Mouse Very Long-chain Acyl-CoA Synthetase in X-linked Adrenoleukodystrophy*

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X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disorder characterized by accumulation of very long-chain fatty acids (VLCFA). This accumulation has been attributed to decreased VLCFA β-oxidation and peroxisomal very long-chain acyl-CoA synthetase (VLCS) activity. The X-ALD gene, ABCD1, encodes a peroxisomal membrane ATP binding cassette transporter, ALDP, that is hypothesized to affect VLCS activity in peroxisomes by direct interaction with the VLCS enzyme. Recently, a VLCS gene that encodes a protein with significant sequence identity to known rat and human peroxisomal VLCS protein has been identified in mice. We find that the mouse VLCS gene (Vlcs) encodes an enzyme (Vls) with VLCS activity that localizes to peroxisomes and is expressed in X-ALD target tissues. We show that the expression of Vlcs in the peroxisomes of X-ALD mouse fibroblasts improves VLCFA β-oxidation in these cells, implying a role for this enzyme in the biochemical abnormality of X-ALD. X-ALD mice, which accumulate VLCFA in tissues, show no change in the expression of Vlcs, the subcellular localization of Vlcs, or general peroxisomal VLCS activity. These observations imply that ALDP is not necessary for the proper expression or localization of Vlcs protein, and the control of VLCFA levels does not depend on the direct interaction of Vlcs and ALDP.

X-linked adrenoleukodystrophy (X-ALD)1 is a progressive neurodegenerative disorder (1) with an incidence of 1 per 17,000 births (2). Clinically, it affects the cerebral white matter, peripheral nerves, adrenal cortex, and testis. Mutations in the X-ALD gene, ABCD1, result in several phenotypic variants that vary in onset age, disease progress rate, and primary affected tissue. These variants can not be attributed to the genotype of the X-ALD gene (3).

ABCD1 was cloned in 1993 (4) and encodes a peroxisomal half ATP binding cassette (ABC) transporter, ALDP. ALDP has been grouped with the ABC protein superfamily on the basis of sequence homology, although its molecular function has not been established.

All male X-ALD patients and the majority of X-ALD female carriers accumulate straight chain saturated very long-chain fatty acids (VLCFA; 24 or more carbons) in their plasma and tissues. This accumulation is associated with decreased VLCFA degradation by peroxisomal β-oxidation as observed in patient fibroblasts. The activity that catalyzes the first step in VLCFA β-oxidation, activation of VLCFA to their CoA derivatives, is decreased in the peroxisomes of X-ALD patient fibroblasts (5–9). This step is catalyzed by very long-chain acyl-CoA synthetase, VLCS. Peroxisomal VLCS activity is the only known enzymatic activity decreased in X-ALD (10–12); however, the ALDP protein does not itself have VLCFA activity. How the loss of ALDP results in reduced peroxisomal VLCS activity is unknown, but several hypotheses have emerged that imply a direct interaction of ALDP with peroxisomal VLCS enzyme (4).

Several X-ALD knockout mice have been generated by our group and others (13–15). All accumulate tissue VLCFA, and fibroblasts derived from these mice have defects in VLCFA β-oxidation. Although X-ALD mice mimic the biochemical phenotype of X-ALD, they fail to exhibit any significant loss of neurological function until a very advanced age (16).

VLCS enzyme was initially isolated from rat liver peroxisomes, and a number of VLCS genes have been subsequently cloned from other organisms (17–19). All of these enzymes have been localized to both peroxisomes and endoplasmic reticulum, sites of VLCS activity in the cell, and shown to have VLCS activity as well as the ability to activate long chain fatty acids. Vlcs has been identified as a mouse gene with a high degree of identity to these enzymes (20, 21) and, as such, warrants investigation in X-ALD.

Our goal in this study is to investigate the functional and physical interactions of Vlcs and ALDP on the biochemical hallmark of X-ALD. We show that Vlcs is a reasonable candidate for the enzyme affected in X-ALD. It is the mouse gene most homologous to rat and human VLCS, it encodes an enzyme with VLCS and long-chain acyl-CoA synthetase (LCS) activity, and Vlcs protein can be found in peroxisomes and endoplasmic reticulum. The overexpression of Vlcs improves the β-oxidation defect in mouse X-ALD fibroblasts when targeted to peroxisomes. Our investigation of Vlcs expression, Vlcs localization, and peroxisomal VLCS activity in the X-ALD mouse demonstrates no differences from wild type. We conclude that ALDP is not necessary for maintaining peroxisomal...
VLCs activity in tissues and that ALDP and Vlcs do not physically or functionally interact to affect VLCPA accumulation.

MATERIALS AND METHODS

Animals

Wild type 129Sve mice were obtained from Taconic, Inc. (Germantown, NY) or bred from Taconic 129Sve stock in the animal facility at Johns Hopkins Medical Institutions. X-ALD mice are from the strain derived by Lu et al. (15) and have been maintained at Johns Hopkins Medical Institutions since their generation. All animals were housed under the same controlled conditions, between 22 and 27 °C, on a 12-h light/dark cycle, with free access to food and water. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (ECC Council Directive 86/609, OJ L 358, 1 DEC.1987; NIH Guide for the Care and Use of Laboratory Animals, U. S. National Research Council, 1996).

Vlc cDNA Cloning

The peptide sequence of rat VLCs (22) was used to search for the EST data base for homologous mouse ESTs. A mouse EST was identified, GenBank accession number AA038113, and obtained from Incyte Genomics (Palo Alto, CA). The EST clone contained 921 base pairs of open reading frame and eight nucleotides of the 3′-untranslated region. This EST clone insert was removed from its vector with EcoRI and NotI and ligated into the same sites of the multiple cloning vector of pCDNA3. The 5′-untranslated region of rat VLCs (17) was used to design a forward PCR primer, 5′-CGCGGCTA-CCGATGCTACGTGACCCAGCAG-3′, with an engineered KpnI site (underlined), and this was used in an RT-PCR reaction to amplify the remaining 5′ end of the cDNA from liver total cDNA with a reverse primer, Vlc R1096, 5′-gtcgctagctggtgatgcgtagaac-3′. The ~1100-bp PCR product encompassed a second naturally occurring KpnI site near its 3′-end. The KpnI sites in the PCR product were used to clone the PCR fragment into a KpnI site near the 5′-end of EST AA038113 now in pCDNA3. The integrity of the final clone was confirmed by DNA sequence analysis, and the full-length open reading frame of Vlc was deposited in GenBank (accession number AF053031).

Northern Blot

Probes for Vlcs and β-actin were generated by random primer labeling of cDNA with [α-32P]dCTP (23). The entire cDNA was released from its vector by restriction digestion and gel-purified. This fragment was labeled with [32P]dCTP using a Rediprime Random Prime Labeling kit (Amersham Biosciences). Probes were purified from unincorporated nucleotide on a Sephadex G-50 column, and incorporation of dCTP was quantitated using a Beckman LS3801 liquid scintillation counter. Probes were heated to 100 °C for 5 min, quickly cooled on ice, and held for 5 min at 4 °C before use. A multiple tissue Northern blot of mouse mRNA samples (BD Biosciences CLONTECH, Palo Alto, CA) was prehybridized in 15 ml of Rapid hyb buffer (Amersham Biosciences) for at least 30 min in a hybridization oven at 68 °C. Two × 106 counts/min of probe were used per ml of hybridization buffer, and the blot was hybridized at 68 °C for 3 h. The blot was washed twice with 0.1 × SSC, 0.1% SDS for 20 min at 65 °C and exposed to film for 1–2 h on an Eastman Kodak Co. AR film with an intensifying screen at ~80 °C. The blot was probed sequentially and between hybridizations was stripped with boiling 0.1% SDS for 30 min and reexposed to film to confirm loss of signal.

RT-PCR

Total RNA prepared from wild-type mouse tissues was used as a template for cDNA synthesis. Superscript II RT kit from Invitrogen was used with oligo(dT) primers for 1 h at 42 °C in a 25-μl reaction with 5 μg of RNA. Two μl of this cDNA was used in a 50-μl PCR for Vlcs or for β-actin. PCR primers used were Vlcs forward (5′-ATGCTGGAGGAAA-GACCCAGAC-3′) and reverse (5′-CACCCTCATCACATCTCCCTTT-C-3′) (233-bp product) and β-actin forward (5′-TGGTAGACCTTCACACCACCAG-3′) and reverse (5′-TTTCTATGGATGCCACAGAGA-3′) (460-bp product).

Cell Culture

All cells were manipulated under sterile conditions and grown at 37 °C in a humid atmosphere of 5% CO2 in air. The X-ALD mouse fibroblast line 140 was described previously (15). X-ALD mouse fibroblasts (line 140) were cultured in 4.5 g/liter glucose, 2 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum. Mouse embryonic fibroblasts were isolated from 12.5–14.5 days post-coitus X-ALD embryos (24). Cells were grown in Dulbecco’s modified Eagle’s medium with 4.5% d-glucose, 2 mM l-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 20% newborn calf serum (In vitrogen). COS-1 cells (gift of C. Thompson) were grown in Dulbecco’s modified Eagle’s medium with 4.5% d-glucose, 2 mM l-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum. Cells harvested for enzyme assays or transfection were washed twice with phosphate-buffered saline (PBS) or Hanks’ buffered salt solution, incubated with 0.25% trypsin at 37 °C, harvested in medium containing 10% fetal calf serum, and pelleted. Cell pellets for enzyme assays were washed twice with PBS or Hanks’ buffered salt solution.

Transfection

Adult X-ALD mouse skin fibroblasts (line 140) were transfected with either empty pcDNA3 or recombinant pcDNA3 vector containing human X-ALD cDNA (pSK2ALD) or Vlcs cDNA (pSkVlcs). For transfection, cells from a T75 tissue culture flask were washed twice with PBS, harvested with trypsin, resuspended in 10 ml of Opti-MEM (Invitrogen), and pelleted by centrifugation at 500 × g for 5 min. The cell pellet was resuspended in 0.5 ml of Opti-MEM with 10 μg of purified plasmid DNA. Transfection was performed using a Cell Porator (In vitrogen) in a 4-mm cuvette at 350 V, 330 microfarads. Cells were resuspended in Dulbecco’s modified Eagle’s medium and replated in a T75 flask. The medium was refreshed the next day, and cells were assayed at day 3.

Mouse embryonic fibroblasts were transfected with pcDNA3, pSK2ALD, pSkVlcs, or both pSk2ALD and pSkVlcs together. For transfection, cells from two 80% confluent T150 flasks between passages 2 and 4 were washed twice with PBS, harvested with trypsin, resuspended in 10 ml of Opti-MEM, and pelleted by centrifugation at 500 × g for 5 min. The cell pellet was resuspended in 0.8 ml of Opti-MEM. Twenty micrograms of endotoxin-free plasmid DNA were added in less than 20 μl of TE buffer. Transfection was performed using an Electro Cell Manipulator 600 (BTX Electroporation Systems, San Diego, CA) in a 4-mm cuvette at 280 V, 1500 microfarads, 129 ohms. Cells were allowed to recover 5–10 min at room temperature, plated in one T75 flask, and grown for 2 days before harvesting for analyses.

Enzyme Assays

Acyl-CoA Synthetase Assays—Acyl-coenzyme A synthetase activity was measured by the method previously described (25). Transiently transfected COS-1 cells were harvested 3 days post-transfection and stored at −80 °C. An aliquot of cells containing 100–200 μg of protein, measured via the method of Lowry (26), was added to a 250-μl reaction containing 20 μl [1-14C]palmitic acid (C16:0; American Radiolabeled Chemicals, St. Louis, MO) or 20 μl [1-14C]lignoceric acid (C24:0; Moravek Biochemicals, Brea, CA). Water-soluble radiolabeled products were quantitated and expressed as n mole of CoA derivative generated in 20 min per μg of protein. Duplicate assays were performed for each measurement, and the average value was reported.

Cellular β-oxidation—β-Oxidation activity was measured as previously described (27). Freshly harvested fibroblast cell pellets were resuspended in 0.25% sucrose, 1 mM Tris-HCl, pH 8.0, 1 mM EDTA and held on ice. The 250-μl reaction contained 100–200 μg of protein, 4 μM [1-14C]lignoceric acid, and 16 μM unlabeled lignoceric acid substrate. Duplicate reactions were measured for each sample, and activity was expressed as n mole of radiolabeled acetate produced/h of protein.

Antibody Production and Western Blot

A fusion protein of maltose-binding protein and the C-terminal 214 amino acids of Vlcs was created using a bacterial expression vector containing maltose-binding protein cDNA (New England Biolabs, Beverly, MA) and mouse Vlcs cDNA. Protein synthesis was induced in BL21 DE3 Escherichia coli by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside and incubation for 4 h at 37 °C. E. coli protein extracts were solubilized in 0.4 M NaCl, purified over a maltose
column, and concentrated to 1 mg/ml. One hundred micrograms of this concentrate was mixed with Freund's complete adjuvant and inoculated into New Zealand White rabbits. A 50-μg boost was given at 2, 3, and 7 weeks. Serum from week 8 was affinity-purified against a glutathione S-transferase (Promega, Madison, WI) Vlcs fusion protein, in which the C-terminal 263 amino acids of Vlcs are fused to glutathione S-transferase.

The purified sera were concentrated and used at 1:200 dilution for immunofluorescence studies and 1:500 dilution for Western blot analyses.

Frozen mouse tissues were disassociated in a Potter-Elvehjem homogenizer in cold PBS with protease inhibitors (Complete inhibitor tablets; Roche Molecular Biochemicals) and 0.1M EDTA. A 5-min centrifugation at 3000 × g at 4 °C removed insoluble material, and the protein concentration of the supernatant was estimated using the method of Lowry (26). Protein samples were diluted to 60 mg of protein/ml and combined with an equal volume of 2× sample buffer for a final concentration of 30 mg protein/ml. Samples were boiled for 3 min and cooled on ice, and 750 μg of protein were loaded per lane on an SDS-8% PAGE gel. Proteins in the gel were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.) in Tris-glycine/methylene transfer buffer for 2 h at 200 mA at room temperature. Proteins were affixed to the membrane by air drying for 15 min after a 30-s soak in methanol. Nonspecific proteins were blocked by pre soaking in a solution of 5% dry milk in PBS for 1 h at room temperature. The blot was then incubated with 0.1% Tween 20 in PBS (PBST) and incubated with primary antibody to the target antigen diluted in 0.01% bovine serum albumin in PBST and 0.02% NaN₃ for 1 h. Primary antibodies were diluted from 1:100 to 1:1000. Rabbit IgG anti-human PEHX14 antibodies were a generous gift from S. Gould and were used at a 1:100 dilution to detect mouse Pex14 protein in peroxisomal membranes (28). Rabbit IgG anti-mouse Vlcs antibodies were used at a 1:100 dilution to detect mouse Pex14 protein. The blot was washed three times with PBST buffer, and a 1:3000 dilution of goat anti-rabbit horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated with the blot for 30 min at room temperature to detect attached primary antibodies. The blot was washed again three times with PBST before being developed with chemiluminescent substrate (Pierce) and exposed to Kodak AR film for 1–60 s to visualize Pex14 and Vlcs proteins.

**Immunofluorescence**

Several aliquots (50 μl) were taken from a 10-ml cell suspension prior to β-oxidation assays, replated onto sterile 1.8-cm glass coverslips, and cultured until they reattached (12–24 h). The coverslips were then removed from medium and washed three times in PBS buffer. Cells were fixed in 3% formaldehyde in PBS for 5 min and rinsed three times with PBS. Fixed cells were either stored in cold PBS with 0.02% NaN₃ at 4 °C for 24 h or processed immediately. Fixed cells were made permeable with a solution of 1% Triton X-100 in PBS for 5 min and rinsed in PBS three times. Cells were incubated with two primary antibodies, one for Vlcs and one for ALDP, diluted 1:200 in PBS, with 0.1% bovine serum albumin for 20 min at room temperature in a humid container to minimize evaporation. The primary antibodies used in this method were rabbit anti-mouse Vlcs (19) or rabbit anti-human VLCS (29), mouse anti-human ALDP (MA2126, Chemicon, Temecula, CA), or rabbit anti-human ALDP (30). After incubation with primary antibody, cells were washed five times in PBS and incubated with the appropriate fluorescently labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) to detect primary antibody bound to antigen. Cells were incubated with secondary antibodies diluted 1:200 in PBS for 20 min at room temperature with 0.1% Tween 20. Secondary antibodies were donkey anti-rabbit or donkey anti-mouse labeled with fluorescein isothiocyanate (green) or rhodamine (red). Coverslips were washed five times in PBS and mounted in a glycerol-based mounting medium, and the cells were photographed with an Axiovision digital camera at 1–10-s exposures with fluorescent light of the appropriate wavelength to excite the secondary antibody label. Control coverslips were processed without each primary antibody as controls.

**Mouse Liver Fractionation**

Linear 35-ml Nycodenz gradients (15–40%) were prepared from 15% (w/v) Nycodenz in buffer containing 0.25 M sucrose, 1 mM MOPS, pH 7.0, and 1 mM EDTA (SME buffer) and 40% (w/v) Nycodenz in MOPS and EDTA only. Gradients were stored overnight undisturbed at 4 °C. Freshly dissected mouse liver (8 weeks old) from male X-ALD or wild type mice were minced and homogenized with one stroke in a Potter-Elvehjem loose pestle homogenizer in cold SME buffer, 10 volumes (ml) to liver weight (g). Nuclei were removed by centrifugation at 3000 × g at 4 °C for 30 s. Equal amounts of X-ALD and wild type supernatant (about 5 ml) were loaded on separate gradients and centrifuged at 29,700 rpm in a Beckman VTi 50 ultracentrifuge for 76 min at 4 °C. Two-milliliter fractions were collected starting from the bottom of the gradient, assayed for catalase activity, and stored at −80 °C for analysis by Western blot and enzyme assays.

**Catalase Assays**

Fractions from Nycodenz gradients were diluted 1:100 in water, and 20- or 40-μl aliquots were assayed for catalase activity in a reaction mixture of 200-μl total volume containing 20 mM imidazole, pH 6.8–7.0, 0.2% Triton X-100, and 0.06% hydrogen peroxide. To standardize the spectrophotometer, a blank control was prepared with no hydrogen peroxide and no sample. After exactly 5 min of incubation at room temperature, the reactions were stopped with 1 ml of stop solution (0.45% TiO₃O₄, 2× H₂SO₄), and absorbance was measured at 405 nm. Catalase activity degrades the hydrogen peroxide in the reaction and prevents the formation of a yellow peroxide-Ti complex. Catalase activity is reported as the decrease in absorbance from that of the negative control.

**RESULTS**

**Cloning of Mouse Very Long-chain Acyl-CoA Synthetase and Sequence Analysis**—The murine very long-chain acyl-CoA synthetase cDNA (Vlcs) encodes a 620-amino acid protein (Vlcs) with 93% identity to rat VLCS and 83% identity to human VLCS (Fig. 1). The N-terminal 22 amino acid sequence of Vlcs is predicted to be an endoplasmic reticulum-targeting signal sequence by analysis with the PSORT algorithm (31). The C-terminal sequence (−KLL) of Vlcs resembles the canonical mammalian peroxisomal targeting signal 1 consensus sequence, -SKL, which functions as a peroxisomal targeting signal 1 in Saccharomyces cerevisiae (32). An AMP binding domain typical of enzymes that form an enzyme-AMP intermediate is predicted for amino acids 220–233, and a VLS-specific motif (33) is present between residues 476 and 499 (Fig. 1).

**Expression of Vlcs cDNA in COS-1 Cells Increases Their VLCS Activity**—To determine whether Vlcs encodes an enzyme with fatty acid acyl-CoA synthetase activity, Vlcs cDNA was expressed in COS-1 cells, and these cells were assayed for increased acyl-CoA synthetase activity toward a representative very long-chain fatty acid, lignoceric acid (C24:0), and long-chain fatty acid, palmitic acid (C16:0) (Fig. 2). As a control, rat acyl-CoA synthetase 1 (rACS-1) (34) cDNA was expressed in COS-1 cells and assayed for activity. Rat ACS-1 has LCS activity but not VLCS activity. As expected, rat ACS-1-expressing COS-1 cells increased their LCS but not VLCS activity. Vlcs increased lignoceric acid acyl-CoA synthetase activity in transiently transfected COS-1 cells 5-fold from 0.51 nmol of C24:0/20 min/mg of protein to 2.47 nmol of C24:0/20 min/mg of protein (Fig. 2A).

Interestingly, Vlcs increased the palmitoyl-CoA synthetase activity of transfected cells 3.5-fold from 14.4 nmol of C16:0/20 min/mg of protein to 51.4 nmol of C16:0/20 min/mg of protein (Fig. 2B). This LCS activity was similar in magnitude to the amount in COS-1 cells transfected with a long-chain synthetase, rat long-chain acyl-CoA synthetase (rACS-1) (Fig. 2B). The increase in LCS activity imparted on COS-1 cells by Vlcs or rat ACS-1 increased the palmitoyl-CoA synthetase activity to a similar extent.

Mouse Liver Fractionation

Linear 35-ml Nycodenz gradients (15–40%) were prepared from 15% (w/v) Nycodenz in buffer containing 0.25 M sucrose, 1 mM MOPS, pH 7.0, and 1 mM EDTA (SME buffer) and 40% (w/v) Nycodenz in MOPS and EDTA only. Gradients were stored overnight undisturbed at 4 °C. Freshly dissected mouse liver (8 weeks old) from male X-ALD or wild type mice were minced and homogenized with one stroke in a Potter-Elvehjem loose pestle homogenizer in cold SME buffer, 10 volumes (ml) to liver weight (g). Nuclei were removed by centrifugation at 3000 × g at 4 °C for 30 s. Equal amounts of X-ALD and wild type supernatant (about 5 ml) were loaded on separate gradients and centrifuged at 29,700 rpm in a Beckman VTi 50 ultracentrifuge for 76 min at 4 °C. Two-milliliter fractions were collected starting from the bottom of the gradient, assayed for catalase activity, and stored at −80 °C for analysis by Western blot and enzyme assays.

**Northem Blot Analysis of Vlcs**—Fig. 3 shows a Northern blot analysis of mouse tissue mRNA probed with radiolabeled cDNA for Vlcs and β-actin. Vlcs message (Fig. 3A) is 2.7 kb as deter-
mined by comparison with RNA species of known length (data not shown). For comparison, the transcript size of the human homologs of Vlcs is 2.4 kb (35). Vlcs message is detected most easily in liver and kidney and, upon longer exposure, in brain and heart. The presence of Vlcs message in brain and heart differs from one previous report (36) but agrees well with another, where the Vlcs transcript is highly expressed in liver and kidney and to a lesser extent in brain and testis (37). In addition to these previous observations, we detect Vlcs message in skeletal muscle, adrenal glands, liver, and brain by RT-PCR (Fig. 3B).

VLCS Activity in Mouse Tissues—Since Vlcs expression is not very strong in brain and adrenal tissues where VLCFA metabolism is expected to be high, wild type mouse tissues were assayed for total VLCS activity. Fig. 3C illustrates the relative amount of VLCS activity in liver, kidney, brain and to a lesser extent in brain and testis (37). In addition to these previous observations, we detect Vlcs message in skeletal muscle, adrenal glands, liver, and brain by RT-PCR (Fig. 3B).

Subcellular Localization of Vlcs—Organelles from mouse liver homogenate were separated by density gradient centrifugation, and peroxisome- and microsome-containing fractions were identified by Western blot analysis with organelle-specific marker antibodies, anti-human PEX14 (peroxisomes; Fig. 4B, second panel) and anti-calreticulin (microsomes; Fig. 4B, third panel). Gradient fractions were assayed for VLCS activity and Vlcs protein. The peroxisome-containing fractions had the highest VLCS-specific activity, but microsomes contain a much greater percentage of the total VLCS activity in the gradient (Fig. 4A). A polyclonal antibody was raised to Vlcs and used to locate this protein in these fractions by Western blot analysis. Vlcs was present in the fractions with VLCS activity (i.e. peroxisomes and microsomes (Fig. 4B, fractions 1-4 and 7-16)) in amounts corresponding to the VLCS activity. Similar experiments on kidney tissue gradients also detect Vlcs protein in both peroxisomal and microsomal fractions (data not shown). Antibodies specific for Vlcs detect a doublet near 70.5 kDa in Western blot analyses of these fractions (Fig. 4B, top panel). Microsomal fractions (fractions 7-16) contain relatively more of the larger species than the smaller; peroxisomal fractions had more of the smaller (fractions 1-4). However, any fraction with detectable Vlcs has some of both species. Similar results are observed in kidney gradient fractions (data not shown). Anti-Vlcs antibodies also detect doublets in Western blot analyses of

FIG. 1. Mouse very long-chain acyl-CoA synthetase cDNA encodes a 620-amino acid protein closely related to rat VLCS and human Vlcs. Protein sequences of mouse Vls, rat VLCS, and human Vlcs have been aligned employing the ClustalW algorithm (47). Predicted AMP-binding motifs as predicted by PROSITE (48) are underlined, and VLCS domains are indicated by a wavy underline (18). A potential endoplasmic reticulum signal sequence as predicted by PSORT II (31) is indicated by italics, and a possible peroxisomal targeting signal 1 targeting signal is shaded at the C terminus.
unfractionated mouse tissue extracts from liver, kidney, testis, and adrenal gland (see Fig. 6).

The origin of this Vlcs doublet is not clear. Mouse liver homogenates were incubated at 37 °C for several hours to see if the smaller species was an artifact of Vlcs degradation, but the ratio of the two species was unchanged. In vitro transcription and translation of Vlcs cDNA using a rabbit reticulocyte lysate system produced a single protein closest in size to the larger species. Incubation of this in vitro transcribed and translated protein with peroxisomes or microsomes did not alter its size (data not shown).

Vlcs Expressed in X-ALD Mouse Fibroblasts Cell Line 140 Improves VLCFA β-Oxidation—To determine if the expression of Vlcs affects peroxisomal VLCFA β-oxidation, X-ALD mouse fibroblasts derived from adult skin (line 140) were transiently transfected with vector only, Vlcs, human ABCD1, or both and assayed for C24:0 β-oxidation. Cells transfected with vector only have VLCFA β-oxidation activity of 0.15 nmol of C24:0/h/mg of protein, but cells transfected with Vlcs cDNA increase their VLCFA β-oxidation ability to 0.23 nmol of C24:0/h/mg. This is similar to the amount of correction achieved with human ABCD1 mRNA, 0.25 nmol of C24:0/h/mg of protein. The combined transfection of mouse X-ALD 140 cells with Vlcs and ABCD1 cDNA results in approximately the same increase in VLCFA β-oxidation (0.26 nmol of C24:0/h/mg of protein), as seen with either alone.

Immunohistochemical localization of Vlcs and ALDP in these cells shows that both proteins are in punctate peroxisomal structures (Fig. 5, B–E).

Improvement of X-ALD Mouse Fibroblast VLCFA β-Oxidation by Vlcs Depends on Its Peroxisomal Targeting—The ability of Vlcs to target peroxisomes and improve VLCFA β-oxidation in mouse X-ALD cells was unexpected. Human X-ALD fibroblasts transfected with human VLCs showed no improvement in VLCFA β-oxidation (19), and VLCs protein targeted the endoplasmic reticulum.2 However, the transfection efficiencies in human cells (20%) were much higher than in mouse 140 cells (8%) and could account for the difference. We determined that transfection efficiencies in X-ALD mouse embryonic fibroblasts were much better (30%) than X-ALD mouse fibroblasts line 140 and repeated the experiment in this line in an attempt to reconcile the conflicting mouse and human data.

X-ALD embryonic fibroblasts transiently transfected with vector alone, Vlcs cDNA, human ABCD1 cDNA, or both were assayed for their ability to β-oxidize C24:0. Unlike our results in the X-ALD mouse 140 line (Fig. 5A), only ABCD1-transfected cells showed any improvement in C24:0 β-oxidation (Fig. 5F). VLCFA β-oxidation was unaffected in cells transfected with Vlcs cDNA. VLCFA β-oxidation in ABCD1/Vlcs double-transfected cells did not improve more than in ABCD1-transfected cells.

2 P. A. Watkins, unpublished observations.
and microsomes, respectively. and anti-calreticulin antibodies were used as markers for peroxisomes. Western blot analysis (Fig. 3). The same fractions were subjected to Western blot analysis (B) with anti-Vlcs antibody. Anti-human PEX14 and anti-calreticulin antibodies were used as markers for peroxisomes and microsomes, respectively.

Unlike the 140 cell line, where Vlcs targets to peroxisomes, in embryonic fibroblasts Vlcs targets a perinuclear reticular compartment presumed to be the endoplasmic reticulum (Fig. 5, G and I). Thus, the difference in the effect of Vlcs on VLCFA β-oxidation is not simply the result of overexpression, but it requires targeting to the peroxisomes. It is unclear why Vlcs targets the peroxisome in one cell line and not the other, but it may be related to the transformation efficiencies of the different cell lines. Whatever the cause, the difference is not explained by the presence or absence of ALDP. In addition, the co-expression of Vlcs and ABCD1 does not change Vlcs targeting to peroxisomes.

The Expression of Vlcs Is Not Changed in X-ALD Mouse Tissues—Western blot analysis with anti-Vlcs antibody detects Vlcs in several wild type and X-ALD mouse tissues (Fig. 7). The amount and tissue location of Vlcs are unchanged in the X-ALD mouse. High levels of Vlcs are present in liver and kidney, whereas less is found in adrenal glands and testis of both X-ALD and wild type mice. Vlcs mRNA was also detected in most of these tissues (Fig. 3). No Vlcs was detected in brain or heart of either X-ALD or wild type mice. Relative amounts of both Vlcs protein species are unchanged in all X-ALD mouse tissues as compared with wild type.

X-ALD Mouse Liver Peroxisomes Have Normal Levels of Vlcs and VLCS Activity—Because decreased peroxosomal VLCS activity has been demonstrated in fibroblasts from X-ALD patients and linked to the decrease in VLCFA β-oxidation and increase in VLCFA, we examined VLCS activity in peroxisomal fractions from X-ALD mouse livers, expecting to observe the same decrease in peroxosomal VLCS activity. Livers taken from male 8-week-old wild type and X-ALD mice were homogenized and separately fractionated by density gradient centrifugation. Catalase activity and the presence of PEX14 on Western blot analysis identified peroxisomal fractions. Fractions were assayed for overall VLCS enzyme activity and standardized by either volume or catalase activity. By either standard, the amount of VLCS activity is equivalent in corresponding wild type and X-ALD mouse liver peroxisomal fractions (Fig. 7, B and C), indicating that the absence of ALDP has no effect on VLCS activity in mouse liver peroxisomes.
Fractions were also assayed by Western blot analysis for Vlcs using anti-Vlcs antibody (Fig. 7A). A comparison of X-ALD and wild type peroxisomal fractions (as indicated by PEX14 antibodies) fails to reveal a dramatic difference in the amount of Vlcs. Thus, the targeting of Vlcs is not dependent on the presence or absence of ALDP.

DISCUSSION

The biochemical phenotype of X-ALD, decreased VLCFA β-oxidation, and peroxisomal VLCS activity implies an interaction between ALDP and Vlcs. Co-transfection experiments in human fibroblasts have suggested a functional but not a physical interaction between ALDP and Vlcs, since Vlcs localization was unaffected in X-ALD cells, but an improvement in VLCS β-oxidation in these cells was not achieved with Vlcs expression alone (19). To examine the nature of the ALDP/Vlcs interaction, we identify an appropriate VLCS enzyme in mouse and show that its expression and the overall VLCS activity in the X-ALD mouse is unchanged.

The predicted protein sequence of mouse Vlcs is nearly identical to rat and human VLCS protein sequences (Fig. 1). The rat gene was identified after isolation of its protein from liver peroxisomes, and the human protein has been shown to localize to both ER and peroxisomes (17, 19). We show here that mouse Vlcs has VLCS activity when expressed in COS-1 cells (Fig. 2), that the relative VLCS to LCS activity in these cells is similar to rat and human VLCS enzymes, and that Vlcs localizes to peroxisomes and the ER (Fig. 4). In addition, Vlcs improves VLCFA β-oxidation in X-ALD cells when targeted to peroxisomes (Fig. 5). We conclude that it is the functional ortholog of the rat and human VLCS genes.

Our transfection of COS-1 cells shows that Vlcs encodes an enzyme with VLCS activity (Fig. 2). Cells expressing Vlcs have an increased ability to activate lignoceric acid, a saturated straight chain very long-chain fatty acid. These cells also have increased long-chain acyl-CoA synthetase activity, implying that Vlcs also accepts long-chain fatty acids as substrates. The magnitude of this long-chain activity is greater than the very long-chain activity; however, this same preference for long chains is observed for both rat and human VLCS. All VLCS enzymes identified and examined to date also have a preference for long-chain over very long-chain fatty acids (19, 22, 38, 39).

Liver and kidney have high levels of Vlcs expression; however, brain and adrenal glands, sites of X-ALD pathology, have lower levels. It was surprising to find so little of this gene expressed in brain and adrenal glands, since these tissues are sites of VLCFA metabolism and as such are expected to have high levels of VLCS activity. A comparison of total VLCS activity in wild type mouse tissues (Fig. 3C) reveals that this assumption is not true. Liver and kidney tissues have much higher VLCS activity than brain or adrenal glands. The relatively low level of VLCS activity in these tissues agrees well with the lower level of Vlcs expression. It is worth noting that VLCFA accumulate in all X-ALD mouse tissues, not just in brain and adrenal glands, implying that the biochemical abnormality exists in all X-ALD tissues and not just those that display an X-ALD pathology. Therefore, increases in VLCFA accumulation that result from loss of peroxisomal interactions of ALDP and Vlcs should be evident in all tissues.

VLCS activity has been localized to peroxisomes and microsomes but not mitochondria (40). Mouse Vls has potential targeting motifs for both of these organelles (Fig. 1) and is present in both peroxisomes and microsomes of liver and kidney (Fig. 4), yet the details of Vlcs targeting are unknown. Antibodies to mouse Vlcs recognize two protein species on Western blot, both around 70 kDa, that differ in size by ~8 kDa (Figs. 4B, 6, and 7A), and unpublished observations confirm the existence of a similar pair of proteins recognized by anti-human VLCS antibody in human liver extracts. We initially hypothesized that each species was organelle-specific. However, mouse liver peroxisomes and microsomes isolated on subcellular fractionation density gradients contain both forms, although in different amounts (Fig. 4B). Peroxisomes have more of the smaller, and microsomes have more of the larger. A similar distribution of Vlcs doublet proteins between peroxisomes and microsomes is observed in kidney gradients prepared by a slightly different method. Vlcs has a putative endoplasmic reticulum signal sequence at its N terminus (Fig. 1). Cleavage of this signal could allow the potential peroxisomal targeting signal 1 (-LKL) at the C terminus to direct Vlcs more efficiently to the peroxisomal matrix and would account for the size difference between the two species. Another possibility is that Vlcs could be post-translationally modified with a phosphate or an acyl group that would change its mobility in SDS-PAGE and be enriched in microsomal or localized protein. The mechanism of Vls distribution to peroxisomes and microsomes and the origin of the Vls doublet are not clear. Since neither the localization (Fig. 7) nor the amount (Fig. 6) of the two forms changes in the absence of ALDP, VLCs distribution and expression are independent of ALDP.

X-ALD fibroblasts have 30% of wild type VLCFA β-oxidation activity and the expression of ABCD1 cDNA rescues this deficiency (41–43). The transient overexpression of other peroxisomal membrane half transporters also rescues VLCFA β-oxidation in X-ALD cells (3, 43–45). This paper demonstrates that Vlcs overexpressed in X-ALD fibroblasts improves VLCFA β-oxidation but only when targeted to peroxisomes. VLCFA β-oxidation activity increases in an adult mouse X-ALD fibroblast line transfected with Vlcs where Vlcs localized to peroxisomes (Fig. 5). However, in mouse X-ALD embryonic fibroblasts transfected with Vlcs, Vlcs targets the endoplasmic reticulum, not peroxisomes, and VLCFA β-oxidation is unaffected (Fig. 5F). These observations imply that the reduction in VLCFA β-oxidation in X-ALD fibroblasts is a result of reduced
peroxisomal VLCS activity and corroborate reports of decreased peroxisomal VLCS activity and VLCFA β-oxidation in human X-ALD fibroblasts.

VLCS activity is decreased in the peroxisomes of X-ALD patient fibroblasts. This decrease is postulated to result in the decrease in saturated VLCFA β-oxidation and accumulation of VLCSA in these patients (6–9). The ABCD1 gene encodes a peroxisomal ABC half transporter, ALDP, with no VLCS activity. It has been hypothesized that the lack of ALDP may affect the expression or localization of VLCS by transporting VLCS protein into the peroxisome, tethering VLCS protein to the peroxisomal membrane, or otherwise targeting VLCS to the peroxisome (4). We have examined Vls in the X-ALD mouse and shown that Vls is unchanged in all aspects tested. Contrary to a previous report employing a different method (46), this study detected no change in the amount or distribution of Vls in X-ALD mouse tissues (Figs. 6 and 7). Although X-ALD mouse mouse liver accumulates VLCFA in excess of wild type, peroxisomes from X-ALD mouse liver have the same amount of Vls as wild type peroxisomes and normal amounts of VLCS activity (Fig. 7), indicating that ALDP is not required for maintaining peroxisomal VLCS activity in mouse liver. Since ALDP is not necessary for the expression of Vls in tissues or for its subcellular distribution, ALDP does not transport VLCS into the peroxisome, tether Vls to the peroxisome, or play any significant part in Vls targeting. Our observations in mouse agree well with observations made in human fibroblasts by Steinberg et al. (19).

In contrast to observations of decreased peroxisomal VLCS activity in human fibroblast peroxisomes (10–12), X-ALD mouse liver peroxisomes have normal amounts of VLCS activity (Fig. 7). The initial report of reduced X-ALD peroxisome VLCS activity from Hashmi et al. (10) discussed the importance of intact peroxisomes in the VLCS assay. They noted that when the peroxisome membrane was compromised, peroxisomal VLCS activity was unchanged in X-ALD, implying a role for ALDP in transport of substrate for peroxisomal VLCS. Since this is the result we observed in mouse liver, it may be that the peroxisomes in our experiment were leaky. Measurements of several peroxisomal marker enzyme activities in X-ALD mouse liver peroxisome fractions from this experiment and others (data not shown) demonstrate that at least with respect to these enzymes (thiolase, acyl-CoA oxidase, and catalase), the peroxisomes are intact. We employed other methods of tissue homogenization and VLCFA β-oxidation assay conditions and observed the same results. We conclude that peroxisomal VLCS activity in X-ALD mouse liver is unchanged and not an artifact of peroxisomal damage.

It may be that the manifestations of any Vls/ALDP interaction differ in mice and humans. Given that both VLCS activity and VLCFA β-oxidation are deficient in human cells as a result of abnormal ALDP, normal VLCS activity in mice may account for the lack of a severe X-ALD mouse phenotype. This is suggested by the ability of Vls cDNA complementation to correct β-oxidation deficiency in X-ALD fibroblasts. In the X-ALD mouse, all aspects of Vls expression and function are unaffected by the absence of ALDP. It is possible that the lack of a severe cerebral phenotype in the X-ALD mouse is due in part to the relatively low level of VLCS activity in mouse brain. If cerebral disease is indeed caused by decreased peroxisomal VLCS activity in the brain due to loss of ALDP in humans and mouse brain has very little VLCS activity to lose, then X-ALD mice may not model the expected change in this activity and fail to develop cerebral disease. Mild neurological defects described in aged X-ALD mice are postulated to be the result of prolonged exposure to the abnormal accumulation of VLCFA (16). Since VLCFA accumulate in X-ALD mice prior to birth and in all tissues (15), despite any evidence that loss of ALDP affects Vls, the accumulation and hence any clinical phenotype are unlikely to be the result of an abnormal Vls and ALDP interaction.

If reduced peroxisomal VLCS activity contributes to X-ALD pathology, then an X-ALD mouse with reduced peroxisomal VLCS activity may model X-ALD better by mimicking the biochemical abnormality observed in human cells. However, VLCFA accumulate in the X-ALD mouse liver despite normal peroxisomal activity. Additional model systems, like a Vls knockout mouse and an X-ALD/Vls double knockout mouse, will be required for the definitive elucidation of Vls interaction with ALDP and the role the two proteins may play in the pathogenesis of X-ALD.

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