Enzymatic Properties of Purified Murine Fatty Acid Transport Protein 4 and Analysis of
Acyl CoA Synthetase Activities in Tissues from FATP4 Null Mice

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Summary

Fatty acid transport protein 4 (FATP4) is an integral membrane protein expressed in the plasma and internal membranes of the small intestine and adipocyte as well as the brain, kidney, liver, skin, and heart. FATP4 has been hypothesized to be bifunctional, exhibiting both fatty acid transport and acyl CoA synthetase activities that work in concert to mediate fatty acid influx across biological membranes. To determine if FATP4 is an acyl CoA synthetase, the murine protein was engineered to contain a C-terminus flag epitope tag, expressed in COS1 cells via adenoviral-mediated infection and purified to near homogeneity using \( \alpha \)-FLAG affinity chromatography. Kinetic analysis of the enzyme was carried out for long chain (palmitic acid, C16:0) and very long chain (lignoceric acid, C24:0) fatty acids as well as for ATP, and CoA. FATP4 exhibited substrate specificity for C16:0 and C24:0 fatty acids with a \( \frac{V_{\text{max}}}{K_{\text{m}}} \) (C16:0) / \( \frac{V_{\text{max}}}{K_{\text{m}}} \) (C24:0) of 1.5. Like purified FATP1, FATP4 was insensitive to inhibition by triacsin C but was sensitive to feedback inhibition by acyl CoA. Although purified FATP4 exhibited high levels of palmitoyl CoA and lignoceroyl CoA synthetase activity, extracts from the skin and intestine from FATP4 null mice exhibit reduced esterification for C24:0, but not C16:0 or C18:1 suggesting that in vivo, defects in very long chain fatty acid uptake may underlie the skin disorder phenotype of null mice.
INTRODUCTION

Studies in multiple tissue types, including cardiomyocytes and adipocytes (1), support the hypothesis that fatty acid transport occurs by a saturable, protein-mediated mechanism and several candidate proteins responsible for FA uptake have been identified. These include the fatty acid binding protein from the plasma membrane (FABPPm), the fatty acid translocase (FAT/CD36), as well as the fatty acid transport protein family of molecules (FATP) reviewed in (2). In mammalian cells, FATP isoforms 1-6 have been identified based on sequence similarity and have distinct tissue-specific distributions of expression (3). When expressed into cultured cells, FATP1 increases fatty acid import and stimulates triacylglycerol synthesis (4). Genetic and biochemical analyses have shown that the FATP homologue in yeast (Fat1p) is required for fatty acid uptake (5) in cells with compromised de novo fatty acid biosynthesis. In yeast, Fat1p functions in concert with acyl CoA synthetases (Faa1p and/or Faa4p) to mediate FA influx (6,7) and from a combination of molecular and cellular studies it has been inferred that they function as part of a FA import complex although such an entity has not been demonstrated.

Previous work from this laboratory has demonstrated that purified FATP1 possesses intrinsic acyl CoA synthetase activity directed to both long and very long chain fatty acids (8). Watkins and colleagues have also shown that FATP3 is also an acyl CoA synthetase but that the protein does not facilitate fatty acid internalization into cultured cells (9). These results suggested that the acyl CoA synthetase and transport functions of FATPs might be separable functions. Indeed, Black and DiRusso have identified yeast FAT1 mutants that are deficient in either transport, or acyl CoA synthetase activity, or both (10). However, the S250A mutant of murine FATP1 as well as multiple yeast FAT1 mutants lacking acyl CoA synthetase activity exhibit greatly
diminished FA influx supporting the hypothesis that that fatty acid uptake into cells is linked, at least in part, to their esterification with coenzyme A in a process termed vectoral acylation (11,12).

The importance of FATP4 in fatty acid utilization has been illustrated through the use of ablated mice that display extreme phenotypes linked to skin biology. FATP4 loss of function due to either a spontaneous transposon insertion in exon 3, or targeted disruption of exon 3, results in mice with a phenotype reminiscent of restrictive dermopathy (13,14). These animals die shortly after birth and have tight, wrinkle-free skin and disrupted skin barrier function. There may be additional phenotypes linked to fat absorption in the intestine (15), but these have not been examined in detail. Moreover, polymorphisms in the human FATP4 locus have been linked to the development of insulin resistance suggesting that this protein may be a major contributor to lipid uptake in the adipocyte as well (16,17).

The objective of the study was to determine if FATP4 is an acyl CoA synthetase, to assess the substrate specificity and intrinsic catalytic efficacy of the purified protein, and to compare those properties to FATP1 in order to determine if fatty acid transport proteins have similar or discretely definable catalytic properties. To that end, murine FATP4 was engineered to contain a C-terminus flag epitope tag, expressed in COS1 cells, and purified to near homogeneity by affinity chromatography. Herein we present data characterizing FATP4 as a high velocity enzyme with specificity for long and very long chain fatty acids as well as the acyl CoA synthetase activity of tissues from wild type and FATP4 ablated mice suggesting that in vivo, the
transport and/or acyl CoA synthetase activity for very long chain fatty acids is critical for FATP4 function.

EXPERIMENTAL PROCEDURES

Reagents—[3H] palmitic acid and [3H] lignoceric acid were obtained from American Radiochemicals Company. All non-labeled fatty acid was obtained from NuChek Prep, Inc., Elysian, MN. Triacsin C was obtained from BIOMOL. Cell culture reagents were obtained from GIBCO. All other reagents were of analytical grade and obtained from Sigma Chemical Co., St. Louis, MO. Dr. Paul Watkins, Kennedy Krieger Institute, kindly provided Baltimore, MD the anti-FATP4 antibody.

Generation of FATP4 Recombinant Adenovirus and Expression in COS1 cells—A recombinant adenovirus expressing both the green fluorescent protein and murine FATP4 was constructed by recombination in Escherichia coli using the methods described by He and colleagues (18). The resulting construct was recombined into pADEasy in E. coli BJ5183 cells recreating the replication-deficient adenovirus genome. Linear constructs of the recombinant adenovirus were transfected (LipofectAMINE; GIBCO-BRL, Gaithersburg, MD) into 293 cells (American Type Culture Collection, Manassas, VA) to allow packaging and amplification of the adenovirus. Large-scale adenovirus preparations from twenty 10-cm plates of infected cells were propagated until approximately 50% of the cells lysed. The cells and media were collected and the
remaining cells lysed by three freeze/thaw cycles. The medium was centrifuged at 20,000 xg for 10 min to pellet the cellular debris and the supernatant containing virus particles was recovered and frozen in aliquots at -70° C.

For protein expression, COS1 cells were plated in 10-cm plates and grown to approximately 80% confluence at 37° C in a 5% CO2 incubator. For infection, the effective concentration of infectious adenoviral particles was experimentally determined by monitoring green fluorescent protein expression and COS1 cell viability seventy-two hours post infection. Adenovirus particles that yielded ~90-100% infection were delivered in 8 mL of DMEM supplemented with 10% FBS per plate. Seventy-two hours post-transfection, cells were harvested by centrifugation, immediately frozen and stored at -70° C.

**Affinity Purification of Recombinant FATP4 Protein**—COS1 cells expressing FATP4-flag were thawed in buffer A (150 mM Tris-HCL, pH 7.5, 250 mM sucrose, and 150 mM NaCl), subjected to five freeze/thaw cycles, and solubilized with 1% n-dodecyl-β-D-maltopyranoside (DDM) for four hours at 4° C. The soluble fraction was separated from debris by centrifugation at 100,000 xg for 1 hour at 4° C, recovered, and glycerol was added to a final concentration of 20%. For purification of recombinant FATP4-flag, α-FLAG matrix beads (Sigma) equilibrated with buffer A was incubated with the protein extract for 6 hours, washed, and eluted with flag peptide in Buffer A containing 0.1% DDM. Eluates were pooled, aliquoted, and stored at -70° C until use. To determine the amount of purified protein, samples were precipitated (19) and protein concentration determined by the bicinchoninic acid method (Pierce) with bovine serum albumin as the standard.
Fatty acyl CoA synthetase assays—Samples were assayed for acyl CoA synthetase activity by the conversion of [3H]-palmitate or [3H]-lignocerate to their CoA derivatives by a modified method from Nagamatsu et al. (20) as described by Hall et al (8). All kinetic studies reported used 0.5-2 µg of purified FATP4 for 2 min at pH 7.5 and 30 mM NaCl in 250 µL of a buffer containing 20 µM fatty acid delivered bound to α-cyclodextrin, 100 mM Tris-HCl (pH 7.5), 10 mM ATP, 5 mM MgCl₂, 200 µM CoA, and 200 µM dithiothreitol. Addition of enzyme purified in the presence of 0.1% DDM resulted in a final concentration of 0.02% DDM in all standard assays. Reactions were terminated with the addition of 1.25 mL of isopropenol:heptane:H₂SO₄ (40:10:1, v/v/v), 0.5 mL of H₂O and 0.75 mL of heptane to facilitate organic phase separation. The aqueous phase was extracted three times with 0.75 mL of heptane to remove unreacted fatty acids and the radioactivity determined by liquid phase scintillation counting. The enzyme activity was stable in the elution buffer and activity was retained for 2 months without significant loss of activity when stored at -70 °C.

Stability of purified FATP4 in DDM-detergent micelles—Purified FATP4 was thawed on ice and then maintained at either 4 °C or 37 °C for various lengths of time and then assayed for acyl-CoA synthetase activity at 37 °C with [3H] lignoceric acid. The zero time point represents assayed activity immediately after thawing.

Competition studies—Due to the unavailability of commercially radiolabeled fatty acids, the fatty acid substrate specificity of the purified FATP4 was indirectly determined by a competitive enzymatic inhibition by the addition of unlabelled fatty acids at a constant concentration of 15
μM in the reaction in addition to either [3H] palmitic acid or [3H] lignoceric acid. A selectivity series for fatty preference was generated by determining the fraction of acyl CoA synthetase activity at 1/2 [S]max.

*Acyl CoA synthetase activity in murine tissues*—Tissues from FATP4 null mice (11) (intestine, liver, lung, brain, and skin) were excised from killed newborn mice, weighed, washed in cold phosphate-buffered saline, and snap-frozen in liquid nitrogen. The tissues were ground to a fine homogenate in a glass homogenizer in a total volume of 800 μL phosphate-buffered saline containing 1% protease inhibitor cocktail P 8340 (Sigma). Protein concentrations were determined by Bradford assay. All mouse procedures were in compliance with the guidelines of the institutional animal care and use committees and in accordance with governmental guidelines.
RESULTS

Previous studies in crude extracts from cells over expressing FATP4 have suggested that the protein is an acyl CoA synthetase (21). To determine if FATP4 exhibits intrinsic fatty acid CoA synthetase activity and to kinetically assess the properties of such a reaction, murine FATP4 was purified and studied. To facilitate purification, FATP4 was C-terminal tagged with a flag epitope and over expressed in COS1 cells by a recombinant adenoviral infection method. To obtain FATP4 protein in a highly purified form that retains biological activity, membranes were extracted with a variety of different detergents and solubilized protein and acyl CoA synthetase activity compared. Figure 1A shows the immunoblotting analysis of the membranes extracted with different detergents. Extraction of membranes with 1% DDM solubilized FATP4 from COS1 to the greatest extent in comparison to the other detergents tested. The degree of FATP4 solubilization obtained for each detergent was generally proportional to the acyl-CoA synthetase activity (Figure 1B) with the exception of C12E8. While the extractable activity obtained with C12E8 was high, the amount of enzyme obtained was relatively low; as such, DDM was chosen for further analyses.

The purification involved detergent extraction of FATP4 from the membrane with 1% DDM and chromatography through α-FLAG-agarose resin. Figure 1C presents the SDS-PAGE analysis of pooled elution fractions collected during a typical FATP4 purification, along with the corresponding immunoblotting analysis with α-FATP4 antibody. The purified FATP4 migrated as a single band on a 7% SDS-polyacrylamide gel and was judged to be at least ~80% pure by silver staining. The level of murine FATP4 enrichment obtained from the α-FLAG column
purification was ~2000-fold as assessed by comparison of the specific activity of the crude cell fraction to the eluted fraction (Table I).

Previous studies on FATP1 have demonstrated that Triton X-100 has an inhibitory effect on its acyl CoA synthetase activity (8), presumably due to sequestration of the substrates into detergent micelles. To determine whether FATP4 acyl CoA synthetase activity was sensitive to DDM, purified enzyme was assayed with increasing concentrations detergent. FATP4 acyl CoA synthetase activity was sensitive to DDM in the reaction buffer, with both C16:0 and C24:0 acyl-CoA synthetase activity decreasing with increasing DDM concentration (Figure 2A). The activity of FATP4 was found to be relatively insensitive to DDM below 0.03% DDM; as such, a final concentration of 0.02% DDM was adopted for all assays. To test the stability of purified FATP4 in DDM-detergent micelles, purified FATP4 was thawed on ice and maintained at either 4°C or 37°C for various times and then assayed for acyl-CoA synthetase activity (Figure 2B). Purified FATP4 in DDM micelles retained activity at 4°C for up to one hour but lost activity quickly when maintained at 37°C.

The FATP4 acyl CoA synthetase activity was optimized with respect to several standard reaction parameters using palmitate as the substrate (Figure 3) although some studies were also conducted with lignocerate (results not shown). Previous studies using FATP1 (8) had shown that the enzymatic parameters for esterification of C16:0 fatty acid were the same for C24:0 fatty acid. As shown in Figure 3, the activity was proportional to the amount of enzyme added to the reaction (3A) as well as time of reaction (3B). In addition, the pH sensitivity of the reaction was evaluated (3C), as was the influence of ionic strength (3D). The purified FATP4 has broad pH
dependence with 7.0–8.5 being generally optimal and enzymatic activity was sensitive to the NaCl concentration when varied from 30 mM to 500 mM. Synthetase activity was modestly activated at lower ionic strength and then was inhibited at salt concentration in excess of 300 mM.

The fatty acid esterification properties of purified FATP4 were measured for two model lipid substrates as well as for CoA and ATP. The apparent $K_m$ values of the purified enzyme were determined for palmitic acid (C16:0), lignoceric acid (C24:0), ATP, and CoA at 37° C (Figure 4). FATP4 demonstrated high affinity toward its substrates and co-substrates exhibiting a $K_m$ of 13 $\mu$M for C16:0, 4.8 $\mu$M for C24:0, 1.4 mM for ATP, and 47 $\mu$M for CoA (Table II). It should be stressed that because these studies are done in the presence of detergent, and using lipid delivered using $\alpha$-cyclodextrin mediated solubilization, the free unbound concentration of fatty acids cannot be determined and the $K_m$ values reported for each fatty acid represent apparent values assuming all lipid was available to the enzyme. Since the solubility of lignoceric acid and palmitic acid vary greatly, this assumption is likely to be incorrect, but represents an experimentally tractable method for analyzing the data and comparing one enzyme to another. The maximal specific activity of ~4300 nmol/ min/ mg was measured for C16:0 and ~1050 nmol/ min/ mg for C24:0 which were 40 and 5-fold greater than those measured for purified FATP1, respectively (8).

Because a large number of different fatty acids are not commercially available in radiolabeled form, the fatty acid substrate specificity of the purified FATP4 was indirectly determined by a competitive enzymatic inhibition by the addition of unlabelled fatty acids in the reaction in addition to either $[^3]$H palmitic acid or $[^3]$H lignoceric acid (Figure 5). Esterification of labeled
lignoceric acid was inhibited only slightly by the addition of 15 μM of two long chain fatty acids, C16:0 and C20:4. In contrast, the conversion of [3H] palmitate to palmitoyl-CoA was decreased by the addition of very long chain fatty acids with the rank order of C24:0 = C20:0 > C16:0. As with the other kinetic analysis, due to the differing solubilities of the various competing fatty acids, true Kᵢ values cannot be determined and a simple selectivity series is presented. As such, these results suggest that the true Kₘ value for lignoceric acid is quite low and that the enzyme is much more specific for very long chain FA rather than long chain FA.

Reports of the cellular concentration of long chain acyl CoA esters vary between 5 and 160 μM (5). To determine whether FATP4 is regulated by feedback inhibition, increasing concentrations of palmitoyl-CoA were titrated into the standard reaction conditions (Figure 6A) and the activity of FATP4 evaluated. At a concentration of 10 μM palmitoyl-CoA, the FATP4 acyl CoA synthetase reaction was inhibited by ~35%, where as 100 μM inhibited the reaction by greater than 90% for both C16:0 and C24:0. Triacsin C has been reported to be a potent competitive inhibitor of ACSL1 and ACSL4 (22), but does not inhibit FATP1 (8). To test whether triacsin C inhibits purified FATP4, various concentrations were added to the standard reaction mixture (Figure 6B) and the conversion of C16:0 and C24:0 to their CoA derivatives evaluated. Surprisingly, Triacsin C had no effect on FATP4 acyl CoA synthetase activity toward C24:0 esterification, but inhibited C16:0 esterification in a dose-dependent manner with an IC₅₀ of ~30 μM. Troglitazone inhibition of CoA synthetase activity paralleled the result with Triacsin C; C24:0 esterification was unaffected by concentrations up to 50 μM and inhibited C16:0 conversion with an IC₅₀ of ~20 μM (Figure 6C). These results are consistent with the proposal.
that FATP4 is more specific for very long chain FA esterification than for long chain esterification.

The phenotype of FATP4 null mice suggests defects in lipid uptake and/or metabolism. To evaluate the relevance of the FATP4 acyl CoA synthetase activity to the phenotype of the null mice, the esterification of a variety of long and very long chain FA was evaluated in extracts from FATP4 null mice (Figure 7). Although the C16:0 esterification activity of purified FATP4 exceeded that for C24:0, there were no statistically significant changes in palmitic or oleic acid esterification measured in any tissue. This is likely due to the presence of other acyl CoA synthetases capable of C16:0 esterification in the crude extract. In contrast, esterification of C24:0 was significantly decreased in the intestine, brain, and skin samples from the FATP4 null mice when compared to wild type littermates, but was identical in the liver and lung samples where additional FATP family members are expressed (3). These results are consistent with the in vitro characterization of FATP4 activity and strongly suggest that in vivo, the loss of FATP4-mediated very long chain fatty acid uptake and/or esterification may underlie the null phenotype.
DISCUSSION

The FATP family of proteins was originally identified by the ability to facilitate fatty acid uptake using a fluorescent fatty acid internalization assay (23). Subsequently, mammalian FATP family members have been shown to catalyze fatty acid uptake using a variety of assays in multiple systems including evaluation in stable cell lines, transient transfection into 293, COS or CHO cells, and complementation of deletions in the yeast orthologue (3,4,24). Moreover, the purified FATP1 is an acyl CoA synthetase and based on work from the yeast system has been postulated to function in a process termed vectoral acylation. Vectoral acylation links CoA dependent esterification of fatty acids to their influx thereby trapping the internalized lipid, preventing its diffusion or transport from the cell. It is unclear if FATPs facilitate both transbilayer movement of fatty acids and esterification or simply esterification of a fatty acid that has diffused across the membrane. In yeast, Fat1p functions in concert with Faa1 and/or Faa4p to facilitate FA uptake. However, yeast FAT1 mutants have been identified with separable transport and esterification activity suggesting that the mammalian FATP family, if functioning similarly, may too be bifunctional (10). This hypothesis does not exclude the participation of other proteins such as CD36 that may function as a fatty acid receptor presenting a high local concentration of fatty acid at the plasma membrane, or ACSL1 another acyl CoA synthetase, that collectively may work in concert with FATP. Vectoral acylation therefore is a functional parallel to the glucose transport-hexokinase system functioning in sugar import. The objective in this study was to evaluate the enzymatic properties of FATP4 and sets the stage for future analysis of the protein in facilitating transbilayer movement.
Molecular and genetic analysis of *Saccharomyces cerevisiae* has indicated that vectoral acylation is mediated by some combination of Fat1p and either Faa1p or Faa4p. Since both *FAT1* and the *FAA* genes encode acyl CoA synthetases, it is not clear what the biochemical role of each protein may be or if one enzyme (*FAA* genes) is specific for one class of FA (long chain FA) while another (*FAT1*) is more specific for very long chain FA. Alternatively, Fat1p may catalyze transbilayer movement of the FA while Faap forms may catalyze CoA dependent esterification. It will be interesting in the future to compare the biochemical properties of the purified yeast Fat1p to yeast Faa proteins and the mammalian FATP family members or to assess their acyl CoA synthetase activity and specificity. The mammalian system is less well defined and although FATP1 and ACSL1 have been co-localized to some regions of the plasma membrane, biochemical evidence demonstrating association under physiological conditions has not been forthcoming.

The current study was undertaken to evaluate the catalytic properties of the FATP4 acyl CoA synthetase reaction. Here we demonstrate that *in vitro*, purified FATP4 has a robust acyl CoA synthetase activity with moderate specificity for long chain fatty acids over very long chain fatty acids. FATP4 is a high velocity enzyme comparable to ACSL1, the traditional enzyme believed to esterify fatty acids broadly in cells. FATP4 prefers long chain fatty acid to very long chain fatty acids, although care must be taken to consider that this conclusion is based on kinetic evaluations that consider all the substrate available to the enzyme. Given the differences in chemical solubility between C16:0 and C24:0, the ability to make absolute statements is tenuous at best.
The kinetic properties of purified FATP4 protein are interesting when compared to those exhibited by ACSL1 purified similarly. Purified his-tagged ACSL1 demonstrated the expected high affinities toward C16:0, ATP, and CoA, with \( K_m \) values of 33 \( \mu \text{M} \), 320 \( \mu \text{M} \), and 6.4 \( \mu \text{M} \) respectively (Table II). Interestingly, ACSL1 also utilized very long chain fatty acids (C24:0) with a \( K_m \) of 18 \( \mu \text{M} \). The maximal velocity of ACSL1 for C16:0 was about 3200 nmol/min/mg, but was greatly reduced for C24:0 to 240 nmol/min/mg. Consistent with the reduced velocity for very long chain fatty acids, ACSL1 was 10-fold more active toward C16:0 than C24:0 as demonstrated by \( V_{max}/K_m \) values of 100 and 13 respectively. In contrast FATP4 exhibited higher activity toward C16:0 and increased specificity for C16:0 (\( V_{max}/K_m \) of 330) over C24:0 (\( V_{max}/K_m \) of 220). For very long chain fatty acids, FATP4 was ~5-fold more active than was ACSL1 while the enzymes had comparable long chain fatty acid esterification activity. In general FATP4 is a more robust acyl CoA synthetase than is ACSL1. The availability of purified FATP4 (this work) and FATP1 (8) allows a future biochemical analysis of FATP proteins in facilitating transbilayer movement independent of esterification.

FATP4 demonstrates a preference for C16:0 over C24:0 in these in vitro studies. However, a number of experimental observations suggest that C24:0 may be the preferred substrate for FATP4 in vitro and in vivo. First, two fatty acid analogs Triacsin C and troglitazone were effective inhibitors of C16:0 esterification but not for C24:0. Second, in competition studies, lignoceric acid could compete for C16:0 conversion; however, C16:0 did not inhibit C24:0 esterification. Finally, tissues from FATP4 null animals were only defective for C24:0 esterification, while C16:0 or C18:1 conversion were unaltered. While the apparent \( K_m \) values for C16:0 and C24:0 were essentially identical, these observations suggest the actual \( K_m \) value
for C24:0 is much lower that that of C16:0.

A comparative analysis of the two purified FATP proteins reveal significant differences between the enzymes. FATP4 is a high velocity acyl CoA synthetase with preference for C24:0 over C16:0. Additionally, the FATP4 reaction is sensitive to a number of potential inhibitors including the product palmitoyl-CoA, and two fatty acid analogs, Triacsin C and troglitazone. On the other hand, FATP1 is comparatively a low velocity enzyme with broad specificity for fatty acids of 16 to 24 carbons. The FATP1 reaction is insensitive to Triacsin C and troglitazone, but subject to product inhibition. The relative abundance and cellular locations of FATP1 and FATP4 in adipocytes are not known. However if both enzymes are located on the plasma membrane, based upon the relative velocities of the two enzymes, FATP4 may be the major contributor to vectoral acylation. This may explain the lack of effects on adipose tissue lipid metabolism observed in FATP1 null mice where the major metabolic effect is centered in muscle (25).

Tissue extracts of the intestine, brain, and skin from FATP4 null mice exhibit reduced esterification for C24:0, but not C16:0 or C18:1 suggesting that in vivo, the very long chain acyl CoA synthetase activity of FATP4 contributes to the skin disorder phenotype of null mice. This conclusion is supported by lipid composition analysis of the FATP4 null mice. The epidermis of the FATP4 -/- mice had a reduced molar content of phosphatidylcholine, phosphatidylethanolamine, and cholesteryl ester, but increased ceramide compared to wild type littermates. Furthermore, detailed analysis of the ceramide species demonstrated the null mice had a significantly lower portion of very long chain fatty acids (C26:0 and C26:0-OH) and an
increased proportion of fatty acids with less than 26 carbons. This shift to shorter chain fatty acids was also observed for phosphatidylcholine and phosphatidylserine. The lack of effects in other tissues is likely due to the expression of other acyl CoA synthetases, including FATP isoforms, in those cell types.

In sum, these results demonstrate that FATP4 exhibits intrinsic acyl CoA synthetase activity and is a high velocity enzyme relative to FATP1 and ACSL1. For long chain fatty acids, the FATP4 enzyme exhibits a $V_{max} / K_m$ similar to ACSL1. This may suggest that ACS family members could functionally compensate for disruptions of FATP for some but not all lipid substrates. However, striking evidence from FATP4 null mice (14) that exhibit a wrinkle-free phenotype reminiscent of essential fatty acid deficiency suggests unique specialized roles for the fatty acid transport proteins in very long chain fatty acids metabolism and that their physiological significance is central to normal lipid homeostasis.
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ABBREVIATIONS:

The abbreviations used are: FATP, fatty acid transport protein; ACS, acyl CoA synthetase; FABP<sub>PM</sub>, fatty acid binding protein from the plasma membrane; FAT/CD36, fatty acid translocase / CD36 antigen; DDM, n-dodecyl-β-D-matlopyranoside, C<sub>12</sub>E<sub>8</sub>, octaethylene glycol monododecyl ether. SD, standard deviation.
Table I.  
FATP4 purification profile

Recombinant FATP4 was expressed and purified as described under “Experimental Procedures”. Fractions were taken at various steps throughout the purification and assayed for lignoceroyl-CoA synthetase activity as described under “Experimental Procedures”. Values are reported as the mean ± standard deviation.

| Step               | Volume (mL) | Protein (mg/mL) | Sp. Act. nmol/min/mg | Fold purification |
|--------------------|-------------|-----------------|----------------------|-------------------|
| Crude extract      | 3.75        | 15.8            | 0.34 ± 0.01          | 1                 |
| Detergent soluble  | 3.75        | 10.3            | 0.6 ± 0.1            | 1.8               |
| α-Flag elution     | 5.0         | 0.005           | 723 ± 68             | 2125              |
Table II.
Comparison of kinetic constants for FATP4, FATP1 and ACSL1
Recombinant FATP4 was expressed, purified, and assayed for lignoceroyl-CoA and palmitoyl-CoA synthetase activities as described under “Experimental Procedures”. Data are representative of at least two individual trials ± standard deviation and should be considered apparent rather than true values due to the presence of detergent in the analysis. The (*) denotes values determined using palmitic acid (C16:0) as substrate.

|           | FATP4       | FATP1*      | ACSL1*     |
|-----------|-------------|-------------|------------|
| **ATP**   |             |             |            |
| $K_m$ (mM)| 1.4 ± 0.1   | 0.85 ± 0.1  | 0.32 ± 0.3 |
| $V_{max}$ (nmol/min/mg) | 3740 ± 90 | 160 ± 0.1   | 5003 ± 100 |
| $V_{max}/K_m$ | 2700    | 190         | 15,600     |
| **CoA**   |             |             |            |
| $K_m$ (µM) | 47 ± 4      | 8.3 ± 1.6   | 6.4 ± 0.7  |
| $V_{max}$ (nmol/min/mg) | 4570 ± 170 | 169 ± 10    | 4602 ± 144 |
| $V_{max}/K_m$ | 100      | 20          | 720        |
| **C16:0** |             |             |            |
| $K_m$ (µM) | 13 ± 3      | 21 ± 5      | 33 ± 4     |
| $V_{max}$ (nmol/min/mg) | 4248 ± 420 | 122 ± 40    | 3232 ± 400 |
| $V_{max}/K_m$ | 330       | 6           | 100        |
| **C24:0** |             |             |            |
| $K_m$ (µM) | 4.8 ± 1.0   | 13 ± 3      | 18 ± 8     |
| $V_{max}$ (nmol/min/mg) | 1051 ± 69  | 243 ± 30    | 240 ± 50   |
| $V_{max}/K_m$ | 220       | 20          | 13         |

# Data from reference 8.
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FIGURE LEGENDS:

Figure 1. Immunoblot analysis of detergent soluble extracts of FATP4 from COS1 cells and SDS-PAGE analysis of purified FATP4. FATP4-Flag plasmid was transfected into COS1 cells and 72 h post-transfection, cells were harvested by centrifugation and extracts prepared in buffer A (150 mM Tris-HCL, pH 7.5, 250 mM sucrose, and 150 mM NaCl) containing either 1% Triton X-100, 2% octaethylene glycol monododecyl ether (C12E8), 1% n-dodecyl-β-D-maltopyranoside (DDM), 2% n-octyl-β-D-glucopyranoside (OG), or 1% 1,2-diheptanoylphosphatidylcholine (DHPC). Panel A, equal volumes of extracts were separated on a 7% SDS-polyacrylamide gel and analyzed for the presence of FATP4 immunochemically using α-FATP4 antibodies. Detection was accomplished using HRP-conjugated goat anti-rabbit IgG secondary antibody and enhanced chemiluminescence. Lane 1, 1% Triton X-100; lane 2, 2% C12E8; lane 3, 1% DDM; lane 4, 2% OG; lane 5, 1% DHPC. Numbers on y-axis represent molecular mass markers in kDa. Panel B, the FATP4 soluble crude extracts were assayed for C24:0 (■) and C16:0 (○) acyl-CoA synthetase activity. Numbers on x-axes correspond to western blot lanes. The values are expressed as the mean ± standard deviation. Data presented is from a representative experiment of three independent determinations. Panel C, silver stain (lane 1) and immunodetection with α-FATP4 antibody (lane 2) of pooled elution fractions from a FATP4 purification. Numbers on y-axes represent molecular mass markers in kDa.
Figure 2. **FATP4 sensitivity and stability in DDM.** Panel A, purified FATP4 was assayed for acyl-CoA synthetase activity with C16:0 (○) or C24:0 (■) in the presence of increasing concentrations of DDM. Initial acyl-CoA synthetase activity is defined as the activity in the presence of 0.02% DDM. Initial C24:0 activity was 194 nmol/min/mg and initial C16:0 activity was 410 nmol/min/mg. Panel B, stability of FATP4 at 4° C (■) and 37° C (▲) was determined by assaying acyl-CoA synthetase activity with C24:0 at various time points. The values are expressed as the mean ± standard deviation. Data presented is from a representative experiment of three independent determinations.

Figure 3. **FATP4 acyl CoA synthetase reaction conditions.** Panel A, acyl CoA synthetase activity as a function of FATP4. Panel B, time course of C16:0 esterification by FATP4. Panel C, effect of pH on FATP4 acyl CoA synthetase activity. Panel D, effect of ionic strength on FATP4 acyl CoA synthetase activity. Data points are expressed as the mean ± standard deviation. Data presented is from a representative experiment of three independent determinations.

Figure 4. **Substrate analysis for purified FATP4 (■).** Panel A, activity as a function of C16:0. Panel B, activity as a function of C24:0. Panel C, activity as a function of ATP with C16:0 as the substrate. Panel D, activity as a function of CoA with C16:0 as the substrate. Data points are expressed as the mean ± standard deviation. Data presented is from a representative experiment of three independent determinations.
Figure 5. Competition studies with various long chain and very long chain fatty acids.

Purified FATP4 was assayed for long chain or very long chain acyl-CoA synthetase activity in the presence of indicated fatty acids at 15 µM. Panel A, C16:0 (○) esterification was evaluated with the following competitor fatty acids: C24:0 (■), C20:0 (×), and C16:0 (●). Panel B, C24:0 (■) esterification was evaluated with the following competitor fatty acids: C20:4 (×) and C16:0 (○). The values are expressed as the mean ± standard deviation. Data presented is from a representative experiment of two independent determinations.

Figure 6 Inhibition of FATP4 synthetase activity. Purified FATP4 was assayed for C16:0 (open symbols) and C24:0 (closed symbols) acyl-CoA synthetase activity in the presence of increasing concentrations of (A) palmitoyl-CoA, (B) Triacsin C, and (C) troglitazone. The values are expressed as the mean ± standard deviation. Data presented is from a representative experiment of three independent determinations.

Figure 7 Acyl CoA Synthetase activity in FATP4 null mice. Tissues from FATP4 null mice and wild type littermates were assayed for (A) C16:0, (B) C18:1, and (C) C24:0 acyl CoA synthetase activity. * denotes p-values <0.05. The values are expressed as the mean ± standard deviation. Data presented is from a representative experiment of three independent determinations.
Enzymatic properties of purified murine fatty acid transport protein 4 and analysis of Acyl CoA synthetase activities in tissues from FATP4 null mice
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