Dual Subcellular Localization in the Endoplasmic Reticulum and Peroxisomes and a Vital Role in Protecting against Oxidative Stress of Fatty Aldehyde Dehydrogenase Are Achieved by Alternative Splicing*

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Fatty aldehyde dehydrogenase (FALDH, ALDH3A2) is thought to be involved in the degradation of phytanic acid, a saturated branched chain fatty acid derived from chlorophyll. However, the identity, subcellular distribution, and physiological roles of FALDH are unclear because several variants produced by alternative splicing are present in varying amounts at different subcellular locations. Subcellular fractionation experiments do not provide a clear-cut conclusion because of the incomplete separation of organelles. We established human cell lines heterologously expressing mouse FALDH from each cDNA without tagging under the control of an inducible promoter and detected the variant FALDH proteins using a mouse FALDH-specific antibody. One variant, FALDH-V, was exclusively detected in peroxisomal membranes. Human FALDH-V with an amino-terminal Myc sequence also localized to peroxisomes. The most dominant form, FALDH-N, and other variants examined, however, were distributed in the endoplasmic reticulum. A gas chromatography-mass spectrometry-based analysis of metabolites in FALDH-expressing cells incubated with phytol or phytanic acid showed that FALDH-V, not FALDH-N, is the key aldehyde dehydrogenase in the degradation pathway and that it protects peroxisomes from oxidative stress. In contrast, both FALDHs had a protective effect against oxidative stress induced by a model aldehyde for lipid peroxidation, dodecanal. These results suggest that FALDH variants are produced by alternative splicing and share an important role in protecting against oxidative stress in an organelle-specific manner.

Plants produce a variety of secondary metabolites, and some of these are potentially toxic to animals (1). Herbivora have developed behavioral and physiological strategies to avoid specific plants and to detoxify any toxins ingested. Detoxification can occur in the mouth and the gut rumen with or without the help of microbes (2). The absorbed toxins must be detoxified in the intestine and liver, but studies on these mechanisms are limited because to date most animal experiments have been carried out using laboratory diets. Recently we found that a nuclear receptor, peroxisome proliferator-activated receptor α (PPARα), is involved in the detoxification by using plant seeds as a diet for mice (3).

PPARα is activated by fatty acid ligands and is an important regulator of lipid metabolism in animals (4). Despite the claimed essential role of this receptor in the liver, the PPARα-null mouse shows little phenotypic change when fed a normal laboratory diet (5, 6). We have examined its extrahepatic roles and found that PPARα induces the expression of 17β-hydroxysteroid dehydrogenase type 11 in the intestine (7). Recent studies on the substrates of 17β-hydroxysteroid dehydrogenase type 11 showed that they include not only glucocorticoids and sex steroids but also bile acids, fatty acids, and branched amino acids (8, 9). So we examined the possibility that PPARα plays a vital role in inducing enzymes for metabolizing secondary metabolites of plants in normal and PPARα knock-out mice fed various plant seeds and found that sesame often killed PPARα knock-out mice but not normal mice (3). A DNA microarray analysis revealed that sesame induces the expression of 17β-hydroxysteroid dehydrogenase type 11 and also various detoxifying enzymes in the intestine and liver in a PPARα-dependent and -independent manner (3). As the enzyme most significantly induced in a PPARα-dependent manner, we identified a fatty aldehyde dehydrogenase, FALDH or ALDH, encoded by the mouse Aldh3a2 gene (this study).

ALDHs comprise a superfamily of NAD(P)⁺-dependent enzymes that catalyze the oxidation of a wide variety of endogenous and exogenous aliphatic and aromatic aldehydes (10). The ALDH3 subfamily enzymes efficiently oxidize middle and long chain aldehydes (10), and one member of this subfamily, FALDH encoded by ALDH3A2, has a distinct role from the dehydrogenase encoded by ALDH3A1 (11, 12). FALDH is essential for the complete breakdown of phytanic acid, a branched fatty acid derived from the chlorophyll molecule (13),

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2 The abbreviations used are: PPARα, peroxisome proliferator-activated receptor α; FALDH, fatty aldehyde dehydrogenase; ALDH, aldehyde dehydrogenase; GFP, green fluorescent protein; RT, reverse transcription; PBS, phosphate-buffered saline; GC-M5, gas chromatography-mass spectrometry; HEP, human embryonic kidney.

3 B. Ashibe and K. Motojima, unpublished data.
and loss of its activity has been proved to be the cause of Sjögren-Larsson syndrome (14). It has been suggested that FALDH protects cells against oxidative stress associated with lipid peroxidation and plays an important role in insulin action (15). Because of these potential roles in protection against oxidative stress and PPARα-regulated expression (16), we speculate that PPARα-dependent induction of FALDH expression plays an important role in the detoxification of the toxic compound(s) directly or indirectly derived from sesame seeds. However, FALDH has not been sufficiently characterized at the protein level.

Phytanic acid, a metabolite of phytol, has a methyl group at the carbon 3 position and must first undergo α-oxidation and then β-oxidation (17, 18). It is well established that α-oxidation occurs exclusively in peroxisomes (18, 19). However, opinions differ on the identity and subcellular localization of the key enzyme converting pristanal to a β-oxidizable pristane acid. Human genetic analyses using FALDH-deficient fibroblasts have shown that the product of the ALDH3A2 gene is responsible for most, if not all, of the activity behind the conversion (13). However, van den Brink and Wanders (18) and Jansen et al. (20) claimed the possibility of the existence of one or more additional aldehyde dehydrogenases reacting with pristanal in peroxisomes, and Masaki et al. (21) reported that FALDH expressed from cDNA in COS-1 cells is exclusively detected in the endoplasmic reticulum (ER). The organization of the mouse and human ALDH3A2 genes is well conserved, and similar alternative splicing patterns of transcripts have been reported (22, 23). The insertion of an additional exon produces a minor variant of FALDH with a distinct carboxyl-terminal domain of unknown function (22). The existence of alternative splicing and variants present in different amounts at various subcellular locations have made it difficult to characterize FALDH at the protein level.

In this study, we analyzed the subcellular distribution and function of the major and various variant forms of mouse FALDH using a mouse FALDH-specific antibody after overexpressing each cDNA in human HEK293 cells under the control of an inducible promoter, thus avoiding problems originating from the extremely similar structures and incomplete separation by subcellular fractionation of various forms of FALDH with large differences in expression levels. Our data suggest that only one specific variant of FALDH is expressed exclusively in peroxisomes and plays an essential role in the efficient degradation of branched chain fatty acids in the peroxisomal α-oxidation system and that it protects cells from the damage induced by lipid peroxidation.

**EXPERIMENTAL PROCEDURES**

**Animals and Treatments**—All procedures involving animals were approved by the Meiji Pharmaceutical University Committee for Ethics of Experimentation and Animal Care. Male C57/BL6J and PPARα-null mice (around 6 weeks old) were maintained under a 12-h light-dark cycle with free access to food and water. After being fed a diet containing the PPARα agonist Wy14,643 [(4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio)acetic acid] (Tokyo-Kasei, Tokyo, Japan) at 0.05% (w/w) or a normal laboratory diet, the mice were killed by cervical dislocation, and portions of the intestine and liver were removed for homogenization.

**Subcellular Fractionation of Mouse Liver Homogenate**—Subcellular fractionation was performed as described previously (24) with some modifications. A homogenate was prepared by one stroke with a Teflon-glass homogenizer at 1000 rpm in 3 volumes of ice-cold homogenization buffer (0.25 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4). A postnuclear supernatant fraction was prepared by centrifugation of the homogenate for 5 min at 1200 g (3). Homogenization buffer was added to the supernatant at up to 10 volumes of the original tissue and recentrifuged for 5 min at 6600 g. The pellet was suspended in 2 volumes of ice-cold homogenization buffer (mitochondrial fraction). The supernatant was recentrifuged for 20 min at 12,500 g to obtain the supernatant (microsomal and cytosolic fraction). The pellet was suspended in 2 volumes of ice-cold homogenization buffer (lysosomal fraction). For isolation of the peroxisome-enriched fraction, 1 ml of the lysosomal fraction was layered onto 7.5 ml of Nycodenz (Sigma–Aldrich) solution (30% Nycodenz in 1 mM EDTA, pH 7.3) and centrifuged for 1 h at 131,000 g (3). The pellet obtained was suspended in 200 μl of Nycodenz solution and used as the peroxisome-enriched fraction. To disassemble peroxisomes, the peroxisome-enriched fraction was diluted with 4 volumes of 12.5 mM sodium pyrophosphate, pH 9.0 or with Triton X-100 to a final concentration of 0.1% as described previously (25) and centrifuged for 1 h at 131,000 g.

**Preparation of an Antibody against Mouse FALDH and Western Blot Analysis**—A rabbit polyclonal antibody against mouse FALDH was prepared using the peptide corresponding to Cys425—Arg439 as the antigen. This sequence is unique to mouse FALDH and common to all FALDH variants (22). The antibody used for immunofluorescence microscopy was purified by affinity purification using a peptide-coupled Sepharose 4B column (EAH-Sepharose 4B, GE Healthcare). The specificity of the antibody was confirmed by an enzyme-linked immunosorbent assay method using the synthesized peptide and then by Western blotting (3). Protein concentration was determined using the Protein Assay Rapid Kit (Wako, Tokyo, Japan), and the same amounts of protein samples were analyzed by SDS-PAGE. The separated proteins were blotted to polyvinylidene difluoride membranes (Immobilon, Millipore), and FALDH was detected by the antibody followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (MP Biochemicals) with SuperSignal West Pico Chemiluminescent Substrate (Pierce). The antibody against the ER membrane protein α-calnexin was purchased from StressGen, and that against the rat peroxisomal membrane protein PMP22 was a gift from Dr. T. Imanaka (Toyama University, Toyama, Japan).

**Isolation of RNA and RT-PCR Analysis**—Total RNA from mouse tissue was prepared by the acid guanidinium thiocyanate-phenol-chloroform method as described previously (26, 27). Total RNA from the cultured cells was obtained using Quick-Gene (Fujifilm, Tokyo, Japan) and QuickGene RNA cultured cell kit S (Fujifilm). Reverse transcription was performed using an ExScript RT reagent kit (Takara Bio, Kyoto, Japan). Real time PCR was performed with a LightCycler 1.5 instrument (Roche Diagnostics) and SYBR ExScript RT-PCR kit.
(Takara Bio) as directed by the manufacturer. The primers for real-time PCR were as follows: 5'-CGGCTACCACATCCAA-GGAA and 5'-GCTGGAATACCCGGGCT for 18 S rRNA, 5'-TGACCTGTATTATTATTTGCAACC and 5'-CGAGCAA- GAGCTCACGATCC for human hypoxanthen phospho- bonyltransferase, 5'-GTCAGCTGCGCAAGTTTCC and 5'-CTCAATCAGCTGATCTCCACT for FALDH-N, 5'-GTCAGCTGGCAGGAGTTTCC and 5'-GAAGCCCAA- CGAGCTTCTTC for FALDH-V, 5'-CAGCCTTGATTGT- CAAGTTTGT and 5'-TCCGATACACGAGGACTCT for FALDH-V2, 5'-CTCTGCCCCTTGGAGGTTGT and 5'-AGGTCAGAAGACTGGTTGTG for FALDH-V3 (22), 5'- TGCACCTTACGCTCAGTCTC and 5'-GATAAGCTCCCAG- CACACT for human FALDH-N, and 5'-ATTGTTACGCGCTG- TGCTT and 5'-TGAATACACGAAAATCAACAGG for human FALDH-V (23).

Isolation of Splice Variants and DNA Sequencing—The PCR primers used for the isolation of splice variants of mouse FALDH were the exon 8 primer 5'-CTCTGCCCCTTGGAGGTTGT and exon 9' primer 5'-GAAGCCCAAACAGGAGTTTCC. Purified RT-PCR products were ligated into pGEM-T Easy (Promega). The cycle sequencing was performed with T7 and SP6 primers in both directions, and the sequences were analyzed using the LIC-4200L-2G sequencer (LI-COR). To annotate each sequence, National Center for Biotechnology Information (NCBI) Blast (www.ncbi.nlm.nih.gov/BLAST/) was used. To confirm that the variants obtained are not splicing intermediates, the sequences were analyzed using the NCBI Blast BLAST tool. After 48 h of transfection, the medium was supplemented with hygromycin B (50 μg/ml) to initiate selection for stably transfected cells. The selected cells were cloned and cultured in medium supplemented with hygromycin B (50 μg/ml) and blasticidin (5 μg/ml). For expression of FALDH, tetracycline (1 μg/ml) was added 24 h before the treatment of the cells with phytol, dodecanal, or phytanic acid.

Immunofluorescence Localization—Stably or transiently transfected HEK293 cells cultured on polylysine-coated cover slips were washed with PBS and then fixed with 4% paraformaldehyde for 30 min at 4 °C. After being washed with PBS, cells were permeabilized with ice-cold methanol for 10 min at 4 °C or with a mixture of acetone and methanol (1:1) for 10 min at 4 °C. The cells were incubated with 5% bovine serum albumin in PBS for 30 min at room temperature to block nonspecific binding of the antibodies for 30 min at room temperature. To detect the Myc-tagged proteins, an antic-Myc mouse monoclonal antibody (Nacalai Tesque, Kyoto, Japan) was used. After a rinse with wash buffer (0.4% Triton X-100 in PBS), the cells were incubated with secondary goat anti-rabbit or anti-mouse IgGs conjugated with fluorescein isothiocyanate (MP Biochemicals) or with Alexa Fluor 594-labeled goat anti-rabbit IgG (Invitrogen). After being washed with wash buffer and rinsed with PBS, the cells were fixed in Mowiol (Sigma-Aldrich), and the specimens were subjected to confocal fluorescence microscopy with a Fluoview FV500 microscope (Olympus, Tokyo, Japan). DsRed containing peroxisome-targeting signal-1 (DsRed-SKL) (28, 29) was used to locate peroxisomes in HEK293 cells.

GC-MS Analysis—GC-MS was performed according to a published method (30) with modifications on an Agilent GC6890 gas chromatograph coupled to a JMS-AM150 mass spectrometer (Jeol, Tokyo, Japan). A 30-m HP-5 column (0.32-mm inner diameter with 0.25-μm film, Agilent) was used with helium as the carrier gas. Samples (2 μl) were injected in the splitless mode. The gas chromatograph oven temperature was programmed as follows: 2 min at 70 °C followed by a rise to 120 °C at 5 °C/min, a rise to 260 °C at 7 °C/min, a pause at 260 °C for 3.5 min, a rise to 300 °C at 15 °C/min, and then 10 min at 300 °C. The identities of eluted aldehydes were confirmed by comparison of the retention times of the authentic standards. The GC-MS analysis was performed to determine the conversion of pristanic acid and stearic acid to their corresponding aldehydes.

After verification by sequencing, the cDNA insert was cloned into pcDNA5/FRT/TO (Invitrogen) by colony screening to construct an expression plasmid for amino-terminally Myc-tagged human FALDH-V. A human FALDH-N (GenBank accession number NM_00382) expression vector was constructed by swapping the RT-PCR fragment obtained by using primers 5'-TTCTCACCATTCA-GCAGCT and 5'-CAACCCGGCATTTGATT after digestion with HindIII in the cDNA fragment and Apal in the vector region of the FALDH-V expression plasmid above.

Generation of Tetracycline-inducible Flip-In T-REx HEK293 Cells—Human HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of air, 5% CO2. To cause Flip-In T-REx HEK293 cells to express FALDH-N, -V, -V2, or -V3 under the control of tetracycline, the cells were transfected with a mixture of DNA of one of the pcDNA5/FRT/TO plasmids and pOG44 (Invitrogen) using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. The selected cells were cloned and cultured in medium supplemented with hygromycin B (50 μg/ml) and blasticidin (5 μg/ml). For expression of FALDH, tetracycline (1 μg/ml) was added 24 h before the treatment of the cells with phytol, dodecanal, or phytanic acid.

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were determined by comparing their retention times and mass spectra with those of the tert-butylidemethylsilyl derivatives of purchased materials (stearic acid from Wako and pristanic acid from Sigma-Aldrich) as standards. The single ion monitoring mode was used for the detection of the $M^+ - 57$ ions for both pristanic acid and stearic acid. The amount of pristanic acid and stearic acid was quantified by integration of the respective peaks.

Pristanic Acid Assay—The cells were cultured with 750 μM phytol with or without tetracycline for 24 h. Pristanic acid was extracted from the cells according to a procedure described previously (30) with a slight modification. After being washed with PBS, the cells were homogenized with a Multi-beads shocker (Yasui Kikai, Osaka, Japan) in PBS. Then 0.1 volumes of 12 M HCl was added and mixed by vortexing. Six volumes of ether was added, and the mixture was stored at 4 °C for 48 h after mixing. The organic layer was collected, and the solvents were evaporated to 2 ml at 40 °C and dried with MgSO$_4$. The solution was transferred to reaction vials, and the solvent was evaporated completely. The extracted compounds were converted to their corresponding tert-butylidemethylsilyl derivatives using 10 μl of N-methyl-N-(tert-butylidemethylsilyl)trifluoroacetamide (Pierce) and 10 μl of pyridine at 80 °C for 1 h. After the derivatization, the solution was directly used as a sample for GC-MS.

Cell Viability Assay—The viability of the HEK293 cells was determined using the Cell Counting Kit-F (Dojindo, Kumamoto, Japan). The assay is based on the activity of living cells to hydrolyze calcein-AM to produce fluorescent calcein (31). The excitation and emission wavelengths of calcein are 485 and 535 nm, respectively, and the intensity of fluorescence is directly proportional to the number of living cells in culture. The fluorescence was quantified with a Typhoon 9410 variable mode imager (GE Healthcare).

ER Stress Response Assay—ER stress was quantified by measuring the amount of variant XBP1 mRNA as described previously (32). Briefly total RNA was extracted from the test cells with Quick-Gene 810 (Fujifilm). The mRNA of the XBP1 variant was quantified by real time PCR using specific primers (5’-GCTGAGTCGCGACGAGGT and 5’-TTGTCCAGAATGCCCACAG). For an internal standard, the amount of human glyceraldehyde-3-phosphate dehydrogenase mRNA was measured using the primers 5’-AGCCACATCGCTACGACAC and 5’-GCCCAATACGACCAATCC.

Statistical Analysis—Data are means ± S.D. Significance was examined by the unpaired t test.

RESULTS

FALDH Was Induced in the Liver of Normal Mice Fed Sesame but Was Far Less Abundant in PPARα-null Mice—Our preliminary analysis to detect the PPARα-dependently induced mRNAs in the liver of normal mice fed with sesame showed that FALDH, in addition to several P450s, was induced several fold.  To confirm this, we prepared a mouse FALDH-specific antibody (see Fig. 4A) and analyzed the changes in the amount of FALDH in the liver of wild-type or PPARα-null mice fed a normal laboratory diet or sesame seeds by Western blotting. As shown in Fig. 1, sesame induced FALDH expression by severalfold in the liver of wild-type mice. In the liver of PPARα-null mice, the basal expression levels of FALDH were low, about a fifth to a tenth of that in the corresponding tissue of wild-type mice, and a small but significant induction by sesame was observed. These results indicated that the induction of FALDH expression by sesame is dependent on the expression of PPARα, which is consistent with a recent publication (16). The sesame seeds may contain a natural ligand for PPARα (3). Identification of this ligand and elucidation of the mechanism for PPARα-independent induction of FALDH expression by sesame are important issues needing to be solved. However, we are first interested in the physiological significance of the induction of FALDH expression and started the characterization of FALDH at the protein level.

Mouse FALDH mRNA Has Several Variants Generated by Alternative Splicing—FALDH is known to have variant forms produced by alternative splicing of the primary transcript (22), but the relationship between the mRNA variants and the subcellular localization of the translation products is still at issue. To detect possible new FALDH isoforms, total RNA was isolated from the liver of mice fed a diet containing the PPARα agonist Wy14,643, and RT-PCR was performed with a left primer in exon 8 and a variant-specific right primer in exon 9 that had been contained in a variant as reported by Lin et al. (22). In addition to a major band corresponding to the known major variant (designated V) several minor products were obtained as shown in Fig. 2. Cloning and sequencing of the products revealed two new variants (designated V2 and V3) whose sequences were not found in the literature, although portions were found in an expressed sequence tag data base (corresponding to expressed sequence tags cx563174 and cx563778, respectively). RT-PCR and sequencing of two variant-specific right primers located in the intron between exons 9 and 9’ (see Fig. 2) and a left primer close to the 5’-untranslated region of FALDH confirmed that none of these are splicing intermediates. Four FALDH mRNA variants analyzed in detail in this study encode distinct carboxyl-terminal amino acid sequences because of frame shifting caused by the insertion of intron sequences. The major normal form encodes FALDH-N (GenBank accession number NP_031463) with a carboxyl-terminal KDQL end that is close to an ER retention signal. On the other hand, the major variant form V encodes FALDH-V (GenBank accession number AAK01551) with a carboxyl-terminal SKH end that is close to the peroxisomal matrix-targeting sig-
Expression level was normalized using the level of 18S rRNA and represented by real time RT-PCR with the specific primer pairs used for RT-PCR. The cDNAs synthesized from total RNA isolated from the liver of C57/BL6J mice fed a diet containing 0.05% Wy14,643 for 5 days were analyzed by RT-PCR. Each PCR product was cloned into the pGEM-T vector and sequenced using T7 and SP6 primers. The expression of all the mRNAs was largely dominated by variant FALDH-V. The levels of FALDH-V2 and -V3 were quite low in mouse liver. The relative ratio of FALDH-V, -V2, or -V3 mRNA to FALDH-N mRNA was estimated by real time PCR as 1.7, 0.7, or 0.03% under normal conditions and 2.3, 0.7, or 0.2% after feeding a diet containing Wy14,643, respectively.

**FIGURE 2.** Two novel splicing variants are generated from the mouse Aldh3A2 gene transcript, and these mRNAs encode FALDH isoforms with distinct carboxy-terminal sequences. The upper part of the figure shows the exon-intron organization of the mouse Aldh3A2 gene. Arrows represent orientations of the primers used for RT-PCR. The cDNAs synthesized from total RNA isolated from the liver of C57/BL6J mice fed a diet containing 0.05% Wy14,643 for 5 days were analyzed by RT-PCR. Each PCR product was cloned into the pGEM-T Easy cloning vector and sequenced using T7 and SP6 primers. The right part of the figure shows the deduced amino acid sequence of differential carboxyl termini. Black boxes represent the common transmembrane domain among the isoforms.

**FIGURE 3.** Expression of all four mRNA isoforms is induced by PPARα agonist Wy14,643 in mouse liver. The four mRNA variants were analyzed by real time PCR using specific primer sets represented by arrows in the upper part of the figure. C57/BL6J mice were fed a diet containing 0.05% Wy14,643 for 5 days (n = 3). The cDNAs were synthesized by reverse transcription of total RNA isolated from the livers using random hexamers as primers. Each mRNA expression level was normalized using the level of 18S rRNA and represented as a ratio relative to the normalized value of the expression in the liver of mice fed a normal diet. Ctrl, control.

The carboxyl-terminal sequences of V2 and V3 (corresponding to expressed sequence tags cc563174 and cc563778, respectively) are also distinct without significant characteristics.

**Expression of All Four FALDH mRNA Sequences Is Induced by a PPARα Agonist**—To examine the possibility of variant-specific transcriptional regulation, the expression levels of four normal and three variant forms of FALDH mRNA were quantitated by real time RT-PCR with the specific primer pairs shown in Fig. 3. The expression of all the mRNAs was largely induced in mouse liver upon administration of a PPARα agonist. Gloerich et al. (16) recently found that the conversion of phytol to phytanic acid is regulated via PPARα using null mice. The present observations support their conclusion. The induction ratios were unequal among the sequences, from 8- to 17-fold the basal level, but it is unlikely that the alternative splicing is coupled with alternative promoter usage in transcription. The changes in the induction ratios may be caused by the differences in the efficiency of alternative splicing and the stability of the mRNA isoforms. Although a precise comparison is not possible by the RT-PCR method, basal levels of each mRNA were largely different; FALDH-N mRNA was most abundant, and the remaining few percent was mostly dominated by variant FALDH-V. The levels of FALDH-V2 and -V3 were quite low in mouse liver. The relative ratio of FALDH-V, -V2, or -V3 mRNA to FALDH-N mRNA was estimated by real time PCR as 1.7, 0.7, or 0.03% under normal conditions and 2.3, 0.7, or 0.2% after feeding a diet containing Wy14,643, respectively.

**FALDH-N and -V Isoforms Are Distributed Differently in the Cell**—To characterize the FALDH isoforms at the protein level, rabbit polyclonal antibodies against mouse FALDH were raised using a synthetic peptide as an antigen corresponding to the sequence from residue 425 to 439 in exon 9. This sequence was chosen to detect all four FALDH isoforms of mice but not those of humans. The antibodies specifically recognized mouse FALDH and the PPARα-dependent induction of FALDH expression in mouse liver was confirmed as shown in Fig. 4A. The antibodies recognized both FALDH-N and -V expressed in HEK293 human cells under the control of a tetracycline-inducible system. The calculated molecular mass of the V form is larger than that of the N form by 2.6 kDa, and the difference was confirmed by Western blotting as shown in Fig. 4B.

Next the subcellular distribution of the four isoforms in human HEK293 cells was examined using mouse-specific antibodies against FALDH in established cell lines with tetracycline-inducible expression of FALDH-N, -V, -V2, or -V3. Masaki et al. (21) reported that FALDH-N expressed from cloned cDNA is located on the endoplasmic reticulum, and our observations support their conclusion as shown in Fig. 5A. The carboxyl-terminal sequence KDQL of FALDH-N may actually function as an ER retention signal. Whereas the carboxyl-terminal sequences of FALDH-V2 and -V3 were not similar to the possible retention signal, they were located on apparently the same membranous structure as FALDH-N. FALDH-N, -V2, and -V3 isoforms are likely to be present in the ER, although they have distinct carboxyl-terminal sequences. These results suggest that the unidentified sequence is important for the other isoforms to remain on the ER. Efficiency to remain in the ER membrane may differ among the isoforms, but this possibility was not examined in the present study. On the other
hand, the localization pattern of FALDH-V was significantly different from that of any other isoform. It was localized to small dotted structures in the cytoplasm (Fig. 5A). We directly examined the possibility of its peroxisomal localization by comparing the patterns of FADH-V and a SKL-tagged dye, DsRed-SKL as a peroxisome marker (28). FALDH-V, but not FALDH-N, co-localized with DsRed-SKL as shown in Fig. 5B. Thus FALDH-V was apparently exclusively present in peroxisomes in HEK293 cells.

In humans, splice variant forms of FALDH mRNA are also known (23). However, the carboxyl-terminal amino acid sequence deduced from a variant mRNA corresponding to mouse FALDH-V is SKQR, and this may not function as a peroxisome-targeting signal. To examine whether or not human FALDH-V is also distributed in peroxisomes, we cloned human FALDH-N and FALDH-V expressed with Myc tag to specifically identify the products. As shown in Fig. 5C, only human FALDH-V was localized to peroxisomes as evidenced by co-localization with DsRed-SKL. Thus we confirmed that one splice variant form of human FALDH is present in peroxisomes.

To further confirm the isoform-specific localization of FALDH, subcellular fractionation of the postnuclear fraction of mouse liver was performed using Nycodenz for the separation of peroxisomes followed by Western blotting.

**FIGURE 4.** FALDH-V protein is distinguishable from the major form FALDH-N on SDS-PAGE. A, the proteins in postnuclear fractions from the liver of C57BL6J mice fed a diet containing 0.05% Wy14,643 for 5 days were separated by SDS-PAGE. Endogenous FALDH-N and FALDH-V were detected by the anti-FALDH peptide antibody (WB). Protein patterns (CBB-stain) are shown to indicate equivalent protein loading and induction of peroxisomal proteins, such as a 70-kDa protein, by Wy14,643. WB, Western blot; CBB, Coomassie Brilliant Blue. B, FALDH-N and FALDH-V were overexpressed in HEK293 cell lines using a tetracycline-inducible system and probed by the mouse-specific antibody. The protein samples were analyzed separately (N and V) and after mixing (N+V) together with the postnuclear fraction from the liver of mice treated with Wy14,643 as a control. The calculated molecular mass is 54.0 kDa for FALDH-N and 56.6 kDa FALDH-V. PNS, postnuclear supernatant fraction.

**FIGURE 5.** The major and two other isoforms of FALDH are located in the endoplasmic reticulum, whereas one isoform, FALDH-V, is found in peroxisomes of HEK293 cells. A, cells overexpressing FALDH-N, FALDH-V, FALDH-V2, or FALDH-V3 were fixed and immunostained with anti-mouse FALDH polyclonal antibody using Alexa 594-labeled secondary antibodies. Representative confocal microscopic images of the localization of FALDH are shown. B, the co-localization experiment was performed by transiently transfecting the FALDH-overexpressing cells with DsRed-SKL to visualize peroxisomes. Mouse FALDH was immunostained with the polyclonal antibody and fluorescein isothiocyanate-labeled secondary antibodies. Representative confocal images of the co-localization are shown. C, the distribution of human FALDH was determined by transiently co-transfecting HEK293 cells with Myc-tagged human FALDH-N or FALDH-V DNA with DsRed-SKL. The transfected cells were fixed and immunostained with anti-Myc monoclonal antibody and fluorescein isothiocyanate-labeled anti-mouse IgG secondary antibodies to visualize human FALDH. Representative confocal microscopic images are shown.

**Peroxisome-specific Aldehyde Dehydrogenase**
Western blot analysis. PMP22 in the recovered fractions after centrifugation were compared with Triton X-100 to examine their solubility. Distributions of FALDH-V and was treated with pyrophosphate to obtain integral membrane proteins or fractions; C, mitochondrial fraction; C, microsomal and cytosolic fractions. B, Nycodenz pellet fraction was separated by SDS-PAGE and analyzed by Western blotting using the anti-FALDH antibody, anti-calnexin antibody for the ER, and anti-PMP22 antibody for the peroxisomal membrane. The peroxisome-enriched fraction and lysosomal fraction (L) were separated using Nycodenz. The peroxisome-enriched fraction was further centrifuged to obtain a pellet (Nycodenz, P) and supernatant (Nycodenz, S). PNS, postnuclear supernatant fraction; M, mitochondrial fraction; C, microsomal and cytosolic fractions. B, Nycodenz pellet fraction was treated with pyrophosphate to obtain integral membrane proteins or with Triton X-100 to examine their solubility. Distributions of FALDH-V and PMP22 in the recovered fractions after centrifugation were compared by Western blot analysis. PPI, pyrophosphate treatment; Triton-X, Triton X-100 treatment; pellet, pellet fractions; sup, supernatant fractions. C, Coomassie Brilliant Blue-stained protein patterns of each fraction are shown to indicate equivalent protein loading and separation of typical peroxisomal proteins by the disassembly treatments in B.

FIGURE 6. FALDH-V is a peroxisomal membrane protein. The livers of mice fed a diet containing Wy14,643 were homogenized and fractionated by differential centrifugation to obtain subcellular fractions as described under “Experimental Procedures.” A, the same amount of protein in each fraction was separated by SDS-PAGE and analyzed by Western blotting using the anti-FALDH antibody, anti-calnexin antibody for the ER, and anti-PMP22 antibody for the peroxisomal membrane. The peroxisome-enriched fraction and lysosomal fraction (L) were separated using Nycodenz. The peroxisome-enriched fraction was further centrifuged to obtain a pellet (Nycodenz, P) and supernatant (Nycodenz, S). PNS, postnuclear supernatant fraction; M, mitochondrial fraction; C, microsomal and cytosolic fractions. B, Nycodenz pellet fraction was treated with pyrophosphate to obtain integral membrane proteins or with Triton X-100 to examine their solubility. Distributions of FALDH-V and PMP22 in the recovered fractions after centrifugation were compared by Western blot analysis. PPI, pyrophosphate treatment; Triton-X, Triton X-100 treatment; pellet, pellet fractions; sup, supernatant fractions. C, Coomassie Brilliant Blue-stained protein patterns of each fraction are shown to indicate equivalent protein loading and separation of typical peroxisomal proteins by the disassembly treatments in B.

Overexpression of FALDH-V Increases the Conversion of Phytohol to Pristanic Acid—To determine which FALDH plays a major role in the conversion of phytol to pristanic acid, the amounts of pristanic acid converted from phytol in the cells overexpressing FALDH-N or -V were compared because we assumed that FALDH-V was the enzyme responsible for the final conversion step in peroxisomes. Fatty acids were extracted from cells incubated with phytol in parallel and were analyzed by GC-MS as shown in Fig. 7. Although it had been a concern that the differences between the cell types might be small because HEK293 cells most likely expressed endogenous FALDH, and pristanic acid was an intermediate in the degradation pathway, overexpression of FALDH-V but not FALDH-N largely increased the amount of pristanic acid. A similar increase of pristanic acid in FALDH-V-overexpressing cells was observed when the cells were incubated with phytanic acid instead of phytol (not shown). These results suggest that FALDH in the peroxisome but not in the ER plays a dominant role in the oxidation of pristanol to pristanic acid.

Overexpression of FALDH-V Protects the Cells from the Damage Caused by Phytanic Acid—To examine the physiological role of FALDH, responses of the cells to the stress caused by phytanic acid were compared between the cells overexpressing FALDH in the ER and those overexpressing FALDH in the peroxisome by measuring cell viability (Fig. 8A) and the extent of
The heterologously expressed variant mouse FALDH from each cDNA without tagging could be detected distinctly in a specific subcellular location. In the present study, using cells inducibly overexpressing each variant of FALDH and a specific antibody, it was shown that one variant of FALDH, FALDH-V, but not the others exclusively localizes in peroxisomes. The GC-MS analysis of the metabolites in the FALDH-expressing cells incubated with phytol or phytanic acid showed that FALDH-V is the key aldehyde dehydrogenase in the phytanic acid degradation pathway, which protects the cells from lipid peroxidation. Finally, the role of FALDH in protecting cells from lipid peroxidation in general was examined by treating the same series of cells as above with a middle chain aldehyde, dodecanal, to simulate a toxic effect of lipid peroxidation products and measuring the changes in cell viability (Fig. 10). Overexpression of either type of FALDH partially protected the cells from the death induced by lipid peroxidation. A similar protective effect of the two FALDH isoforms against oxidative stress caused by dodecanal was noteworthy when compared with previous results showing a predominant effect of FALDH-V against the stress caused by phytanic acid. Both FALDHs are likely to play an important role in protecting the intracellular membrane from lipid peroxidation by localizing to distinct regions of the cell.

**DISCUSSION**

The identity and subcellular localization of the key aldehyde dehydrogenase in the phytanic acid degradation pathway are controversial (12, 18, 20, 21) because several variants produced by alternative splicing are present in varying amounts and different subcellular locations. In the present study, using cells inducibly overexpressing each variant of FALDH and a specific antibody, it was shown that one variant of FALDH, FALDH-V, but not the others exclusively localizes in peroxisomes. The GC-MS analysis of the metabolites in the FALDH-expressing cells incubated with phytol or phytanic acid showed that FALDH-V is the key aldehyde dehydrogenase in the degradation pathway. It also has a protective role against oxidative stress induced by lipid peroxidation.
large, our strategy dominated previous strategies. Mouse FALDH-V has a possible peroxisomal matrix-targeting signal-1 (SKH instead of SKL) at the carboxyl terminus as suggested by Lin et al. (22). We confirmed its peroxisomal membrane localization morphologically (Fig. 5B) and biochemically (Fig. 6). Furthermore human FALDH-V, which has an SKQR sequence at the carboxyl terminus, was also proved to localize in peroxisomes (Fig. 5C). Honsho and Fujiki (33) reported that not the carboxyl-terminal sequence but a short, positively charged intervening loop sequence and flanking hydrophobic segments are required for peroxisomal membrane localization of human peroxisomal membrane protein PMP34. These suggest that sequences other than the carboxyl-terminal sequence of FALDH-V is important for peroxisomal targeting.

Peroxisomal localization of FALDH-V is important when we consider the ultimate cause of Sjögren-Larsson syndrome. Kelson et al. (12) could detect aldehyde dehydrogenase activity in the ER fraction of human cells but not in peroxisomes, whereas Jansen et al. (20) identified aldehyde dehydrogenase activity in rat liver peroxisomes and claimed it to be one or more additional aldehyde dehydrogenases. However, our result endorses the view that the peroxisomal aldehyde dehydrogenase converting pristanal to pristanic acid is FALDH-V (Fig. 7). The evidence for the existence of another peroxisomal aldehyde dehydrogenase is the 10–15% residual activity of pristanal dehydrogenase in mutant fibroblasts from patients (20). Although the types of mutations were not described in their study, most of them may be missense mutations (34), and such mutated enzymes, in general, are not null but have some residual activities in vivo. The reported remaining activities of aldehyde dehydrogenase determined using FALDH mutant cells could be those of FALDH itself. Our present data strongly suggest that the aldehyde dehydrogenase in peroxisomes is FALDH-V, and it converts pristanal to pristanic acid. Thus we propose from our present data together with published data (18, 20, 35) that all the steps of degradation of phytanic acid to pristanic acid are carried out in peroxisomes as summarized in Fig. 11. To confirm this, it will be necessary to quantitatively correlate the expression levels and activities of each FALDH isoform using cells totally free of endogenous human FALDH. To this end, we are now trying to knock down endogenous FALDH in HEK293 cells.

FALDH has a wide substrate specificity (12) and plays an important role that is not restricted to the pathway of phytol degradation as evidenced by several studies (36–38) including the present study showing protection against the cytotoxicity of a middle chain aldehyde (15), such as dodecanal (Fig. 10). In the case of dodecanal, the expressions of FALDH-N in the ER and FALDH-V in peroxisomes are equally effective at protecting cells (Fig. 10). This result suggests that a model lipid peroxidation product, dodecanal, exerts its toxicity at both the ER and peroxisomes as summarized in Fig. 12, and an additive effect of overexpression of both FALDH-N and FALDH-V can be expected. Distinct expression of two FALDH isoforms in either the ER or peroxisomes is achieved in vivo by alternative splicing, and this mechanism is evolutionarily conserved at least in mice and humans, suggesting its essential role in oxidative stress protection. The ratio of the expression levels of two mRNAs for these isoforms did not significantly change under various conditions (Fig. 3), and the
major step to regulate the expression of these mRNAs would be at the transcriptional level. Recent studies demonstrated that expression of FALDH is regulated by PPARα and its ligand (16) as we also confirmed in the present study (Figs. 3 and 4), although the precise molecular mechanism has not been elucidated (39, 40). Furthermore phytol and/or its metabolites were shown to activate PPARα probably by acting as ligands (41–43). These observations indicate the autocatalytic nature of this detoxifying system for xenobiotics; a potentially toxic molecule binds to the nuclear receptor PPARα to activate transcription of the gene(s) for its own detoxifying enzyme(s). PPARα is a sensor of not only fatty acids but also some xenobiologic toxic molecules and plays a vital role in the detoxifying system.

In addition to xenobiotics, oxidation of potentially toxic endobiotics, such as very long chain fatty acids, branched chain fatty acids, and bile acid intermediates, takes place in peroxisomes via the production of a large amount of \( \text{H}_2\text{O}_2 \), up to 35% of the total in the liver. Oxidation-driven detoxification is effective, but it is accompanied by the production of reactive oxygen species. PPARα is well known to largely activate transcription of the genes for oxidation enzymes, but the expression of the enzymes needed to decompose reactive oxygen species such as catalase is not largely activated. These disproportional increases of oxidases and reactive oxygen species are due to oxidative stress and the expression of the enzymes required to decompose reactive oxygen species such as catalase is not largely activated. This disproportional increase of oxidases and of reactive oxygen species-decomposing enzymes should cause oxidative stress in peroxisomes, leading to lipid peroxidation and protein adduction (44). In this context, a large induction of FALDH-V by PPARα on peroxisomal membranes is reasonable (see Fig. 12). The proposed vital role of FALDH-V in protecting peroxisomes from oxidative stress can be examined by overexpressing the oxidases in FALDH-V knockdown cells.

Demozay et al. (15) found a reduction of FALDH expression in both insulin-resistant and diabetic rodent models and suggested its importance in insulin action by protecting against oxidative stress associated with lipid peroxidation leading to deregulation of insulin action. A malignant effect of increased oxidative stress in accumulated fat is also emphasized to be an important pathogenic mechanism of obesity-associated inflammation, diabetes, and metabolic syndrome (45). For this reason, the physiological role and the control of expression of FALDH at the whole body level should be studied further, and the possibility that compounds targeting FALDH are candidates for new drugs to treat obesity-associated diseases (15) needs to be examined.

We started the study on FALDH because its expression was induced in the intestine and liver of mice fed sesame seeds (3). The present results suggest a protective role of FALDH against oxidative stress induced by lipid peroxidation partly explain the toxicity of sesame only in PPARα-null mice because FALDH is far less abundant in the liver of the mutant mice than of the wild-type mice (Fig. 1). However, induction of FALDH expression alone cannot explain the toxicity. Sesame seeds induce expression of several detoxification enzymes including many P450s and hypoglycemia in PPARα-dependent and -independent manners (3). We speculate that relationships between other ligands and nuclear receptors also exist in detoxification systems. To clarify the causal linkage between a compound in sesame seed and a malignant effect on metabolism at the molecular level, we are establishing an assay system to replicate the response in animals using an isolated compound. We think that animals should have evolved metabolizing systems for the secondary metabolites in plant seeds, and further understanding of these systems may lead to the development of a new field of research.

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