

# Identification of the Major Intestinal Fatty Acid Transport Protein

Andreas Stahl,\* David J. Hirsch,\*†  
Ruth E. Gimeno,‡ Sandhya Punreddy,‡ Pei Ge,‡  
Nicki Watson,\* Shraddha Patel,\* Mariana Kotler,\*  
Alejandra Raimondi,‡ Louis A. Tartaglia,‡  
and Harvey F. Lodish\*†§

\*The Whitehead Institute for Biomedical Research  
9 Cambridge Center  
Cambridge, Massachusetts 02142

†Department of Biology  
Massachusetts Institute of Technology

‡Millennium Pharmaceuticals, Incorporated  
640 Memorial Drive  
Cambridge, Massachusetts 02139

## Summary

While intestinal transport systems for metabolites such as carbohydrates have been well characterized, the molecular mechanisms of fatty acid (FA) transport across the apical plasmalemma of enterocytes have remained largely unclear. Here, we show that FATP4, a member of a large family of FA transport proteins (FATPs), is expressed at high levels on the apical side of mature enterocytes in the small intestine. Further, overexpression of FATP4 in 293 cells facilitates uptake of long chain FAs with the same specificity as enterocytes, while reduction of FATP4 expression in primary enterocytes by antisense oligonucleotides inhibits FA uptake by 50%. This suggests that FATP4 is the principal fatty acid transporter in enterocytes and may constitute a novel target for antiobesity therapy.

## Introduction

Fats, mainly in the form of di- and triglycerides, contribute over 40% of the caloric content of western diet (Clandinin et al., 1991). Efficient intraluminal digestion and absorption predominantly in the jejunum and also ileum allow less than 5% of the ingested lipids to escape with feces (Carey et al., 1983). Lipases, mainly pancreatic lipase, liberate fatty acids from the lipid droplets in the small intestine (Verger et al., 1996), which then form mixed micelles with bile acids. Long chain fatty acids (LCFAs) are absorbed by the epithelial cells of the small intestinal villi termed enterocytes (Windler and Greten, 1989), reesterified, and incorporated into chylomicrons as triglyceride. Chylomicrons are formed in the ER of enterocytes and undergo exocytosis at the basolateral side of the cell where they subsequently enter the lymphatic system (Tso and Balint, 1986).

The uptake of many nutrients including amino acids, di- and tripeptides, and many vitamins and minerals is mediated by energy-coupled transporters on the apical side of the enterocyte while other molecules, such as

fructose, enter the epithelial cells via facilitated diffusion (Thorens, 1993). Glucose entry into the enterocyte is mediated by SGLT1, a sodium-coupled glucose transporter in the brush border membrane of enterocytes (Hediger et al., 1987). Most of the glucose taken up by enterocytes is not modified and exits the cell following a concentration gradient through Glut 2, a facilitative glucose transporter expressed basolaterally (Thorens, 1992). Subsequent glucose transport in other organs such as liver, muscle, and kidney is then mediated by various members of the Glut family (Thorens, 1996).

Although there are well characterized examples of transporter families for amphipathic molecules such as bile acids (Suchy et al., 1997; Shneider, 1998), it was initially believed that LCFAs are absorbed by the intestinal epithelial cells through mere diffusion (Green and Riley, 1981; Ling et al., 1989). However, there is now ample evidence that, in addition to this diffusion component, the intestine (Stremmel, 1988b; Gore et al., 1994), liver (Stremmel, 1989), heart (Sorrentino et al., 1988; Stremmel, 1988a), adipose tissue (Schaffer and Lodish, 1994), and other organs express a saturable and competent LCFA transport system (Abumrad et al., 1998).

Using an expression cloning strategy, our lab had previously identified a membrane protein, fatty acid transport protein (FATP), from murine adipocytes that facilitates the uptake of LCFAs (Schaffer and Lodish, 1994). Subsequently, we reported the discovery of a large family of FATPs characterized by the presence of a FATP signature sequence (Faergeman et al., 1997; Hirsch et al., 1998). Human and mouse FATPs have unique expression patterns and are found in major organs of fatty acid metabolism such as adipose tissue, liver, heart, and kidney (Hirsch et al., 1998). So far, five distinct FATPs in mice and six different FATPs in humans have been identified and designated mmFATP1 through mmFATP5 and hsFATP1 through hsFATP6, respectively (Hirsch et al., 1998). Here, we show that a member of this novel family, FATP4, mediates the efficient uptake of fatty acids by enterocytes.

## Results

### FATP4 Is a Functional Fatty Acid Transporter

Full-length hsFATP1 and hsFATP4 cDNAs were identified by searching Millennium's databases using the BlastX algorithm (Altschul et al., 1990). A full-length mmFATP4 was amplified by PCR from a liver library. Alignments of human and mouse FATP1 and FATP4 showed 91% identity between hs- and mmFATP4, while the homology to the closest related gene, FATP1, in the same species was significantly less (62% identity), clearly demonstrating that hsFATP4 is indeed the homolog of mmFATP4. During the preparation of this paper, another group (Fitscher et al., 1998) independently cloned hsFATP4 and reported an amino acid sequence identical to ours.

Using a mammalian expression vector, we generated 40 stable 293 cell lines expressing hsFATP4 and 20 cell

§ To whom correspondence should be addressed (e-mail: lodish@wi.mit.edu).

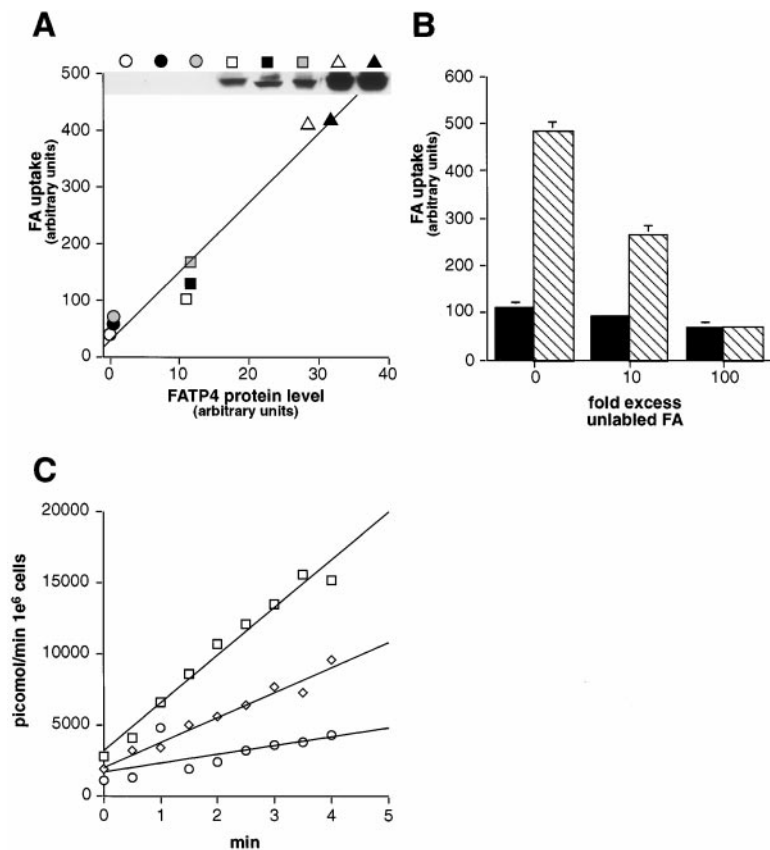


Figure 1. FATP4 Mediates LCFA Uptake

(A) Mammalian expression constructs containing either hsFATP4 (squares and triangles) or empty control vector (circles) were stably transfected into 293 cells. Short-term uptake of Bodipy-FA in the presence of BSA was determined by FACS. The mean fluorescence of the viable cell population is expressed in arbitrary fluorescence units. FATP4 protein expression was determined by densitometry of anti-FATP4 Western blots, shown in the insert, and is expressed in arbitrary units.

(B) Short-term uptake of Bodipy-palmitate (1  $\mu$ M), either by control cells (black bars) or FATP4-expressing cells (hatched bars), was measured in the presence of 0, 10, or 100  $\mu$ M unlabeled palmitate. FA uptake was quantified by FACS and expressed in arbitrary fluorescence units.

(C) The rate of [ $^3$ H]palmitate uptake by 293 cells, which were stably transfected with a construct for either human FATP4 (diamonds) or an empty vector (circles), was compared to that of isolated enterocytes (squares).

lines transfected with a control plasmid. The ability of the different cell lines to take up FA, as assessed by uptake assays using the fluorescently labeled Bodipy-palmitate, correlated well with their FATP4 expression levels determined by Western blotting (Figure 1A). All 20 vector control clones showed amounts of Bodipy-FA uptake similar to each other and to untransfected 293 cells. In contrast, among the 40 FATP4-transfected clones, a large number ( $\sim$ 20) showed an approximately 2-fold increase in Bodipy-FA uptake compared to any of the vector controls, and three had a 5- to 10-fold increase in Bodipy-FA uptake.

Several of the cell lines with the highest amount of Bodipy-FA uptake as well as isolated primary enterocytes were used to measure the uptake of radiolabeled FAs. Short-term uptake by 293 cells and enterocytes of all FAs tested was linear (Figure 1C). hsFATP4 expression enhanced the rate of palmitate uptake approximately 3-fold over 293 cells transfected with vector alone (Figure 1C) and also accelerated the uptake of oleate but not of linolate, arachidonate, octanoate, butyrate, or cholesterol (Table 1). Isolated primary enterocytes showed a similar preference for palmitate and oleate and absence of transport of arachidonate, octanoate, and butyrate, but displayed a more robust transport of linolate and cholesterol than the transfected 293 cells.

To further characterize the substrate specificity of FATP4, we measured the uptake by stably transfected 293 cells of 5  $\mu$ M Bodipy-FA in the presence of a 20-fold molar excess (i.e., 100  $\mu$ M) of FAs, FA derivatives,

and lipid-soluble vitamins and hormones. Both saturated and nonsaturated fatty acids containing 10–26 C atoms strongly competed for uptake of Bodipy-palmitate (Figure 1B and Table 2) and thus are presumed to be substrates of FATP4. In contrast, fatty acids with eight or fewer C atoms did not compete and thus are presumed not to be FATP4 substrates. Similarly, esters of long chain FAs and other hydrophobic molecules tested had no effect on uptake of Bodipy-palmitate.

#### FATP4 Is Strongly Expressed by the Enterocytes of the Small Intestine

To determine the cellular distribution of FATP4 in E18.5 C57/B16 embryos, in situ hybridization with a non-cross-reacting probe was used. On embryo whole mounts, strong mmFATP4 expression could be seen in the small intestine (Figure 2A, left panel). Microscopy of counterstained sections showed that FATP4 mRNA was present at high levels in the enterocytes of the intestinal villi (Figure 2A, right panels). In situ hybridization of cross sections of the ileum and duodenum from 8-week-old adult mice also demonstrated a strong expression of FATP4 in enterocytes (Figure 2B). The highest expression levels of FATP4 were seen in the enterocytes of the jejunum and ileum, and lower, but significant, amounts were detected in the epithelial cells of the duodenum. However, FATP4 mRNA was undetectable in any other cell type of the small intestine such as mesenchymal, endothelial, and smooth muscle cells and was completely absent from the colon (Figure 2B, left six panels).

Table 1. Uptake of Different Substrates by FATP4-Expressing Cell Lines and Enterocytes

Fatty Acid	293 Cells		FATP4 Specific <sup>a</sup>	Enterocytes <sup>a</sup>
	Control <sup>a</sup>	Stably Expressing FATP4 <sup>a</sup>		
Palmitate	564	1695	1131	3036
Oleate	662	1122	459	117
Linolate	640	673	33	116
Arachidonate	3	5	2	0
Octanoate	0	0	0	5
Butyrate	0	50	50	73
Cholesterol	319	345	26	531

Uptake of different substrates by enterocytes and by control and stable FATP4-expressing 293 cells. The rates of uptake for the indicated fatty acids was measured over 4 min taking measurements every 30 s. All fatty acids were at a concentration of 10  $\mu$ M in HBS containing 5 mM taurocholate.

<sup>a</sup>Uptake measured as pmol/min 10<sup>6</sup> cells.

Interestingly, while the expression of FATP4 in the mature enterocytes of the villi was exceptionally high, it was low or undetectable in the undifferentiated precursor cells in the crypts between the villi (Figures 2A, right panel, and 2B, upper left four panels). No signals above background were detected for mmFATP1, mmFATP3 (data not shown), and mmFATP5 in any of the intestinal

tissues (Figure 2B, right six panels). mmFATP2 was detected by in situ hybridization at low levels in the epithelial cells of the ileum, jejunum, and duodenum. Northern blot analysis of hsFATP1 through hsFATP6 in human ileum and jejunum confirmed the notion from the murine data that only FATP4 is expressed at appreciable levels in the small intestine (Figure 3).

Table 2. Competition of Bodipy-FA Uptake by FATP4-Expressing Cells

Fatty Acids	Formula	Competition
Butyric acid	C <sub>4</sub> H <sub>8</sub> O <sub>2</sub>	–
Caproic acid	C <sub>6</sub> H <sub>12</sub> O <sub>2</sub>	–
Caprylic acid	C <sub>8</sub> H <sub>16</sub> O <sub>2</sub>	–
Capric acid	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>	++
Lauric acid	C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>	++
Myristic acid	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	++
Palmitic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	++
Stearic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	+
Oleic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	++
Linoleic acid	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	++
Arachidic acid	C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>	++
Lignoceric acid	C <sub>24</sub> H <sub>48</sub> O <sub>2</sub>	++
Cerotic acid	C <sub>26</sub> H <sub>52</sub> O <sub>2</sub>	++
Fatty Acid Derivatives		
Palmitic acid methyl ester	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	–
Stearic acid methyl ester	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	–
Oleic acid ethyl ester	C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>	–
Oleic acid oley ester	C <sub>36</sub> H <sub>68</sub> O <sub>2</sub>	–
Oleoyl CoA	C <sub>39</sub> H <sub>68</sub> N <sub>7</sub> O <sub>17</sub> P <sub>3</sub> S	–
Cholesteryl oleate	C <sub>45</sub> H <sub>78</sub> O <sub>2</sub>	–
Lipid-Soluble Vitamins and Hormones		
Retinoic acid (pro-vitamin A)	C <sub>20</sub> H <sub>28</sub> O <sub>2</sub>	±
Ergocalciferol (vitamin D2)	C <sub>28</sub> H <sub>44</sub> O	–
Tocopherol (vitamin E)	C <sub>29</sub> H <sub>50</sub> O <sub>2</sub>	–
3-Phytylmenadione (vitamin K1)	C <sub>31</sub> H <sub>46</sub> O <sub>2</sub>	–
Prostaglandin E2	C <sub>20</sub> H <sub>32</sub> O <sub>5</sub>	–

Competition for bodipy-FA uptake by FATP4-expressing cells by different hydrophobic compounds. The uptake of 5  $\mu$ M Bodipy-FA, C1-Bodipy-C12, was measured in the presence a 20-fold molar excess (i.e., 100  $\mu$ M) of the indicated fatty acids or fatty acid derivatives. The maximal 100% inhibition was defined as the amount of Bodipy-FA incorporated in the presence of 200  $\mu$ M lauric acid which was on average 18%  $\pm$  5% that of untreated cells. –, 0%–30% inhibition by the indicated substance;  $\pm$ , 30–50% inhibition; +, 50%–70% inhibition; ++, 70%–100% inhibition.

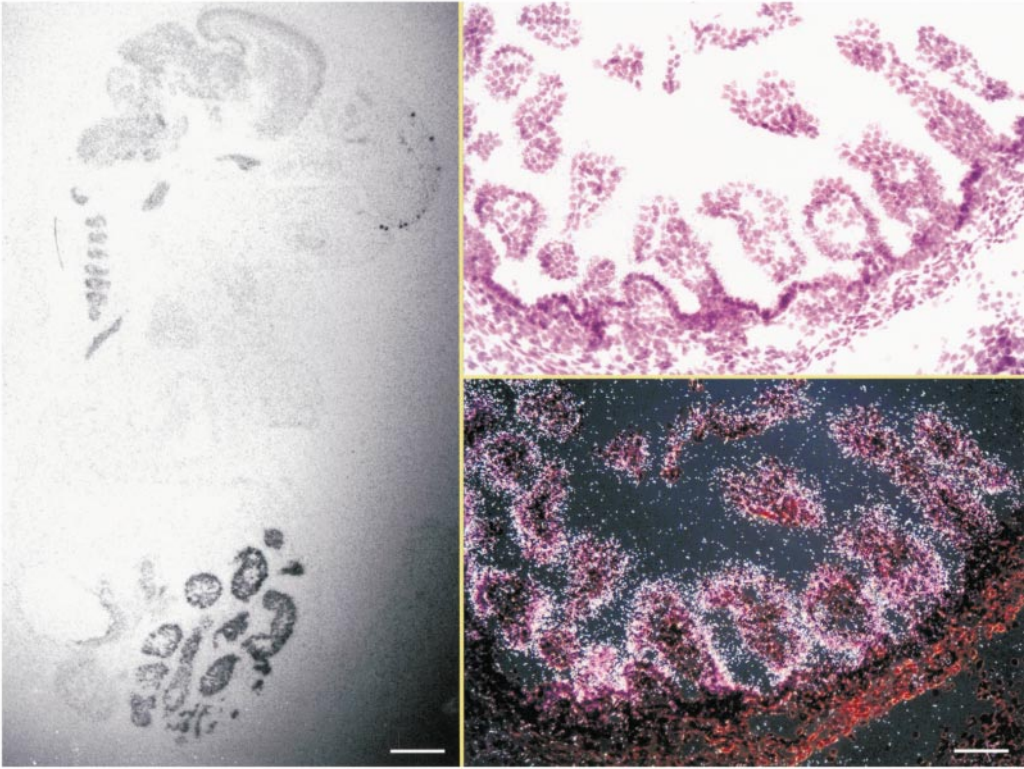
#### The FATP4 Protein Is Localized to the Apical Side of Enterocytes

To further characterize the FATP4 protein and its localization, we raised a polyclonal antiserum against a GST fusion protein of the C terminus of mmFATP4 in rabbits. The antiserum showed only weak cross-reactivity with the GST fusion proteins of C termini of other FATP family members in Western blot experiments (Figure 4A). In Western blot experiments with lysates from isolated enterocytes from three different adult Balb C mice, the antiserum exclusively recognized a single approximately 70 kDa band, which is in accordance with the predicted molecular weight of 72 kDa for mmFATP4 (Figure 4B). This signal was specific for FATP4 since none was obtained with preimmune serum (data not shown), and, more importantly, it could be abolished by preincubation of the anti-FATP4 serum with FATP4-GST fusion protein (Figure 4B).

Immunofluorescence microscopy of fresh frozen unfixed sections of adult mouse small intestine with the FATP4-specific antiserum confirmed the expression of FATP4 in the epithelial cell layer of the villi (Figure 5B), while incubation with a preimmune serum from the same rabbit demonstrated only weak background fluorescence (Figure 5A). At higher magnifications (Figures 5C and 5D), it was apparent that FATP4 is preferentially localized to the apical side of the enterocyte that faces the lumen of the small intestine. Similar observations were made for the localization of hsFATP4 in the human ileum and jejunum (data not shown). Further analysis of the subcellular localization of FATP4 in enterocytes by deconvolution microscopy confirmed that the transporter is localized to the apical side of the enterocyte including the brush border membrane (Figure 5E).

Immunoelectron microscopy of fresh frozen sections through the small intestine using a FATP4-specific polyclonal antiserum showed a gradient of protein distribution starting from the apical side of the paranuclear

**A**



**B**

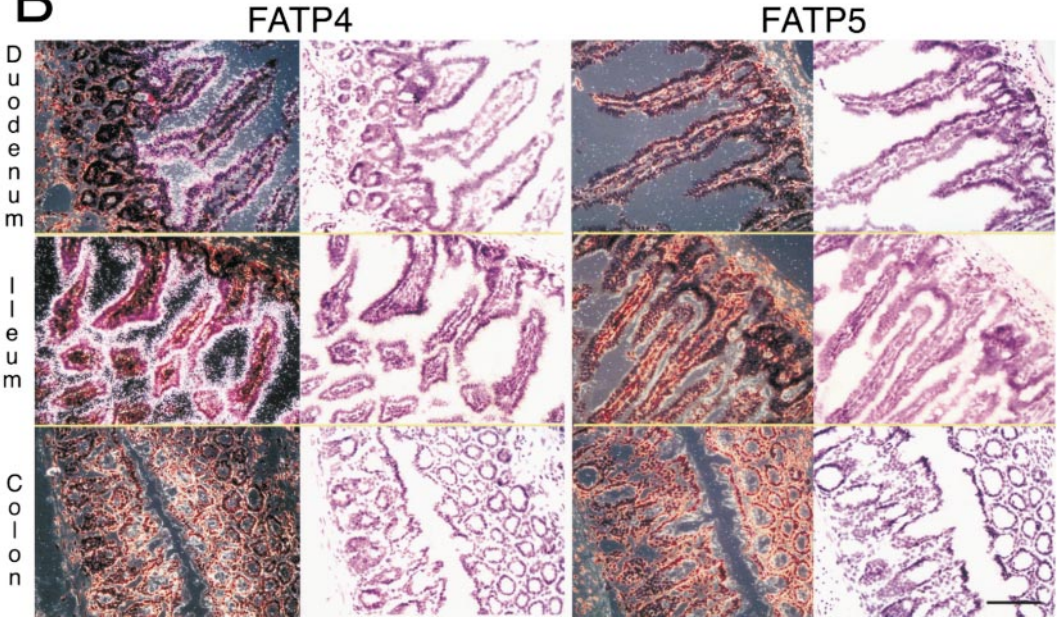


Figure 2. FATP4 Is Localized in Enterocytes by In Situ Hybridization  
(A) In situ hybridization of a section through a whole E18.5 embryo (left panel) and sections through the small intestine of E18.5 embryo (right panels) with a FATP4-specific riboprobe shown at 200 $\times$  in phase contrast and dark field.  
(B) In situ hybridization of a section through duodenum, ileum, and colon of adult mice with FATP4- and FATP5-specific riboprobes shown at 200 $\times$  in dark field and phase contrast.

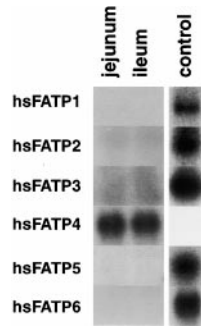


Figure 3. FATP4 Is the Prevalent Member of the FATP Family in the Ileum and Jejunum

Northern blot analysis of the expression patterns of hsFATP1 through hsFATP6 in the ileum and jejunum. Probes were from unique regions of the genes and did not cross-react with other FATP members. As a control, all probes were hybridized under similar conditions to mRNAs from a variety of FATP-expressing tissues. FATP1, heart; FATP2, kidney; FATP3, lung; FATP5, liver; FATP6, heart.

region and being most prominent in the microvilli of the brush border membrane and unidentified membranous structures underlying this area (Figure 6A). FATP4 molecules could be consistently detected in association with the plasma membrane of microvilli in the enterocyte brush border (Figures 6B and 6C).

The apical localization of FATP4 and its exclusion from the basolateral face of the enterocyte is indicative of a role of the transporter in absorption of dietary fatty acids rather than the import of fatty acids from the blood.

#### FATP4 Is Required for Efficient Uptake of Fatty Acids by Enterocytes

To demonstrate the importance of FATP4 for the absorption of dietary lipids by the small intestinal epithelium,

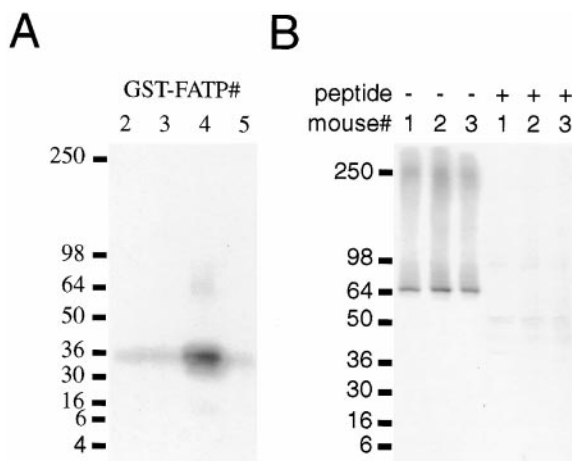


Figure 4. The FATP4 Protein Is Specifically Detected by a Polyclonal Antiserum

(A) Western blot of GST fusion proteins of the C termini of FATP2 through FATP5 with a polyclonal antiserum against the C terminus of FATP4.

(B) Western blot detection of FATP4 in enterocyte lysates from three different mice without or with preincubation of the antiserum with the antigen. Molecular mass standards are indicated in kDa.

we developed an antisense-based approach to modulate the expression of FATP4 in primary cultures of enterocytes. To this end, mouse enterocytes were isolated from the small intestine and cultured *ex vivo* for 48 hr. Although cells did not reattach to the tissue culture treated plastic, viability after 2 days was usually greater than 90%. LCFA uptake was measured at 37°C by incubating enterocytes with a solution of mixed micelles consisting of radiolabeled FAs and the bile acid taurocholate, thereby mimicking the presentation of fatty acids in the intestine after the cleavage of triglycerides by pancreatic lipase. At a 50  $\mu$ M concentration, uptake of [ $^3$ H]oleate by cultured enterocytes was linear over a 10 min time interval (Figure 7A) and was temperature dependent (data not shown). Incubation of enterocytes for 48 hr with a 100  $\mu$ M solution of a phosphothioate oligonucleotide corresponding to nucleotides 10–28 of mmFATP4 in the sense orientation had no effect on the rate of oleate uptake, when compared to untreated cells (Figure 7A). However, incubation with the corresponding antisense oligonucleotide reduced the rate of enterocyte oleate uptake by approximately 50%. In contrast, a mismatched control oligonucleotide with identical base composition had no effect on FA uptake (Figure 7B). The cell viability in all cases was comparable (approximately 90%). FATP4 antisense treatment affected only FATP4-mediated uptake processes since two FATP4 antisense oligonucleotides (nucleotides 10–28 and 22–42) inhibited palmitate and oleate but not methionine uptake and incorporation (Figure 7C). Further, we evaluated the dose response of antisense inhibition and its correlation with FATP4 protein levels. Incubation with increasing concentrations of the FATP4 antisense oligonucleotide resulted in increased reductions of [ $^3$ H]oleate uptake and a proportionate decrease in the level of FATP4 protein; at the highest concentration of oligonucleotide tested, both were decreased by 60% (Figure 7B). Thus, [ $^3$ H]oleate incorporation by enterocytes is proportional to the level of FATP4 protein, and we conclude that FATP4 accounts for most FA transport by isolated enterocytes.

#### Discussion

While intestinal transport systems for metabolites such as carbohydrates have been well characterized, the molecular mechanisms of fatty acid transport across the apical plasmalemma of enterocytes have remained largely unclear. Here, we identify FATP4 as the principal fatty acid transporter in the small intestine that mediates the efficient uptake of dietary fatty acids.

FATP4 is expressed at high levels on the apical side of mature enterocytes in the small intestine. Stable overexpression of FATP4 in 293 cells significantly enhances the uptake of long chain fatty acids with the same specificity as enterocytes. FATP4 expression enhanced uptake of palmitate and, to a lesser extent, that of oleate, but not of FAs with fewer than ten C atoms. Isolated enterocytes exhibited a similar preference for palmitate over other tested FAs and, identically to FATP4-expressing 293 cells, did not incorporate significant amounts of octanoate, arachidonate, or butyrate. FATP4-specific uptake of the essential FA linolate by transfected 293

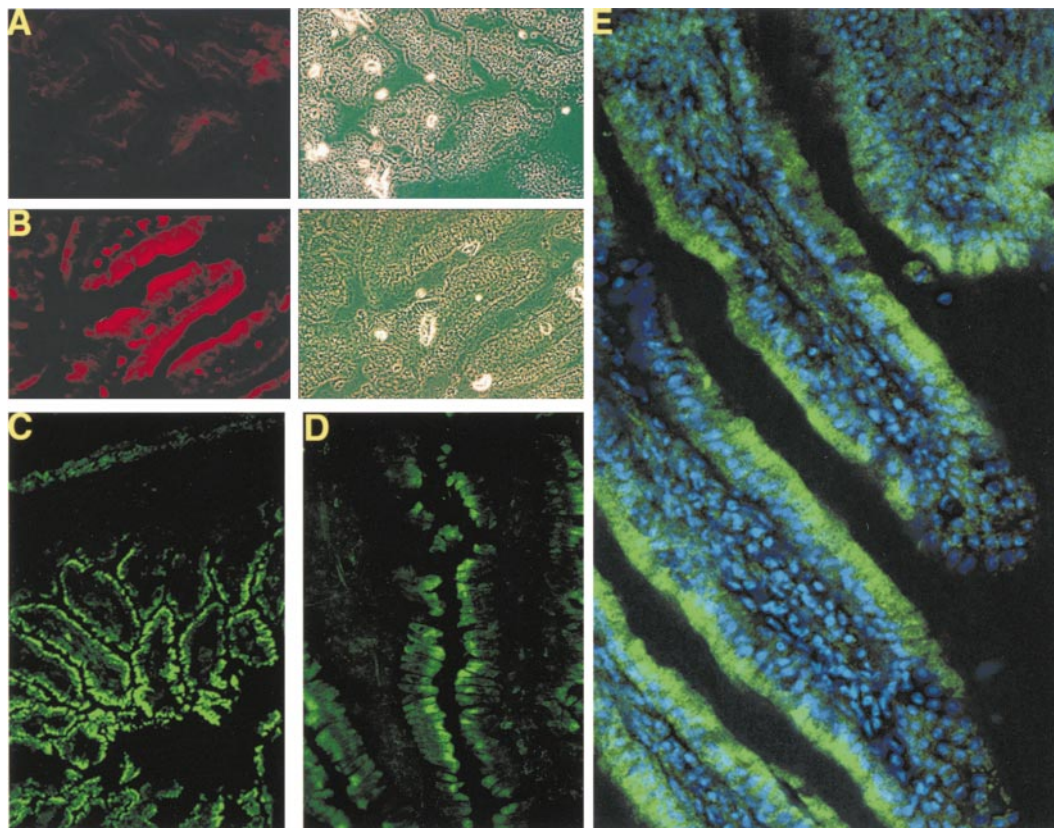


Figure 5. FATP4 Is Located on the Apical Face of Enterocytes

Fluorescent and phase contrast pictures of murine small intestine. Fresh frozen thin sections were either incubated with preimmune (A) or anti-FATP4 serum (B). Confocal laser microscopy images of FATP4 in the small intestine at 20 $\times$  (C) and 63 $\times$  (D). Deconvolution microscopy of a small intestinal thin section (E) stained with anti-FATP4 (green) and DAPI (blue) at a 40 $\times$  magnification.

cells was significantly lower than that of enterocytes. However, uptake of linolate, as well as oleate, by control-transfected 293 cells was higher than uptake of these FAs by enterocytes, potentially masking small increases in linolate and oleate uptake due to FATP4 expression. The 293 cell line is derived from a kidney carcinoma, and kidney expresses high amounts of FATP2 and smaller levels of FATP1 (Hirsch et al., 1998). The expression of FATP1 and FATP2 in 293 cells is currently unknown but could potentially be responsible for the observed differences between untransfected 293 cells and enterocytes.

Both competition and uptake assays demonstrate that FATP4 has a clearly defined substrate specificity and can mediate the transport of a wide range of physiologically relevant FAs. Short chain FAs up to a chain length of C10 are not transported, while saturated and unsaturated long chain and very long chain FAs are efficient substrates. Interestingly, arachidonate was inefficiently transported both by FATP4-expressing cell lines and enterocytes, while stable expression of FATP1 in 3T3 cells did mediate a pronounced increase in arachidonate uptake (Schaffer and Lodish, 1994), hinting at distinct differences in substrate specificity between the different FATPs. Further, while modifications at the end of the aliphatic chain, such as a Bodipy group, are tolerated, esterifications or other modifications of the carboxyl group led to an abrogation of transport.

Although FATP4 is present at low levels in a variety of tissues, most notably the brain (our unpublished data; Fitscher et al., 1998), it is the major FATP protein expressed in the small intestine as found by Northern blot and in situ hybridization analysis. The epithelial cells of the ileum and the jejunum are the principle sites of dietary fatty acid absorption (Windler and Greten, 1989). FATP4 was expressed at high levels in the enterocytes in these locations as judged by in situ hybridization while it was absent from all other intestinal cell types. The notion that FATP4 is involved in the absorption of dietary fatty acids rather than solely the supply of an energy source to the enterocytes themselves was further supported by the observation that FATP4 mRNA and protein levels were high in the differentiated enterocytes along the villi but low or absent in the undifferentiated progenitor cells in the crypts that have a high metabolic rate but are not involved in the absorption of nutrients. Further, FATP4 was absent from the colon, which is known to play only a marginal role in the absorption of long chain fatty acids (Windler and Greten, 1989). Interestingly, our preliminary data show that a FATP isoform in *D. melanogaster* is localized in the mid- and hindgut of the larvae (data not shown) implicating FATPs also in nutritional uptake by invertebrates.

Nutritional transporters such as glucose transporters and other membrane proteins can be targeted to either of two distinct plasma membrane compartments in en-

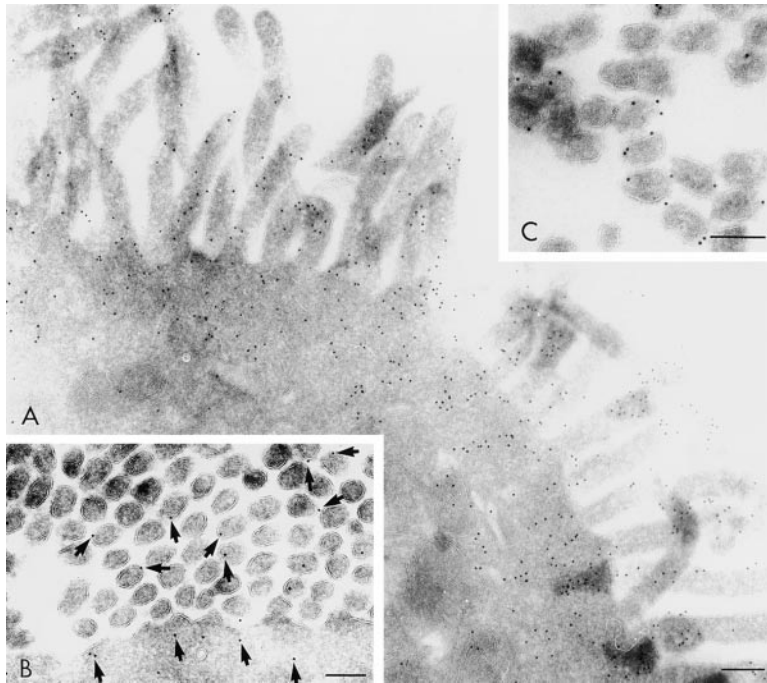


Figure 6. FATP4 Is Present in the Microvilli  
Immunoelectron microscopy of fresh frozen murine intestinal cells. Fresh frozen unfixed microsections of murine ileum were incubated with FATP4-specific antiserum, which was detected by 10 nm gold conjugated secondary antibodies. Longitudinal section through brush border membrane ([A], bar = 0.21  $\mu$ M). Cross section through microvilli ([B], bar = 0.3  $\mu$ M); arrows indicate the position of the gold particles. High magnification cross section through microvilli ([C], bar = 0.12  $\mu$ M).

terocytes (Rodriguez-Boulan and Nelson, 1989; Stevens, 1992). Apical transporters are involved in the uptake of nutrients from the lumen of the intestine and are often energy coupled. In contrast, basolateral transporters allow the absorbed molecules to leave the enterocytes and enter the blood stream, as is the case for sugars, while exocytosis at the basolateral allows

larger particles such as chylomicrons to leave the enterocyte and enter the lymphatics. We demonstrate here that FATP4 in enterocytes is found predominantly in the apical compartment, including the microvilli of the brush border membrane, suggesting that FATP4 is involved in the uptake of dietary fatty acids from the intestinal lumen into the enterocyte.

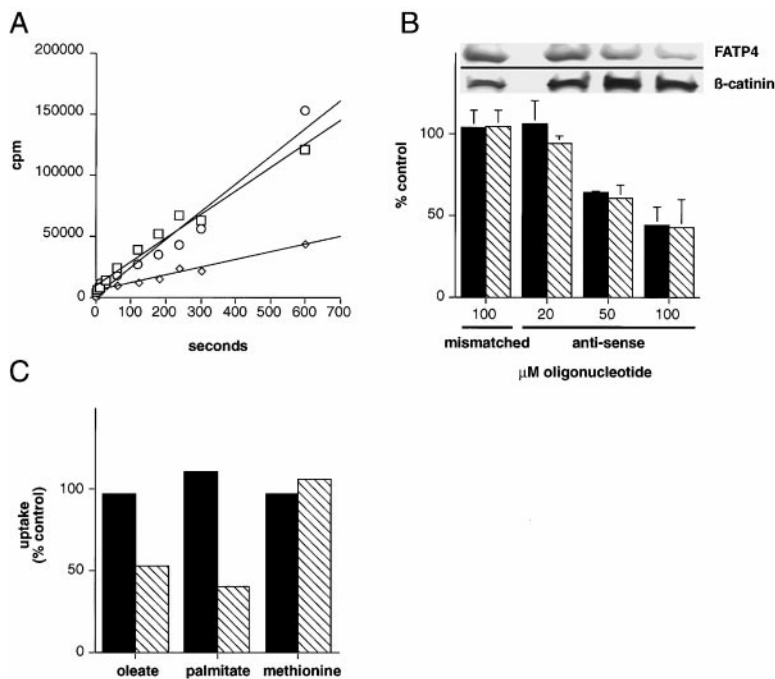


Figure 7. FATP4 Is Required for Efficient LCFA Uptake by Enterocytes

(A) The kinetics of [ $^3$ H]oleate incorporation by enterocytes isolated from the small intestine of mice were measured after incubation for 48 hr in tissue culture either without oligonucleotides (squares) or with 100  $\mu$ M FATP4-specific sense (circles) or antisense (diamonds) oligonucleotides.

(B) Isolated enterocytes were incubated for 48 hr with increasing concentrations of the FATP4 antisense oligonucleotide or with 100  $\mu$ M of a randomized control oligonucleotide with identical nucleotide composition to the FATP4 antisense oligonucleotide. The uptake of oleate by the enterocytes was then measured over a 5 min time interval (solid bars). In parallel, the levels of FATP4 protein and, as a loading control,  $\beta$ -catenin, were determined by Western blotting and quantitated using densitometry (hatched bars). FA uptake and FATP4 protein levels were normalized to that of untreated cells. The averages and standard deviations of four independent experiments are shown.

(C) Uptake rates of [ $^3$ H]oleate, [ $^3$ H]palmitate, and [ $^{35}$ S]methionine by primary enterocytes were measured after 48 hr incubation with either of two FATP4 antisense (solid bars) or randomized control oligonucleotide (hatched bars) at a concentration of 100  $\mu$ M and expressed as percent of untreated cells.

The fact that FATP4 is a functional long chain fatty acid transporter highly expressed on the apical side of enterocytes in the small intestine strongly suggests that it is involved in the transporter-mediated uptake of dietary fatty acids. However, the overall contribution of FATP4 to the absorption of dietary lipids could not be concluded from these data alone. Therefore, we used an antisense oligonucleotide-based approach to modulate FATP4 expression in primary enterocytes. Phosphothioate-modified oligonucleotides have been successfully employed to modulate expression of a number of genes in different cell types (Agrawal and Zhao, 1998; Gewirtz et al., 1998) including the downregulation of membrane transport proteins (Oberbauer et al., 1996). Primary enterocytes have been successfully isolated before from many sources including rat and hamster (Pinkus, 1981; Velasco et al., 1986; Mircheff and van Corven, 1990). After isolation, enterocytes demonstrate saturable and temperature-dependent uptake of LCFA (Gore et al., 1994; our unpublished data). FATP4 antisense treatment of the enterocytes greatly reduced their ability to take up LCFA, while the corresponding sense control oligonucleotide had no effect. Concentration-dependent reduction of the level of FATP4 protein expression by antisense oligonucleotides was closely correlated with a reduction in FA uptake. A 60% reduction in the level of FATP4 protein led to a corresponding 60% reduction in the rates of oleate and palmitate uptake, indicating that FATP4 is indeed responsible for the majority of long chain FA uptake into enterocytes. The notion that FATP4 antisense oligonucleotides reduced LCFA uptake specifically by inhibiting FATP4 expression was further supported by the fact that a chemically identical control oligonucleotide with scrambled sequence had no effect and by the observation that other energy-dependent uptake processes, such as amino acid uptake, were not altered by the FATP4 antisense oligonucleotide treatment. Further, two different FATP4 antisense oligonucleotides, both immediately downstream of the translational initiation site, inhibited FA uptake into enterocytes, while their respective mismatched controls had no effect. Three additional oligonucleotides further downstream of the FATP4 gene were also tested but showed only marginal (10%–15%) inhibition of FA uptake; however, in all cases, incubation with the corresponding control mismatched oligonucleotides also had no inhibitory effect (data not shown). These data show, for the first time, that the downmodulation of an endogenous FATP in primary cells results in a significantly reduced rate of FA uptake, illustrating the importance of FATPs for this process.

Besides the FATP family, several other molecules have been implicated in the binding and transport of fatty acids. A homolog of the human CD 36 scavenger receptor (FAT) binds a large number of hydrophobic molecules including fatty acids (Baillie et al., 1996) and is localized to adipocytes, myocytes, mammary cells, and enterocytes (Sfeir et al., 1997). However, it is absent from liver but highly expressed in organs not associated with FA metabolism such as spleen (Abumrad et al., 1993). Plasma membrane fatty acid-binding protein (FABPpm), a 43 kDa molecule, transiently increases fatty acid uptake in 3T3 fibroblasts and is expressed in several tissues including heart, liver, and small intestine

(Stremmel et al., 1985a, 1985b; Sorrentino et al., 1988). Further, antibodies against FABPpm were reported to decrease oleate uptake of perfused jejunal segments (Stremmel, 1988b). However, FABPpm was subsequently identified as the mitochondrial isoform of aspartate aminotransferase (Stump et al., 1993), and it remains unclear how this mitochondrial enzyme could participate in the transport of fatty acids across the plasma membrane. FATP4 expression correlates closely with FA uptake in 293 cells and enterocytes, and FATP4 can mediate the uptake of all physiological long chain FAs tested, demonstrating that FATP4 is the major FA transporter in the small intestine. However, it is likely that efficient FA uptake requires many components upstream as well as downstream of FATPs, possibly including extracellular scavenger receptors such as CD36, and intracellular molecules, like LCFA CoA ligases and fatty acid-binding proteins.

Over 55 million people in the US alone suffer from obesity (Van Itallie, 1996), contributing significantly to obesity-related health problems such as hypertension and type 2 diabetes (Ernst et al., 1997). Lipids in the average western diet contribute up to 40% of total calories, and efforts have been made to reduce the caloric input either by substitution of lipids with nondigestible analogs (Lawson et al., 1997) or by inhibiting pancreatic lipase (Guercioli, 1997). Since enterocytes can only absorb free fatty acids but not triglycerides, inhibition of hydrolysis of lipids by lipases blocks their uptake. However, recent clinical studies have only shown a modest effect of such treatments (Davidson et al., 1999). An alternative or additional treatment could be direct inhibition of fatty acid uptake (Thomson et al., 1997), which could be theoretically achieved by blocking FATP4 function. Our antisense data have shown that such treatments could in principle result in drastic reduction of LCFA uptake by the small intestine, making FATP4 an attractive target for future antiobesity drugs.

#### Experimental Procedures

##### Isolation of hsFATP1 and mmFATP4

Clones encoding full-length human FATP1 and FATP4 were obtained from a heart and a spleen cDNA library, respectively, by searching Millennium databases for sequences similar to murine FATP1–5 coding regions using the BlastX algorithm (Altschul et al., 1990).

##### Generation of Cell Lines Stably Expressing hsFATP4

A DNA fragment containing the entire hsFATP4-coding sequence, as well as 100 nucleotides of the 5' and 50 nucleotides of the 3' untranslated region, was inserted into the vector pIRES-neo (Clontech). The resulting construct or a vector control (pIRES-neo) was transfected into 293 cells using the lipofectamine method (GIBCO-BRL) according to the manufacturer's direction. Cells that had taken up the DNA were selected with 1 mg/ml G418 (GIBCO-BRL). Single colonies were picked 1 to 2 weeks after transfection and grown in medium containing 0.8 mg/ml G418. Colonies were screened for ability to take up fatty acids by measuring uptake of a fluorescently labeled fatty acid (Bodipy-FA) as described (Hirsch et al., 1998).

##### Northern Blotting

Human mRNA blots were obtained from Invitrogen. Blots were probed with <sup>32</sup>P-labeled DNA probes using the Rapid-Hyb buffer (Amersham) according to the manufacturer's instructions. Probes were generated by PCR from either the 3' untranslated regions or the poorly conserved 5' translated regions of human FATPs.

#### In Situ Hybridization

Tissues were collected from 8-week-old C57/Bl6 mice. Tissues were fresh frozen, cut on a cryostat at 10  $\mu$ m thickness, and mounted on Superfrost Plus slides (VWR). Sections were air dried for 20 min and then incubated with ice-cold 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) for 10 min. Slides were washed twice for 5 min each with PBS, incubated with 0.25% acetic anhydride/1 M triethanolamine for 10 min, washed with PBS for 5 min, and dehydrated with 70%, 80%, 95%, and 100% ethanol for 1 min each. Sections were incubated with chloroform for 5 min. Hybridizations were performed with <sup>35</sup>S-radiolabeled ( $5 \times 10^7$  cpm/ml) cRNA probes generated from the 3' untranslated regions of mouse FATPs by PCR followed by in vitro transcription in the presence of 50% formamide, 10% dextran sulfate, 1 $\times$  Denhardt's solution, 600 mM NaCl, 10 mM DTT, 0.25% SDS, and 100  $\mu$ g/ml tRNA for 18 hr at 55°C. After hybridization, slides were washed with 10 mM Tris-HCl (pH 7.6), 500 mM NaCl, 1 mM EDTA (TNE) for 10 min, incubated in 40  $\mu$ g/ml RNase A in TNE at 37°C for 30 min, washed in TNE for 10 min, incubated once in 2 $\times$  SSC at 60°C for 1 hr, once in 0.2 $\times$  SSC at 60°C for 1 hr, and once in 0.2 $\times$  SSC at 65°C for 1 hr, and dehydrated with 50%, 70%, 80%, 95%, and 100% ethanol. Localization of mRNA transcripts was detected by dipping slides in Kodak NBT-2 photoemulsion and exposing for 7 days at 4°C, followed by development with Kodak Dektol developer. Slides were counterstained with haematoxylin and eosin and photographed. Controls for the in situ hybridization experiments included the use of a sense probe that showed no signal above background in all cases.

#### Immunofluorescence and Immunogold Electron Microscopy

Unfixed mouse small intestine was washed with Hanks' buffered salt solution containing 1 mM EDTA, infused with 2.3 M sucrose solution, and embedded in O.C.T., 4583 compound. The material was thick sectioned (15  $\mu$ m–40  $\mu$ m). The sections were washed in PBS containing 1% BSA and 0.075% glycine to block nonspecific binding. Primary and secondary antibodies were diluted in PBS with 10% FCS and incubated for 1 hr. The sections were mounted in 90% glycerol/PBS containing 1 mg/ml paraphenylenediamine and examined with a Bio-Rad MRC 600 confocal, mounted on a Zeiss Axioscop.

For the immunogold labeling, the tissue was fixed with 2% paraformaldehyde in PBS for 10 min, after which it was cryoprotected by infiltration with 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.4), containing 20% polyvinylpyrrolidone, and then mounted on aluminum cryo nails and frozen in liquid nitrogen (Tokuyasu, 1986). Ultrathin sections were collected on carbon/formvar-coated nickel grids. The primary antibody (anti-FATP4) was diluted in 10% FCS in PBS and incubated overnight at 4°C, followed by donkey anti-rabbit IgG-gold (12 nm) (Jackson labs) for 1 hr. The sections were stained in 2% neutral uranyl acetate (20 min) and absorption stained with 2% uranyl acetate in 0.2% methylcellulose containing 3.2% polyvinyl alcohol. The sections were examined with a Philips EM 410 electron microscope.

#### Enterocyte Isolation and Antisense Oligonucleotide Treatment

Enterocytes from male and female 2- to 8-month-old BALB/c mice were isolated following standard procedures (Pinkus, 1981). In brief, small intestines (duodenum, ileum, and jejunum) were removed, rinsed with Hanks' buffered salt solution (HBS; GIBCO-BRL), cut into approximately 1 cm long sections, and incubated in 50 ml HBS containing 0.1 M sucrose (Bio-Rad) and 20 mM EDTA. Intestinal sections were gently stirred for 10 min. The detached enterocytes were filtered through sterile cheesecloth (VWR) and pelleted by centrifugation. The cells were then gently resuspended in RPMI 1640 (GIBCO-BRL) containing 10% FCS and 0.01 mg/ml transferrin. Concentrated solutions of oligonucleotides were added to the enterocytes to yield the indicated final concentrations and the cells were incubated for 48 hr at 37°C, 5% CO<sub>2</sub>. Two antisense oligonucleotides with their respective controls were used showing comparable results. The sequences of the phosphothioate oligonucleotides were: mmFATP4-S1, GGAGCCTCTGGTGGGGG; mmFATP4-AS1, CCC CCACCAGAGAGGCTCC; mmFATP4-Control1, CCACCCCGGAAAG CCTGC; mmFATP4-AS2, GGAGAACAGTAGGCCCCAC; mmFATP4-Control2, GAGCCCGCCACCGTAGAGACA.

#### LCFA Uptake Assays

Bodipy-FA uptake assays using FACS were performed as described previously (Hirsch et al. 1998) and also adapted to a 96-well format. LCFA uptake assays with enterocytes or with stably transfected 293 cells were done as follows. Mixed micelles of radiolabeled FA (NEN) and taurocholate (Sigma) in HBS were generated by brief sonication at 37°C. Equal volumes of cells and micelle solution were mixed, resulting in a final FA concentration of 25  $\mu$ M for antisense assays and 10  $\mu$ M for substrate specificity assays. Final taurocholate concentration was 5 mM. Cells were incubated for the indicated amount of time at 37°C. The assay was stopped by transferring the cells onto filter paper followed by extensive washes with ice-cold HBS containing 0.1% BSA using a cell harvester (Brandell). Incorporated oleate was then determined by  $\beta$ -scintillation counting (Beckman).

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