FATP facilitates ATP-dependent LCFA transport.

The IYTSGTTG PK motif has been proposed to play a role in ATP binding by a number of proteins (24–28). Many proteins with this motif catalyze formation of an amide or ester linkage to the carboxyl group of their substrates via adenylated intermediates. The IYTSGTTG PK motif has been proposed to form a phosphate binding loop in which lysine binds the γ-phosphate of the nucleoside triphosphate (25). The motif does have numerous amino acid residues with hydroxyl groups on their side chains that may participate in hydrogen bonding with distant residues to form a nucleotide binding fold. In luciferase, for which the crystal structure is known (29), the IYTSGTTG PK motif lies in a loop connecting antiparallel strands 6 and 7 of β-sheet A, and the side chain of S198 is predicted to form a hydrogen bond with another residue located across the putative nucleotide binding cleft.

In all proteins containing the IYTSGTTG PK motif, Ser-250 is invariant. In the present study, we chose to mutate FATP serine 250 in such a way as to remove a potential hydrogen bond donor residue that may be important in creating a nucleotide binding fold. Our studies provide the first demonstration of a mutation in any putative mammalian fatty acid transporter which impairs transport function and the first demonstration in any protein that mutation of the central serine in the IYTSGTTG PK motif specifically impairs nucleotide binding. The configuration of amino acid residues surrounding the adenine ring in the ATP-bound state of FATP is likely to be significantly different from that found in the peptide synthetases, since peptides from tyrocidine synthetase I that are cross-linked to the photoaffinity probe 2-azido-ATP (G373-K384, W405-R416, and L483-K494) are not found in FATP (30). Furthermore, tyrocidine synthetase can only be labeled by 2-azido-ATP (G373-K384, F376-L387, X388, and F389) (31, 32), in which X is any amino acid and Φ is a hydrophobic amino acid. In peptide synthetases, but not in FATP, the IYTSGTTG PK motif is associated with a TGD motif, which is implicated in Mg2+-ATP binding in ATPases (27, 33, 34).

Some, but not all, proteins containing the IYTSGTTG PK motif have CoA synthetase activity. Notable exceptions include the peroxisomal peripheral membrane protein Pcs60p (35) and luciferase. Despite 40% sequence identity between rat FATP (21) and rat very long-chain acyl-CoA synthetase (36), FATP lacks the 25-amino acid signature motif, DGWLTHTDIXWXXPGX(V)IKIDRRKK, previously identified in fatty acyl-CoA synthetases (37) and lacks the QVKLGXGXEIXXXE motif found in many proteins that form adenylated intermediates (33, 34). Our prior studies suggest that FATP facilitates cellular uptake of LCFAsthat are not net enzymatic modification of the substrate (11). Moreover, membrane fractions from our FATP expressing cell lines do not have long chain acyl-CoA synthetase activity.2

In addition, during starvation when lipolysis and net efflux of LCFAsthat are not net enzymatic modification of the substrate (11). Moreover, membrane fractions from our FATP expressing cell lines do not have long chain acyl-CoA synthetase activity.2

Several potential mechanisms for FATP action involving ATP binding can be envisioned. FATP may be an active transporter that uses energy from ATP hydrolysis to drive LCFA transport across the plasma membrane. The energy from ATP hydrolysis may be used to effect a conformational change in FATP that facilitates flipping of its LCFA substrate from the exoplasmic to the cytosolic leaflet of the plasma membrane. Other potential mechanisms of FATP action involving ATP binding.
The precipitation was carried out in the presence of 100 µM serum directed against FATP residues 628–640. Control immunoprecipitation was performed using a polyclonal rabbit antisera directed against FATP residues 628–640. After SDS-PAGE, gels were dried and incorporation of [32P] was detected by autoradiography. [32P] incorporation was quantified using an LKB laser densitometer. The bar graph displays [32P] incorporation in arbitrary units as a function of cell line. Error bars reflect standard deviation for assays performed in triplicate. These results were confirmed in three independent experiments.

The bar graph displays 32P incorporation in arbitrary units as a function of cell line. Error bars reflect standard deviation for assays performed in triplicate. These results were confirmed in three independent experiments.

It is unknown whether FATP mediates ATP hydrolysis. FATP-mediated transport is not inhibited by well characterized inhibitors of P-type (100 µM ouabain (Ref. 39)), F-type (20 µg/ml oligomycin; Ref. 40), or V-type (1 µM bafilomycin A1; Refs. 41 and 42) ATPases (data not shown). In addition, we detect no difference in ATPase activity in plasma membranes from NIH 3T3 cells and from cells overexpressing FATP (data not shown). Use of permeabilizing agents to introduce [α-32P]8-azido-ATP or non-hydrolyzable ATP analogs into our fibroblast cell lines would render difficult subsequent measurements of LCFA transport to determine whether photo-labeling or non-hydrolyzable substrates inhibit FATP function. Deterministic assessment of potential ATPase activity will require purification of FATP in its native conformation. Since FATP is a multiple membrane-spanning protein, such experiments are beyond the scope of the present study.

Our studies show that the serine residue in the IYTSGTTGPK motif, which is present in other ATP-dependent proteins, is critical for FATP function and nucleotide binding. Analysis of nucleotide binding sites in proteins for which crystal structures have been established indicates that at least two and as many as five non-contiguous peptide regions may be involved in FATP's nucleotide binding domain through the PK motif, which is present in other ATP-dependent proteins. Definitive assessment of potential ATPase activity will require purification of FATP in its native conformation. Since FATP is a multiple membrane-spanning protein, such experiments are beyond the scope of the present study.

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