

The Human *FA2H* Gene Encodes a Fatty Acid 2-Hydroxylase*

Received for publication, June 15, 2004, and in revised form, August 12, 2004
Published, JBC Papers in Press, August 27, 2004, DOI 10.1074/jbc.M406649200

Nathan L. Alderson‡, Barbara M. Rembiesa‡, Michael D. Walla§, Alicja Bielawska‡,
Jacek Bielawski‡, and Hiroko Hama‡¶

From the ‡Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425 and §the Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208

2-Hydroxysphingolipids are a subset of sphingolipids containing 2-hydroxy fatty acids. The 2-hydroxylation occurs during *de novo* ceramide synthesis and is catalyzed by fatty acid 2-hydroxylase (also known as fatty acid α -hydroxylase). In mammals, 2-hydroxysphingolipids are present abundantly in brain because the major myelin lipids galactosylceramides and sulfatides contain 2-hydroxy fatty acids. Here we report identification and characterization of a human gene that encodes a fatty acid 2-hydroxylase. Data base searches revealed a human homologue of the yeast ceramide 2-hydroxylase gene (*FAH1*), which we named *FA2H*. The *FA2H* gene encodes a 372-amino acid protein with 36% identity and 46% similarity to yeast Fah1p. The amino acid sequence indicates that FA2H protein contains an N-terminal cytochrome b_5 domain and four potential transmembrane domains. FA2H also contains the iron-binding histidine motif conserved among membrane-bound desaturases/hydroxylases. COS7 cells expressing human FA2H contained 3–20-fold higher levels of 2-hydroxyceramides (C16, C18, C24, and C24:1) and 2-hydroxy fatty acids compared with control cells. Microsomal fractions prepared from transfected COS7 cells showed tetracosanoic acid 2-hydroxylase activities in an NADPH- and NADPH:cytochrome P-450 reductase-dependent manner. FA2H lacking the N-terminal cytochrome b_5 domain had little activity, indicating that this domain is a functional component of this enzyme. Northern blot analysis showed that the *FA2H* gene is highly expressed in brain and colon tissues. These results demonstrate that the human *FA2H* gene encodes a fatty acid 2-hydroxylase. FA2H is likely involved in the formation of myelin 2-hydroxy galactosylceramides and -sulfatides.

Sphingolipids are a large class of lipids found in all eukaryotic cells and are involved in a variety of cellular processes. The structural diversity of sphingolipids stems from over 300 known distinct head groups as well as modifications of the hydrophobic ceramide moiety. One of the common modifications of the ceramide moiety is 2-hydroxylation of the *N*-acyl chain. Sphingolipids with 2-hydroxy fatty acid are found in

most organisms including plants, yeast, worms, vertebrate animals, and some bacterial species.

In mammals, 2-hydroxy fatty acid-containing sphingolipids are uniquely abundant in nervous and epidermal tissues. In mammalian central and peripheral nervous systems, galactosylceramides and sulfatides (3-sulfate ester of galactosylceramide) are major lipid components of myelin (1, 2). These glycosphingolipids contain a high proportion (~50%) of 2-hydroxy fatty acid (3) and are critical components of myelin (4, 5). In mammalian epidermal tissues, there are several unique, very long chain ceramides with 2-hydroxy fatty acids, which are critical for the permeability barrier function of epidermis (6, 7).

Several biophysical studies (8–10) demonstrated that the 2-hydroxyl group in sphingolipids has a profound effect in the lipid organization within membranes because of its hydrogen-bonding capability. The participation of the 2-hydroxyl group in hydrogen bonds with neighboring lipids was shown by analysis of the crystal structure of synthetic glycosphingolipids with 2-hydroxyoctadecanoic acid (8) and by analysis of the phase transition temperature (9). The monolayer behavior of synthetic ceramides showed that the 2-hydroxyl group promotes condensation to a close-packed arrangement (10). These studies provide a physical basis for the effects of 2-hydroxysphingolipids in biomembranes.

The 2-hydroxylation of sphingolipid *N*-acyl chains occurs during *de novo* ceramide synthesis and is catalyzed by the enzyme fatty acid 2-hydroxylase (also known as fatty acid α -hydroxylase). Biochemical properties of this enzyme have been studied in rat brain postnuclear (11) or microsomal fractions (12). The rat brain fatty acid 2-hydroxylase requires molecular oxygen, Mg^{2+} , pyridine nucleotides (NADPH or NADH), and a microsomal electron transport system (13, 14). Insensitivity to carbon monoxide indicates that the rat brain fatty acid 2-hydroxylase is not a P-450 enzyme but another type of mixed function oxidase (11).

In the yeast *Saccharomyces cerevisiae* most sphingolipids contain 2-hydroxy fatty acid. The 2-hydroxylation is dependent on the *FAH1* (also known as *SCS7*) gene, which has been identified as a gene containing a cytochrome b_5 -like sequence (15) and as a suppressor of the Ca^{2+} -sensitive phenotype of *csg2* mutants (16). Yeast *fah1* mutants show increased resistance to pore-forming antifungal agents, presumably because of altered plasma membrane properties (17). Yeast Fah1p is a member of the membrane-bound desaturase/hydroxylase family with the conserved histidine motif (HX_(3–4)HX_(7–41)HX_(2–3)HHX_(61–189)(H/Q)X_(2–3)HH), which is thought to coordinate a non-heme di-iron cluster at an active site (13). The reactions catalyzed by the enzymes in this family require electron donors and molecular oxygen (13). For yeast Fah1p, the terminal electron donor is likely the intramolecular cytochrome b_5 domain. It is notable that the predicted properties of yeast Fah1p are

* This study was supported by National Institutes of Health Grant 1 P20 RR 17677-01 and an Atorvastatin Research Award (to H. H.) sponsored by Pfizer, Inc. 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.

The amino acid sequences of the protein can be accessed through NCBI Nucleotide Database under NCBI accession number NM_024306.

¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashely Ave., Charleston, SC 29425. Tel.: 843-792-6949; Fax: 843-792-8568; E-mail: hama@muscedu.

consistent with the biochemical properties of rat brain fatty acid 2-hydroxylase. Therefore, it is a reasonable assumption that mammalian fatty acid 2-hydroxylases are encoded by *FAH1* homologues.

Here we report identification and characterization of the human *FA2H* gene, which was identified based on the sequence similarity to the yeast *FAH1* gene. Fatty acid 2-hydroxylase activity was found in COS7 cells expressing the human *FA2H* gene.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). [α - 32 P]dCTP was purchased from PerkinElmer Life Sciences. Deuterated (3,3,5,5- D_4)-tetracosanoic acid was purchased from Larodan Fine Chemicals (Malmö, Sweden). A synthetic peptide corresponding to the C-terminal 20 amino acids of FA2H (NH₂-KLWDYCFHTLTPEKPHLKTQ-COOH) was synthesized by AnaSpec (San Jose, CA). Anti-hFA2H polyclonal antibodies for the C-terminal peptide were generated in rabbits and affinity purified using immobilized antigen. Horseradish peroxidase (HRP)¹-linked anti-FLAG M2 monoclonal antibodies (A8592) were purchased from Sigma. HRP-linked sheep anti-mouse IgG (NXA931), HRP-linked donkey anti-rabbit IgG (NA934), and the ECL Western blotting detection reagents were purchased from Amersham Biosciences. Goat anti-rabbit cytochrome *b*₅ antibodies (RDI-CYTOB5abG) and HRP-linked donkey anti-goat IgG antibodies (RDI-705035147) were purchased from Research Diagnostics, Inc. (Flanders, NJ). Purified human cytochrome *b*₅ was purchased from PanVera (Madison, WI). Purified human NADPH:P-450 reductase and NADPH regenerating system solutions were purchased from BD Biosciences.

Cell Culture—COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose and L-glutamine, sodium pyruvate, and 10% fetal bovine serum. Cells were maintained at 5% CO₂ at 37 °C.

Human FA2H Expression Plasmid—A human gene with significant similarity to the yeast *FAH1* gene was identified by BLAST analyses. A human *FA2H* cDNA clone was purchased from the American Type Culture Collection (ATCC no. MGC-10804). The original data base entry indicated the open reading frame starting at the methionine 93 (Fig. 1). The *FA2H* open reading frame was amplified accordingly by PCR using a pair of oligonucleotides (5'-GCAGGGATCCATGGAGAACGAGCCTGTAGC-3' and 5'-TGGGAATTCCTACTGCGTCTTCAGGTGGGG-3'), which were digested with BamHI and EcoRI and cloned into pBluescript II vector, resulting in pBS-hFA2HΔN. Later, a sequence comparison among the human, mouse, and rat homologues revealed an N-terminal extension of 92 amino acids corresponding to a cytochrome *b*₅ domain. To create a full-length recombinant human *FA2H* containing the N-terminal cytochrome *b*₅ domain, a 276-bp NcoI fragment corresponding to the N-terminal 92 amino acids was inserted into the NcoI site of pBS-hFA2HΔN, resulting in pBS-hFA2H. Following sequence verification, the two versions of *FA2H* (hFA2HΔN and hFA2H) were subcloned into the mammalian expression vector pcDNA3 (Invitrogen), resulting in pcDNA3-hFA2HΔN and pcDNA3-hFA2H, respectively. To introduce an N-terminal FLAG tag, the BamHI-EcoRI fragment of pBS-hFA2H was subcloned into pYES2-FLAG (18), resulting in pYES2-FLAG-hFA2H. The FLAG-hFA2H fragment was cut out with HindIII and EcoRI and subcloned into pcDNA3, resulting in pcDNA3-FLAG-hFA2H.

Transfection—COS7 cells were transfected with pcDNA3, pcDNA3-hFA2H, pcDNA3-hFA2HΔN or pcDNA3-FLAG-hFA2H using FuGENE 6 transfection reagent (Roche Applied Science). Cells were harvested 24 h after transfection by trypsin-EDTA treatment for lipid analyses or were directly lysed in 6-well culture dishes for Western blot analyses.

Cell Fractionation—COS7 cells were transfected in 6-well culture dishes. Twenty-four hours after transfection, cells were harvested by trypsin-EDTA treatment, resuspended in a solution of 10 mM Tris-HCl, pH 7.4, 1% glycerol, and 1 mM phenylmethylsulfonyl fluoride, and lysed by freezing-thawing followed by sonication (amplitude 29%, 5 s on, 0.1 s off, 12 cycles) (Fisher Sonic Dismembrator Model 500). Cell lysate was centrifuged at 1000 × *g* for 5 min to remove unbroken cells and large debris. The supernatant (low speed supernatant) was centrifuged at 100,000 × *g* for 2 h to separate the supernatant (cytosolic fraction) and the pellet (membrane fraction).

Western Blot Analysis—COS7 cells were transfected in 6-well culture dishes. Twenty-four hours after transfection, cells were washed twice with phosphate-buffered saline, lysed with 100 μl of SDS-PAGE lysis buffer/well (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 25 μg/ml phenylmethylsulfonyl fluoride), and mixed with an equal volume of 2× SDS-PAGE sample buffer. Following electrophoresis, proteins were blotted onto nitrocellulose membranes and incubated with HRP-linked anti-FLAG M2 monoclonal antibodies (1:500) or anti-human FA2H polyclonal antibodies (1:1000) followed by incubation with HRP-linked donkey anti-rabbit IgG (1:5000). Protein bands were detected using ECL Western blotting detection reagents. For detection of cytochrome *b*₅, goat anti-rabbit cytochrome *b*₅ antibodies and HRP-linked donkey anti-goat IgG antibodies were used. Purified human cytochrome *b*₅ was used as a positive control.

Ceramide Determination by Liquid Chromatography/Tandem Mass Spectrometry—Approximately 1 × 10⁶ COS7 cells were transfected with pcDNA3 or pcDNA3-hFA2H, harvested by trypsin-EDTA treatment, and washed with Hanks' balanced salt solution and phosphate-buffered saline. Internal standards were added to the cells, and lipids were extracted with ethyl acetate/isopropanol/water (60:30:10, v/v). A small aliquot of each lipid extract was set aside for phosphate quantitation. Lipid extracts were evaporated to dryness and reconstituted in 100 μl of methanol. The reconstituted samples were injected onto a Thermo Finnigan Surveyor liquid chromatography system with a BDS Hypersil C8 column (150 × 3.2 mm, 3-μm particle size) and eluted with a mobile phase consisting of 1 mM methanolic ammonium formate and 2 mM aqueous ammonium formate. Electrospray ionization-tandem mass spectroscopic analysis of ceramides was performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer operating in a multiple reaction monitoring positive ionization mode. Peaks of the target analytes and internal standards were collected and processed using the Xcalibur software system. Calibration curves were constructed by plotting peak area ratios of the target analyte to their respective internal standard against concentration using a linear regression model. Lipid phosphates were determined by the method of Van Veldhoven and Bell (19). Ceramide contents were normalized to the lipid phosphate.

Fatty Acid Determination by Gas Chromatography/Mass Spectrometry—COS7 cells were transfected with pcDNA3 or pcDNA3-hFA2H, harvested, and lysed as described above. Crude cell lysate (0.5 ml) was mixed with a set of internal standards (C15, C17, C19, C21, and C23 fatty acids), and free fatty acids were extracted three times with 1 ml of diethyl ether. Combined diethyl ether extracts were dried under nitrogen. To prepare fatty acid methyl esters, 1 ml of anhydrous methanolic HCl was added to each sample and incubated at 65 °C for 45 min, and samples were dried under nitrogen. To prepare trimethylsilyl derivatives of hydroxyl groups, 100 μl of Tri-Sil reagent (Pierce) was added to each sample and incubated for 30 min at room temperature. One to 2 μl of each sample was applied to a Hewlett-Packard 5890 gas chromatograph with a Restek RTX-5 column (5% diphenyl, 95% dimethyl polysiloxane, 0.25 mm inner diameter, 0.25 μm D.F., 30 m). The injection port and the transfer line were maintained at 250 °C, and the oven temperature was increased from 110 to 300 °C at 10 °C/min. Mass spectra data were obtained on a VG-70S magnetic sector mass spectrometer. Peaks of the target analytes and internal standards were collected and processed using the Opus software system (Micromass Information Systems, Modesto, CA). Calibration curves were constructed by plotting peak area ratios of the target analytes to their respective internal standard against concentration, using linear regression analysis.

Fatty Acid 2-Hydroxylase Assay—Membrane fractions were prepared as described under "Cell Fractionation." Membrane pellets were resuspended in 1 ml of the lysis buffer by brief sonication in a bath sonicator. The assay mixture contained microsomal fractions (100 μg of proteins), 2.7 mM Tris-HCl, pH 7.6, 1.28 mM NADP⁺, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, 0.2 unit of glucose 6-phosphate dehydrogenase, 1 μg of human NADPH:cytochrome P-450 reductase, and 1 μg (2.7 nmol) of (3,3,5,5- D_4)-tetracosanoic acid (stock solution was prepared as 10 μg/ml in 1.5 mM α -cyclodextrin) in a total volume of 1.5 ml. The assay mixture was placed in a 50-ml polypropylene tube and incubated at 37 °C for 2 h with shaking (100 rpm) to facilitate the diffusion of oxygen. At the end of incubation, 50 pmol of C23 fatty acid (internal standard) was added to each sample, and fatty acids were extracted three times with 2 ml diethyl ether. The combined diethyl ether extracts were brought to dryness under a stream of nitrogen. Fatty acids were derivatized and quantified as described under "Fatty Acid Determination by GC/MS." The ions monitored for 2-hydroxy-(3,3,5,5- D_4)-tetracosanoic acid had a mass of 415 and 459, corresponding to M-15 and M-59, respectively. The

¹ The abbreviations used are: HRP, horseradish peroxidase; GC/MS, gas chromatography/mass spectrometry.

Human	1	.MAPAPPPAASFSPEVQRRLAAGACWVR.RGARLYDLSSFVRHHPGGEOLRRARAGODI
Yeast	1	MSTNTSKTLELFSKKTVEHNTANDCWVTYQNRKLYDVTRELFSEHPGGDESILLDYAGKDI
Human	59	SADL.DGPPHRHSANARRWLE.QYVVGELRGEQOCMS...ENEPEVALE.....ETQKT
Yeast	61	TEIMKDSVDVHEHSDSAYEILEDEYLLIGYLATDEEAPRLLTNKNHKVEVQLSADGTEFDST
Human	107	D....PAMEPRFKVVDNDKD....LVDWRKPLLWVGHGLGKEDWVHQPVTRPTRL.
Yeast	121	TFVKELPABEKLSIATDYNDYKHKHFLDLNRPLLQILRSDFKKDFYVDO.IHRPRHYG
Human	156FHSDLLEGLSKTWWVSVPLLWVPLVLYLSWSYRTEFAQGNV.RLFTSETTEYTVA
Yeast	180	KGSAPLFGNLEPLIKTAWVVPVAVLPPVVV....YHMGVALKNNQLFACE.....
Human	210	VPKSMFPGLEMLGFLWLSLIEYLTHRFLFMKP.PSDSYLIMLHFVMHGCHHKAPDGS
Yeast	228LFCVGVFVWTLIEYGLHRFLFHFDDWLPESNIAFATHLLHGCHHYLPMDKY
Human	269	RLVFPVPVPSLVIGVFLYLCMLLEPAVGGTVFAGGLLGVLYDMDHYLHFGSPHKGSY
Yeast	280	RLVMPPTLFLVILCAFFYKLVFALLPLYWAYAGFAGGLFGVYCYDECHFFLHHSK..LPPE
Human	329	LYSLKAHEVKKHFAHOKSGFGISTKLWDYCEHT.LTPEKPHLKTQ.. 372
Yeast	338	MRKLLKHYLEHHYKNYQLGFGVTSWFWDVEGTYLCPDAPLSKMKYE 384

FIG. 1. Sequence comparison between human FA2H and yeast Fah1p. The two sequences were aligned using the GCG Pileup program. Identical and conserved amino acids are highlighted in black and gray, respectively. The eight histidines in the consensus sequence (HX₍₃₋₄₎HX₍₇₋₄₁₎HX₍₂₋₃₎HHX₍₆₁₋₁₈₉₎(H/Q)X₍₂₋₃₎HH) are marked by asterisks underneath the sequence. Predicted transmembrane domains of human FA2H are marked by solid lines above the sequence.

activities were calculated as picomoles of 2-hydroxy-(3,3,5,5-D₄)-tetra-*sanoic acid*/mg of protein/min.

Northern Blot Analysis—Premade Northern blots containing poly(A)⁺ RNA from human tissues (Clontech) were probed with FA2H cDNA or a human β -actin control cDNA. The FA2H cDNA probe was a 862-bp fragment obtained by PCR with oligonucleotides FA2H-FBam (5'-GCAGGGATCCATGGAGAACGAGCCTGTAGC-3') and FA2H-RR1 (5'-TGGGAATTCTCACTGCGTCTTCAGGTGGGG-3'). Probes were labeled with [α -³²P]dCTP using the Megaprime DNA labeling system (Amersham Biosciences). Hybridization was performed according to the manufacturer's instructions.

RESULTS

Identification of Human FA2H Gene—BLAST sequence analyses identified several human cDNA sequences that had significant similarity to yeast *FAH1* (also known as *SCS7*), a gene required for 2-hydroxylation of sphingolipid-associated very long chain fatty acids (15, 16, 20). All the cDNA clones were derived from the same gene located in the human chromosome 16 (NCBI locus identification, 79152), which we named *FA2H* for fatty acid 2-hydroxylase. The *FA2H* gene product is a 372-amino acid protein (42.8 kDa) that has 36% identity and 46% similarity to yeast *Fah1p* (Fig. 1).

Yeast *Fah1p* contains an N-terminal cytochrome *b*₅ domain (NCBI conserved domain data base accession no. 22935), four potential transmembrane domains, and the characteristic histidine motif conserved among membrane-bound desaturases/hydroxylases (consensus: HX₍₃₋₄₎HX₍₇₋₄₁₎HX₍₂₋₃₎HHX₍₆₁₋₁₈₉₎(H/Q)X₍₂₋₃₎HH) (13, 15, 20). These histidines are thought to coordinate the non-heme di-iron cluster at an active site (13). Similarly, human FA2H protein contains a cytochrome *b*₅ domain at its N terminus (amino acids 1–92). Within this domain is the conserved heme-binding domain (His-Pro-Gly-Gly), suggesting that it is functional as an electron carrier. The cytochrome *b*₅ domains of yeast *Fah1p* and human FA2H lack a membrane anchor, which is present in the microsomal cytochrome *b*₅. The C-terminal three quarters of FA2H are highly homologous to the sphingolipid fatty acid hydroxylase domain (NCBI conserved domain data base accession no. 9675). This domain is found in previously identified *FAH1* homologues in *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, and *Caenorhabditis elegans* (15), as well as in hypothetical proteins in the fungi *Magnaporthe grisea* and *Neurospora crassa*, the malaria mosquito *Anopheles gambiae*, *Drosophila melanogaster*, the nematode *C. briggsae*, *Xenopus laevis*, *Mus musculus*, and *Rattus norvegicus* (NCBI conserved domain architecture retrieval tool). All of these proteins contain the eight conserved histidines (Fig. 1, consensus sequence asterisks) and an N-terminal cytochrome

*b*₅ domain with the exception of the *A. thaliana* homologue, which lacks a cytochrome *b*₅ domain (15).

2-Hydroxyceramides and 2-Hydroxy Fatty Acids in COS7 Cells Expressing Human FA2H—To show that human FA2H is involved in the formation of 2-hydroxysphingolipids, the levels of ceramides and 2-hydroxyceramides in COS7 cells transfected with pcDNA3 or pcDNA3-hFA2H were determined. Total cellular lipids were extracted 24 h after transfection, and ceramides were fractionated on a reversed-phase high pressure liquid chromatography column and quantified by a tandem mass spectrometer. Fig. 2 shows the levels of ceramides and 2-hydroxyceramides with five different fatty acid chain lengths. Ceramides with normal (non-hydroxy) fatty acids are comparable with or slightly decreased in FA2H-expressing cells compared with the control cells (Fig. 2A). Cellular 2-hydroxyceramides in FA2H-transfected cells increased 3–20-fold, except for 2-hydroxyceramide C20:0 (Fig. 2B). The levels of 2-hydroxyceramides continued to increase after 24 h and reached as much as 40-fold over control cells in 72 h in the case of C16:0 ceramide (data not shown). These results provide evidence that FA2H is involved in the formation of 2-hydroxysphingolipids.

The fatty acid 2-hydroxylation is believed to occur prior to ceramide synthesis in mammals. However, it has not been clearly demonstrated whether 2-hydroxylation occurs on free fatty acids. To determine whether 2-hydroxylation occurs on free fatty acids, we measured free 2-hydroxy fatty acids in control and FA2H-transfected cells along with non-hydroxy fatty acids. Because free 2-hydroxy fatty acids were expected to be minor species, a high-resolution GC/MS assay was developed for the quantification of the fatty acids. 2-Hydroxy fatty acids were derivatized as trimethylsilyl ether of methyl esters. Fig. 3 shows the mass spectrum of trimethylsilyl ether of 2-hydroxytetra-*sanoic acid* methyl ester. Electron impact ionization generated two distinct ions with masses of 411 and 455 (Fig. 3, inset). The limit of detection by this method was at the femtomol level. Using this method, non-hydroxy and 2-hydroxy fatty acids were quantified in transfected COS7 cells. As shown in Fig. 4A, non-hydroxy fatty acid levels were indistinguishable between control and FA2H-transfected cells. Consistent with the presence of 2-hydroxyceramides shown in Fig. 2, low levels of 2-hydroxy fatty acids were present in control cells. Among the six different free 2-hydroxy fatty acids analyzed, the levels were 2.3–5.4-fold higher in FA2H-transfected cells over control cells, except for C20 fatty acid (1.5-fold). The highly elevated free 2-hydroxy fatty acid levels are consistent with the possi-

FIG. 2. Ceramide levels in transfected COS7 cells. Ceramides were extracted from COS7 cells 24 h after transfection with pcDNA3 (open bars) or pcDNA3-hFA2H (filled bars) and analyzed by liquid chromatography/mass spectrometry. The levels of ceramides in each sample were normalized by the lipid phosphate content. The average and standard deviation of four independent transfection experiments are shown. *A*, ceramides with non-hydroxy fatty acids are shown. *B*, ceramides with 2-hydroxy fatty acids are shown.

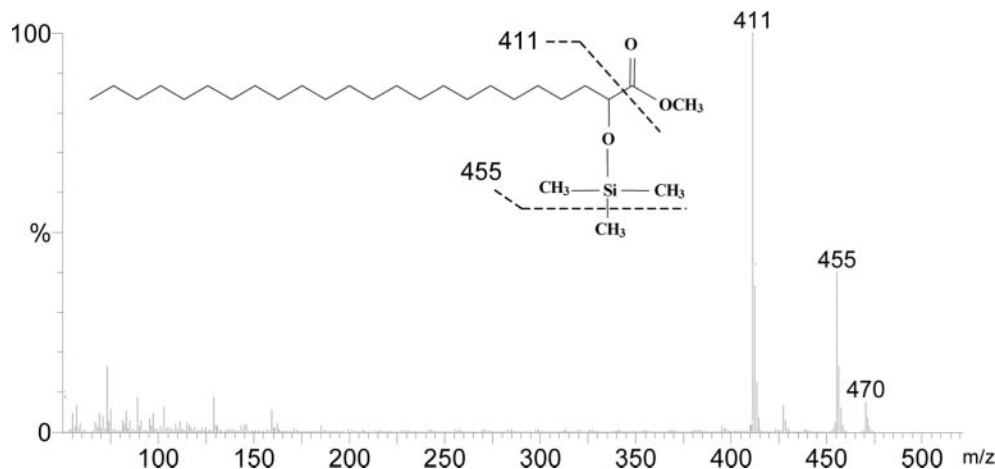
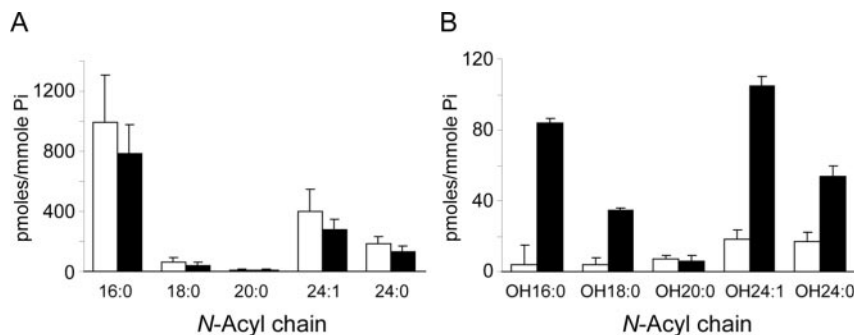


FIG. 3. Mass spectrum of trimethylsilyl ether of 2-hydroxytetracosanoic acid methyl ester. The two prominent ions with masses of 455 and 411, derived from the fragmentations, are indicated in the inset. The corresponding ions generated by the same fragmentation of 2-hydroxy(3,3,5,5-D)-tetracosanoic acid methyl ester have masses of 459 and 415, respectively, which are the target analytes for the *in vitro* fatty acid 2-hydroxylase assay (see Fig. 6).

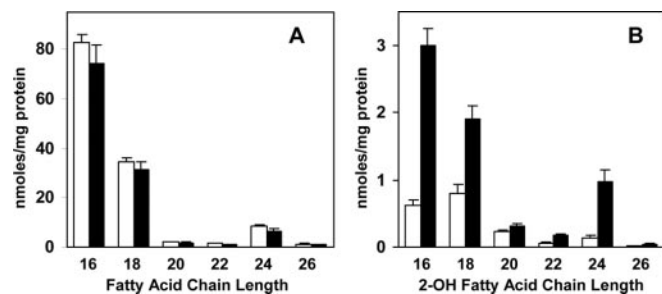


FIG. 4. Fatty acids and 2-hydroxy fatty acids in transfected COS7 cells. Fatty acids were extracted from COS7 cell transfected with pcDNA3 (open bars) or pcDNA3-hFA2H (filled bars). Non-hydroxy fatty acids (*A*) and 2-hydroxy fatty acids (*B*) were quantified by GC/MS as described under "Experimental Procedures." The average and standard deviation of three measurements are shown.

bility that FA2H-dependent 2-hydroxylation occurs on free fatty acids.

Western Blot Analysis—The amino acid sequence of human FA2H indicated that it was a membrane-bound, 43-kDa protein. There was another potential translation initiation site at methionine 93, which had been noted as a translation initiation site in some data base entries for the same gene. Interestingly, methionine 93 is located between the N-terminal cytochrome b_5 domain and the sphingolipid fatty acid hydroxylase domain. It is conceivable that translation initiation at methionine 93 produces a catalytically active 33-kDa protein, based on the fact that the *Arabidopsis* homologue, lacking a cytochrome b_5 domain, is active when expressed in yeast (15). To test whether the cytochrome b_5 domain is necessary for catalytic activity of recombinant human FA2H, full-length and truncated FA2H, starting from methionine 1 and methionine 93, respectively,

were constructed. FLAG-tagged FA2H was also constructed to confirm the presence of the N-terminal portion with anti-FLAG antibodies. When expressed in COS7 cells, the three versions of FA2H were 43, 33, and 44 kDa, as expected (Fig. 5A). There was no detectable endogenous FA2H in COS7 cells, indicating low abundance of endogenous FA2H in these cells and/or poor conservation of the C-terminal portion of FA2H, which was used to generate the antibodies. Western blot with anti-FLAG monoclonal antibodies detected FLAG-tagged FA2H, confirming that the entire N-terminal portion is present in this construct (Fig. 5B). The overexpressed FA2H was exclusively fractionated in the membrane fraction (Fig. 5C) as expected from the presence of four putative transmembrane domains. Anti-cytochrome b_5 antibodies did not cross-react with the full-length human FA2H expressed in COS7 cells (data not shown).

In Vitro Fatty Acid 2-Hydroxylase Activity—To show that FA2H-dependent 2-hydroxylation occurs on free fatty acids, we have developed a sensitive and specific *in vitro* assay using GC/MS for the detection of reaction products. Based on previous studies (14, 21) of the rat brain enzyme, fatty acid 2-hydroxylation is coupled with a microsomal electron transport system involving NADH:cytochrome b_5 reductase or NADPH:cytochrome P-450 reductase. The requirement of an electron transport system is also predicted from the presence of the cytochrome b_5 domain in the human FA2H protein. Because NADPH is a more effective electron donor than NADH for the rat brain enzyme (21), our assay system included purified recombinant human NADPH:cytochrome P-450 reductase and an NADPH regeneration system (NADP⁺, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase). With these components, electron transport would occur in the following sequence: NADPH → NADPH:cytochrome P-450 reductase → cytochrome b_5 domain of FA2H → the catalytic di-iron of FA2H.

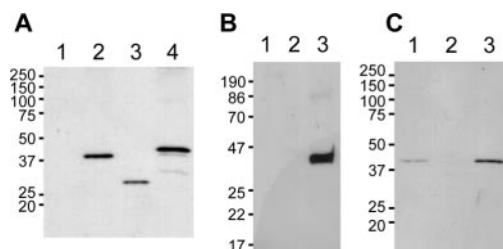


FIG. 5. Western blot analysis. A, cell extracts were prepared from COS7 cells transfected with pcDNA3 (lane 1), pcDNA3-hFA2H (lane 2), pcDNA3-hFA2H Δ N (lane 3), or pcDNA3-FLAG-hFA2H (lane 4). Cell extracts were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and incubated with anti-hFA2H polyclonal antibodies. B, cell extracts were prepared from COS7 cells transfected with pcDNA3 (lane 1), pcDNA3-hFA2H (lane 2), or pcDNA3-FLAG-hFA2H (lane 3). The blot was incubated with anti-FLAG monoclonal antibodies. C, crude cell lysate (lane 1), the cytosolic fraction (lane 2), and the membrane fraction (lane 3) were prepared from COS7 cells transfected with pcDNA3-hFA2H. Proteins (30 μ g/well) were separated by SDS-PAGE, blotted, and incubated with anti-hFA2H polyclonal antibodies.

Because human FA2H is a membrane-bound protein as shown above, microsomal fractions of transfected COS7 cells were used as enzyme sources.

To distinguish exogenously added substrate from endogenous fatty acids present in microsomal fractions, we used a deuterated fatty acid, (3,3,5,5- D_4)-tetracosanoic acid. When deuterated tetracosanoic acid was incubated with microsomes, an NADPH regeneration system, and NADPH:cytochrome P-450 reductase and analyzed by GC/MS, a new lipid co-eluted with trimethylsilyl ether of 2-hydroxytetracosanoic acid methyl ester. The molecular ion of this compound had a mass of 474. Fragmentation of the compound generated two distinct ions with masses of 459 and 415, which are 4 atomic mass units larger than the corresponding ions generated from a non-deuterated 2-hydroxytetracosanoic acid derivative (Fig. 3). Based on the retention time, the mass of the molecular ion, and the fragmentation pattern, we concluded that the lipid species is a deuterated 2-hydroxytetracosanoic acid derivative.

Consistent with the cellular ceramide and fatty acid analyses, control COS7 cells (transfected with an empty vector pcDNA3) had a low level of fatty acid 2-hydroxylase activity (Fig. 6). When full-length or FLAG-tagged FA2H was expressed, the cells had 6.4- and 6.1-fold higher tetracosanoic acid 2-hydroxylase activities, respectively, compared with the control. When the truncated FA2H lacking the cytochrome b_5 domain was expressed, the cells had only 1.3-fold higher activity over control, indicating that cytochrome b_5 domain is required for efficient hydroxylation. The 2-hydroxylase activities were dependent on NADPH and NADPH:cytochrome P-450 reductase, indicating a successful reconstitution of an electron transport system (Fig. 6B). The assay mixture did not contain ATP or CoA, and the activities were not affected by the ceramide synthase inhibitor fumonisins B₁ (data not shown), demonstrating that FA2H-dependent fatty acid 2-hydroxylation does not require acyl-CoA or acyl-CoA-dependent ceramide synthesis.

FA2H Is Highly Expressed in Brain—2-Hydroxysphingolipids are most abundant in the brain because the major myelin lipids galactosylceramides and sulfatides contain high levels of 2-hydroxy fatty acids. If human FA2H is responsible for producing these 2-hydroxysphingolipids, the expression of the FA2H gene is also expected to be high in the brain. To test this possibility, FA2H expression in various human tissues were analyzed. FA2H mRNA was detected as a single band of \sim 3 kb (Fig. 7). The brain had the highest expression of FA2H. The colon also had a high level of FA2H expression. FA2H expres-

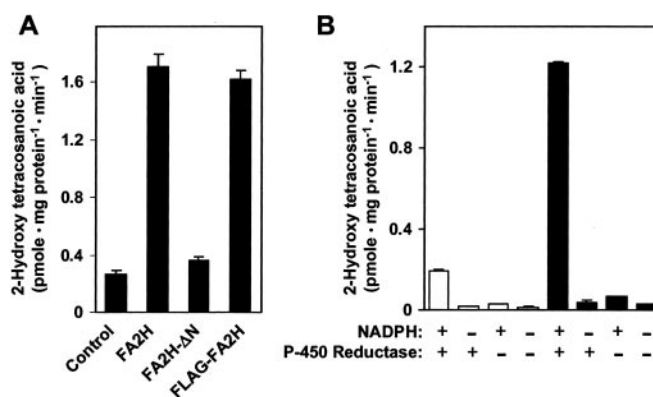


FIG. 6. Fatty acid 2-hydroxylase activities of transfected COS7 cells. A, microsomal fractions were prepared from COS7 cells transfected with pcDNA3, pcDNA3-hFA2H, pcDNA3-hFA2H Δ N, or pcDNA3-FLAG-hFA2H. The assay mixtures contained 100 μ g of microsomal proteins in the presence of an NADPH regeneration system and human NADPH:P-450 reductase. Deuterated 2-hydroxy tetracosanoic acid formed during incubation was quantified by GC/MS. The average and standard deviation of triplicate measurements are shown. B, microsomal fractions were prepared from COS7 cells transfected with pcDNA3 (open bars) or pcDNA3-hFA2H (filled bars). The assay mixtures contained 100 μ g of microsomal proteins in the presence (+) or absence (-) of an NADPH regeneration system and human NADPH:P-450 reductase as indicated. The mean and range of duplicate measurements are shown.

sion was detected in testis, prostate, pancreas, and kidney but to lesser extents.

DISCUSSION

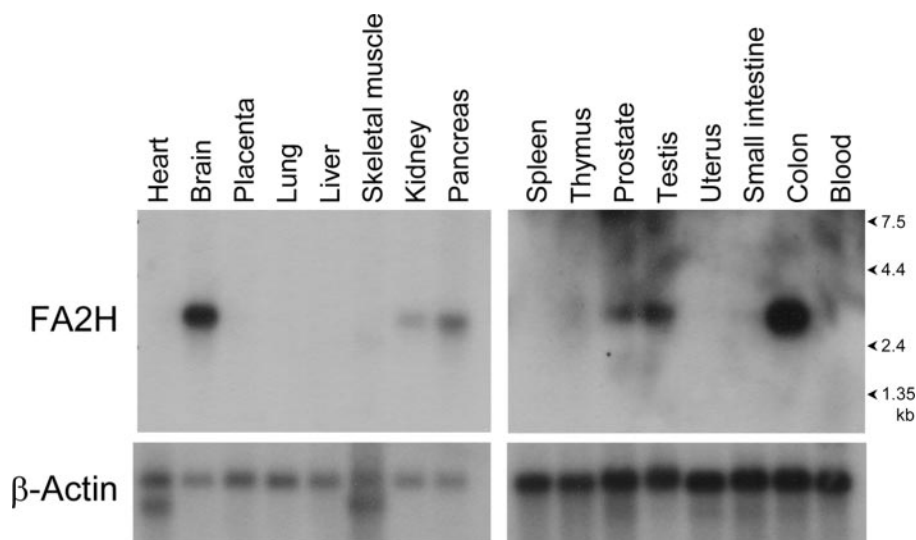
In this report, we provide evidence that the human FA2H gene encodes a fatty acid 2-hydroxylase. This is the first mammalian fatty acid 2-hydroxylase gene to be characterized. We also provide evidence that free fatty acid is a direct substrate of this enzyme.

Earlier studies on the rat brain fatty acid 2-hydroxylase by Kishimoto and colleagues showed that the rat brain enzyme is a microsomal mixed function oxidase that requires molecular oxygen, pyridine nucleotides (NADPH or NADH), and a microsomal electron transport system (11, 12, 14, 21). Involvement of a cytochrome P-450 enzyme was not implicated because fatty acid 2-hydroxylase was not inhibited by carbon monoxide (11). The fatty acid 2-hydroxylase activities were detectable in the brain but not in liver, kidney, spleen, or heart (11). The characteristics of human FA2H described in the present report are consistent with these previous observations for the rat brain fatty acid 2-hydroxylase.

The deduced amino acid sequence of human FA2H indicates that it is a membrane-bound enzyme with a characteristic histidine motif, HX₍₃₋₄₎HX₍₇₋₄₁₎HX₍₂₋₃₎HHX₍₆₁₋₁₈₉₎(H/Q)X₍₂₋₃₎HH (13). This histidine motif is conserved among a family of membrane-bound desaturases and hydroxylases, including yeast Fah1p and its homologues (15, 16). Based on the Mössbauer studies (22) of the *Pseudomonas oleovorans* alkane ω -hydroxylase, the eight histidine residues are thought to coordinate the catalytic di-iron clusters. All of the uncharacterized FA2H homologues contain this motif and appear to use the same reaction mechanism for fatty acid 2-hydroxylation.

Yeast Fah1p and human FA2H and their homologues, except for the *Arabidopsis* homologue (15), also contain a cytochrome b_5 domain (15, 16). A cytochrome b_5 domain is also present in a number of fatty acid and sphingolipid desaturases (23-25), suggesting that an intramolecular cytochrome b_5 domain is a common molecular architecture for lipid biosynthetic enzymes that catalyze oxidative reactions. Truncated human FA2H lacking the cytochrome b_5 domain has a significantly lower

FIG. 7. **FA2H expression in human tissues.** Premade Northern blots containing poly(A)⁺ RNA from human tissues (~2 μ g/lane) were probed with ³²P-labeled human FA2H or β -actin cDNA. The smaller mRNA species in the β -Actin panels, particularly abundant in heart and skeletal muscle, are mRNAs of other isoforms of actin that cross-hybridize with the β -actin probe.



activity than the full-length FA2H, indicating that the cytochrome b_5 domain is a functional component of an electron transport chain. When the intramolecular cytochrome b_5 domain is absent, electron transport is presumably carried out by a microsomal cytochrome b_5 at a very low efficiency. It is interesting to note that despite the significant sequence similarity to the microsomal cytochrome b_5 , the cytochrome b_5 domain of human FA2H did not cross-react with anti-cytochrome b_5 antibodies. It has also been shown that anti-cytochrome b_5 antibodies did not inhibit the rat brain fatty acid 2-hydroxylase (14), which also contains a cytochrome b_5 domain according to the rat genome data base. Thus, there appears to be some structural diversity between the microsomal cytochrome b_5 and intramolecular cytochrome b_5 domains, which may account for the inefficient interaction between the microsomal cytochrome b_5 and the catalytic domain of FA2H.

The fractionation experiment indicated that human FA2H is a membrane protein. Although the precise subcellular localization of FA2H has not been determined, it is predicted to be localized in the endoplasmic reticulum, where fatty acid elongation and ceramide synthesis occur. The four putative transmembrane domains of human FA2H are likely oriented in such a way that both N and C termini face the cytoplasm as proposed for the yeast Fah1p (15, 16). This orientation places the cytochrome b_5 domain and the histidine motif on the same (cytoplasmic) side of the membrane. The cytochrome b_5 domain of FA2H lacks the C-terminal membrane anchor that is present in the microsomal cytochrome b_5 . Instead, the cytochrome b_5 domain is linked to a hydrophilic stretch of the sphingolipid fatty acid hydroxylase domain. This hydrophilic region may serve as a flexible arm that allows the cytochrome b_5 domain to interact with cytochrome reductases and the catalytic di-iron cluster.

Previous biochemical studies showed that the rat brain fatty acid 2-hydroxylase requires pyridine nucleotides (11) and that NADPH is more effective than NADH (21). Because NADPH is a poor substrate for microsomal NADH:cytochrome b_5 reductase (26), NADPH:cytochrome P-450 reductase is likely involved in electron transport to the cytochrome b_5 domain of the rat brain fatty acid 2-hydroxylase. Thus, there appear to be two electron transport systems functioning in fatty acid 2-hydroxylation. One transport system is from NADPH via NADPH:cytochrome P-450 reductase to the cytochrome b_5 domain of FA2H, and the other system is from NADH via NADH:cytochrome b_5 reductase to the cytochrome b_5 domain of FA2H. We opted for the readily available purified human NADPH:cyto-

chrome P-450 reductase and NADPH regeneration system to reconstitute the former electron transport system in the *in vitro* assay. A large increase in tetracosanoic acid 2-hydroxylase activity was seen in control and FA2H-transfected cells when both NADPH and NADPH:cytochrome P-450 reductase were present (Fig. 6), indicating that the endogenous cytochrome P-450 reductase is not sufficient to support maximal FA2H activities measured *in vitro*. Adding an exogenous NADPH:cytochrome P-450 reductase in the assay would greatly improve the chance of detecting fatty acid 2-hydroxylase activities in various tissues and organisms previously thought to have no activities. Our results also suggest a possibility that cellular NADPH:cytochrome P-450 reductase levels (and perhaps NADH:cytochrome b_5 reductase levels) may significantly influence the formation of 2-hydroxy fatty acids and 2-hydroxysphingolipids *in vivo*.

In the previous studies on the rat brain fatty acid 2-hydroxylase, free fatty acids or acyl-CoA was used as a substrate in the *in vitro* assays (11, 12, 21). However, the putative reaction products 2-hydroxy fatty acid or 2-hydroxy-acyl-CoA were never detected. Instead, the products were detected as 2-hydroxyceramide. Therefore, there is a possibility that free fatty acids are first incorporated in ceramides prior to 2-hydroxylation, which appears to be the case with *S. cerevisiae* (27) and *Tetrahymena pyriformis* (28). On the other hand, tetracosanoyl sphingosine (C24 ceramide) was not hydroxylated in the same assay, suggesting that ceramide could not be 2-hydroxylated (11). However, tetracosanoyl sphingosine is highly hydrophobic and difficult to solubilize in aqueous solutions without detergent. There is a possibility that C24 ceramide did not properly come into contact with the enzyme during the assay. Thus, the question of whether a fatty acid is 2-hydroxylated before or after conversion to a ceramide remained unanswered (11, 14). In the present study, we have shown that free 2-hydroxy fatty acids accumulate in FA2H-transfected COS7 cells. Additionally, 2-hydroxytetracosanoic acid was detected as a reaction product in our *in vitro* assay with tetracosanoic acid. Because ATP and CoA were not included in the assay mixture, tetracosanoic acid could not have been converted to a CoA ester and further to ceramide by acyl-CoA-dependent ceramide synthase. Ceramide synthesis by reverse ceramidase activities should also be negligible without exogenously added sphingoid bases. Thus, the results in this study demonstrate that human FA2H converts free fatty acids to 2-hydroxy fatty acids, although we have not tested whether acyl-CoA and ceramide could also serve as substrates. Further analyses for substrate specificity would require purified enzyme preparations that are devoid of

acyl-CoA synthase, acyl-CoA thioesterase, ceramide synthase, and ceramidase activities.

The fatty acid and ceramide profiles of FA2H-transfected COS7 cells indicate that FA2H can use fatty acids with various chain lengths. In the previous *in vitro* studies (11, 29), however, 2-hydroxylase activities were not detected with stearic acid (C18:0), presumably because of lower sensitivity of the previous assays. Considering the relative abundance of palmitic acid (C16:0) *in vivo*, fatty acids longer than palmitic acid must be better substrates for FA2H.

The Northern blot analysis demonstrated that the *FA2H* gene is highly expressed in the human brain, consistent with the idea that FA2H is the fatty acid 2-hydroxylase involved in the formation of 2-hydroxy galactosylceramides and 2-hydroxy sulfatides in myelin. Studies of knock-out mice that were devoid of galactosylceramides and sulfatides provide evidence that these glycolipids are critical for myelination (30, 31). Interestingly, the major glycolipid in the mutant mice was 2-hydroxy glucosylceramide, which is not found in normal myelin (30, 31). It appears that 2-hydroxy fatty acids of these glycolipids are crucial for myelination and myelin function. Identification of FA2H as fatty acid 2-hydroxylase allows us to study the functions of 2-hydroxy glycolipids in myelin and other tissues.

Acknowledgments—We thank Drs. John Baynes and Susan Thorpe for allowing the use of laboratory space and Dr. Yi-Te Hsu for helpful discussions.

REFERENCES

- Nonaka, G., and Kishimoto, Y. (1979) *Biochim. Biophys. Acta* **572**, 432–441
- Norton, W. T., and Cammer, W. (1984) in *Myelin* (Morell, P., ed) pp. 147–195, Plenum Press, New York
- Hoshi, M., Williams, M., and Kishimoto, Y. (1973) *J. Neurochem.* **21**, 709–712
- Stoffel, W., and Bosio, A. (1997) *Curr. Opin. Neurobiol.* **7**, 654–661
- Coetzee, T., Suzuki, K., and Popko, B. (1998) *Trends Neurosci.* **21**, 126–130
- Wertz, P. W., and Downing, D. T. (1983) *J. Lipid Res.* **24**, 759–765
- Downing, D. T. (1992) *J. Lipid Res.* **33**, 301–313
- Pascher, I., and Sundell, S. (1977) *Chem. Phys. Lipids* **20**, 175–191
- Boggs, J. M., Koshy, K. M., and Rangaraj, G. (1988) *Biochim. Biophys. Acta* **938**, 361–372
- Lofgren, H., and Pascher, I. (1977) *Chem. Phys. Lipids* **20**, 273–284
- Hoshi, M., and Kishimoto, Y. (1973) *J. Biol. Chem.* **248**, 4123–4130
- Akanuma, H., and Kishimoto, Y. (1979) *J. Biol. Chem.* **254**, 1050–1060
- Shanklin, J., and Cahoon, E. B. (1998) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 611–641
- Shigematsu, H., Hisanari, Y., and Kishimoto, Y. (1990) *Int. J. Biochem.* **22**, 1427–1432
- Mitchell, A. G., and Martin, C. E. (1997) *J. Biol. Chem.* **272**, 28281–28288
- Haak, D., Gable, K., Beeler, T., and Dunn, T. (1997) *J. Biol. Chem.* **272**, 29704–29710
- Hama, H., Young, D. A., Radding, J. A., Ma, D., Tang, J., Stock, S. D., and Takemoto, J. Y. (2000) *FEBS Lett.* **478**, 26–28
- Mao, C., Xu, R., Szulc, Z. M., Bielawski, J., Becker, K. P., Bielawska, A., Galadari, S. H., Hu, W., and Obeid, L. M. (2003) *J. Biol. Chem.* **278**, 31184–31191
- Van Veldhoven, P. P., and Bell, R. M. (1988) *Biochim. Biophys. Acta* **959**, 185–196
- Dunn, T. M., Haak, D., Monaghan, E., and Beeler, T. J. (1998) *Yeast* **14**, 311–321
- Shigematsu, H., and Kishimoto, Y. (1987) *Int. J. Biochem.* **19**, 41–46
- Shanklin, J., Achim, C., Schmidt, H., Fox, B. G., and Munck, E. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2981–2986
- Mitchell, A. G., and Martin, C. E. (1995) *J. Biol. Chem.* **270**, 29766–29772
- Sperling, P., Zahringer, U., and Heinz, E. (1998) *J. Biol. Chem.* **273**, 28590–28596
- Napier, J. A., Michaelson, L. V., and Sayanova, O. (2003) *Prostaglandins Leukotrienes Essent. Fatty Acids* **68**, 135–143
- Strittmatter, P. (1959) *J. Biol. Chem.* **234**, 2665–2669
- Dickson, R. C., Wells, G. B., Schmidt, A., and Lester, R. L. (1990) *Mol. Cell. Biol.* **10**, 2176–2181
- Kaya, K., Ramesha, C. S., and Thompson, G. A., Jr. (1984) *J. Biol. Chem.* **259**, 3548–3553
- Murad, S., Chen, R. H., and Kishimoto, Y. (1977) *J. Biol. Chem.* **252**, 5206–5210
- Coetzee, T., Fujita, N., Dupree, J., Shi, R., Blight, A., Suzuki, K., and Popko, B. (1996) *Cell* **86**, 209–219
- Bosio, A., Binczek, E., and Stoffel, W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13280–13285