



Published in final edited form as:

J Biol Chem. 2004 September 3; 279(36): 37789–37797. doi:10.1074/jbc.M406225200.

Mammalian Wax Biosynthesis:

I. IDENTIFICATION OF TWO FATTY ACYL-COENZYME A REDUCTASES WITH DIFFERENT SUBSTRATE SPECIFICITIES AND TISSUE DISTRIBUTIONS*

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Abstract

The conversion of fatty acids to fatty alcohols is required for the synthesis of wax monoesters and ether lipids. The mammalian enzymes that synthesize fatty alcohols have not been identified. Here, an *in silico* approach was used to discern two putative reductase enzymes designated FAR1 and FAR2. Expression studies in intact cells showed that FAR1 and FAR2 cDNAs encoded isozymes that reduced fatty acids to fatty alcohols. Fatty acyl-CoA esters were the substrate of FAR1, and the enzyme required NADPH as a cofactor. FAR1 preferred saturated and unsaturated fatty acids of 16 or 18 carbons as substrates, whereas FAR2 preferred saturated fatty acids of 16 or 18 carbons. Confocal light microscopy indicated that FAR1 and FAR2 were localized in the peroxisome. The FAR1 mRNA was detected in many mouse tissues with the highest level found in the preputial gland, a modified sebaceous gland. The FAR2 mRNA was more restricted in distribution and most abundant in the eyelid, which contains wax-laden meibomian glands. Both FAR mRNAs were present in the brain, a tissue rich in ether lipids. The data suggest that fatty alcohol synthesis in mammals is accomplished by two fatty acyl-CoA reductase isozymes that are expressed at high levels in tissues known to synthesize wax monoesters and ether lipids.

Wax esters are abundant neutral lipids that coat the surfaces of plants, insects, and mammals. They are composed of long chain alcohols esterified to fatty acids and have the chemical property of being solid at room temperature and liquid at higher temperatures. Waxes play essential biological roles in preventing water loss, abrasion, and infection and are produced commercially at levels approaching 3 billion pounds per year for use in polishes, cosmetics, and packaging. In some mammals, wax esters constitute ~30% of sebum and meibum, the oils secreted by the sebaceous and meibomian glands onto the surfaces of the skin and eye, respectively (1,2).

Although the enzymes of wax biosynthesis in mammals have not been isolated, the components of the pathway can be inferred from work in plants (3,4) and mammalian tissue extracts (5). As indicated in Scheme 1, two catalytic steps are required to produce a wax monoester, including reduction of a fatty acid to a fatty alcohol and subsequently, the trans-esterification of the fatty alcohol to a fatty acid. The first step is catalyzed by the enzyme fatty acyl-CoA reductase (FAR),¹ which uses the reducing equivalents of NAD(P)H to convert a fatty acyl-

*This work was supported in part by National Institutes of Health Grant HL20948 (to D. W. R.) and Robert A. Welch Foundation Grant I-0971 (to D. W. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[†]Supported in part by National Institutes of Health Medical Scientist Training Grant GM08014.

CoA into a fatty alcohol and CoASH. cDNAs specifying fatty acyl-CoA reductases have been identified in the jojoba plant (6), the silkworm moth (7), wheat (8), and in a microorganism (9); however, the biochemical properties and subcellular localizations of these enzymes have not been reported.

Fatty alcohols have two metabolic fates in mammals: incorporation into ether lipids or incorporation into waxes. Ether lipids account for ~20% of phospholipids in the human body and are synthesized in membranes by a pathway involving at least seven enzymes (10). The second step of this pathway is catalyzed by the enzyme alkyl-dihydroxyacetone phosphate synthase, which exchanges an *sn-1* fatty acid in ester linkage to dihydroxyacetone phosphate with a long chain fatty alcohol to form an alkyl ether intermediate. Once produced, ether lipids are precursors for platelet activating factor, for cannabinoid receptor ligands, and for essential membrane components in cells of the reproductive and nervous systems (10,11).

In the current study a bioinformatics approach was used to identify mouse (m) and human (h) cDNAs encoding two fatty acyl-CoA reductase isozymes designated FAR1 and FAR2. The biochemical properties and subcellular localizations of recombinant FAR enzymes expressed in cultured mammalian and insect cells were defined, and tissue distributions were delineated. An accompanying paper reports the isolation and characterization of a wax synthase enzyme that catalyzes the second step of the mammalian wax biosynthetic pathway (12).

EXPERIMENTAL PROCEDURES

Bioinformatics and cDNA Cloning

Fatty acyl-CoA reductase protein sequences from the jojoba plant (*Simmondsia chinensis* (6)) and the silkworm moth (*Bombyx mori* (7)) were compared with those in the protein data base using the program BLASTP (13) to identify potential mouse and human reductase sequences. Two proteins with ~30% sequence identity to the plant and insect enzymes were identified, and cDNAs for each were obtained. A mouse fatty acyl-CoA reductase 1 cDNA (mFAR1, IMAGE clone 3495305, GenBank™/EBI Data Bank accession number BC007178) in the pCMV-SPORT6 vector was obtained from the Mammalian Gene Collection (IRAV) (Invitrogen). A mouse fatty acyl-CoA reductase 2 cDNA (mFAR2, IMAGE clone 6809131, GenBank™/EBI Data Bank accession number BC055759) in the pYX-Asc vector was obtained from Open Biosystems (Huntsville, AL). The cDNA insert in this plasmid was released by digestion with the restriction enzymes EcoRI and NotI, purified by agarose gel electrophoresis and extraction (QIAquik gel extraction kit, Qiagen, Valencia, CA), and ligated into the pCMV6 vector (GenBank/EBI Data Bank accession number AF239250).

¹The abbreviations used are:

FAR	fatty acyl-coenzyme A reductase
BSA	bovine serum albumin
CMV	cytomegalovirus
CT	threshold value
HEK 293	human embryonic kidney 293
PBS	phosphate-buffered saline.

A human fatty acyl-CoA reductase 1 cDNA (hFAR1, GenBank™/EBI Data Bank accession number AY600449) in the pCMV6-XL6 vector was purchased from Origene Technologies (Rockville, MD). A human fatty acyl-CoA reductase 2 cDNA (hFAR2, IMAGE clone 4732586) in the pDNR-LIB vector was obtained from Invitrogen. The cDNA insert in this plasmid was released by digestion with the restriction enzymes EcoRI and XhoI and then treated with the Klenow fragment of *Escherichia coli* DNA polymerase I and the four deoxynucleoside triphosphates to generate blunt ends. The engineered hFAR2 cDNA insert (nucleotides 62–2235 of GenBank™/EBI Data Bank accession number BC022267) was purified by gel electrophoresis and extraction (QIAquick gel extraction kit) and ligated into the SmaI site of the pCMV6 vector. DNA sequence analysis confirmed the identity and structure of the hFAR2 cDNA.

Construction of FLAG Epitope-tagged Expression Plasmids

An expression plasmid (pCMV·SPORT6-FLAG-mFAR1) encoding a FLAG epitope-tagged version of the mouse FAR1 protein was assembled as follows. The FAR1 cDNA was amplified from the pCMV·SPORT6-mFAR1 template described above by PCR using the oligonucleotide primers 5'-GTACCTGTCGACCCACCATGGATTACAAGGATGACGACGATAAGAGACAAGTCTGGATGGTTTCAATCC-3' and 5'-ATTATGCGGCCGCGGTCTTCAGTATCTCATAGTGCTG-3'. The DNA was digested with the restriction enzymes SalI and NotI and ligated into the pCMV·SPORT6 vector (Invitrogen). The mFAR1 encoded by the resulting plasmid has the FLAG epitope (amino acid sequence DYKDDDDK) at the amino terminus.

An expression plasmid (pCMV·SPORT6-FLAG-mFAR2) encoding a FLAG epitope-tagged version of the mouse FAR2 protein was assembled as follows. The mFAR2 cDNA was amplified from the pCM·V6-mFAR2 template described above by the PCR using the oligonucleotide primers 5'-GTACCTGTCGACCCACCATGGATTACAAGGATGACGACGATAAGATGTCCATGATCGCAGCTTTCTAC-3' and 5'-ATTATGCGGCCGCTGTTCTTAGACCTTGAGTGTGCTG-3'. The DNA product was digested with the restriction enzymes SalI and NotI and ligated into the pCMV·SPORT6 vector (Invitrogen). This engineering placed the FLAG epitope at the amino terminus of the encoded mouse FAR2 protein.

Baculovirus Expression Vectors

A baculovirus recombinant donor plasmid with a mouse FAR1 cDNA in the pFastBAC HTC vector (Invitrogen) was constructed by amplifying the FAR1 cDNA insert in the vector pCMV·SPORT6-mFAR1 described above using the oligonucleotide primers 5'-GCCTATGTCGACGGACAAGTCAGGATGGTTTCAATCC-3' and 5'-ATTATGCGGCCGCGGTCTTCAGTATCTCATAGTGCTG-3'. The modified cDNA was digested with the restriction enzymes SalI and NotI, purified by agarose gel electrophoresis and QIAquick gel extraction, and ligated into pFastBAC HTC.

A mouse FAR2 cDNA baculovirus recombinant donor plasmid was constructed by amplifying the cDNA from the pCMV6-mFAR2 plasmid using the oligonucleotide primers 5'-GCCTATGTCGACGAACCATACAGGAACGGAGGAATC-3' and 5'-ATTATGCGGCCGCGTATCTGAGGTTCCAGATGATGGG-3'. The resulting DNA product was digested with the restriction enzymes SalI and NotI and ligated into pFastBAC HTC.

A baculovirus donor plasmid containing the mouse Δ^4 -3-oxosteroid 5β -reductase cDNA (nucleotides 48–1053 of GenBank™/EBI Data Bank accession number BC018333) was constructed as follows. Hepatic cDNA was amplified via PCR using the oligonucleotide primers 5'-CAGAAGCTTCAGATTCTTCTCTACG-3' and 5'-TGTTTCAGTATTCGTCATGAAATGGG-3'. The resulting cDNA product was ligated into the TOPO TA vector (Invitrogen) and propagated in *E. coli*. The cDNA insert in this plasmid was excised by digestion with the restriction enzyme EcoRI, purified by agarose gel electrophoresis and the QIAquick gel extraction kit, and then ligated into the pFastBAC HTC vector.

After construction of the donor plasmids, infectious *Autographica californica* nuclear polyhydrosis baculovirus stocks were generated and titrated in *Spodoptera frugiperda* (Sf9) cells using the Bac-to-Bac baculovirus expression system kit (Invitrogen).

Preparation of Bovine Serum Albumin (BSA)-conjugated Fatty Acids

Capric acid (decanoic acid, C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), homo- γ -linolenic acid (C20:3), and arachidonic acid (C20:4) were purchased from Sigma Chemical Co., dissolved in ethanol at a concentration of 62 mM, and then precipitated by the addition of 5 M NaOH to a final concentration of 0.25 M. The ethanol was evaporated under a stream of N₂ gas, and precipitated fatty acids were resuspended in 4 ml of 0.9% (w/v) NaCl by stirring and heating at 80 °C. An aliquot (4.16 ml) of 24% (w/v) BSA dissolved in H₂O was added and the solution stirred at room temperature for 10 min. Thereafter, 0.9% NaCl was added to bring the total volume to 10 ml. The resulting stocks contained 5 mM fatty acid, 0.5% (w/v) NaCl, and 10% (w/v) BSA. [1-¹⁴C]Palmitic acid and [1-¹⁴C]oleic acid dissolved in ethanol were purchased from PerkinElmer Life Sciences. [1-¹⁴C]Capric acid, [1-¹⁴C]lauric acid, [1-¹⁴C]myristic acid, [1-¹⁴C]palmitic acid, [1-¹⁴C]-stearic acid, [1-¹⁴C]linoleic acid, [1-¹⁴C]homo- γ -linolenic acid, and [1-¹⁴C]arachidonic acid dissolved in ethanol were purchased from American Radiolabeled Chemicals. An aliquot of radiolabeled fatty acid was dried under a stream of N₂ gas and resuspended in the corresponding 5 mM solution of unlabeled fatty acid made as above to generate a stock containing 5 mM BSA-conjugated fatty acid and 0.36–0.4 mM 1-¹⁴C-labeled BSA-conjugated fatty acid.

FAR Enzyme Assay in Transfected Cells

On day 0, human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were plated at a density of 4×10^5 cells/60-mm dish in low glucose Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin sulfate. On day 2, cells were transfected with 3.5 μ g of a plasmid mixture containing 0.5 μ g of pVA1 and 3 μ g of pCMV-SPORT6-mFAR1, pCMV6-XL6-hFAR1, pCMV6-mFAR2, or pCMV6-hFAR2 expression plasmid using the FuGENE 6 reagent. Approximately 23 h after transfection, the cell medium was aspirated and replaced with 2.25 ml of fresh Dulbecco's modified Eagle's medium supplemented with 33.3 μ M BSA-conjugated palmitic acid and 2.4 μ M BSA-conjugated [1-¹⁴C]palmitic acid. After a further 24 h of incubation, cells were washed once with phosphate-buffered saline (PBS), harvested with a rubber policeman into 2 ml of PBS, and utilized for thin layer chromatography (TLC) as described below.

FAR Enzyme Assay in Baculovirus-infected Sf9 Cells

Sf9 cells were plated on day 0 at a density of 2.5×10^6 cells/60-mm dish in Sf-900 II SFM medium (Invitrogen). Approximately 4 h after plating, the medium was replaced with Sf-900 medium supplemented with 50 units/ml penicillin and 50 μ g/ml streptomycin sulfate, and the cells were infected with recombinant baculovirus at a multiplicity of infection of 5–10 for 26

h. Thereafter, the medium was replaced with 2 ml of plating medium supplemented with 37.5 μM BSA-conjugated fatty acids and 2.7–3 μM BSA-conjugated 1- ^{14}C -labeled fatty acids, and the infected cells were returned to the incubator for an additional 28 h. The cells were washed with 2 ml of PBS and then scraped into 2 ml of PBS using a rubber policeman prior to analysis by TLC.

TLC

Fatty acid metabolites in transfected or infected cells harvested into 2 ml of PBS were extracted into 8 ml of chloroform:methanol (2:1, v/v). The organic layer was dried under a stream of nitrogen, and the lipid residue was resuspended in 50 μl of hexane and spotted on prescored 150 Å silica gel plates (Whatman). Metabolites were resolved by chromatography in one of two solvent systems. Solvent system 1 involved development for 30 min in hexane:ether:formic acid (65:35:2, v/v/v). Solvent system 2 employed development for 30 min in hexane followed by drying of the plate in air for 15 min and a second development for 40 min in toluene. Radiolabeled metabolites on the plates were detected either by exposure to Biomax MR film (Eastman Kodak) or phosphorimaging using Fuji BAS-TR2040 screens (Fuji Medical Systems, Tokyo, Japan) and the Storm 820 imaging system (Amersham Biosciences). Lipid standards were purchased from Sigma Chemical Co., dissolved in ethanol (palmitic acid, stearic acid, hexadecanol, octadecanol, 1-oleoyl-racemic glycerol, (S)1,2-diolein, and glyceryl trioleate) or chloroform (dipalmitin and glyceryl tripalmitate) at final concentrations of 10 mM, and aliquots of 5 μl were chromatographed on the plates in lanes adjacent to those containing radiolabeled lipids. Standards were visualized by spraying the TLC plates with 0.1% (w/v) 2',7'-dichlorofluorescein in ethanol followed by examination under ultraviolet light (14).

Preparation of Sf9 Cell Membranes

On day 0 of an experiment, Sf9 cells were inoculated at a density of 500,000 cells/ml in 120 ml of Sf-900 II SFM medium. On day 1, the cultures were infected at a multiplicity of infection of 2–4 with the indicated recombinant baculovirus and cultured an additional 48 h. Cells were collected by centrifugation at $1,000 \times g$ for 5 min at 4 °C in a desktop centrifuge, and the cell pellets were washed once with 30 ml of PBS. Cell pellets were resuspended in 3 ml of hypotonic lysis buffer (10 mM Hepes-KOH, pH 7.6, 1.5 mM MgCl_2 , 10 mM KCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, supplemented with one minicomplete protease inhibitor mixture tablet (Roche Applied Science)/10 ml), incubated on ice for 10 min, and then lysed by passage through a 23-gauge needle 20 times. The nuclei were removed by centrifugation at $1,000 \times g$ for 5 min at 4 °C, and the resulting supernatant was centrifuged at $130,000 \times g$ for 30 min at 4 °C in a TLA120.2 rotor in a TL-100 ultracentrifuge (both from Beckman Coulter, Inc., Fullerton, CA). The membrane pellets were resuspended in assay buffer (0.3 M sucrose, 0.1 M Tris-HCl, pH 7.4, 1 mM EDTA, and protease inhibitors as described above).

Assay of Cofactor Preference

FAR enzyme activity was measured in a volume of 500 μl of 0.3 M sucrose, 0.1 M Tris-HCl, pH 7.4, 1 mM EDTA, 2.5 mM dithiothreitol, 5 mM MgCl_2 , one minicomplete protease inhibitor mixture tablet (Roche Applied Science)/10 ml, 0.8 mg/ml BSA, 98 μM palmitoyl-CoA, 7 μM [^{14}C]palmitoyl-CoA (PerkinElmer Life Sciences), and 2.5 mM β -NADPH or β -NADH. Aliquots (75 μg) of Sf9 cell membrane protein isolated from cells infected with either baculovirus expressing the steroid 5 β -reductase or the mFAR1 enzyme were added, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 100 μl of 6 N HCl and 0.9 ml of PBS, and lipids were extracted into 6 ml of chloroform:methanol (2:1, v/v). TLC on dried and resuspended lipids was performed as described above.

Assay of Palmitoyl-CoA versus Palmitic Acid Preference

These experiments were done as described under “Assay for Cofactor Preference” except that the reaction mixtures contained 2.5 mM β -NADPH and either 98 μ M palmitoyl-CoA and 7 μ M [1-¹⁴C]palmitoyl-CoA (obtained from PerkinElmer Life Sciences) or 2.9 μ M BSA-conjugated [1-¹⁴C]palmitic acid and 40 μ M BSA-conjugated palmitic acid with or without 1 mM ATP and 100 μ M CoA. Aliquots (75 μ g) of Sf9 cell membrane protein isolated from cells infected with either baculovirus expressing the steroid 5 β -reductase or the mFAR1 enzyme were added to the mixture, and the tube was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 100 μ l of 6 N HCl, and the lipids were separated by TLC as described above.

Immunocytochemistry

On day 0 of an experiment, Chinese hamster ovary-K1 cells (American Type Culture Collection) were plated at a density of 1×10^5 cells in 6-well dishes containing 22-mm² glass coverslips in Dulbecco’s modified Eagle’s medium and Ham’s F-12 (50:50 mix) medium supplemented with 5% fetal calf serum (v/v), 100 units/ml penicillin, and 100 μ g/ml streptomycin sulfate. On day 1, cells were transfected with 1 μ g of plasmid DNA (pCMV6, pCMV·SPORT6-FLAG-mFAR1, or pCMV·SPORT6-FLAG-mFAR2) using FuGENE 6 reagent. After 18 h, cells were washed twice with ice-cold Dulbecco’s PBS and then fixed in methanol at -20 °C for 10 min. Cells were washed three times with ice-cold PBS and then incubated in a blocking solution (PBS containing 1% (w/v) BSA (Sigma)) for 1 h at room temperature. Cells were incubated with primary antibodies (SKL rabbit polyclonal antibody (15) at 1:1,000 dilution and/or FLAG M2 mouse monoclonal antibody (Sigma) at 1:2,500 dilution) for 8 h at 4 °C. The cells were then rinsed three times for 5 min each in PBS containing 0.1% (w/v) BSA and incubated for 1 h with secondary antibodies (Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) and/or Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes)) both at 1:1,000 dilution in PBS containing 0.1% (w/v) BSA. Cells were rinsed three times with PBS containing 0.1% (w/v) BSA, twice with PBS, and then twice with deionized distilled H₂O. The coverslips were mounted on a glass slide using a ProLong Antifade Kit (Molecular Probes) and then examined using a 63 \times 1.3 NA PlanApo objective on a model 510 Laser Scanning Confocal microscope (Carl Zeiss, Inc., Göttingen, Germany).

Real Time PCR

Total RNA was prepared from mouse tissues using RNA STAT-60 (Tel-Test, Friendswood, TX). Aliquots (100 μ g) of RNA were treated with deoxyribonuclease I (RNase-free, Ambion, Inc., Austin, TX). cDNA synthesis was initiated from 2 μ g of deoxyribonuclease I-treated RNA using random hexamer primers and Taqman reverse transcription reagents (Applied Biosystems). For each of the real time PCRs, which were carried out in triplicate, cDNA synthesized from 20 ng of deoxyribonuclease I-treated RNA was mixed with 2 \times SYBR Green PCR Master Mix (Applied Biosystems). Samples were analyzed on an Applied Biosystems PRISM 7900HT Sequence Detection System. Oligonucleotide primer sequences used in these experiments were: mFAR1 mRNA, 5'-TTAATGTTTATGCTCAAAACCTAGTGAAC-3' and 5'-CCCCTTCTACCCTTTGCA-3', mFAR2 mRNA, 5'-CCCGACAAACTAATTTAACTCACTAA-3' and 5'-GCTTGGTGCTGCCTTTCTCT-3', and cyclophilin mRNA, 5'-TGGAGAGCACCAAGACAGACA-3' and 5'-TGCCGGAGTCGACAATGAT-3'.

RESULTS

The sequences of two eukaryotic fatty acyl-CoA reductase enzymes were used as probes of the mammalian data base. The first, from the jojoba plant *S. chinensis* (6), identified two mouse and human proteins with ~30% sequence identity. The second fatty acyl-CoA reductase sequence was from the silk-worm moth *B. mori* (7). A search for orthologs of this insect enzyme

in the mammalian data base revealed the same two mouse and human proteins as did searches with the plant enzyme sequence, again with low sequence identity. The fact that the plant, insect, mouse, and human sequences shared the same short regions of amino acid identity (Fig. 1) suggested that the mammalian enzymes were potential fatty acyl-CoA reductases. The two enzymes were tentatively named mouse and human fatty acyl-CoA reductase 1 and 2 (FAR1 and FAR2).

Comparisons between cDNA sequences and genomic DNA revealed that the mouse FAR1 enzyme was encoded by a gene on chromosome 7 F1 and contained at least 13 exons and 12 introns. The mouse FAR2 gene on chromosome 6 G3 contained at least 12 exons and 11 introns. The structures of the two genes were similar, both in overall size and in the positions of the introns, 10 of which interrupted the coding portions of the enzymes at the same positions (*arrowheads*, Fig. 1). Two introns in the FAR1 gene were located in the 5'-noncoding portion of the transcribed mRNA, and the FAR2 gene contained one intron in this region. The human FAR1 and FAR2 genes contained at least 12 exons and 11 introns and were present on autosomes (FAR1 = chromosome 11 p15.2; FAR2 = chromosome 12 p11.23).

The amounts of FAR1 and FAR2 mRNAs were determined in different tissues of the mouse by real time PCR (Fig. 2). The FAR1 mRNA had the broadest distribution, being present at readily detectable levels ($C_T = 16.8$ – 28) in the 20 tissues or cell lines examined (Fig. 2, *top panel*). The tissue with the highest level of FAR1 mRNA was the preputial gland ($C_T = 16.8$), a specialized sebaceous gland located near the tail of the animal. In contrast to the FAR1 mRNA, the FAR2 mRNA was present at generally lower levels in a smaller number of tissues. The two highest expressing tissues were the eyelid ($C_T = 21.3$) and skin ($C_T = 23.2$) (Fig. 2, *bottom panel*). An abundance of FAR2 mRNA in these tissues is consistent with a role for this reductase isozyme in lipid synthesis within the meibomian glands of the eyelid and the sebaceous glands of the skin. Both FAR1 and FAR2 mRNAs are present in the brain where large quantities of ether lipids are synthesized. RNA blotting experiments indicated an abundant FAR1 mRNA of 4.2 kb in the preputial gland together with a minor species of 3.8 kb. Similarly, an abundant FAR2 mRNA of 2.7 kb was detected in eyelid RNA along with minor species of 1.7 and 3.3 kb (data not shown).

Complementary DNAs encoding mouse and human FAR1 and FAR2 were cloned into mammalian expression vectors as described under "Experimental Procedures." Expression of FAR1 and FAR2 cDNAs in HEK 293 cells resulted in the conversion of BSA-conjugated [14 C]palmitate into [14 C]hexadecanol (Fig. 3, *lanes 2–5*), whereas transfection of a vector lacking a cDNA insert did not produce hexadecanol (*lane 1*), indicating that HEK 293 cells have negligible levels of endogenous fatty acyl-CoA reductase activity. Several conclusions were reached from these results. First, the putative mammalian FAR cDNAs identified by bioinformatics encoded *bona fide* fatty acyl-CoA reductases. Second, there are two fatty acyl-CoA reductase isozymes in the mouse and human as well as other mammalian genomes (see "Discussion"). Third, comparisons of the results in Fig. 3, *lanes 2 and 3*, with those in *lanes 4 and 5* indicated that the expressed mouse and human FAR1 enzymes were more active than the corresponding FAR2 enzymes in transfected HEK 293 cells.

In an effort to increase expression of FAR1 and FAR2, the mouse cDNAs were recombined into baculovirus expression vectors as described under "Experimental Procedures." As expected, FAR1- and FAR2-expressing viruses produced higher levels of enzyme activity in intact insect cells compared with those obtained in HEK 293 cells, but the levels of FAR1 enzyme activity in the Sf9 cells remained ~5–10-fold higher than those of FAR2 despite equivalent levels of expression (data not shown). These results suggested that the difference in activity between the recombinant FAR1 and FAR2 enzymes reflected the intrinsic properties of the proteins and was not the result of an anomaly between the mammalian and insect cell

expression systems. For these reasons the two expression systems were used interchangeably in subsequent experiments to characterize FAR1 and FAR2.

Centrifugation experiments with cell lysates from FAR1 baculovirus-infected Sf9 cells showed that reductase enzyme activity sedimented with the membrane fraction. Assays with membrane preparations showed that enzyme activity with palmitoyl-CoA substrate was optimal over a range of magnesium (3–32 mM) and KCl (0–200 mM) concentrations and between pH 7.0 and 8.0. When the assay buffer had a pH greater than 8.0, considerable nonenzymatic conversion of fatty acyl-CoA to fatty alcohol was observed. No inhibition of FAR1 activity was detected in the presence of excess product (0.5 mM hexadecanol). Based on these findings, the standard FAR1 enzyme assay was performed in a buffer of pH 7.4 containing 3.0 mM MgCl₂ and 0 mM KCl for 30 min at 37 °C.

In contrast to the results obtained with membranes from FAR1-expressing cells, FAR2 enzyme activity was lost upon lysis of either baculovirus-infected Sf9 cells or transfected HEK 293 cells (data not shown). FAR2 enzyme activity was not detected with the inclusion of different fatty acid substrates, NAD(P)H cofactors, or detergents in the assay buffer, and variations in the ionic conditions or in the incubation time failed to resuscitate FAR2 enzyme activity. Thus, we were only able to analyze FAR2 enzyme activity in whole cells.

Fatty acyl-CoA reductases catalyze a concerted reaction in which the thioester bond of the fatty acyl-CoA substrate is cleaved, and the resulting fatty acid is reduced to an alcohol by transfer of electrons from an NAD(P)H cofactor (16). A series of experiments was performed to determine the substrate and cofactor utilized by mouse FAR1. As shown by the data in Fig. 4A, conversion of palmitate to hexadecanol by membranes from FAR1 baculovirus-infected Sf9 cells required the presence of ATP and CoA in the reaction mixture (*lane 4*). If the preparations were incubated with palmitoyl-CoA, the reaction proceeded in the absence of ATP and CoA (*lane 6*). These results suggested that FAR1 utilized fatty acyl-CoA esters as substrates instead of free fatty acids. Furthermore, because the synthesis of hexadecanol was approximately the same when palmitoyl-CoA or palmitate plus ATP and CoA was used as substrate, the data indicated that the Sf9 cell extracts were saturating for acyl-CoA synthetase enzyme(s) that form the CoA derivatives of fatty acids.

Recombinant mouse FAR1 enzyme required NADPH as a cofactor (Fig. 4B, *lane 7*). No activity was measured when 2.5 mM NADH was substituted in the reaction mixture (*lane 6*), and the inclusion of NADH at this concentration did not inhibit the ability of NADPH to serve as a cofactor (*lane 8*). Membranes prepared from Sf9 cells infected with a baculovirus expressing an unrelated enzyme, steroid 5 β -reductase, contained no fatty acyl-CoA reductase activity when these cofactors were present alone or in combination (*lanes 1–4*).

To determine the fatty acid substrate preferences of the FAR enzymes, Sf9 cells were infected with mouse FAR1 or FAR2 cDNA-containing baculoviruses or a control virus expressing the mouse steroid 5 β -reductase cDNA, and the cells were incubated with BSA-conjugated ¹⁴C-labeled fatty acids of different carbon chain length or saturation (Fig. 5). The FAR1 enzyme preferred C16, C18, C18:1, and C18:2 fatty acids and was less active against other lipids (*top panel*). With longer exposures of the TLC plate to x-ray film, activity was observed when C10–C14 substrates were added to the medium. All fatty acids tested were incorporated into lipids having the same mobility as monoacylglycerols by an endogenous insect activity and to a lesser extent into diacylglycerol products, indicating that they gained access to biosynthetic enzymes in the infected cells. Experiments done with microsomal membranes from FAR1-expressing Sf9 cells produced similar results with respect to fatty acid substrate preference (data not shown).

In contrast to the substrate preference of the FAR1 enzyme, the FAR2 enzyme showed a more narrow specificity for fatty acids, acting with partiality toward saturated C16 and C18 lipids (Fig. 5, *middle panel*). Longer exposures of the TLC plate to x-ray film showed weak activity against the shorter saturated fatty acids. The control-infected cells expressing steroid 5 β -reductase did not reduce any of the fatty acids tested, although all substrates were incorporated into other lipid products by endogenous enzymes (*bottom panel*).

The subcellular localizations of the FAR1 and FAR2 enzymes were determined by immunocytochemistry (Fig. 6). The mouse cDNAs were engineered to contain FLAG epitopes at the amino termini of the encoded proteins and the resulting modified cDNAs cloned into pCMV expression vectors. The introduction of the FLAG epitope did not affect the fatty acyl-CoA reductase activities of the two modified enzymes when the constructs were expressed in HEK 293 cells (data not shown). Transfection of the DNAs into Chinese hamster ovary-K1 cells followed by staining with anti-FLAG monoclonal antibody showed that both the FAR1 (Fig. 6E) and FAR2 enzymes (Fig. 6H) localized to peroxisomes distributed throughout the cytoplasm of expressing cells. The identification of these vesicular bodies as peroxisomes was confirmed by costaining with a polyclonal antiserum directed against a targeting sequence (serine-lysine-leucine, SKL) present in many peroxisomal enzymes (15). As seen in Fig. 6, A, D, and G, this antiserum recognized the same type of subcellular organelle in all cells on the coverslip, and when these rhodamine images were merged with the fluorescein images generated with the anti-FLAG antibody, many peroxisomes in transfected cells were observed to express both antigens (Fig. 6, F and I).

DISCUSSION

In the current study, we identify two mammalian fatty acyl-CoA reductase enzymes that convert a variety of fatty acids to fatty alcohols. The two FAR isozymes are ~58% identical in sequence and are encoded by genes with similar exon-intron structures located on different chromosomes. The mouse FAR1 mRNA is most abundant in the preputial gland and present at lower levels in many other organs and cells. The highest levels of FAR2 mRNA are detected in tissues that are rich in sebaceous glands (eyelid and skin). FAR1 acts on fatty acids of different chain lengths and degrees of saturation, whereas FAR2 prefers saturated C16 and C18 fatty acids as substrates. The FAR enzymes are localized to the peroxisome as judged by immunocytochemistry in transfected cells. The distinct biochemical properties and tissue distributions of the two fatty acyl-CoA reductases suggest that these isozymes perform different functions in lipid metabolism.

Pairwise sequence comparisons between the mammalian FAR enzymes and the previously defined plant and insect orthologs reveal ~30% amino acid identity (Fig. 1). Searches with the mouse protein sequences indicate putative fatty acyl-CoA reductases in many species, including the toad (*Xenopus laevis*, GI28277293), mosquito (*Anopheles gambiae*, e.g. GI31197903, and many others), rat (GI34859004), zebra fish (GI28278322), fruit fly (*Drosophila melanogaster*, e.g. GI24654209 and many others), and nematode (*Caenorhabditis elegans*, GI17570463). The prospective reductases are 33% (mosquito) to 96% (rat) identical in sequence to the mouse FAR enzymes, with identity extending over the complete length of the compared proteins. Homologous sequences include so-called "male sterility proteins" that are implicated in lipid synthesis and the formation of the pollen cell wall in plants (17), and in the case of wheat, they have been shown to have fatty acyl-CoA reductase enzyme activity (8).

Comparisons between the known and presumed reductases show that only a small number of amino acids are conserved across species. For example, among mouse, plant, and insect proteins, only 61 amino acids (~13%) are identical (Fig. 1). Thirteen of the highly conserved

residues are either glycines or prolines, which may play structural roles in these proteins. No obvious NADPH cofactor binding or catalysis sites were found among the conserved sequences.

In mice and humans, the FAR1 and FAR2 isozymes share ~58% sequence identity and are encoded by genes of similar structure, suggesting that they arose from a common evolutionary precursor via duplication. This genetic event is presumably ancient as apparent orthologs for each isozyme are present in several species for which complete genome sequences are available, including the puffer fish (*Fugu rubripes*, FRUP00000132990 and FRUP00000130769) and the rat (XP_215022.2 and NW_047696.1).

The conservation of two isozymes in distantly related species represents one line of evidence that each FAR protein has a different biological function. This idea is supported further by their different fatty acid substrate preferences (Fig. 5) and their differential expression in tissues (Fig. 2). FAR1 is distributed broadly and acts on fatty acids that vary in size and saturation, suggesting that this isozyme plays a general role in the synthesis of fatty alcohols. In contrast, the narrow distribution and substrate preference of the FAR2 isozyme are indicative of a more specialized function. Some support for this division of labor is to be found in the ether lipids (plasmalogens) of tissues expressing the FAR1 enzyme, which have diverse structures consistent with the production and incorporation of a variety of fatty alcohols into this class of lipids (10). Furthermore, the fatty alcohol composition of waxes secreted by the sebaceous glands of mouse skin is different from those of the preputial gland (2), which may reflect the differential expression and substrate specificities of the FAR1 and FAR2 enzymes in these tissues.

In the experiments reported here, the FAR1 enzyme was consistently more active in reducing fatty acids than the FAR2 enzyme when assayed in intact cells (*e.g.* Fig. 3). The reason for this difference was not ascertained but did not appear to be the result of discrepancies in expression level as judged by immunoblotting (data not shown), substrate preference (Fig. 5), or differences in subcellular localization (Fig. 6). Furthermore, FAR2 enzyme activity was lost upon lysis of the cells and could not be preserved or restored by several different treatments. The FAR2 enzyme may require a protein cofactor for activity which is not present in HEK 293 or Sf9 cells. In support of this possibility, a soluble protein identified as a member of the fatty acid-binding protein family was reported to enhance reductase activity in extracts of mouse preputial glands (18). This effect was shown to be caused by the ability of the protein to bind fatty acyl-CoAs, thereby decreasing the effective concentration of the lipid below the critical micelle value and lessening the detergent effects of the substrate. Although BSA was included in all reactions to buffer fatty acyl-CoA concentrations, and no feedback inhibition by substrate was observed with the FAR1 enzyme, we cannot rule out the possibility that FAR2 requires a unique accessory protein for full activity. Future expression cloning experiments with cDNA libraries from tissues such as the eyelid that express high levels of the FAR2 enzyme may identify a stimulatory factor.

Both the FAR1 and FAR2 enzymes are localized to the peroxisome (Fig. 6) and are found in the pellet fraction of high speed centrifugations, suggesting that they are bound to the membrane of this organelle. Hydropathy plots and other sequence analysis programs do not reveal classical transmembrane domain profiles within the reductases, thus we do not know whether they are integral membrane proteins or tightly bound to the phospholipid bilayer of the peroxisome. Similarly, it is not immediately evident how the FAR proteins are imported into this organelle because sequence prediction programs that identify peroxisomal proteins (mendel.imp.univie.ac.at) do not reveal a type 1 targeting sequence, and visual scanning fails to uncover a conserved type 2 consensus sequence (19).

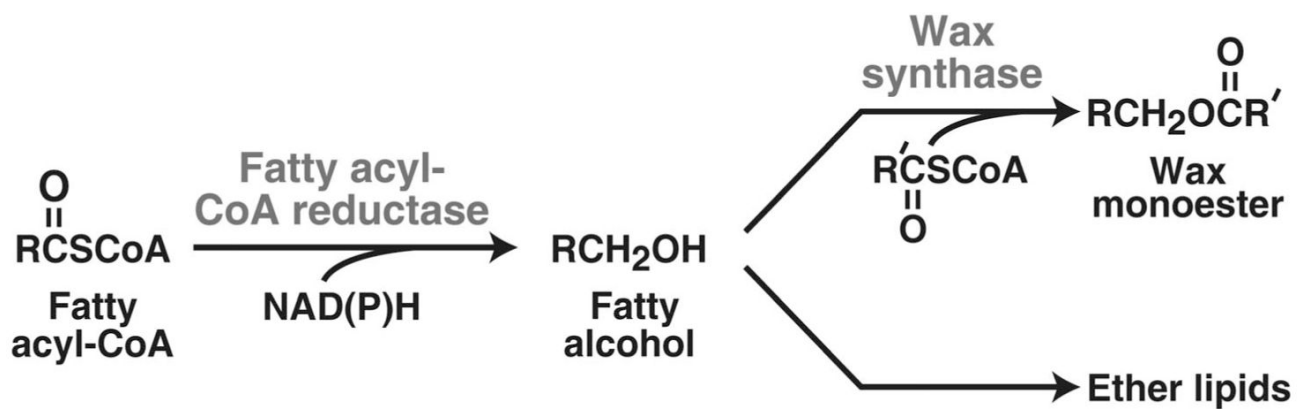
The presence of FAR enzymes in the peroxisome is consistent with a central role in the production of fatty alcohols for ether lipid biosynthesis. Three of the seven enzymes involved in synthesis of ether lipids are found in this organelle, including alkyl-dihydroxyacetone phosphate synthase, which replaces an *sn-1* fatty acid in ester linkage to dihydroxyacetone phosphate with a long chain fatty alcohol to form an alkyl ether intermediate (10). Colocalization of the reductase and synthase within the peroxisome obviates the need for interorganellar transport of the fatty alcohol and presumably facilitates the synthesis of ether lipids. In contrast, the synthesis of wax monoesters by the wax synthase enzyme (Scheme 1), which is localized in the endoplasmic reticulum (see accompanying paper (12)), requires transport of the fatty alcohol across two lipid bilayers. Whether the movement of fatty alcohols represents a controlling step in the synthesis of these and other classes of lipids, and how transport is accomplished, are questions to be answered in future studies.

Acknowledgments

We thank Jill Fairless and Lisa Beatty for expert assistance with tissue culture; Joel Goodman for antiserum against the SKL epitope and advice on peroxisomes; Helen Yin, Greg Graf, Manuel Martinez, Wei-Ping Li, Mike Roth, and James Waddle for guidance and access to confocal microscopes; and Jonathan Cohen, Joel Goodman, Helen Hobbs, and Jay Horton for critical review of the manuscript.

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SCHEME 1.
Catalytic steps required to produce a wax monoester.

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Mouse FAR1 -----MVSIPPEYEGNILLTGCATGCGLWLLERLLRSCFVNSVYVYVQKAGQ 50
Mouse FAR2 -----MSMIAAFYSNLSLLDGCATGCGLWLLERLLRSCFVNSVYVYVQKAGQ 50
Insect FAR MSHNGTLDHYQTVREPYDGSVFTDGCATGCGLWLLERLLRSCFVNSVYVYVQKAGS 60
Plant FAR -----MEEMGSILEFLDNAILVGGAGSGLKATFVEKVLRSQENVKKLLVLLBATDDE 53

Mouse FAR1 TPQBRVE-EILSSKLDRLRDNPD----FRKRTIAINSELTPQKLALSEE-DKEI IID 103
Mouse FAR2 TLQBRVF-QILNSKLEKVKVCPN----VHRKTRPISADLNQRDFALSKE-DVQELLS 103
Insect FAR NTEBEMR-KYLDQPHSRKYEHPE----YFKRIPISGDITAPKLGLCDE-ERNLILN 113
Plant FAR TAALQLQNEVFGKELKVKLQNLGANFYSFVSEKVTVVPGDITGEDLCLKDVNLKEMMR 113

Mouse FAR1 STNVIFHCATVRFNENLRDAVQLQVIATRQLLLLAQQMKNLEVFMHVSITAMAYCNRK-- 161
Mouse FAR2 CTNLIIFHCATVRFDAHLREAVQLQVATATQQLLLMASQMPKLEAPFHISFASNCNLS-- 161
Insect FAR EVSIVIHSAASVKLNHLKFTLNTLVGGTMKVLVLELVKEMKNLAMPVYVYVSTASNTSQR-- 171
Plant FAR EIDVVVNLKATINFIERYDVSLLELVYGAKYVLDFAKCKNRKLEVHVSITAMVSGEKNQL 173

Mouse FAR1 HIDEVYVP-----PPVDPKLLID-SLQWMDGLVNDITPKLIGDR----- 200
Mouse FAR2 HIDEVYVP-----CPVEPKIID-SMQLLDDSIIEEITPKLIGDR----- 200
Insect FAR ILEEKLYP-----QSLNLEIQKFAEYHYLQKNDENMKFIGNH----- 211
Plant FAR ILEKPYVMGESLNGRLGLDINVEKKLVEAKINLQAGATEKSIKSTMKDMGIERARHWG 233

Mouse FAR1 -ENNYIYFKALGEVVVQEGAKLNVAIVRSEIVGASWRSEFFGWIDNFGNPSGLFIAAGK 259
Mouse FAR2 -ENNYIYFKALGEVVVQEGSNLNVAIVRSEIVGATWQSEFFGWIDNFGNPSGLIATAGK 259
Insect FAR -ENNYIYFKALGEVNLVAEEHGEIPTIIRHPSHITASABEVRGCFVDSWSGATAMAFAALK 270
Plant FAR WENNYIYFKALGEMLLMQYKGDIPLTIRHPTITSTFKSEFFGWVEGVRTIDNVVYVYK 293

Mouse FAR1 GILRTMRASNALADLVFVWVWNTSLAAAWYSGVNRPRNIMVACTTGSTNFPHWGEVE 319
Mouse FAR2 GILRSIKATPMAVDYLDFVWVWNTSLAVGWYTAVHRPKSTLLESTSGNLEGNWYFWMG 319
Insect FAR GWNMIWYTGEEINDELELWQKTLVW--IAKYKPKFQVTVVHTTSDLEHGISIRRF 328
Plant FAR SRLRCMLCGPSTIIDLELWQKTLVWATIVAMVAHANQRVVEFVTEHVSSADNEMKLSALP 353

Mouse FAR1 YHVIKFKRNLQEQAFRRPNVN---LTSN-HLLYHYIAVSHKAEFLYDIY----LRM 370
Mouse FAR2 LQVLATIEKLEFESAFRRPNAD---FTTS-NFTTHYWNVTSHRVBAIYDFY----LRL 370
Insect FAR IKLSEFASKNETSNAAPFAATT---LLTKQPLIKLVTFLMQTTEAFLADLW----MKT 380
Plant FAR EMAHRYFTKNEWINDRNPVHVGRAMVSSFSFPHLYLTLNFLLEKVLKELIANTIFCQWF 413

Mouse FAR1 TGRSPRMMKTIIRLHKAMVLEYFTSNVWVWNTDNDVNMVWNLQNLN--EDKKTENIDVRQL 428
Mouse FAR2 TGRKPRMLKLMNRLKTIISMLEYFINHSEWSTNNTMELSELSP--EDQRVNFVDRQL 428
Insect FAR QRKEAKFVKQHNLVRSRDQLEFFTSQSWLLRCERARVSAALS--SDRAVRFCDPSTI 438
Plant FAR KGKYMCLKRKTLLRLVLDIYKPLFFQGFDDMNTKRIIAAKESIVEADMVYFAPRAI 473

Mouse FAR1 HNAEIT-IENYCNCFKKYVLEEMSGLPAARKHLNLRNIRYGFNTLIVLILWRIPARSQ 487
Mouse FAR2 NNAEIT-IENYVLCYKYLKEDLAGIPKAKQHLRRLRNIRYHFNLTALFLIWRLLIARSQ 487
Insect FAR DNDQV-LPIYFEGHNKHLFPKNL----- 460
Plant FAR NNAEITFLKTHFELVVEHVLN----- 493

Mouse FAR1 MARNIWFVVSICYKFLSYFRASSTMY 515
Mouse FAR2 MARNVWFFIVSFCYKFLSYFRASSTLKV 515
Insect FAR -----
Plant FAR -----

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FIG. 1. Amino acid sequences of mouse, insect, and plant FAR enzymes

A, the deduced sequences of the proteins were aligned using the ClustalW (version 1.82) software program with identities indicated by *black boxes*. Amino acids are numbered on the *right*. *Arrowheads* above the mouse FAR1 sequence indicate the positions of 10 introns in the coding portion of the gene. The introns of the FAR2 gene were in the same positions. The GenBank™/EBI Data Bank accession numbers for the mouse FAR1 and FAR2 cDNA sequences are BC007178 and BC055759, respectively. Those for the human FAR1 and FAR2 cDNAs are AY600449 and BC022267, respectively.

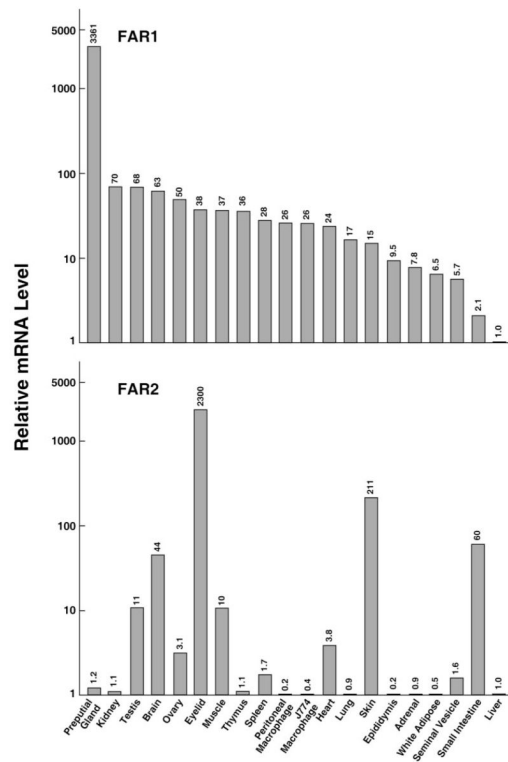


FIG. 2. Tissue distributions of mouse FAR1 and FAR2 mRNAs

The relative levels of each reductase mRNA were determined by real time PCR in the tissues and cell types indicated on the *bottom* of the figure using cyclophilin mRNA levels as a reference standard. The data for a given FAR mRNA were normalized to the threshold values (C_T) determined in the liver (FAR1 mRNA = 27.4, FAR2 mRNA = 28.0) and then expressed on a log₁₀ scale. This experiment was repeated twice on different days using the same preparations of tissue RNAs, which were isolated from pools of animals (organ samples) or dishes (cell samples).



FIG. 3. Expression of mouse and human FAR enzymes in HEK 293 cells

Plasmid DNAs encoding the indicated FAR enzymes or a vector alone control (*pCMV*) were introduced into HEK 293 cells grown in 60-mm dishes. Approximately 24 h after transfection, fresh Dulbecco's modified Eagle's medium containing 33.3 μM BSA-conjugated palmitic acid and 2.4 μM BSA-conjugated [$1\text{-}^{14}\text{C}$]palmitic acid was added to the cells. After an additional 24-h incubation, cells were washed once with PBS, harvested, and lipids extracted with chloroform:methanol in preparation for TLC. Chromatography on silica gel plates was performed for 0.5 h in solvent system 2, and the silica gel plate was exposed to Kodak BioMax MR film. The positions to which palmitate and hexadecanol standards migrated on the TLC plate are indicated on the *right*.

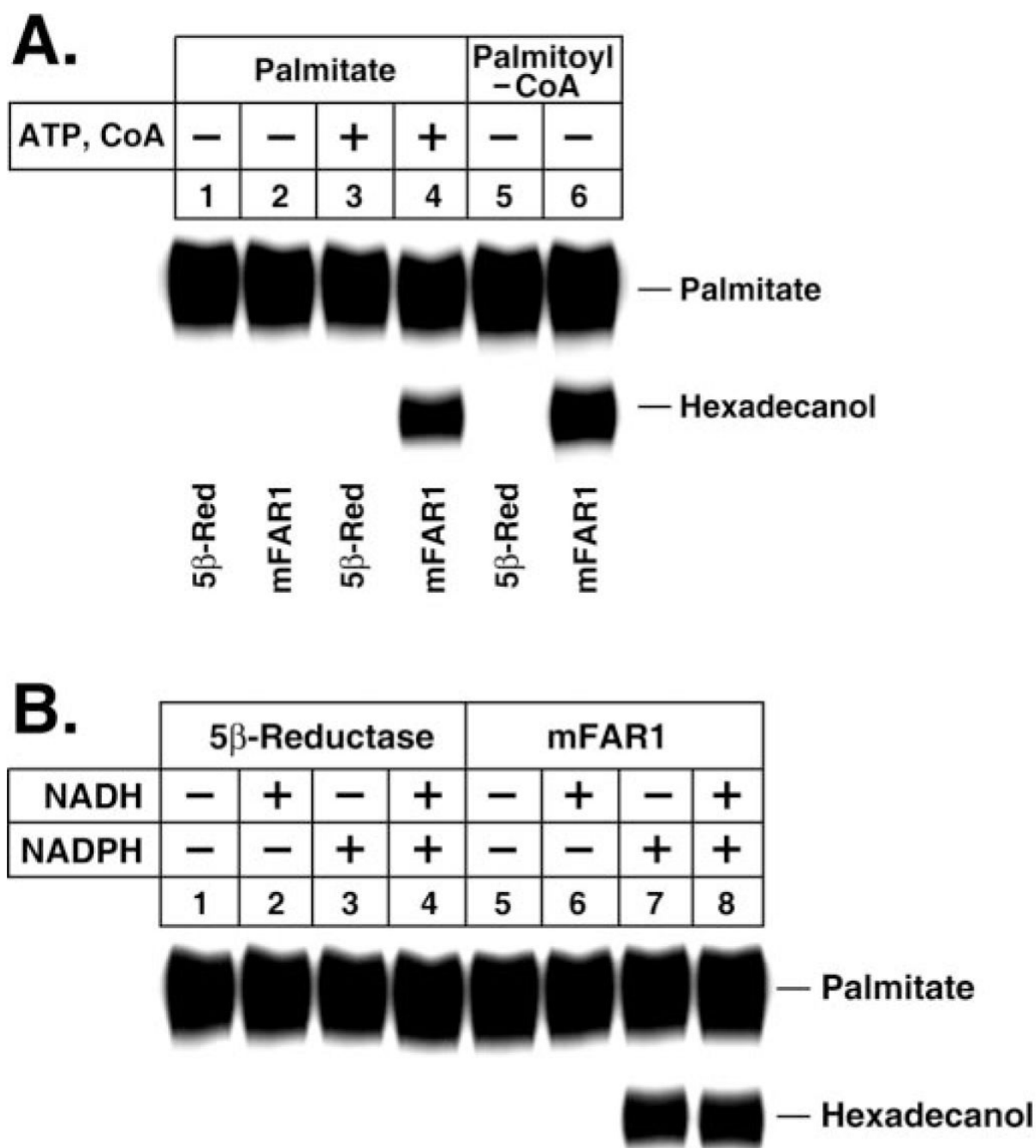


FIG. 4. Substrate and cofactor preferences of mouse FAR1 enzyme

A, insect cells (Sf9) in suspension were infected with baculovirus vectors encoding either a control protein (steroid 5 β -reductase, 5 β -Red) or the mouse FAR1 enzyme (mFAR1) for 48 h at a multiplicity of infection of 2–4. Cell membranes were prepared as described under “Experimental Procedures,” and aliquots (75 μ g of protein) were incubated for 30 min at 37 °C in a reaction containing 2.5 mM β -NADPH and either 2.9 μ M BSA-conjugated [1- 14 C]palmitic acid and 40 μ M BSA-conjugated palmitic acid, with or without 1 mM ATP and 100 μ M CoA, as indicated (lanes 1–4), or 98 μ M palmitoyl-CoA and 7 μ M [1- 14 C]palmitoyl-CoA (lanes 5 and 6). The reaction was stopped by the addition of 100 μ l of 6 N HCl, lipids were extracted and separated by TLC using solvent system 1, and radioactivity was detected by exposing the plate

to x-ray film. The positions to which palmitate (substrate) and hexadecanol (product) migrated are shown on the *right*. *B*, Sf9 insect cells were infected with the indicated baculovirus vectors, and cell membranes were prepared. Aliquots (75 μg of protein) were incubated in reactions containing 98 μM palmitoyl-CoA, 7 μM [1- ^{14}C]palmitoyl-CoA, and 2.5 mM β -NADPH, 2.5 mM β -NADH, or 2.5 mM β -NADPH and 2.5 mM β -NADH, for 30 min at 37 °C. Lipids were extracted and analyzed as described in *A*. The experiments of *A* and *B* were repeated at least twice on separate days.

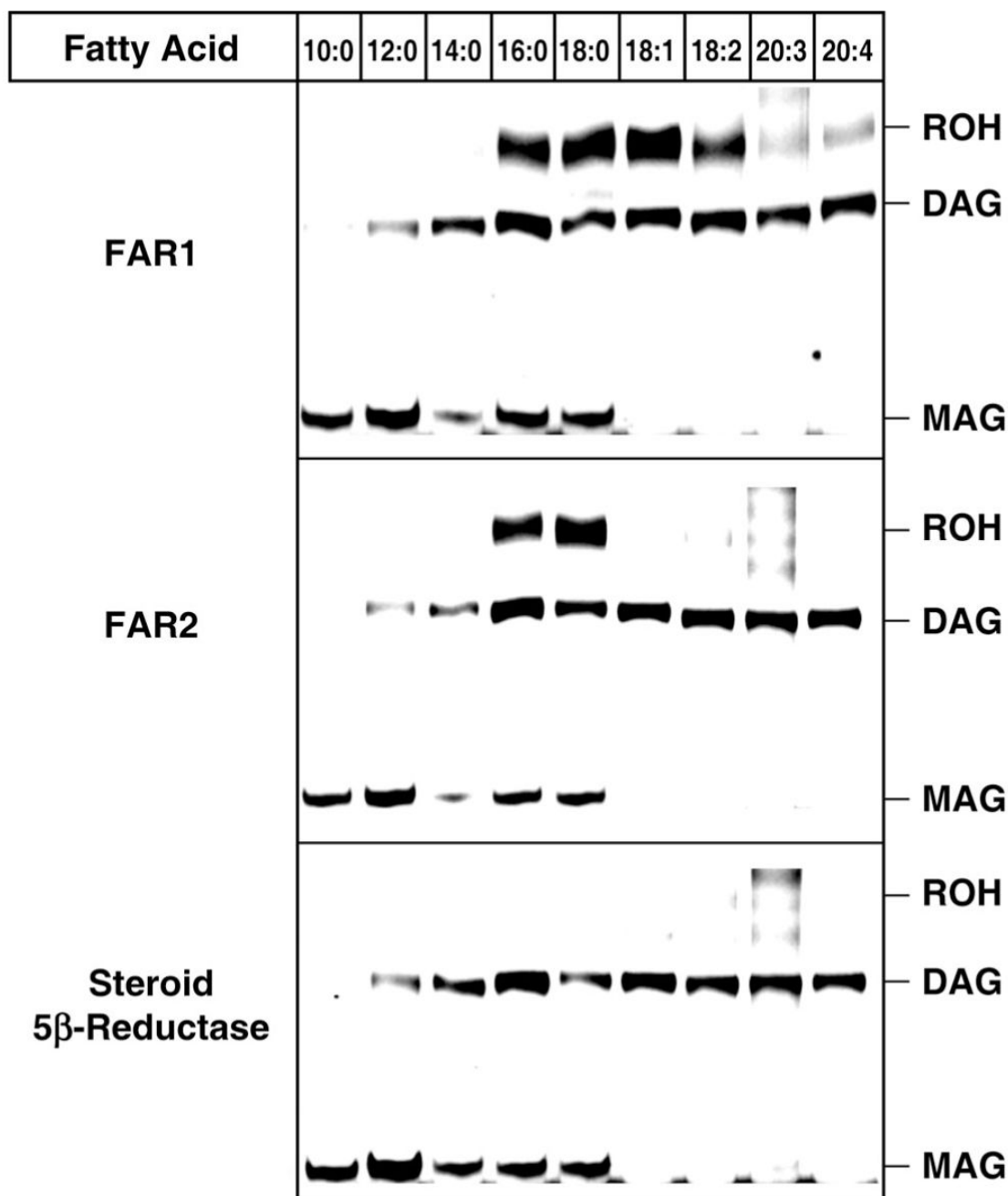


FIG. 5. Substrate preferences of mouse FAR1 and FAR2 enzymes

Baculovirus vectors encoding the fatty acyl-CoA reductase proteins or the control protein steroid 5 β -reductase were used to infect adherent Sf9 cells at a multiplicity of infection of 5–10. Approximately 26 h after infection, intact cells were incubated with the indicated ^{14}C -radiolabeled fatty acids conjugated to BSA. The final concentrations of fatty acids in the medium were 37.5 μM unlabeled lipid and 2.7–3 μM ^{14}C -radiolabeled lipid. The cells were returned to the incubator for an additional 28 h, and thereafter lipids were extracted for TLC. The positions to which fatty alcohol (ROH), diacylglycerol (DAG), and monoacylglycerol (MAG) standards migrated on the plates developed in solvent system 1 are indicated on the right of the autoradiograms. Several radiolabeled compounds of unknown structure were

present in the preparation of radiolabeled homo- γ -linolenic acid (20:3) used in these experiments, which were repeated two times.

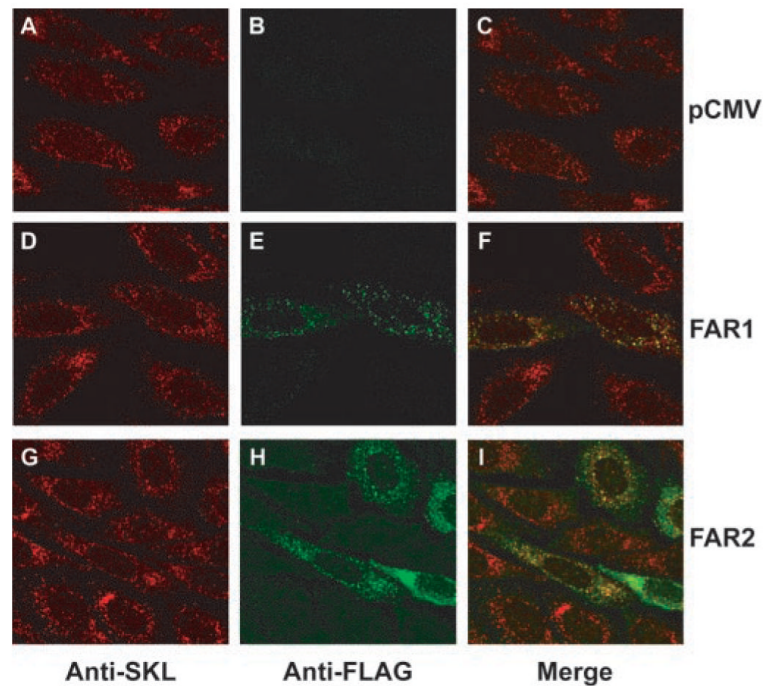


FIG. 6. Subcellular localization of mouse FAR1 and FAR2 enzymes

Chinese hamster ovary-K1 cells were transfected with the indicated expression vectors encoding nothing (*pCMV*), FLAG epitope-tagged FAR1, or FLAG epitope-tagged FAR2 enzymes. After transient expression, cells were fixed, permeabilized, and incubated with a rabbit polyclonal antiserum directed against the SKL epitope of peroxisomal proteins (*Anti-SKL*) and a mouse monoclonal antibody directed against the FLAG epitope (*Anti-FLAG*). *A*, *D*, and *G*, detection of rabbit polyclonal antibodies directed against SKL epitope with rhodamine-conjugated goat anti-rabbit antiserum. *B*, *E*, and *H*, detection of mouse monoclonal antibody directed against FLAG epitope with fluorescein-conjugated goat anti-mouse antiserum. *C*, *F*, and *I*, merged images of rhodamine and fluorescein signals. Double indirect immunofluorescence microscopy was performed on a Zeiss 510 Laser Scanning Confocal microscope using a 63×1.3 NA PlanApo objective. The FLAG-FAR1 enzyme is detected in the peroxisome (*middle panels*). The FLAG-FAR2 enzyme is present in peroxisomes of cells expressing low levels of the protein and in the peroxisomes and endoplasmic reticulum of cells expressing high levels of the protein (*bottom panels*). These data are representative of two separate experiments.