Fatty acid transport protein 4 (FATP4) is a fatty acyl-CoA synthetase that preferentially activates very long chain fatty acid substrates, such as C24:0, to their CoA derivatives. To gain better insight into the physiological functions of FATP4, we established dermal fibroblast cell lines from FATP4-deficient wrinkle-free mice and wild type (w.t.) mice. FATP4−/− fibroblasts had no detectable FATP4 protein by Western blot. Compared with w.t. fibroblasts, cells lacking FATP4 had an 83% decrease in C24:0 activation. Peroxisomal degradation of C24:0 was reduced by 58%, and rates of C24:0 incorporation into major phospholipid species (54–64% decrease), triacylglycerol (64% decrease), and cholesterol esters (58% decrease) were significantly diminished. Because these lipid metabolic processes take place in different subcellular organelles, we used immunofluorescence and Western blotting of subcellular fractions to investigate the distribution of FATP4 protein and measured enzyme activity in fractions from w.t. and FATP4−/− fibroblasts. FATP4 protein and acyl-CoA synthetase activity localized to multiple organelles, including mitochondria, peroxisomes, endoplasmic reticulum, and the mitochondria-associated membrane fraction. We conclude that in murine skin fibroblasts, FATP4 is the major enzyme producing very long chain fatty acid-CoA for lipid metabolic pathways. Although FATP4 deficiency primarily affected very long chain fatty acid metabolism, mutant fibroblasts also showed reduced uptake of a fluorescent long chain fatty acid and reduced levels of long chain polyunsaturated fatty acids. FATP4-deficient cells also contained abnormal neutral lipid droplets. These additional defects indicate that metabolic abnormalities in these cells are not limited to very long chain fatty acids.

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demonstrate that a lack of FATP4 causes significant alterations in cellular lipid metabolism. We observed reduced fatty acid uptake in mutant cells and decreased flux of VLCFA through both catabolic and anabolic pathways. We conclude that FATP4 is the principal VLCFA-activating enzyme in skin fibroblasts.

EXPERIMENTAL PROCEDURES

Materials and General Methods—Cell culture medium was from Mediatech, and fetal bovine serum was from Gemini Biochemicals. [1-14C]Palmitic acid and [1-14C]lignoceric acid were from Moravek Biochemicals. Mouse monoclonal antibodies against ATP synthase and protein disulfide isomerase and rabbit polyclonal antibodies to histone-H3 and actin were from Chemicon. Rabbit polyclonal anti-manganese-superoxide dismutase was from Stressgen. Antibodies to PMP70 (sheep) and Pex13p (rabbit) were gifts from D. A. Bernlohr. Secondary antibodies for immunofluorescence, donkey anti-rabbit IgG fluorescein isothiocyanate conjugate, donkey anti-mouse IgG rhodamine conjugate, and donkey anti-sheep IgG rhodamine conjugate were all from Jackson ImmunoResearch. Secondary antibody for Western blotting, donkey anti-rabbit IgG fluorescein isothiocyanate conjugate, was from Santa Cruz Biotechnology. Alexa fluor 594-conjugated cholera toxin B-subunit was from Molecular Probes. Lipid standards for thin layer chromatography were all from Avanti Polar Lipids, except for phosphatidyl ethanolamine, tripalmitin, dioleoyl-sn-glycerol, and cholesteryl palmitate, which were from Sigma. Protein was determined by the method of Lowry et al. (21). Analyses of total cellular fatty acids as their methyl esters (following acid methanolysis) by gas chromatography/mass spectrometry were obtained through the Kennedy Krieger Institute Mental Retardation Research Center and were performed by the Peroxisomal Diseases Laboratory using previously described methods (22, 23). Statistical significance was determined using Student’s t test.

Establishment of Fibroblast Cell Lines—Skin samples were obtained under sterile conditions from the dorsum of newborn w.t. and Slc27a4+/− mice on the day of birth. Skin was washed with sterile phosphate-buffered saline and stored at 4 °C in complete cell culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics) until processed. The samples were cut into small (~1 mm²) pieces, which were attached to the bottom of cell culture dishes by scoring with a scalpel blade. Complete medium was added, and the dishes were incubated in a 5% CO₂ atmosphere in a humidified 37 °C incubator. After a few days, monolayer cell growth near the attached skin pieces was observed, and the culture medium was changed. Once colonies reached a size of ≥5 mm in diameter, they were harvested by gentle trypsinization and replated in new dishes. Several cell lines were established from two different w.t. and two different Slc27a4+/− mice. The cells were subcultured weekly (1:3). For experiments, the cells were used at passage ≤15. For cell fractionation and lipid metabolism studies, the cells were harvested by gentle trypsinization, washed with phosphate-buffered saline, and resuspended in 0.25 M sucrose containing 10 mM Tris, pH 7.5, and 1 mM EDTA. Assay of fatty acid β-oxidation rates (see below) of passage 3 cells versus passage 15 cells gave identical results (data not shown).

Subcellular Fractionation—Cell suspensions were homogenized using a precision ball bearing homogenizer that efficiently disrupts the plasma membrane without significant organelle damage (24). Preparation of fractions enriched in nuclei, mitochondria, peroxisomes, endoplasmic reticulum, and cytosol by differential centrifugation was exactly as described (20). The crude mitochondrial pellet was subfractionated into a purified mitochondrial fraction and a mitochondria-associated membrane (MAM) fraction by centrifugation through a Percoll gradient as described previously (20).

FATP4 Antibody Production—To produce an antigen for immunization of rabbits, a fusion construct encoding the C-terminal 35 amino acids of FATP4 in frame with glutathione S-transferase was prepared. A 108-bp fragment was amplified by PCR using FATP4 full-length cDNA5 as template with forward primer 5′-ATGCCGAAATTCCGCTATTGTGAAAGACCCG-3′, which incorporates an EcoRI site (underlined), and reverse primer 5′-TATTCTCGAGTCACAGCTTCTCCTCGCCTGC-3′, which incorporates an XhoI site (underlined) just after the TGA stop codon (reverse complement shown in bold). This fragment was cloned in frame into the EcoRI and XhoI sites of the pGEX-His-T3 vector (Amersham Biosciences). After transfer to Escherichia coli strain BL21-DE3, induction of fusion protein expression and subsequent purification was as described previously (9). Immunization and bleeding of rabbits was done by Cocalico Biologicals (Reamstown PA). The antibody was affinity-purified using previously described methods (9). Briefly, dilute antiserum was first passed over a glutathione-Sepharose column to remove anti-glutathione S-transferase antibodies. Anti-FATP4 antibodies were then bound to and eluted from a Sepharose column containing covalently linked fusion protein.

Indirect Immunofluorescence and Western Blotting—For immunofluorescence studies, the cells were cultured on glass coverslips and grown to ~50% confluence. The cells were fixed with 3% formaldehyde, permeabilized with 1% Triton X-100, and incubated with primary antibodies and fluorescent-conjugated secondary antibodies as previously described (25). For labeling of plasma membranes, the cells were fixed, but not permeabilized, and incubated for 1 h with Alexa fluor 594-conjugated cholera toxin B-subunit (4 μg/ml in phosphate-buffered saline). After extensive washing, the cells were permeabilized with Triton X-100 (25) prior to incubation with anti-FATP4 antibody. For Western blots, the protein concentrations of samples to be compared were first normalized, and equal amounts were loaded in each lane. Electrophoresis was carried out according to the method of Laemmli (26). After transfer to nitrocellulose membrane and incubation with primary and secondary antibodies, proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce).

5 Jia, Z., Pei, Z., Maiguel, D., Toomer, C. J., and Watkins, P. A. (2007) J. Mol. Neurosci., in press.
Acyl-CoA Synthetase and Oxidation Assays—Activation of [1-14C]palmitic acid and [1-14C]lignoceric acid to their CoA derivatives was assayed as previously described (27). Briefly, labeled fatty acid (20 μM final concentration; ~20,000 dpm/nmol) solubilized with α-cyclodextrin (10 mg/ml in 10 mM Tris, pH 8.0) was incubated for 20 min at 37 °C in a total volume of 0.25 ml with 40 mM Tris, pH 7.5, 10 mM ATP, 10 mM MgCl₂, 0.2 mM CoA, 0.2 mM dithiothreitol, and cell suspension or subcellular fraction as indicated in the figure legends. The reactions were terminated by the addition of Dole’s solution (28), unreacted substrate was removed by heptane extraction, and radioactivity in the aqueous phase was quantitated by liquid scintillation counting. Activation of the fluorescent fatty acid C₁₀-BODIPY 500/510 C₁₂ by frozen/thawed suspensions of COS-1 cells following transfection for 2 days with FATP4 cDNA or empty vector was assayed similarly, except that fluorescence intensity of the aqueous phase was measured using a Turner model 450 fluorometer.

Oxidation of [1-14C] fatty acids to water-soluble products was also measured as previously described (27). Briefly, fatty acids were solubilized with α-cyclodextrin as described for ACS assays and incubated in 20 mM Tris, pH 8.0, for 1 h at 37 °C with freshly harvested cell suspensions and required cofactors (8.5 mM ATP, 8.5 mM MgCl₂, 1 mM NAD, 0.17 mM FAD, 2.5 mM carnitine, 0.16 mM CoA, 1 mM malate). The reaction was carried out under hypotonic conditions in which substrates and cofactors can readily permeate the plasma membrane. The reactions were terminated by the addition of 50 μl of 1 N KOH. After saponification by incubating for 1 h at 37 °C in 2 ml of 0.1 M KOH in chloroform/methanol (2:1), 0.5 ml of 0.7 N HCl was then added to acidify the mixture and to cause a phase separation. The upper aqueous phase was discarded, and the lower phase was washed twice with theoretical upper phase (29), before drying under nitrogen. Following solubilization with 50 μl of hexane/diethyl ether (1:1), duplicate 20-μl aliquots were applied to Whatman Lk6D plates.

All thin layer chromatography plates were preswashed with chloroform/methanol (1:1), and for phospholipid analysis only, the plates were subsequently wetted with 2.3% boric acid in ethanol and dried for 5 min. All of the plates were dried for 15 min at 100 °C. Solvent systems used were hexane/diethyl ether/acetic acid (80:20:1) for neutral lipids, chloroform/ethanol/water/triethylamine (30:35:7:35) for phospholipids, and hexane/diethyl ether/acetic acid (90:10:1) for ceramide. Labeled lipids were detected by phosphorimaging analysis (Fuji-BAS 2500) and identified by comparison with authentic standards. For quantification of phosphorimaging data, known amounts of [1-14C]palmitic acid (200–2500 dpm) were spotted on a blank area of TLC plates following development to generate a standard curve (r² > 0.98). Graphpad Prism software was used to convert phosphorimaging densities to dpm using the standard curve. In these studies, <20% of the available substrate was consumed during the 2-h incubation. Neutral lipid standards were detected by exposure to iodine vapor, and phospholipid standards were detected under ultraviolet light after spraying the plates with primuline (0.005% (w/v) in acetone/water (4:1)). To verify that radioactivity in neutral and polar lipids in cell suspensions labeled with [1-14C]lignoceric acid remained as intact C24:0 and was not shorter chain fatty acids resulting from recycling of [1-14C]acetyl-CoA generated from β-oxidation of lignoceric acid, Folch extracts were subjected to acid methanolysis (30). Methyl esters were applied to high performance reverse phase TLC plates (Analtech) and developed in the solvent system chloroform/methanol/water (7:15:1) (31). For standards, authentic [1-14C]fatty acids containing 16–24 carbons were converted to their respective methyl esters using the same protocol. Other than methylignocerate, methylpalmitate was the only radioactive species detected. Quantitation of the methylpalmitate produced indicated that it accounted for <2% of the radioactivity associated with neutral and polar lipids. Thus, we conclude that at least 98% of radioactivity incorporated into complex lipids remained in the form of C24:0.

Cellular Uptake of Fluorescent Fatty Acid and Staining of Neutral Lipid Droplets—For fatty acid uptake measurements, the fluorescent fatty acid C₁₀-BODIPY 500/510 C₁₂ (4,4-
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FIGURE 1. FATP4 protein is deficient in Slc27a4<sup>-wrfr</sup> fibroblasts. Total cellular extracts from two different control (+/+ and two different FATP4-deficient Slc27a4<sup>-wrfr</sup> (−−) mouse skin fibroblast lines were subjected to SDS-PAGE and Western blot analysis using affinity-purified FATP4 antibody. The positions of the molecular mass markers are shown on the left.

difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid) (Molecular Probes number D3823) was used as described previously (11). The cells were grown as monolayers on glass coverslips. After washing with warm phosphate-buffered saline, C<sub>1</sub>-BODIPY 500/510 C<sub>12</sub> in dimethyl sulfoxide was added to a final concentration of 20 μM, and the cells were incubated 2 min at 37 °C. After washing, the coverslips were mounted and viewed using a 20× objective.

Vesicles containing neutral lipids were stained with the fluorescent dye BODIPY 493/503 (Molecular Probes number D3922) as described (32, 33). This BODIPY fluorophor is a lipophilic dye that has an affinity for neutral lipids and is not a fatty acid analog. Briefly, cell monolayers on glass coverslips were fixed and washed as for indirect immunofluorescence. BODIPY 493/503 in dimethyl sulfoxide was added to a final concentration of 20 μM, and the cells were incubated 10 min at 37 °C. After washing, the coverslips were mounted and viewed using a 100× objective. All of the exposures were taken under identical conditions.

RESULTS

Slc27a4<sup>-wrfr</sup> Fibroblasts Lack FATP4 Protein—To investigate the effect of FATP4 deficiency on cellular lipid metabolism, skin fibroblast lines were produced from two w.t. mice and two Slc27a4<sup>-wrfr</sup> littermates. Initial characterization of Slc27a4<sup>-wrfr</sup> mice revealed the absence of detectable Slc27a4 mRNA, and it was predicted that little if any stable FATP4 protein would be produced. No FATP4 protein was detected when samples of whole cells were analyzed by Western blot (Fig. 1), confirming this prediction.

Activation of Long Chain Fatty Acids (LCFA) and VLCFA by FATP4-deficient Fibroblasts—When FATP4 was overexpressed in COS-1 cells, membrane extracts had a greater fold increase in activation of VLCFA than LCFA (16). To determine how a lack of endogenous FATP4 deficiency affected activation of LCFA and VLCFA, ACS activity using palmitic acid (C16:0) and lignoceric acid (C24:0) as substrates was measured in w.t. and Slc27a4<sup>-wrfr</sup> fibroblasts. Mutant cells lacking FATP4 had a 38% decrease in palmitate activation (p < 0.0001) and an 83% decrease in activation of the VLCFA, lignocerate (p < 0.0001), as compared with w.t. fibroblasts (Fig. 2). This finding suggests that FATP4 is the major VLCFA-activating enzyme in skin fibroblasts. To rule out the possibility that another enzyme capable of activating VLCFA might also be deficient in these cells, we analyzed w.t. and mutant cells by Western blot. The only enzymes previously reported to activate VLCFA are ACSVL1 (FATP2; SLC27A1), ACSVL2 (FATP6; SLC27A6), ACSVL3 (FATP3; SLC27A3), FATP1 (SLC27A1), ACSB (FATP5; SLC27A5), and ACSBG1 (5–10).

Decreased Fatty Acid Oxidation Rates in FATP4-deficient Fibroblasts—To understand the downstream consequences of FATP4 deficiency in skin fibroblasts, we first investigated the fatty acid β-oxidation pathway. It is generally accepted that LCFA degradation occurs primarily in mitochondria, whereas VLCFA degradation is mainly a peroxisomal process (34). Oxidation rates for C16:0 were slightly lower in mutant fibroblasts than w.t., but this decrease was not statistically significant (Fig. 4). In contrast, there was a 58% decrease (p < 0.05) in the VLCFA degradation rate in cells lacking FATP4 (Fig. 4). Because the β-oxidation assay is done under hypotonic conditions in which substrates and cofactors can readily traverse the plasma membrane, decreases observed in mutant fibroblasts were not the result of defective fatty acid transport into the cells.

Altered Fatty Acid Composition of FATP4-deficient Fibroblasts—Decreased ability of FATP4-deficient fibroblasts to degrade VLCFA could lead to accumulation of these potentially toxic fatty acids in cells. Therefore, we examined the total fatty acid composition of cultured normal and FATP4-deficient cells following acid methanlysis to cleave acyl groups from complex lipids. There was a slight (10%) but statistically significant decrease in total saturated fatty acids in mutant fibroblasts, which was primarily due to a 25% decrease in the level of...
In contrast, the relative levels of total FATP4 and ACSVL2, 50% mutant fibroblasts, the level of linoleic acid (C18:2) was increased 25 and 105%, respectively, in FATP4-deficient (−/−) cells as compared to control (+/+). There was no increase in the quantity of saturated VLCFA (C23:0–C30:0). On the other hand, there were some unexpected changes in cellular mono- and polyunsaturated fatty acids. The level of arachidonic (C20:4) fatty acids was 36–38% lower in the mutant cells. However, whereas the levels of arachidonic (C20:4), palmitoleic (C16:1), and eicosapentaenoic (C20:5) fatty acids were polyunsaturated, the level of linoleic acid (C18:2) was unchanged (Table 1). Similarly, levels of eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acids were significantly lower in FATP4-deficient cells. The level of α-linolenic acid (C18:3n3), although very low compared with the other ω3 fatty acids, was actually higher in the mutant cells (Table 1).

**Incorporation of VLCFA, but Not LCFA, into Complex Lipids Is Decreased in FATP4-deficient Fibroblasts**—We next investigated whether a lack of FATP4 in fibroblasts affected the synthesis of complex lipids. Incorporation of the VLCFA linoleic into both nonpolar and polar lipids was severely impaired in fibroblasts from FATP4-deficient mice (Fig. 5). Incorporation of C24:0 into triacylglycerol and cholesterol esters was reduced by 64 and 58%, respectively, in mutant fibroblasts. Similarly, decreased incorporation of this VLCFA into several phospholipid species, including phosphatidyl choline (54% decrease), lyso phosphatidyl choline (78% decrease), phosphatidyl serine (64% decrease), and phosphatidyl ethanolamine (56% decrease), were also observed. In contrast, no significant decreases in the incorporation of the LCFA, palmitate, into these lipid species was observed between w.t. and FATP4-deficient fibroblasts (Fig. 5); in fact, palmitate incorporation into triacylglycerol was increased ~50% in mutant cells. Phosphatidyl serine synthesis from C16:0, diacylglycerol synthesis from C24:0, and phosphatidyl inositol synthesis from either substrate were essentially undetectable in our assays (data not shown). In a separate set of experiments, we investigated incorporation of labeled LCFA and VLCFA into ceramide by control and FATP4-deficient fibroblasts. However, no significant differences were found (Fig. 5). All in all, these studies are consistent with a significant impairment of VLCFA incorporation into neutral and glycerophospholipids in FATP4-deficient fibroblasts.

![Figure 3: Expression of other very long chain ACSs in FATP4-deficient fibroblasts](image)

*FIGURE 3. Expression of other very long chain ACSs in FATP4-deficient fibroblasts.* Total cellular extracts and endoplasmic reticulum-enriched fractions (P fractions; see “Experimental Procedures”) from control (+/+ and FATP4-deficient (−/−) cells were subjected to SDS-PAGE and Western blot analysis. Antibodies used were directed against ACSs reported to activate VLCFA substrates, including FATP4 (SLC27A4), FATP1 (SLC27A1), ACSVL1 (FATP2; SLC27A2), ACSVL2 (FATP6; SLC27A6), ACSVL3 (FATP3; SLC27A3), ACSB (FATP5; SLC27A5), and ACSBG1. For FATP1, ACSVL1, ACSVL3, ACSB, and ACSBG1, 30 μg of either whole cell suspension or P fraction was loaded. For FATP4 and ACSVL2, 50 μg of either whole cell suspension or P fraction was loaded.

![Figure 4: Effect of FATP4 depletion on LCFA and VLCFA β-oxidation rates](image)

*FIGURE 4. Effect of FATP4 depletion on LCFA and VLCFA β-oxidation rates.* Degradation of the LCFA, C16:0, and the VLCFA, C24:0, to water-soluble products was measured in two control (+/+ ) and two mutant (−/−) fibroblast lines as described under “Experimental Procedures.” This experiment was performed three times. The results are presented as the means ± S.E.

| Control  | FATP4-deficient | p value | FATP4-deficient |
|----------|-----------------|---------|-----------------|
| All saturated fatty acids | 36.5 ± 1.1 | <0.01 | 90 |
| C16:0 | 18.6 ± 0.5 | n.s. | 97 |
| C18:0 | 16.6 ± 1.5 | <0.02 | 75 |
| All saturated VLCFA | 1.4 ± 0.1 | n.s. | 98 |
| All ω9 fatty acids | 27.5 ± 3.4 | <0.05 | 125 |
| C18:1 (ω9) | 19.4 ± 2.1 | 26.6 ± 2.7 | <0.01 | 137 |
| All ω5 + ω7 fatty acids | 5.7 ± 1.1 | 11.6 ± 2.8 | <0.01 | 205 |
| C16:1 (ω7) | 1.4 ± 0.4 | 3.8 ± 1.2 | <0.01 | 271 |
| C18:1 (ω7) | 4.0 ± 0.7 | 7.5 ± 1.6 | <0.01 | 188 |
| All ω6 fatty acids | 17.2 ± 1.9 | 10.6 ± 3.2 | <0.02 | 62 |
| C18:2 (ω6) | 2.1 ± 0.3 | 2.4 ± 0.7 | n.s. | 114 |
| C20:4 (ω6) | 12.3 ± 1.9 | 6.4 ± 2.7 | <0.02 | 52 |
| C22:5 (ω6) | 0.32 ± 0.07 | 0.17 ± 0.07 | <0.05 | 53 |
| All ω3 fatty acids | 6.6 ± 0.4 | 4.2 ± 1.1 | <0.01 | 64 |
| C18:3 (ω3) | 0.01 ± 0.00 | 0.04 ± 0.02 | <0.02 | 40 |
| C20:5 (ω3) | 0.85 ± 0.12 | 0.48 ± 0.06 | <0.01 | 56 |
| C22:6 (ω3) | 3.1 ± 0.3 | 2.1 ± 0.7 | <0.05 | 68 |

**TABLE 1: Fatty acid analysis of normal and FATP4-deficient mouse fibroblasts**

The cells were cultured to confluence, harvested by trypsinization, and subjected to gas chromatography/mass spectrometry. Saturated fatty acids included compounds containing from 9 to 30 carbons, whereas saturated VLCFA included only those containing 23–30 carbons. With the exception of C20:3 and all ω6, ω7, and ω9 fatty acids were monounsaturated. All ω6 and ω3 fatty acids were polyunsaturated. Two different control fibroblast lines and two different FATP4-deficient lines were analyzed on two separate occasions. The data from the two control lines and two mutant lines were combined, and the results are presented as the means ± S.E., n.s., not significant.
Subcellular Localization of FATP4 in Fibroblasts—Because deficiency of FATP4 affected lipid metabolic pathways known to be associated with different subcellular organelles, we investigated the subcellular localization of the endogenous enzyme in w.t. fibroblasts. Indirect immunofluorescence studies using affinity-purified anti-FATP4 antibody indicated that the protein was mainly in punctate to elongated membranous structures that resembled fibroblast mitochondria; some nuclear immunostaining was also observed (Fig. 6A). Double labeling with antibody against the mitochondrial membrane protein ATP synthase revealed partial, but not complete, colocalization with FATP4 (Fig. 6, B and C). When Scl27a4<sup>−/−</sup> fibroblasts were incubated with the same antibodies, no FATP4 immunostaining was observed (Fig. 6D), confirming the Western blot result shown in Fig. 1. ATP synthase was readily detected in the mutant cells (Fig. 6, E and F). In contrast, immunostaining of FATP4 and either the peroxisomal marker PMP70 (Fig. 6, G–J) or the endoplasmic reticulum marker protein disulfide isomerase (Fig. 6, J–L) in w.t. cells showed significantly less overlap than observed with ATP synthase. In contrast to previous reports suggesting that FATP4 resides in the plasma membrane (18, 19), no FATP4 immunoreactivity was detected in plasma membrane of mouse skin fibroblasts (Fig. 6, M–O).

The low level of FATP4 immunoreactivity in peroxisomes and endoplasmic reticulum was surprising in light of the reduced VLCFA degradation (a peroxisomal process; Fig. 4) and the decreased incorporation of lignoceric acid into complex lipids (involving endoplasmic reticulum enzymes; Fig. 5) observed in mutant fibroblasts lacking FATP4 protein. Therefore, we used differential centrifugation to fractionate control and mutant fibroblasts to explore this issue further. Crude nuclear (N), heavy mitochondrial (M), light mitochondrial (L), endoplasmic reticulum (ER), and S (soluble, or cytosolic) fractions were prepared and analyzed by Western blot. FATP4 was most abundant in the N and M fractions (Fig. 7), consistent with the result of immunofluorescence studies (Fig. 6A). A small amount of FATP4 was also detected in the L fraction. Although the nuclear marker, histone H3, was only found in the N fraction, the mitochondrial marker enzyme, manganese-superoxide dismutase, was found in both the N and M fractions, suggesting that the N fraction has some mitochondrial contamination.

Although the low level of FATP4 in the peroxisome-enriched L fraction may be sufficient to supply activated VLCFA for degradation by peroxisomal β-oxidation, the inability to detect this enzyme in control fibroblast endoplasmic reticulum was not consistent with the profound effect of FATP4 deficiency on neutral lipid and phospholipid synthesis. We therefore hypothesized that endogenous FATP4 might reside in the MAM fraction, because this fraction has been shown to contain enzymes that participate in complex lipid synthesis (35, 36). The crude M fraction was subfractionated into a purified mitochondrial fraction and MAM. Western blotting clearly indicated that FATP4 was in both purified mitochondria and MAM, whereas the mitochondrial marker manganese-superoxide dismutase was only found in purified mitochondria. This finding raises the possibility that MAM-associated FATP4 could provide activated VLCFA for synthesis of certain neutral lipids and phospholipids in skin fibroblasts.

ACS Activity of Fibroblast Subcellular Fractions—To determine whether the location of FATP4 correlated with its enzyme activity, we assayed the same subcellular fractions from w.t. and Scl27a4<sup>−/−</sup> fibroblasts for their ability to activate LCFA and VLCFA substrates. Fractions enriched in nuclei, mitochondria, peroxisomes, and endoplasmic reticulum all had considerable capacity to activate both C16:0 and C24:0 (Fig. 8). The cytosolic fraction had low activity, as expected. When purified mitochondria and MAM were prepared from the crude mitochondrial fraction, both also exhibited ACS activity with both substrates. The mean decreases in C24:0 activation in mitochondrial, peroxisomal, and endoplasmic reticulum-enriched fractions from FATP4-deficient fibroblasts were 62, 60, and 77%, respectively, whereas the decreases for C16:0 activation in these fractions were 26, 20, and 22%, respectively. Similar decreases with both substrates were also seen in purified mitochondria and in MAM fractions.

The ACS activity attributable to FATP4 in the M, L, and P fractions using the C24:0 substrate (Fig. 8, compare +/+ versus −/−) was inconsistent with the levels of FATP4 detected either by immunofluorescence (Fig. 6) or Western blot (Fig. 7). In particular, we were initially unable to detect FATP4 in the P fraction despite its higher activity there than in the M fraction, where the protein was readily visualized. As noted earlier, sev-
eral other enzymes with reported very long chain ACS activity can be found in mouse skin fibroblasts, including ACSVL1, ACSVL2, ACSVL3, ACSB, and ACSBG1 (Fig. 3, left columns). We therefore investigated which of these are also found in the P fraction and determined whether the level of any was decreased in FATP4-deficient fibroblasts. Whereas ACSB and ACSBG1 were not present in this fraction, ACSVL1, ACSVL2, and ACSVL3 were easily detected; however, the levels of the latter three enzymes were not decreased in the mutant fibroblasts (Fig. 3, right columns). Furthermore, when the amount of P fraction loaded on the gel was 5-fold higher than what was loaded in the experiment shown in Fig. 7, we were able to detect FATP4 immunoreactivity (Fig. 3). Nonetheless, the discrepancy between the levels of FATP4 in the different subcellular fractions as judged by immunoreactivity and its apparent activity as judged by loss of function in the mutant fibroblasts remain unresolved.

Fatty Acid Uptake in FATP4-deficient Fibroblasts—In addition to their documented enzyme activity, members of the very long chain ACS family have been implicated in fatty acid uptake, particularly of LCFA (11–13). To determine whether a lack of endogenous FATP4 affected the ability of skin fibroblasts to take up fatty acids, we incubated control and mutant cells with the fluorescent LCFA C1-BODIPY 500/510 C12 and visualized the cells by fluorescence microscopy. Control fibroblasts readily took up the BODIPY FA (Fig. 9, A and B), whereas uptake by the mutant fibroblasts was barely detectable (Fig. 9, C and D). Using COS-1 cells 2 days post-transfection with either FATP4 cDNA or empty vector, we confirmed that C1-BODIPY 500/510 C12 is a substrate for FATP4; activation of this fatty acid was 5-fold higher in FATP4-expressing cells than in vector-transfected cells (data not shown). These observations are consistent with previous findings in COS-7 cells overexpressing FATP4 (11).

Lipid Droplets in FATP4-deficient Fibroblasts—We previously reported that the lower squamous layers of the epidermis of FATP4-deficient mouse skin had numerous oil red O-staining neutral lipid droplets, whereas few if any were detected in the epidermis of normal littermates (15). Therefore, we looked for neutral lipid-containing droplets in fibroblasts from control and FATP4-deficient mice. The majority (~90%) of control fibroblasts contained numerous, small vesicles that stained with the neutral
lipid-specific fluorescent dye, BODIPY 493/503 (Fig. 9, E and F). In contrast, staining was more variable in the mutant cells. Approximately half of the cells lacked lipid droplets, but those that did generally had much larger vesicles than seen in control cells (Fig. 9, G and H). To determine whether the apparent increase in lipid seen in some mutant cells was the result of increased de novo lipid synthesis, we incubated control and mutant fibroblasts with [1-14C]acetate. The rates of acetate incorporation into total lipids were similar in control (0.24 nmol/20 min/mg of protein) and mutant (0.26 nmol/20 min/mg of protein) fibroblasts.

DISCUSSION

Investigating the metabolic function of a protein is often done by comparing control cells to cells experimentally manipulated to overexpress the protein. However, if the protein under investigation is an enzyme whose activity is not rate-limiting for downstream processes, little insight into the role of the protein in these processes will be gained. Another concern with this type of study is that the subcellular location of the overexpressed protein may differ from that of the endogenous protein, potentially affecting its participation in downstream metabolic processes. A better understanding of the physiological role of an enzyme can often be achieved by loss-of-function studies using cells lacking the endogenous protein. To investigate the role of FATP4 in lipid metabolism, we produced fibroblast cell lines from the skin of wild type mice and littermates with a
genetic deficiency of FATP4 (15). These cells proved to be excellent models in which to study FATP4 function.

FATP4 overexpression studies in COS-1 cells indicated that VLCFA substrates were preferred over LCFA (16). This observation was confirmed in the present study, because FATP4 deficient fibroblasts had less than 20% of the capacity of control cells to activate the VLCFA C24:0 but >60% residual capacity to activate the LCFA, C16:0. The magnitude of the FATP4 defect was somewhat surprising because these cells contain several other enzymes capable of activating VLCFA, particularly ACSVL1, ACSVL2, ACSVL3, and ACSB (Fig. 3). None of these enzymes were found to be decreased in FATP4-deficient cells. FATP1 was not detected in either control or mutant cells, and although ACSBG1 was originally reported to activate VLCFA when overexpressed in COS-1 cells (5), we subsequently reported that the endogenous enzyme preferentially activated the LCFA, C16:0 (20). These results are also consistent with the reduced esterification of C24:0, but not C16:0, in FATP4-deficient fibroblasts (Fig. 2), which account for the residual 17% of the degradation rate of control cells, suggesting that VLCFA might accumulate in these cells. However, fatty acid analysis of these cells revealed that cells lacking FATP4 have normal VLCFA levels. Incorporation of VLCFA into several lipids, including triacylglycerol, cholesterol esters, and major phospholipid species, was reduced by more than 50% in FATP4-deficient cells. Thus, FATP4 appears to provide activated VLCFA for most major lipid catabolic and anabolic pathways of skin fibroblasts. Interestingly, incorporation of C24:0 into ceramide was not reduced in mutant fibroblasts. This suggests that one of the other very long chain ACSs expressed in fibroblasts (Fig. 3), which account for the residual 17% of VLCFA activation in FATP4-deficient fibroblasts (Fig. 2), is specific for directing C24:0 into the ceramide synthetic pathway. Another consideration is that keratinocytes, not fibroblasts, are thought to be the main source of ceramides needed for skin barrier function (39).

Abnormal lipid metabolism in fibroblasts from Scl27a4<sup>−/−</sup> mice was further demonstrated by differences in lipid droplet morphology. These vesicles stained with the fluorescent dye, BODIPY 493/503, which is specific for neutral lipids such as triacylglycerol and cholesterol esters (32). In control fibroblasts, lipid droplets were generally smaller and were found in most cells. In mutant fibroblasts, although only approximately half the cells had lipid droplets, the vesicles were typically larger and were more clustered. Although this observation is reminiscent of the oil red O-positive neutral lipid vesicles that were found in the skin of Scl27a4<sup>−/−</sup> mice (15), the latter were in suprabasal keratinocytes and not fibroblasts. The nature and origin of the lipids in the large vesicles of mutant fibroblasts has not been resolved. However, it is unlikely that the lipids arose from increased de novo synthesis, because the rates of [14C]acetate incorporation were similar in both control and FATP4-deficient cells. We also observed that incorporation of labeled palmitate into triacylglycerol was increased in mutant cells, raising the intriguing possibility that the large vesicles are the result of increased triacylglycerol uptake and storage.

Members of the very long chain ACS family (SLC27A1–6) that includes FATP4 have been characterized as fatty acid transporters as well as enzymes (13, 14, 18). The need for protein mediators of fatty acid translocation across the plasma membrane is controversial. In addition to the FATPs, proteins such as fatty acid translocase/CD36 and plasma membrane fatty acid-binding protein have been postulated to play a role in fatty acid transport (40–42). On the other hand it is clear that fatty acids can cross lipid bilayers by passive diffusion (43, 44). Many studies implicating FATPs in fatty acid transport were done by overexpressing the proteins, a situation in which the subcellular site of the FATP may not parallel its endogenous location. We recently investigated endogenous ACSVL3 (FATP3) in mouse Leydig MA-10 cells and found that depletion of this protein by RNA interference did not affect cellular uptake of LCFA (9). We also demonstrated that endogenous ACSVL3 was not found in the plasma membrane of these cells. On the other hand, it was recently reported that when FATP4 was exogenously overexpressed in COS-7 cells, it localized to the endoplasmic reticulum and not the plasma membrane, but its presence was sufficient to drive the uptake of the fluorescent LCFA, C<sub>1</sub>-BODIPY 500/510 C<sub>12</sub> (11). In the present study of fibroblasts, we found no plasma membrane immunostaining of FATP4, yet the absence of FATP4 from internal organellar membranes in mutant cells resulted in a dramatic decrease in C<sub>1</sub>-BODIPY 500/510 C<sub>12</sub> uptake. We also obtained direct evidence that the BODIPY fatty acid is a substrate for FATP4 by assaying activation of this substrate in COS-1 cells overexpressing FATP4. The fact that in both cases internal FATP4 is capable of driving fatty acid uptake suggests that FATP4 facilitates transport in a metabolic fashion rather than by acting as a transporter per se. We and others have proposed that ACSs including the FATPs facilitate fatty acid uptake by initially “trapping” them as their CoA thioesters (9, 10, 45, 46). These fatty acyl-CoAs can then participate in downstream metabolic pathways. The net effect of esterification and subsequent metabolism is a reduction in the intracellular free fatty acid concentration, thus allowing additional fatty acids to enter the cell by passive diffusion. The results of the study shown in Fig. 9 are consistent with this hypothesis.

We were unable to resolve completely the precise subcellular location(s) of endogenous FATP4 in fibroblasts. It was somewhat surprising that the protein was found associated with several organelles, including mitochondria, peroxisomes, and MAM. The peroxisomal location was consistent with a role for FATP4 in VLCFA β-oxidation, and the MAM location may explain the function of FATP4 in complex lipid synthesis. However, despite fairly robust very long chain ACS activity in endoplasmic reticulum-enriched P fractions from control cells, which was reduced in FATP4-deficient cells, the amount of immunoreactive FATP4 in this fraction was very low. One possible explanation for this was that the level of another ACS capable of activating VLCA was also reduced in mutant fibro-
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blasts, but Western blots of P fractions did not reveal differences for ACSVL1, ACSVL2, or ACSVL3 (Fig. 3). Because our antibody is specific for a rather small epitope (the C-terminal 35 amino acids) of FATP4, it is possible that the endoplasmic reticulum protein was processed by a C-terminal truncation event. This seems unlikely, because colleagues using antibody raised to the C-terminal 15 amino acids of FATP4 detected overexpressed protein in endoplasmic reticulum fractions of HeLa cells (11). Furthermore, when we overexpressed human FATP4 cDNA in FATP4-deficient mouse fibroblasts, we detected protein in the endoplasmic reticulum by immunofluorescence that was associated with increased very long chain ACS activity (data not shown). Complete resolution of the question will require development of new antibodies raised against more internal epitopes. It is also possible that, in addition to FATP4 or other very long chain ACS, activity of an ACS belonging to the long chain family or an as yet uncharacterized ACS could be decreased in the mutant fibroblasts; however, to the best of our knowledge, other such enzymes capable of activating VLCFA have not been reported.

Analysis of the fatty acid composition of control and FATP4-deficient fibroblasts revealed some unexpected differences. Although no changes in VLCFA levels were found, significantly deficient fibroblasts revealed some unexpected differences. To the best of our knowledge, other such enzymes capable of activating VLCFA have not been reported.

In conclusion, the results of studies with mouse skin fibroblasts deficient in FATP4 revealed that this protein plays a significant role in VLCFA homeostasis in these cells. Although fibroblasts only constitute a small percentage of cells present in normal skin, the severity of lipid metabolic abnormalities reported here suggests that FATP4 may play a major role plays in VLCFA metabolism in other cell types such as keratinocytes. These abnormalities begin to provide the mechanistic basis for defective barrier function and other skin defects in the Slc27a4<sup>−/−</sup> mouse.

Acknowledgments—We thank Dr. Stephen Gould (Johns Hopkins University) for antibodies to PMP70 and Pex13p, Dr. David Bernlohr (University of Minnesota) for antibody to FATP1, and Dr. Ronald Schnaar (Johns Hopkins) for Alexa fluor 594-conjugated cholera toxin B-subunit.

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