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Cloning of Human Very-Long-Chain Acyl-Coenzyme A Dehydrogenase and Molecular Characterization of Its Deficiency in Two Patients

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Summary

Two overlapping cDNA clones (1,991 bp and 736 bp, respectively) encoding the precursor of human mitochondrial very-long-chain acyl-coenzyme A dehydrogenase (VLCAD) were cloned and sequenced. The cDNA inserts of these clones together encompass a region of 2,177 bases, encoding the entire protein of 655 amino acids, including a 40–amino acid leader peptide and a 615–amino acid mature polypeptide. PCR-amplified VLCAD cDNAs were sequenced in cultured fibroblasts from two VLCAD-deficient patients. In both patients, a 105-bp deletion encompassing bases 1078–1182 in VLCAD cDNA was identified. The deletion seems to occur due to exon skipping during processing of VLCAD pre-mRNA. This is the first demonstration of a mutation causing VLCAD deficiency. Quantitative cDNA expression of normal human VLCAD was performed in the patients' fibroblasts, using vaccinia viral system, which demonstrated that the deficiency of the normal VLCAD protein causes impaired long-chain fatty acid β-oxidation activity in the patients' fibroblasts. In patient fibroblasts, raising VLCAD activity to ~20% of normal control fibroblast activity raised palmitic acid β-oxidation flux to the level found in control fibroblasts, which may offer important information for the rational design of future somatic gene therapy for VLCAD deficiency.

Introduction

In mammals, fatty acids are oxidized by two major systems, namely the mitochondrial and peroxisomal β-oxidation systems. The first step in the mitochondrial and peroxisomal β-oxidation cycle is catalyzed by acyl-coenzyme A (CoA) dehydrogenase and acyl-CoA oxidase (AOX), respectively. Four distinct acyl-CoA dehydrogenases, i.e., short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), and very-long-chain acyl-CoA dehydrogenase (VLCAD), have been identified in the mitochondrial β-oxidation system (Rhead 1991; Coates and Tanaka 1992*a*, 1992*b*; Engel 1992; Hale and Bennett 1992; Izai et al. 1992). cDNAs encoding human SCAD and MCAD have been cloned, and mutation analyses of the two deficiencies, particularly of the latter, have been extensively performed (Coates et al. 1988; Naito et al. 1990; Yokota et al. 1991; Tanaka et al. 1992; Andresen et al. 1994). On the other hand, mutations of the LCAD and VLCAD genes underlying their deficiencies have not yet been identified for two reasons. First, VLCAD was identified only 2 years ago (Izai et al. 1992), and the primary sequence of human VLCAD was hitherto unknown. Second, there has been clinical and biochemical uncertainty concerning the identity of the LCAD and VLCAD deficiencies, because of the overlap of the substrate specificity of the two enzymes (Hale et al. 1985, 1990*a*, 1990*b*; Indo et al. 1991*a*).

In the last two years, several patients with inherited VLCAD deficiency have been identified by this and other laboratories (Aoyama et al. 1993; Bertrand et al. 1993). Also, in three patients previously diagnosed as having LCAD deficiency on the basis of activity assays, the LCAD sequence was found to be perfectly normal, while immunoblot analysis indicated that these patients were in fact deficient in VLCAD rather than in LCAD (Yamaguchi et al. 1993). Recently, we reported detailed biochemical and immunochemical studies of seven patients having VLCAD deficiency (Aoyama et al., in press).

Clinically notable features of these patients were also summarized: all patients were found to have hepatocellular dysfunction, severe lipid storage in organs, and cardiac disease; at least four patients presented with hypertrophic cardiomyopathy. Additionally, the cDNA-encoding rat VLCAD has been cloned, and the im-

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portant role of VLCAD as a rate-limiting enzyme in long-chain fatty acid β-oxidation system has been demonstrated (Aoyama et al. 1994*c*).

We report here the isolation and sequencing of cDNAs encoding the entire precursor of human VLCAD, the first identification of mutations in the VLCAD gene in two patients with VLCAD deficiency, and the restoration of long-chain fatty acid β-oxidation activity in the patients' fibroblasts by means of cDNA-expression of normal human VLCAD.

Material and Methods

Material

An antibody that reacts with human VLCAD was prepared using purified human VLCAD protein (Aoyama et al., in press). cDNAs encoding rat VLCAD (Aoyama et al. 1994*c*) and human AOX (Aoyama et al. 1994*b*) were prepared as described, respectively. Human liver cDNA libraries prepared in the λgt 11 expression vector were obtained from Dr. Frank J. Gonzalez of the National Institutes of Health (Aoyama et al. 1989*c*). Reagents used for both DNA sequencing and amino acid sequencing of proteolytic peptides were purchased from Applied Biosystems. Vaccinia virus strain WR, pSC 11, and human TK^- 143 cells were obtained from Dr. Bernard Moss of the National Institutes of Health. CV-1 and hepatoma H4IIEC3 cells were from the American Type Culture Collection. Electron transfer flavoprotein (ETF) was prepared from rat liver mitochondria (Furuta et al. 1981). [1-^{14}C]Palmitic acid was obtained from American Radiolabeled Chemicals. Sodium 2-[5-(4-chlorophenyl)pentyl]-oxirane-2-carboxylate (POCA) was a generous gift from Byk Gulden Pharmazeutika.

Source of the Cells and Culture Method

The case histories of the two VLCAD-deficient cells, patient 2 and 3, together with five others (Aoyama et al., in press), have been reported elsewhere (Aoyama et al. 1993; Yamaguchi et al. 1993). Skin fibroblasts were cultured in the medium containing Dulbecco's modified Eagle's medium, 10% (v/v) FCS, 0.1 mM nonessential amino acids, 1 × antibiotic-antimycotic solution (GIBCO/BRL Oriental) and 4.5 mg D-glucose/ml.

cDNA Cloning Method

Four-hundred thousand independent plaques from the human liver cDNA library were screened using the rat VLCAD cDNA (RVL-57) (Aoyama et al. 1994*c*) as a probe (Maniatis et al. 1982). After positive clones were obtained, another human liver cDNA library was screened using the longest cDNA insert in previously identified positive clones as a probe. Phage DNA of positive clones was then prepared using the plate-lysate method (Maniatis et al. 1982). The cDNA insert from an *Eco*RI digest of the phage DNA was subcloned into pTZ18U plasmid vector (TOYOBO). Plasmid DNA was isolated in large scale, using DNA preparation kits (Qiagen).

DNA Sequencing

Serial sequence deletions of the clone were carried out via exonuclease III digestion, using Kilo-sequence deletion kits (Takara), on the basis of the method described by Henikoff (1984). DNA sequencing was performed directly on the cDNA insert in the plasmid by the dideoxy-sequencing method (Sanger et al. 1977) using Dye primer cycle sequencing kits (Applied Biosystems).

RNA Preparation and Northern Blot Analysis

Total RNA was extracted from fibroblasts, using the guanidinium isothiocyanate procedure (Chomczynski and Sacchi 1987). Twenty micrograms each of total RNA were denatured in formaldehyde/formamide and were electrophoresed in 1% agarose gel containing formaldehyde, and RNA was transferred to a nylon membrane (Hybond-N^+; Amersham) by capillary blotting (Sambrook et al. 1989). Prehybridization and hybridization were carried out according to Amersham protocol.

Amplification of cDNA by PCR

Two micrograms of total RNA were reverse-transcribed with Murine leukemia virus reverse transcriptase and random hexanucleotide primers according to manufacturer's instructions. One twentieth of the cDNA product was annealed to 1 pmol each of the sense and antisense primers, and 40 cycles of PCR reaction were performed using a DNA Thermal Cycler (Idaho Technology). Each cycle consisted of 5-s denaturation at 94°C, 2-s annealing at 52°C, and 1-min extension at 75°C. The final extension was for 10 min. The PCR products were purified by electrophoresis using 1% agarose gel. The purified fragments were directly used for DNA sequencing.

Amino Acid Sequence Analysis

Native human VLCAD protein (Aoyama et al., in press) and its proteolytic polypeptides prepared by endoproteinase Glu-C or endoproteinase Lys-C (Boehringer Mannheim) treatment were electrophoresed on 10% polyacrylamide gel containing sodium dodecyl sulfate and were transferred to a polyvinylidene difluoride membrane (Bio-Rad). Amino-terminal sequence was determined using a model 477A gas-phase sequencer (Applied Biosystems) and an in-line model 120A phenylthiohydantoin analyzer.

Vaccinia Viral Expression of VLCAD cDNA

The vaccinia virus system was used to express VLCAD cDNA. The cDNA insert was first inserted into the re-

combination plasmid pSC11 (Chakrabarti et al. 1985). The recombinant plasmid was allowed to integrate into the vaccinia virus, using methods described by Aoyama et al. (1989*a*, 1989*c*, 1990). Final identification of vaccinia virus producing VLCAD protein was carried out by immunoblotting of cell lysate protein, using a polyclonal antibody.

Other Methods

Palmitoyl-CoA dehydrogenation activities by mitochondrial acyl-CoA dehydrogenases (Aoyama et al. 1994*c*) and by peroxisomal AOX (Aoyama et al. 1994*b*; Souri et al. 1994) were measured as described elsewhere, respectively. Palmitic acid β-oxidation activity in fibroblasts was measured as described by Aoyama et al. (1994*c*).

Results

Isolation and Nucleotide Sequence of cDNAs Encoding Human VLCAD

Approximately 4×10^5 independent recombinants from a human liver cDNA library were screened using the rat VLCAD cDNA (Aoyama et al. 1994*c*) as a probe. After tertiary screening, five positive clones were isolated. One of them (HVL-1), ~2.0 kb in length, was subcloned into the *Eco*RI site of pTZ18U plasmid vector and sequenced. HVL-1 encoded a 604–amino acid peptide, which represented 92% of the entire coding region of VLCAD, lacking ~150 bases at the 5′ end of the coding region.

To obtain a full-length clone, we further screened ~2×10^5 recombinants from another human liver cDNA library, using HVL-1 as a probe, and obtained 55 positive plaques. Analysis of sizes and restriction maps of inserts from five of them indicated that one of them (HVL-2), a 736-bp cDNA, extended further than any other clones at the 5′ end. HVL-2 was subcloned into the *Eco*RI site of pTZ18U plasmid vector and sequenced. A fragment of HVL-2 digested with *Sph*I was inserted into the fragment of pTZ18U containing HVL-1 partially digested with *Eco*RI and *Sph*I to prepare the full-length clone.

The nucleotide sequence of human VLCAD cDNA is shown in Figure 1 along with the deduced amino acid sequence. The nucleotide sequence covered by HVL-1 and HVL-2 encompasses 2,177 bp, including 33 bp in the 5′-noncoding region, followed by the entire coding region of 1,965 bp and 179 bp in the 3′-noncoding region. A short poly (A) tail was present at the end of the 3′-noncoding region. The size of human VLCAD clone (2,177 bp) is similar to that (2,117 bp) of rat VLCAD (Aoyama et al. 1994*c*). As shown in figure 1, the amino-terminal sequence of native human VLCAD protein and sequences of six proteolytic polypeptides obtained by endoproteinase Glu-C, endoproteinase Lys-C or elastase digestion (total of 122 amino acid residues) perfectly match those deduced from the cDNA sequence. Only Gly-43, deduced from the cDNA sequence, was a mismatch with Asp-43, determined from the amino-terminal sequence of the native protein, probably because of individual differences. These results confirm the identity of the cDNA clone and the fidelity of the reading frame. A comparison of the predicted sequence of the precursor VLCAD to the amino-terminal sequence data of the purified mature protein showed that Ala-41 is the amino terminus and that the segment encompassing the first 40 residues constitutes the leader sequence in the precursor. When the amino acid sequence of the leader peptide was compared with that of rat VLCAD (Aoyama et al. 1994*c*), the sequence similarity is fairly high (68%), and the content of basic residues is similar, as noted in other mitochondrial enzymes (Hay et al. 1984; Matsubara et al. 1989). Both the termination residue (Try-40) of the leader peptide and the characteristic basic residue (Arg-38) locating 3 residues upstream from the cleavage site are conserved. Amino acid sequence of the mature human VLCAD protein was compared with that of rat VLCAD (Aoyama et al. 1994*c*); the sequence similarity is high (86%).

Mutation Analysis in Two Patients

Seven patients with VLCAD deficiency have been identified using anti-human VLCAD antibody in our laboratory (Aoyama et al., in press). All of the patients' fibroblasts exhibited common features, such as very low content of VLCAD protein, low VLCAD-mediated palmitoyl-CoA dehydrogenation activity, impaired palmitic acid β-oxidation flux, and poor biosynthesis of VLCAD protein. In pulse-chase experiments, smaller-size VLCAD proteins were detected in the fibroblasts from three patients (patients 2, 3, and 5). Since these three patients could possibly have a common deletion of the VLCAD protein, mutation analysis using VLCAD mRNA was performed, focusing on these three patients. However, unlike patients 2 and 3, the cDNA sample from patient 5 was not amplified at all by PCR, indicating that the mutation in his cells was clearly different from those in the two others. Patient 5 was thereby excluded from the mutation analyses described here.

Ten sense primers, each encompassing (−22)-(−3), 200-219, 402-421, 606-625, 814-833, 1023-1042, 1239-1258, 1456-1475, 1653-1672, and 1842-1861, and 10 antisense primers, each encompassing 239-220, 441-422, 645-626, 853-834, 1062-1043, 1280-1261, 1495-1476, 1692-1673, 1881-1862, and 1988-1969, were synthesized on the basis of the sequence data in figure 1. cDNA samples from control and patients 2 and 3 were, respectively, amplified by PCR using appropriate combinations of the sense and the antisense primers. As

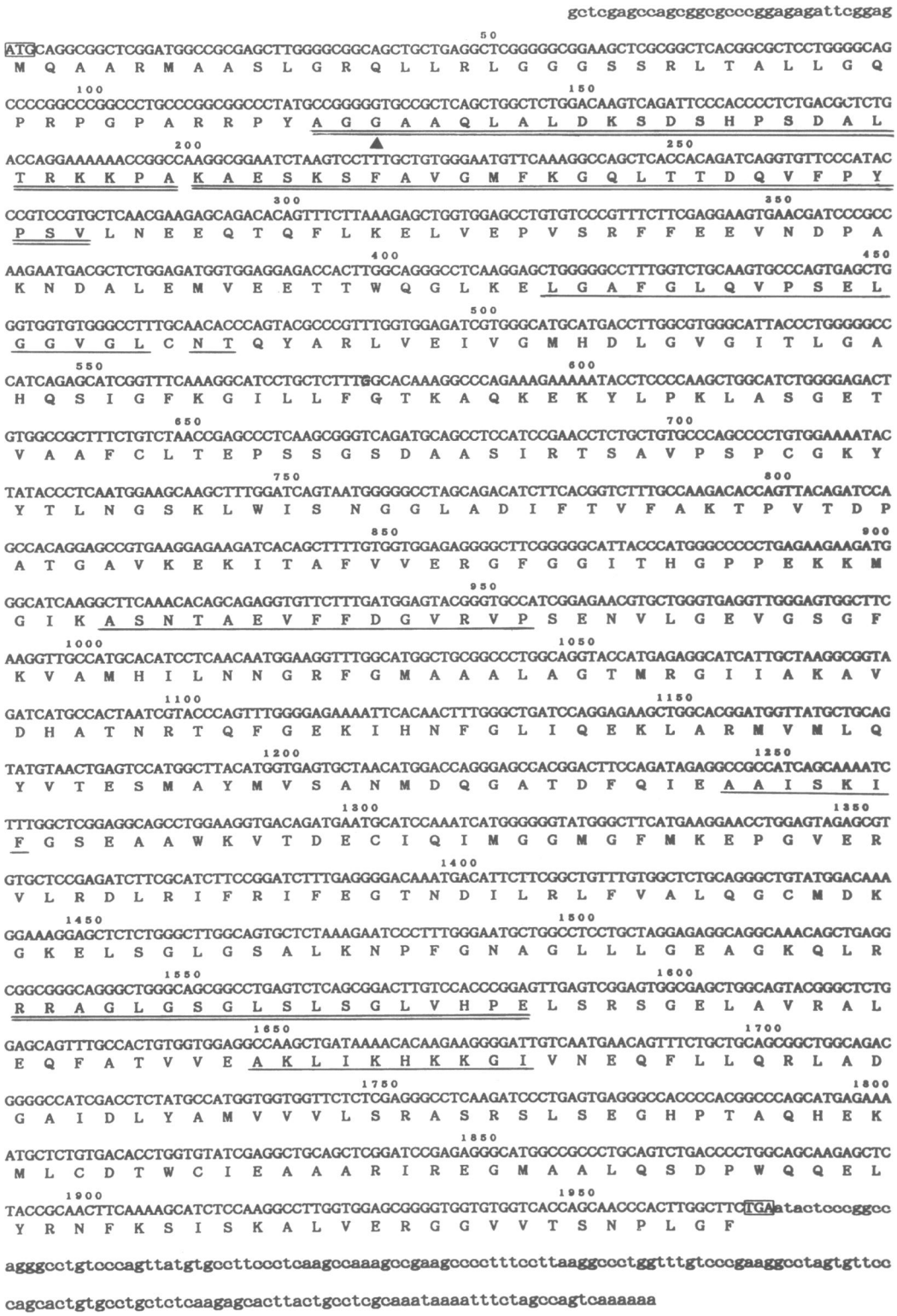


Figure 1 Nucleotide and deduced amino acid sequences of human VLCAD cDNA. The numbering of nucleotides starts at the first residue of the coding sequence. Both the initiation and termination codon are boxed. Underlined amino acid sequences matched those obtained from the four proteolytic peptides of denatured human VLCAD. Double-underlined amino acid sequences, except Gly-43 (indicated with a blackened triangle [▲]), matched those obtained from the amino-terminal and two proteolytic peptides of native human VLCAD.

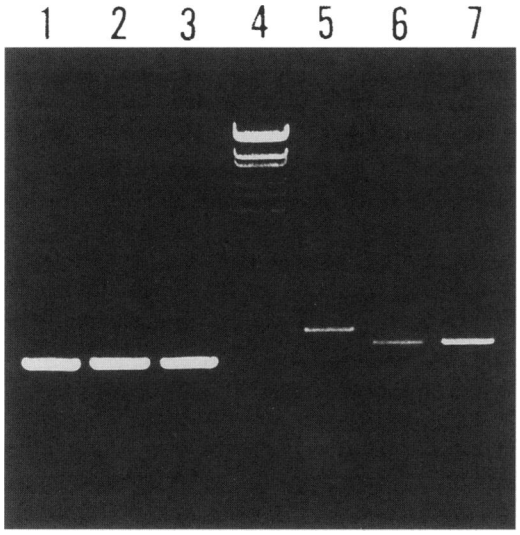


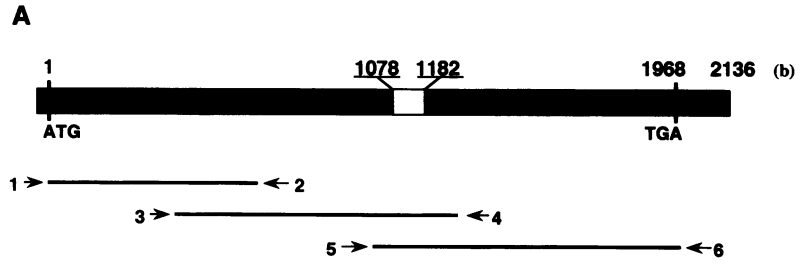
Figure 2 Agarose gel electrophoresis of PCR-amplified cDNA fragments. cDNAs prepared from control and patient 2 and 3 were amplified using the pair of primers, as described in Methods. The amplified products were directly applied to 1% agarose gel containing ethidium bromide. The primers used are: *A*, 5′-CCCCAAGCTGGCATCTGGGG-3′ (606-625); *B*, 5′-GCCTCTCATGGTACCTGCCA-3′ (1062-1043); and *C*, 5′-GGCTGCCTCCGAGCCAAAGA-3′ (1280-1261). Lane 1, Control (primer A and B). Lane 2, Patient 2 (A and B). Lane 3, Patient 3 (A and B). Lane 4, DNA molecular weight standard marker, λDNA-*Eco* T14I digest. Lane 5, Control (A and C). Lane 6, Patient 2 (A and C). Lane 7, Patient 3 (A and C).

shown in figure 2, cDNA fragments from control and patients 2 and 3 amplified using the sense (606-625) and the antisense (1062-1043) primers were similar in size. However, both cDNA fragments from patients 2 and 3 amplified using the sense (606-625) and the antisense (1280-1261) primers were ~100 bp smaller than those from the control group. There was no difference in size among cDNA fragments from control and patients 2 and 3 amplified in the regions encompassing nucleotide (−22)-1062 and 1239-1988, respectively (data not shown). These data indicate the existence of the deletion(s) in the region bounded by nucleotides 1062-1239. As described in figure 3, three cDNA fragments were then synthesized by PCR using cDNA samples from patients 2 and 3, to determine nucleotide sequence. A 105-bp deletion at nucleotide position 1078-1182 was found in both patients 2 and 3. The nucleotide sequence of the remaining region, excepting the 105-bp deletion, was identical to that in control. These data suggested homozygosity for the deletion in both patients. Eight sets of the sense and the antisense primers located around the nucleotide position 1078-1182 were used for PCR-amplification of genomic DNA from both patients and controls, in order to identify the cause of the 105-bp deletion on the VLCAD gene. However, these attempts were fruitless, despite changing the PCR conditions, suggesting the existence of large introns in this region. Genomic Southern blot analysis was then performed to characterize the 105-bp deletion. As shown in figure 4*A*, a cDNA probe encompassing nucleotide position 1078-1182 strongly hybridized with two genomic DNA fragments, ~4 kb in size, from both patients and control. *Pst*I digestion of genomic DNA should have yielded the two fragments, because the region at nucleotide position 1078-1182 contains a *Pst*I site. The same nylon sheet was then used for hybridization with a cDNA probe at nucleotide position 402-591. A single strong band was observed in each sample (fig. 4*B*), and the intensity of the band in each sample figure 4*B* was roughly proportional to that in figure 4*A*. These data suggested that the 105-bp deletion occurred due to exon skipping during processing of VLCAD pre-mRNA.

The content of VLCAD mRNA in fibroblasts from control and patients 2 and 3 was measured. The relative contents in control and in patients 2 and 3 were 100, 2, and 14, respectively (fig. 5). In a parallel experiment, the relative contents of mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein large subunit mRNA (Kamijo et al. 1993, 1994) in them were 100, 18, and 86, respectively (data not shown). The cell line from patient 2 seems to have an impaired ability to synthesize both these mRNAs because of its high passage number (>36), which may make the interpretation ambiguous. Nevertheless, these data suggest that both patients' fibroblasts contained significantly less VLCAD mRNA than control fibroblasts, which may reflect the instability of VLCAD mRNA containing the 105-bp deletion.

cDNA-Expression of VLCAD in Fibroblasts

The full-length human VLCAD cDNA was inserted into a vaccinia virus vector as outlined in Methods. Skin fibroblast cells from patients 2 and 3 and from controls were infected with the recombinant virus particles, and cell lysates were then analyzed by immunoblotting with anti-VLCAD antibody. As shown in figure 6, comparable amounts of VLCAD polypeptide appeared in fibroblasts from each of two controls and patients 2 and 3 (lanes 1, 2, 3, and 8, respectively) after infection by VLCAD-recombinant virus alone. The amount of the expressed VLCAD protein was estimated to be ~0.15%–0.2% of the total cell protein. The size of the expressed VLCAD was identical to that of purified human VLCAD, suggesting that most of VLCAD protein accumulating in fibroblasts lost the leader peptide (40 amino acid residues) and was localized in mitochondria. To examine the effect of a phased restoration of VLCAD activity at the cell level, skin fibroblasts from patient 3 were treated with vaccinia viruses, using a quantitative cDNA-expression technique (Aoyama et al. 1995). The content of the expressed VLCAD propor-



B

Primer	Sequence	Position
1	5'-CGGCGCCCGGAGAGATTCGG-3'	(-22)-(-3)
2	5'-ACAGAAAGCGGCCACAGTCT-3'	645-626
3	5'-GGGCCTCAAGGAGCTGGGGG-3'	402-421
4	5'-GGCTGCCTCCGAGCCAAAGA-3'	1280-1261
5	5'-GTTTGGCATGGCTGCGGCCC-3'	1023-1042
6	5'-CAGGCCCTGGCCGGGAGTAT-3'	1988-1969

Figure 3 Strategy for PCR amplification. Nucleotide numbers are indicated in fig. 1. *A*, DNA fragments, synthesized with three pairs of primers, as described in Methods. *B*, Sequence of oligonucleotide primers.

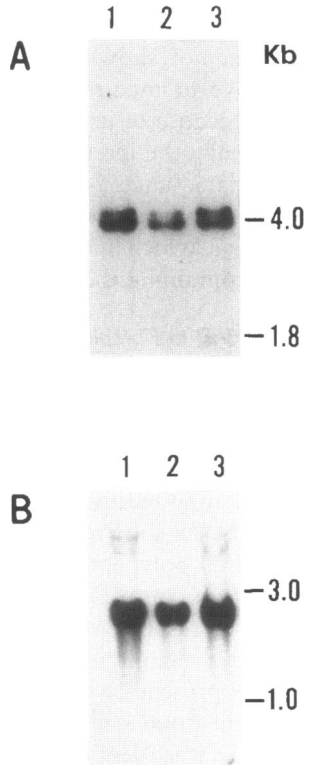


Figure 4 Southern blot analysis of VLCAD gene. Genomic DNA from fibroblasts was digested with *PstI*, electrophoresed in 1% agarose gel, and transferred to a nylon membrane. Prehybridization and hybridization were carried out according to the Amersham protocol. *A*, ^{32}P-labeled cDNA probe at nucleotide position 1078–1182. *B*, ^{32}P-labeled cDNA probe at nucleotide position 402–591. Lane 1, Patient 2. Lane 2, Patient 3. Lane 3, Control. X-ray film was exposed for 7 d at −80°C.

tionally increased according to a change in the ratio (wild-type virus/VLCAD-recombinant virus) for the viral plaque–forming units. Relative contents of the expressed VLCAD at ratios 100/0, 95/5, 75/25, 50/50, and 0/100 were 0.05, 6, 21, 43, and 100, respectively (lanes 4–8). The content at a wild-type/VLCAD recombinant ratio of 95/5 was similar to that found in control fibroblasts.

Skin fibroblasts from patients 2 and 3 and from two

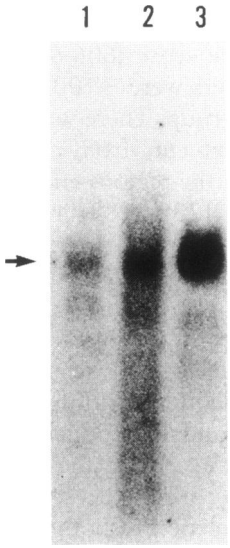


Figure 5 Northern blot analysis of VLCAD mRNA in fibroblasts. Twenty micrograms of total RNA were electrophoresed and blotted onto a nylon membrane. The nylon membrane was hybridized with ^{32}P-labeled insert of human VLCAD (HVL-1). Lane 1, Patient 2. Lane 2, Patient 3. Lane 3, Control. X-ray film was exposed for 14 d at −80°C. An arrow indicates position of VLCAD mRNA.

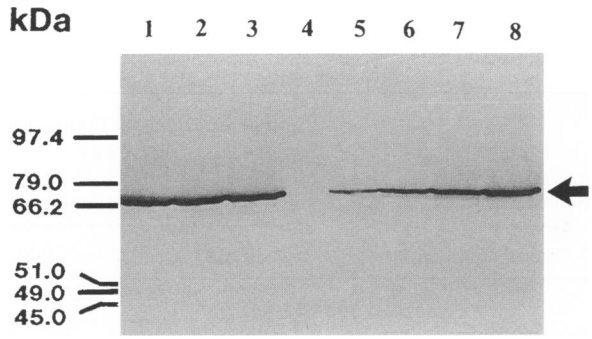


Figure 6 cDNA-expression of VLCAD in fibroblasts. Fifty micrograms of cell lysate were electrophoresed on a 10% gel and transferred to a nitrocellulose membrane. The membrane was then developed using anti-human VLCAD antibody and alkaline phosphatase-conjugated second antibody. Lane 1, Control 1 infected with the viruses (wild-type virus/VLCAD-recombinant virus: 0/100). Lane 2, Control 2 (0/100). Lane 3, Patient 2 (0/100). Lane 4, Patient 3 (100/0). Lane 5, Patient 3 (95/5). Lane 6, Patient 3 (75/25). Lane 7, Patient 3 (50/50). Lane 8, Patient 3 (0/100). Arrow indicates position of VLCAD protein.

controls were infected with the viruses and were then used for the assays of palmitoyl-CoA dehydrogenation and palmitic acid β-oxidation. On comparing palmitoyl-CoA dehydrogenation activity after infection with (wild-type virus/VLCAD-recombinant virus: 100/0) with that after infection with (0/100), the activity in patients 2 and 3 and in controls 1 and 2 was enhanced 100-, 128-, 15-, and 20-fold, respectively (table 1). Palmitic acid β-oxidation flux in mitochondria alone in patients 2 and 3 and controls 1 and 2 was also enhanced 9.3-, 7.5-, 1.2-, and 1.1-fold, respectively (table 1), indicating that introducing normal VLCAD into the patients' fibroblasts causes recovery of the β-oxidation flux to the level seen in control fibroblasts and that, in the control fibroblasts, the β-oxidation activity increases only slightly, because of the quantitative restriction resulting from limiting amounts of the other mitochondrial β-oxidation enzymes, such as the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein.

A phased supplementation of VLCAD activity in fibroblasts from patient 3 was done. Palmitoyl-CoA dehydrogenation activity proportionally increased, from 0.4 ± 0.2 to 51.3 ± 4.7 nmol ETF reduced/min/mg according to a change in the wild-type/VLCAD-recombinant virus ratio (table 1), which is compatible with the results in figure 6. The enhancement of palmitic acid β-oxidation flux in mitochondria alone was saturated after a very small amount of the supplementation (ratio: 99/1), to ~20% of VLCAD activity existing in control fibroblasts (table 1). These results are consistent with the known characteristics of VLCAD, such that it has a 10-fold higher specific activity than that of LCAD toward palmitoyl-CoA as substrate (Izai et al. 1992) and is a rate-limiting enzyme in long-chain fatty acid β-oxidation system (Aoyama et al. 1994*c*).

Peroxisomal AOX can catalyze palmitoyl-CoA dehydrogenation (Hashimoto 1992; Aoyama et al. 1994*a*, 1994*b*) and therefore might contribute to the recovery of the impaired β-oxidation ability in VLCAD deficiency. In order to examine this question, the four cell lines were infected with a recombinant virus containing human AOX cDNA. A similar level of overexpression among the four cell lines was achieved, and AOX-dependent palmitoyl-CoA dehydrogenation activity in patients 2 and 3 and in controls 1 and 2 was increased 22-, 19-, 28-, and 20-fold, respectively (table 1). The AOX-dependent desaturation activity after AOX cDNA expression was no more than 4%–6% of the VLCAD-dependent activity after VLCAD cDNA expression, but it was more than one third of the acyl-CoA dehydrogenase–dependent activity in control fibroblasts infected by wild-type virus. Mitochondrial palmitic acid β-oxidation flux in the four cell lines was not increased at all by the AOX cDNA-expression, but that in peroxisomes alone was enhanced 1.3–1.9-fold by its expression (table 1). Raising AOX activity in the patients' fibroblasts caused poor recovery of the β-oxidation activity, because of localization of the expressed AOX into peroxisomes (Aoyama et al. 1994*a*, 1994*b*) and the low contribution of peroxisomal β-oxidation system to palmitic acid β-oxidation activity in fibroblasts.

Discussion

Human VLCAD is orthologous to rat VLCAD (Aoyama et al. 1994*c*), because of a high amino acid sequence similarity (86%) between them, the existence of ~180 extra amino acid residues at the carboxy terminus of unknown function, and conservation of several important amino acid residues. In the previous report (Aoyama et al. 1994*c*), we pointed out four important residues in rat VLCAD, on the basis of comparison to the sequence and x-ray crystallographic model of MCAD (Kim and Wu 1988; Kim 1991). Glu-462 seems to be the catalytic residue that abstracts the α-proton (C_2-H) from the substrate; Trp-249 seems to cover the dimethylbenzene side of the flavin ring where ETF interacts with the dehydrogenase to accept electrons from the reduced VLCAD; and two residues, consisting of Thr-217 and Ser-251, are positioned around the flavin ring, forming hydrogen bonds. As shown in figure 1, these residues are all conserved in human VLCAD, as in MCAD and SCAD; therefore, the molecular environment around the substrate and flavin adenine dinucleotide in human VLCAD is similar to that in rat VLCAD, human MCAD (Kelly et al. 1987), and human SCAD (Naito et al. 1989).

Table 1

cDNA Activity of VLCAD and AOX in Skin Fibroblasts

Host Cell	Type of Virus Infected (%)	Palmitoyl-CoA Dehydrogenation Activity (nmol ETF reduced/min/mg)[a]	Palmitic Acid β-Oxidation Activity (n = 3) (pmol/h/.1 mg cell protein	
			−POCA[b]	+POCA[b]
VLCAD dependent:				
Patient 2	WT (100)	.5 ± .2	220 ± 30	106 ± 16
	VLCAD (100)	50.1 ± 3.6	982 ± 58	124 ± 18
Patient 3	WT (100)	.4 ± 0.2	156 ± 16	75 ± 12
	WT (99)+VLCAD (1)	.9 ± .2	878 ± 69	108 ± 25
	WT (97)+VLCAD (3)	2.6 ± .3	932 ± 87	97 ± 20
	WT (95)+VLCAD (5)	3.5 ± .2	897 ± 53	102 ± 17
	WT (75)+VLCAD (25)	10.6 ± 1.2	944 ± 80	123 ± 26
	WT (50)+VLCAD (50)	27.2 ± 2.3	995 ± 72	104 ± 21
	VLCAD (100)	51.3 ± 4.7	962 ± 76	112 ± 19
Control 1	WT (100)	3.2 ± 0.4	869 ± 77	133 ± 16
	VLCAD (100)	46.9 ± 4.1	1006 ± 71	108 ± 24
Control 2	WT (100)	2.9 ± .5	744 ± 53	117 ± 20
	VLCAD (100)	57.0 ± 3.3	837 ± 65	139 ± 16
		(nmol/min/mg)[c]		
AOX dependent:				
Patient 2	WT (100)	.05 ± .01	56 ± 16	75 ± 12
	AOX (100)	1.08 ± .15	213 ± 20	137 ± 10
Patient 3	WT (100)	.07 ± .01	220 ± 30	106 ± 16
	AOX (100)	1.32 ± .10	231 ± 27	183 ± 24
Control 1	WT (100)	.05 ± .02	869 ± 77	133 ± 16
	AOX (100)	1.40 ± .19	891 ± 66	170 ± 21
Control 2	WT (100)	.06 ± .01	774 ± 53	117 ± 20
	AOX (100)	1.17 ± .23	805 ± 50	221 ± 19

[a] Skin fibroblasts were infected with the indicated vaccinia virus, harvested 24 h later, sonicated, and assayed for VLCAD-dependent activities, as described by Aoyama et al. (1994*c*). Values are means ± SD (n = 3).

[b] β-Oxidation toward long-chain fatty acid is known to be catalyzed by both mitochondrial and peroxisomal enzymes. POCA, a potent inhibitor of mitochondrial fatty acid β-oxidation, was used to discriminate β-oxidation activity in mitochondria from that in peroxisomes. The activity without POCA (−POCA) corresponds to that in both mitochondria and peroxisomes and that with POCA (+POCA) to that in peroxisomes alone. Therefore, the activity of (−POCA) minus that of (+POCA) corresponds to that in mitochondria alone.

[c] Skin fibroblasts were treated as described above and assayed for AOX-dependent activities as described by Aoyama et al. (1994*a*) and Souri et al. (1994). Values are means ± SD (n = 4).

A 105-bp deletion encompassing bases 1078–1182 in VLCAD cDNA has been found both in patients 2 and 3, and the deletion seems to occur due to exon skipping during processing of VLCAD pre-mRNA. The finding is the first demonstration of mutations in human VLCAD deficiency. The deletion predicts the in-frame deletion of 35 amino acids, beginning with Val-360 of the precursor VLCAD. In this segment of 35 amino acids, VLCAD has 34%, 29%, and 23% sequence similarity with SCAD, MCAD, and LCAD, respectively (Kelly et al. 1987; Naito et al. 1989; Indo et al. 1991*b*). It is interesting that the segment is in one of the regions exhibiting particularly high sequence homology among all the acyl-CoA dehydrogenases (Aoyama et al. 1994*c*). Among MCAD-deficient patients, an A-to-G transition at position 985 in the coding region of the gene is a highly prevalent mutation, found in 89% of all variant MCAD alleles (Yokota et al. 1991; Tanaka et al. 1992). This mutation results in a glutamate being substituted for the normal lysine at position 304 of the mature MCAD. The variant MCAD is unstable and is undetectable in patients' tissues (Coates et al. 1992; Saijo et al. 1994). It has been shown that the variant MCAD is unable to form a tetramer because of to disruption of intramito-

chondrial folding and assembly (Saijo et al. 1994). Leu-389 in VLCAD corresponds to Lys-304 in MCAD, and the region from Ile-379 to Met-388 in VLCAD has high sequence homology (50%) with the corresponding region in MCAD. The region containing the 11 amino acids from Ile-379 to Leu-389 is therefore likely to relate to intramitochondrial folding and assembly. The deletion of 35 amino acids encompassing these 11 amino acids in patients 2 and 3 would make the protein unstable, thus accounting for the small amounts of VLCAD protein in patients' fibroblasts and the detection of faint bands migrating faster than the normal VLCAD protein in pulse-chase experiments (Aoyama et al., in press). In addition, rather low content of VLCAD mRNA in the patients' cells, because of the instability of the variant VLCAD mRNA (fig. 5), may also relate to the low levels of the smaller VLCAD protein.

The existence of the 105-bp/35-amino acid deletion can be readily visualized by the agarose gel electrophoresis of PCR-amplified VLCAD cDNA, as shown in figure 2. Since the deletion causes disappearance of a *Pst*I site, agarose gel electrophoresis of *Pst*I-digested, PCR-amplified VLCAD cDNAs also conveniently demonstrates the existence of this deletion. The development of simple detection methods using PCR will facilitate rapid diagnosis of this disorder, including carrier detection and prenatal diagnosis.

In fibroblasts from patients 2 and 3, the infection with the recombinant vaccinia virus caused overexpression of the precursor VLCAD protein. Most of the precursor proteins were converted into the mature proteins, mainly localized in mitochondria, as is the case with rat VLCAD (Aoyama et al. 1994*c*). In patient fibroblasts, raising VLCAD activity to ~20% of normal control fibroblast activity raised palmitic acid β-oxidation flux to the level found in control fibroblasts, directly confirming that the greatly reduced level (>80%) of normal VLCAD protein and activity causes the impaired long-chain fatty acid β-oxidation flux in the fibroblasts. This result may offer important information for the rational design of future somatic gene therapy for VLCAD deficiency. Since vaccinia virus can infect most kinds of mammalian cells (Aoyama et al. 1989*b*; Ozaki et al. 1993), including lymphocytes, amniotic cells, and amniochorial cells, and can efficiently produce the encoded protein, a method for rapid diagnosis using the viral cDNA-expression system and subsequent measurement of fatty acid β-oxidation activity might be developed in the future.

Since peroxisomal AOX can catalyze dehydrogenation of long-chain and very-long-chain fatty acyl-CoAs, as does mitochondrial VLCAD (Hashimoto 1992; Aoyama et al. 1994*a*, 1994*b*) and is the rate-limiting enzyme in the peroxisomal very-long-chain fatty acid β-oxidation system (Aoyama et al. 1994*a*), supplementation of AOX to the patients' fibroblasts might be expected to cause considerable recovery of palmitic acid β-oxidation activity as a whole. As expected, the overexpression of AOX in the patients' cells enhanced the peroxisomal β-oxidation activity but did not affect the mitochondrial β-oxidation activity at all. This is consistent with the previous data demonstrating that the expressed AOX was localized in peroxisomes alone (Aoyama et al. 1994*a*, 1994*b*). The small increase in the total β-oxidation flux in the patients' cells seems to be due to the relatively small peroxisomal contribution, which catalyzes <13% of the total β-oxidation flux in control fibroblasts (table 1). Peroxisomal β-oxidation in hepatoma H4IIEC3 cells catalyzes 13% and 27% of the total β-oxidation of palmitic acid and stearic acid, respectively, and, in hepatoma Hep G2, 34% and 18%, respectively (Aoyama et al. 1994*c*). Although the contribution of peroxisomal β-oxidation to the total β-oxidation flux of long-chain fatty acids in human liver or heart is unknown, it is assumed to be low, judging from our results at cell level described above. Therefore, the administration of peroxisome proliferators, such as di-(2-ethylhexyl) phthalate and clofibrate, to patients with VLCAD deficiency is not expected to cause significant recovery of the impaired β-oxidation activity. The induction of mitochondrial LCAD and MCAD in these patients, which can catalyze palmitoyl-CoA dehydrogenation (Hashimoto 1992), may be more effective in recovering this impaired activity. Since detailed knowledge concerning chemical induction of mitochondrial acyl-CoA dehydrogenases is limited (Izai et al. 1992; Aoyama et al. 1994*c*), further information will be required to develop this approach as a useful therapy for VLCAD deficiency.

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