Molecular Cloning of cDNA Encoding Rat Very Long-chain Acyl-CoA Synthetase*

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The cDNA encoding rat very long-chain acyl-CoA synthetase (VLACS) was cloned, using degenerative primers synthesized according to the partial amino acid sequences of the peptide fragments of the purified rat liver enzyme. The longest cDNA insert was 2972 base pairs with a 1860-base pair open reading frame encoding 620 amino acids. The calculated molecular mass of 70,692 daltons was consistent with size of the purified enzyme. In Northern blot analysis, a single band was detected at the position of about 3 kilobases, corresponding to the size of the cloned cDNA. cDNA-directed expression in Escherichia coli resulted in accumulation of expressed protein, as an inclusion body. An antibody was raised using this expressed protein to characterize the cDNA and the enzyme. The subcellular localization of VLACS in peroxisomes and microsomes was demonstrated in Western blot analysis. The specific activity and the substrate specificity of the cDNA expressed enzyme in COS-1 cells were consistent with those of the purified rat enzyme. The predicted amino acid sequence of VLACS had a high sequence similarity to fatty acid transport protein (Schaffer, J. E., and Lodish, H. F. (1994) Cell 79, 427–436), and was considered to have domains for adenylation and thioester formation. The entire structure of VLACS was dissimilar to that of long-chain acyl-CoA synthetase (Suzuki, H., Kawarabayashi, Y., Kondo, Y., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T., and Yamamoto, T. (1990) J. Biol. Chem. 265, 8681–8685), except for the domains.

In mammals, very long-chain fatty acids (VLCFAs) containing more than 22 carbon atoms are oxidized mainly in peroxisomes but not in mitochondria (1, 2). Activation of VLCFAs to their CoA thioesters is apparently a crucial step and this step is catalyzed by very long-chain acyl-CoA synthetase (VLACS). VLACS activity localizes in peroxisomes and microsomes but not in mitochondria (3).

X-linked adrenoleukodystrophy (X-ALD) is the most common hereditary disease among the peroxisomal diseases and is characterized by progressive demyelination of the white matter and by adrenocortical insufficiency (4, 5). A marked biochemical deterioration in subjects with this disease is a defect of oxidation of VLCFAs. Since fibroblasts from these patients showed a very low activity in formation of very long-chain fatty acyl-CoAs, it has been considered that a new synthetase, VLACS being different from long-chain acyl-CoA synthetase (6) must be present, and that the phenotypic etiology of X-ALD may be derived from a defect of this enzyme (7–9). The adrenoleukodystrophy protein (ALDP) gene has been cloned, and it was thought to be a disease-causing candidate for X-ALD (10). Various point mutations, deletions, and insertions in the gene have been identified in these patients (11–21). Retroviral transfer of normal ALDP cDNA into patient’s fibroblasts enhanced VLCFA oxidation activity to the level found in control fibroblasts (22). These data indicate that ALDP is responsible for at least a part of X-ALD. The function of ALDP and the relation between ALDP and VLCFA oxidation activity are unknown. It is thus important to examine the exact localization in cells and the role of VLACS, catalyzing a crucial reaction in peroxisomal VLCFA oxidation, and the relation between ALDP and VLACS, including possible participation of 70-kDa peroxisomal membrane protein (23), assigned as one of the ATP-binding cassette transporters (10, 23). VLACS was recently purified from rat liver peroxisomal membrane (24). Although our attempts to prepare a specific antibody were unsuccessful, the purified VLACS did provide partial amino acid sequence data of protease-digested polypeptides, and this made it feasible to begin the cloning of VLACS cDNA. We report here the isolation and sequencing of cDNA encoding rat VLACS, a comparison of the sequence to those of the related enzymes, the subcellular localization, and the biosynthesis of functional VLACS in COS-1 cells.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and MEGALABEL™ were purchased from Takara Shuzo (Kyoto, Japan). [γ-32P]ATP, [α-32P]dCTP, nylon membrane (Hybond N™), and Multiprime DNA Labeling System™ were obtained from Amersham (Amersham Co., Amersham, United Kingdom). A clofibrate-induced rat liver λgt 11 cDNA library constructed with oligo(dT) primer, provided by Dr. Frank J. Gonzalez at the National Institutes of Health (25), and a di(2-ethylhexyl)phosphate (DEHP)-treated rat liver λgt 11 cDNA library constructed with random primers were used for the screening. [1-14C]Palmitic acid (2 GBq/mmol) and [1-14C]Lignoceric acid (2 GBq/mmol) were purchased from CEA (France). Palmitic acid and lignoceric acid were obtained from Sigma. Nycodenz was purchased from Nycomed AS (Oslo, Norway). Oligonucleotide probes for screening and
primers for polymerase chain reaction (PCR) or sequencing were synthesized by a Biotechnology Company (36). Two primers were synthesized according to the partial amino acid sequences of VLACS peptide fragments described by Uchida et al. (24). Probe 1, 5'-ATCTGGCCTGCTTACAC-3', reverse primer; 5'-ATTGCGGCCGCTACGGC-TTCAGGTCTTAT-3'. The PCR product was inserted into the NotI restriction site of a mammalian expression vector pCDM8 (pCDM8 VLACS) (Invitrogen, NV Leek, The Netherlands). The recombinant plasmid (pCDM8VLACS) was extracted from E. coli strain MC106P3, using a Qiagen Plasmid MidiKit™ (Qiagen, Hilden, Germany). Five mg of the plasmid was transfected into COS-1 cells (34) at 60–70% confluence in 6-cm diameter dishes, by the calcium-phosphate procedure (35). The transfected cells were incubated at 37 °C for 16 h, and then cultured in fresh Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 10% (v/v) fetal calf serum. After 48 h, the cells were harvested and then suspended in 300 μl of a solution containing 50 mM Tris-HCl (pH 8.5), 100 mM NaCl, 1 mM ATP, 1 mM MgCl2, 0.1% Triton X-100. The suspension was sonicated, kept on ice for 30 min, and then used for the assay of acyl-CoA synthetase activity toward [1-14C]palmitic acid and [1-14C]lignoceric acid.

**Data Base Searching**—For the homology search for the amino acid sequence of VLACS, we used FASTA and BLAST programs through the DNA Data Bank of Japan.

**Other Methods**—The bands detected in Northern and Western blot analyses were quantified using Densitograph Version 3 (ATTO Co., Tokyo, Japan). Protein concentration was determined using a BCA protein determination kit™ (Pierce, Rockford, IL). For the DEHP treatment, rats were provided laboratory chow containing 2% (w/w) DEHP for 2–3 weeks.

### RESULTS

**Isolation of cDNAs Encoding VLACS**—Approximately 8 x 10⁵ independent plaques in a λgt 11 cDNA library constructed with oligo(dT) primer were screened with probe 2. After tertiary screening, 2 positive clones were obtained. The longer clone about 1.7 kb in length (VLACS 1) was subcloned into pBluescript II SK+ and then sequenced. VLACS 1 encoded a 458-amino acid polypeptide and its amino acid sequence contained the two internal sequences, determined by Uchida et al. (24). The shorter clone was confirmed to be part of the longer one, by sequencing. In the next step, about 6 x 10⁵ independent clones in a λgt 11 cDNA library constructed with random primers were screened, using probe A. A clone, about 0.9 kb in length (VLACS 2) was obtained and was found to have sequence overlapping with that of VLACS 1. We then screened about 6 x 10⁵ independent clones in the oligo(dT)-constructed cDNA library, using probe B. The longest clone, about 3.0 kb in length (VLACS 3), was then finally obtained. The insert was subcloned into EcoRI-digested pBluescript II SK+ vector and sequenced.

**Nucleotide Sequence and Putative Amino Acid Sequence of VLACS**—Fig. 1 shows the nucleotide sequence and the putative amino acid sequence of VLACS 3. Three parts of the deduced amino acid sequence coincided with the NH₂-terminal sequence and the two internal sequences, determined using the purified VLACS (24). The nucleotide sequence consisted of 2972 bp, including 794 bp at the 5'-noncoding region, followed by the 1860-bp open reading frame encoding 620 amino acids, and 318 bp at the 3'-noncoding region. The nucleotide sequence flanking the initiation codon, GCCACCGCGCATG, corresponded to the consensus sequence of the eukaryotic initiation site (36). A consensus polyadenylation signal, AATAAA, was located 13 bp upstream from the poly(A) tail.

The calculated molecular mass of VLACS was 70,692 Da, a value in good agreement with that of the purified VLACS (70 kDa), determined by SDS-polyacrylamide gel electrophoresis (PAGE). In the deduced amino acid sequence, we found no peroxisomal targeting signal-1, a carboxy-terminal tripeptide motif (37), and peroxisomal targeting signal-2, a cleavable sequence located at the amino terminus (38).

**Size of VLACS mRNA**—Eight mg of total RNA extracted from the rat liver was electrophoresed, transferred onto a nylon membrane, and hybridized with VLACS 3. A single band about 3 kb long was detected (Fig. 2, lane 1), this size agreed with that of the cloned cDNA (VLACS 3). The same results were...
obtained when using probes A and B, respectively (data not shown). The VLACS mRNA content in the DEHP-treated rat liver was increased about 5-fold over that in the control rat liver (Fig. 2, lane 2).

Expression of VLACS-His in E. coli—A fusion protein of VLACS-His was expressed in BL21, as described under “Experimental Procedures.” Two h after addition of IPTG, an aliquot of the culture was subjected to SDS-PAGE. An expressed protein band was seen for only BL21, transformed with pET16b VLACS-His and treated with IPTG (Fig. 3, lane 3). As the expressed protein was difficult to solubilize due to formation of inclusion bodies, the protein was solubilized with 6M urea, then purified on affinity chromatography with a nickel column. The purified fusion protein showed a slightly slower migration mobility than that of purified rat VLACS (Fig. 3, lanes 4 and 5).

The expressed protein was used to prepare an antibody. The raised antibody was confirmed to cross-react with purified VLACS (Fig. 4 A). Immunotitration of the purified native VLACS was then examined, using a 5-fold volume of an equivalent amount of the antibody, estimated by immunoprecipitation experiments, under denaturing conditions, as described below. There was no formation of a VLACS-antibody complex.

Fig. 1. Nucleotide sequence of the VLACS cDNA and the predicted amino acid sequence. Underlined amino acid sequences matched the partial amino acid sequences determined for the purified enzyme. The square box indicates in-frame stop codon. The putative polyadenylation signal has a double underline.
fore, both antibodies are useful to study subcellular localization of VLACS. The expression plasmid (pCDM8 VLACS) with the entire coding region of VLACS and the vector (pCDM8) itself were transfected into COS-1 cells, respectively. The lignoceryl-CoA synthetase activity in lysate of the cells transfected with vector alone was 3.7 ± 0.7 microunits/mg, much the same value as that in control cells. The activity in a lysate of the cells transfected with pCDM8 VLACS increased about 50-fold (179 ± 23 microunits/mg). The palmitoyl-CoA synthetase activities in the cells transfected with pCDM8 and pCDM8 VLACS were 115 ± 19 and 302 ± 33 microunits/mg, respectively. The amount of expressed VLACS was then quantified by immunoblot analysis (Fig. 5). The specific activities as lignoceryl-CoA synthetase and palmitoyl-CoA synthetase were estimated to be 45 and 50 milliunits/mg, respectively, and the ratio of palmitoyl-CoA synthetase activity to lignoceryl-CoA synthetase activity was 1.1. The two specific activities and the ratio of the purified rat VLACS were 39 and 58 milliunits/mg and 1.5, respectively (24), indicating that the catalytic properties of the cDNA-expressed VLACS are similar to those of the purified preparation.

Subcellular Localization of VLACS—VLACS were considered to localize in peroxisomes and microsomes but not in mitochondria, as deduced from enzyme activities in subcellular fractions (3). Immunoblot analysis was then performed using rat liver subcellular fractions; peroxisomal, microsomal, and mitochondrial fractions. As shown in Fig. 6, the immunoreactive signal for VLACS was clearly observed in both peroxisomal and microsomal fractions but not in mitochondrial fractions. On the other hand, the signal for LACS was found in all the three subcellular fractions. A doublet of immunoreactive VLACS was observed in both peroxisomal and microsomal fractions. Although in Fig. 6 the doublet bands are apparent in microsomes but not peroxisomes, the ratio of the signal intensities of the two bands was not the same among different preparations of these fractions. Therefore, the smaller band may be produced by proteolytic modification of the larger one in the liver.

Contents of mRNA and Protein of VLACS in Rat Organs—Total RNA was extracted from rat brain, liver, kidney, heart, lung, and skeletal muscle. A band, about 3 kb, was found only in liver and kidney, on Northern blot analysis (Fig. 7A). VLACS mRNA was electrophoresed.
A full-length VLACS cDNA clone was obtained from a rat liver cDNA library, using degenerative primers designed based on partial amino acid sequences of peptide fragments from rat liver VLACS. This clone was confirmed to encode VLACS, sequence analysis revealed the existence of the regions corresponding to the peptide fragments from the purified VLACS, the calculated molecular mass of the coding region corresponded to that of the purified VLACS, the size of mRNA in Northern blot analysis was similar to that of the full-length clone, the DEHP-induction motif of the mRNA was similar to that of VLACS activity in rat liver (24), the antibody raised against cDNA-expressed protein in E. coli specifically cross-reacted with the purified VLACS, and the specific activities and the substrate specificity of the cDNA-expressed protein in COS-1 cells were similar to those of the purified VLACS, respectively.

On considering subcellular localization of acyl-CoA synthetases, distributions of LACS and VLACS seem to be important. LACS was found to localize in peroxisomes, mitochondrial outer membranes, and microsomes (39). VLACS was estimated to be present in peroxisomes, but absent in mitochondria due to subcellular distribution of lignoceroyl-CoA synthetase activity (24). In the present study, VLACS was detected only in peroxisomes and microsomes (Fig. 6), and the calculated specific lignoceroyl-CoA synthetase activities in peroxisomal and microsomal fractions were much the same value of the purified VLACS, which is compatible with previous observations (24).

VLACS has been considered a peroxisomal membrane protein (24), as well as 70-kDa peroxisomal membrane protein and ALDP, therefore, VLACS is expected to have a targeting signal into peroxisomes. In mammalian peroxisomal proteins, peroxisomal targeting signals 1 and 2 were identified (37, 38) and they seem to be specific to peroxisomal matrix protein. VLACS, as well as 70-kDa peroxisomal membrane protein and ALDP, did not have these signals, hence a hitherto unknown targeting signal(s) specific to peroxisomal membrane proteins may exist. In hydropathy analysis (45), VLACS has no typical transmembrane region (data not shown), unlike 70-kDa peroxisomal membrane protein and ALDP. These results can serve as a good base for future projects such as a topological study of VLACS on peroxisomal membrane proteins and interaction of VLACS with ALDP and other peroxisomal membrane proteins, and this will be important to understand the mechanism related to deficiency of the peroxisomal VLACS activity in X-ALD fibroblasts (8). VLACS cDNA and the anti-VLACS antibody will be good tools for such studies.

VLACS, LACS, and FATP seem to belong to the firefly luciferase family as there are two specific domains. Interestingly, VLACS has high sequence similarity to FATP (Fig. 8). However, there was no sequence homology in the NH₂-terminal region between VLACS and FATP (1–41 amino acids in VLACS and 1–69 amino acids in FATP, Fig. 8). The NH₂-terminal sequence of FATP (Fig. 8, underlined) is thought to encode a signal sequence to cytoplasmic membranes. The poor homology seems to reflect the difference concerning subcellular localization of the two proteins.

Proteins in the firefly luciferase family generally show a high sequence divergence in their entire region, except for the two conserved domains, if they have different functions (42). Since VLACS and FATP have different functions, it is the first case in the family that a high similarity through broad regions was
observed between the two proteins with different functions. An additionally cloned clone with FATP cDNA was shown to encode mouse LACS. Interestingly, this LACS was found to have function not only as activation of fatty acids but also as active uptake of long-chain fatty acids (41). Attention needs to be given to whether VLACS also functions as VLCFAs transporter.

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