

Large Deletion of the Peroxisomal Acyl-CoA Oxidase Gene in Pseudoneonatal Adrenoleukodystrophy

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Abstract

We have cloned the cDNA encoding human peroxisomal acyl-CoA oxidase, the first enzyme in the peroxisomal β -oxidation of very long chain fatty acids. Its nucleotide sequence was found to be highly homologous (85%) to the rat cDNA counterpart.

An 88% homology between rat and human was found in the COOH-terminal end of the cDNA which includes the Ser-Lys-Leu peroxisomal targeting signal common to many peroxisomal proteins. The gene spans ~30–40 kb and is poorly polymorphic. Southern blot analyses were performed in two previously reported siblings with an isolated peroxisomal acyl-CoA oxidase deficiency (pseudoneonatal adrenoleukodystrophy). A deletion of at least 17 kb, starting downstream from exon 2 and extending beyond the 3' end of the gene, was observed in the two patients. These observations provide a molecular basis for the observed acyl-CoA oxidase deficiency in our family. In addition, our study will enable the characterization of the genetic defect in unrelated families with suspected acyl-CoA oxidase disorders. (*J. Clin. Invest.* 1994. 94:526–531.) Key words: lipid metabolism • inborn errors • β -oxidation • genetic code

Introduction

Peroxisomes are subcellular organelles with an important function in cellular metabolic processes including the β -oxidation of very long chain fatty acids (1) and the synthesis of bile acids (2) and ether-phospholipids (3). The importance of peroxisomes for cellular metabolism is stressed by the discovery of a number of serious inborn errors of peroxisomal metabolism in humans (4–7).

Disorders with defective peroxisome assembly such as the Zellweger cerebro-hepato-renal syndrome (7), neonatal adrenoleukodystrophy (4), and infantile phytanic acid storage disease (5) are characterized by a decreased number or an absence

of morphologically detectable peroxisomes in liver and other tissues. Multiple enzymatic abnormalities, resulting in an accumulation of bile acid intermediates, very long chain fatty acids (VLCFA),¹ pipecolic, pristanic, and phytanic acids (age-dependent), and severe impairment of plasmalogen biosynthesis, are observed in these disorders (6).

Peroxisomal β -oxidation of fatty acids is catalyzed by three enzymes that are immunologically distinct from the analogous mitochondrial enzymes: (1) acyl-CoA oxidase; (2) a trifunctional enzyme containing enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA isomerase activities (previously named bifunctional enzyme; 8); and (3) peroxisomal β -ketothiolase. The peroxisomal β -oxidation pathway appears to be active toward saturated VLCFA (1). An impairment of this pathway is responsible for the elevated plasma and tissue VLCFA values in patients.

Recently, several patients with defective peroxisomal functions have been described in which peroxisomes were not decreased in number in various tissues. These patients were diagnosed initially with either Zellweger syndrome or neonatal adrenoleukodystrophy based on their clinical and pathological manifestations. However, in contrast to the disorders of peroxisome assembly, these patients appeared to have an isolated defect of the peroxisomal β -oxidation. One patient with clinical features similar to those of Zellweger patients was found to have an isolated deficiency in peroxisomal thiolase protein. No large DNA rearrangement involving the human *peroxisomal thiolase* gene was found in this patient (9). Another patient with a single enzyme defect of peroxisomal VLCFA metabolism, namely trifunctional enzyme deficiency, had been diagnosed originally as having neonatal adrenoleukodystrophy (10).

We have described two siblings with pseudoneonatal adrenoleukodystrophy who exhibited decreased VLCFA oxidation associated with an isolated deficiency of fatty acyl-CoA oxidase activity (11), the first enzyme of the peroxisomal β -oxidation system. We have now initiated an investigation aiming to clone the human *acyl-CoA oxidase* cDNA and to determine the genetic defect in the two siblings. In this study, we report the sequence of the human *acyl-CoA oxidase* cDNA and show that the genetic lesion underlying the acyl-CoA oxidase deficiency involves a large DNA deletion in the two patients.

Methods

Patients. Two previously reported siblings born to consanguineous healthy parents presented clinical manifestations very similar to those

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1. Abbreviations used in this paper: SSPE, sodium chloride sodium phosphate EDTA buffer; VLCFA, very long chain fatty acids.

Table I. Oligonucleotides Used in PCR Amplification

Primer	Sequence 5' to 3'	Coordinates
1-H13[3]	AAATGCATCAACCAAAGCAAC	1830–1810
2-R10[5]	GTCTGGCCAACATGGTGGAC	1408–1428
3-H11[3]	AAGGTTTTTTGCAGCAATTTT	1506–1486
4-R8[5]	CAGTATAAACTCTTCCCGCTC	982–1002
5-H8[3]	TTCGTTAATCCGGTGATAGGT	1071–1051
6-R6[5]	ACTGTCGGGGATATCGGTCCC	664–684
7-H6[3]	CTGGGCATACTTCATCAGCAT	774–754
8-R1[5]	ATGAACCCCGACCTGCGCAAG	1–21
9-H1[5]	TCCGCCAGCTTCAACCCGGAG	31–51
10-H2[3]	TGCCAAACTCCCTCATCTTC	235–216

of patients affected by neonatal adrenoleukodystrophy, namely severe hypotonia, mental retardation, seizures, and no dysmorphic features. Standard karyotypes were normal. In contrast to neonatal adrenoleukodystrophy, hepatic peroxisomes were enlarged in size but not decreased in number. A $C_{26:0}/C_{22:0}$ ratio of 0.076 and 0.16 was observed in the plasma of each patient as opposed to a ratio of 0.016 (± 0.028) in control plasma, and a ratio of 1.577 and 1.047 in patients' fibroblasts compared with 0.08 (± 0.03) in control fibroblasts. The parents' VLCFA ratios were comparable with those of controls. Immunoblotting experiments on liver tissue from the patients revealed no immunologically reactive material using anti-acyl-CoA oxidase antibodies. The accumulation of VLCFA appeared to be associated with an isolated deficiency of the fatty acyl-CoA oxidase, the enzyme that catalyzes the first step of the peroxisomal β -oxidation. Plasmalogen biosynthesis in cultured skin fibroblasts and plasma levels of di- and trihydroxycoprostanic acid, phytanic acid, and pipecolic acid were normal (11).

cDNA cloning and sequencing. A λ -gt10 liver cDNA library (120,000 independent recombinant plaques; 12) was screened using PCR-amplified exon 13 of the rat acyl-CoA oxidase gene (5' primer 5'-GGGAGCATCATCACAGGGGCT-3', 3' primer 5'-CTCTGTTTT-GTTCAGTGGGGA-3'; 13). The two primary clones selected (insert size of 200 bp) were subcloned in phage M13mp18 for sequencing by the dideoxy chain termination method of Sanger. A second screening failed to give larger clones. For this reason a different strategy consisting of amplification of reverse-transcribed specific human mRNA was initiated.

Total human liver RNA (10 μ g) was reverse transcribed to cDNA, using a 21-bp specific primer complementary to the 3' end of the human clones obtained previously, using reverse transcriptase (GIBCO BRL, Gaithersburg, MD) as recommended by the manufacturer. For second-strand cDNA synthesis and PCR amplification of subsequent fragments (Taq-polymerase, 2.5 U; Perkin-Elmer Cetus Instruments, Norwalk, CT), forward primers derived from the rat cDNA sequence were selected. Sequence analysis of these fragments provided information for reverse transcription and amplification of the next fragment. This procedure of using specific human oligonucleotides as backward primers and specific rat oligonucleotides as forward primers was repeated. To obtain the 5' end of the cDNA, these fragments served as probes for screening a larger human liver cDNA λ -gt11 library (14). One single recombinant containing an incomplete insert was selected and used as probe for screening another library (human B cell line cDNA library in pCDM8). A cDNA clone of 2.1 kb encoding the complete protein was obtained.

Primers used. The synthetic oligonucleotides used in PCR sequencing analysis of the human acyl-CoA oxidase cDNA are shown in Table I. The coordinates (numbering starts at ATG codon) are based on the human (H) (Fig. 1) or the rat (R; 13) cDNA sequence.

Southern blotting. Genomic DNA was obtained from lymphoblastoid cell lines by standard methods. Control and patients' genomic DNA (8 μ g) were digested with either BglII or EcoRI restriction enzymes, electrophoresed in a 0.7% agarose gel, and transferred to Hybond N⁺

membranes (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Filters were prehybridized for 1 h and hybridized overnight in a hybridization mix containing 5 \times sodium chloride phosphate EDTA buffer (SSPE), 5 \times Denhardt's solution, 0.5% SDS, and 100 μ g/ml herring sperm DNA. Filters were washed in 2 \times SSPE/0.1% SDS, 1 \times SSPE/0.1% SDS, and 0.1 \times SSPE/0.1% SDS as recommended by the manufacturer.

For hybridizing the Southern blots, the full length cDNA was digested with the restriction enzyme DraI, giving a 5' fragment (nucleotides 0–264) and a 3' fragment (nucleotides 265–2092), and both fragments were labeled using the Amersham labeling kit and ³²P-labeled dCTP. An average of 2.10⁶ cpm/ml was used per blot. Blots were scanned with a phosphorimager.

Results

Isolation of the human acyl-CoA oxidase cDNA. To study the molecular defect in our patients with an acyl-CoA oxidase deficiency, we set out to clone a full-length human acyl-CoA oxidase cDNA. To that end, a λ -gt10 human liver cDNA library was screened with a probe representing exon 13 of the rat acyl-CoA oxidase gene. The two short human clones (± 200 bp) obtained had an 84% homology to the rat cDNA sequence. Further screening of this library failed to give larger clones. Therefore, we amplified reverse-transcribed human liver mRNA using primers based on the rat cDNA sequence. Most of the cDNA sequence was obtained in three amplification steps using primers 1-H13[3] and 4-R8[5], 3-H11[3] and 6-R6[5], and 7-H6[3] and 8-R1[5]. This yielded overlapping fragments of 849, 843, and 774 bp, respectively. The 5' end, however, remained elusive.

Screening of a λ -gt11 human liver cDNA library (containing large inserts) was carried out using PCR fragments as probes and gave an incomplete 1.9-kb insert. Since this insert did not include the 5' end of the cDNA, a third library (a B cell line cDNA library) was screened. The 2.1-kb insert obtained from this last library encoded the complete protein sequence.

The B cell cDNA sequence contained a region of 160 nucleotides (nucleotides 270–429; Fig. 1), which was different from the cDNA sequence of the 1.9-kb liver cDNA clone. Further PCR amplification using primers 9-H1[5] and 7-H6[3] on liver mRNA gave a 774-bp fragment with either sequences though never both together, whereas amplification of lymphocyte mRNA gave only one type of sequence. The sequence found only in liver mRNA was called type I, and the sequence found in both liver and lymphocytes mRNA was called type II (Fig. 2).

The nucleotide sequence of the human acyl-CoA oxidase cDNA (Fig. 1) was found to be highly homologous to its rat cDNA counterpart. The homology between the two sequences averaged 85% at the nucleotide level and 89% at the amino acid level (Fig. 3). As in the rat (13), two species of acyl-CoA oxidase cDNA were found in the liver differing in the region of nucleotides 270–429 (type I and II; Fig. 2). These two regions had a 52% homology to each other at the nucleotide level and each had a 91% homology to their rat counterpart.

Southern blot analysis of the acyl-CoA oxidase gene in control and patients with an acyl-CoA oxidase deficiency. Southern blot analysis of total human genomic DNA digested with a number of restriction enzymes and using the full-length cDNA as the hybridization probe indicated that the chromosomal acyl-CoA oxidase gene is at least 30–40 kb long and


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1  . . . . . K . . . A . . . T . . . . . I . . . . . P . N . . . . .
36  I E N M L I L N D P D F Q H E D L N F L T R S Q R Y E V A V R K K S A I M
71  . . . . . Y . . . S . . . R . . . E I M W F K K L H L V N F V E P V R L N Y S
106 M F I P T L L N Q G T T A Q K E K W L L S S K G L Q I I G T Y A Q T E
141 M G H G T H L R G L E T T A T Y D P E T Q E F I L N S P T V T S I K W
176 W P G G L G K T S N H A I V L A Q L I T K G K C Y G L H A F I V P I R
211 E I G T H K P L P G I T V G D I G P K F G Y D E I D N G Y L K M D N H
246 R I P R E N M L M K Y A Q V K P D G T Y V K P L S N K L T Y G T M V F
281 V R S F L V G E A A R A L S K A C T I A I R Y S A V R H Q S E M K P G
316 E P E P Q I L D F Q T Q Q Y K L F P L L A T A Y A P Q F V G A Y M K E
351 T Y H R I N E G I G Q G D L S E L P E L H A L T A G L K A P T S W T A
386 N T G I E A C R M A C G G H G Y S H C S G L P N I Y V N F T P S C T F
421 E G E N T V M M L Q T A R F L M K S Y D Q V H S G K L V C G M V S Y L
456 N D L P S Q R I Q P Q Q V A V W P T M V D I N S P E S L T E A Y K L R
491 A A R L V E I A A K N L Q K E V I H R K S K E V A W N L T S V D L V R
526 A S E A H C H Y V V V K L F S E K L L K I Q D K A I Q A V L R S L C L
561 L Y S L Y G I S Q N A G D P L Q G S I M T E P Q I T Q V N Q R V K E L
596 L T L I R S D A V A L V D A F D F Q D A T L G S V L G R Y D G N V Y E
631 N L P E W A R K N S P L N K A E V H E S Y H K H L K S L O S K L

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Figure 3. Comparison between the predicted amino acid sequences of the human and the rat *acyl-CoA oxidase* type I. The human amino acid sequence is given in single letter code. Amino acid identity between the rat and human sequence is indicated by a dot above the human sequence and mismatches by the corresponding rat amino acid. The numbering indicated is that of the human amino acid sequence.

lymphoblastoid cell lines from the siblings with an *acyl-CoA oxidase* deficiency, their parents, and controls, was digested with restriction enzymes *Bgl*II, *Eco*RI, and *Hind*III and probed with the cDNA. A partial deletion of the *acyl-CoA oxidase* gene was detected in the patients, regardless of the restriction enzyme used. Patients' DNA digested with restriction enzyme *Eco*RI lacked the 8.0-, 5.5-, and 2.4-kb fragments when probed with the 3' fragment including the type II region (nucleotides 265–2092; Fig. 4A). The same blot was reprobed with a 5' fragment from the human clone, homologous to the first two exons of the rat gene (nucleotides 0–264). A 16-kb fragment was now found to be present in patients as well as in controls (Fig. 4B). This observation was confirmed by PCR amplification using oligonucleotides 9-H1[5] and 11-H2[3], both situated at the 5' end of the cDNA, on genomic DNA from patients and controls. A 500-bp fragment was found to be present both in the probands and in the controls. This fragment was sequenced and apparently included an intron at a position equivalent to the rat intron 1.

Although the exact boundaries of the deletion remain to be determined, we conclude that the deletion in the patients spans most of the gene as observed by Southern blot analysis. However, the first two exons were left intact. These results indicate that the *acyl-CoA oxidase* deficiency in this family is due to a large deletion in the *acyl-CoA oxidase* gene.

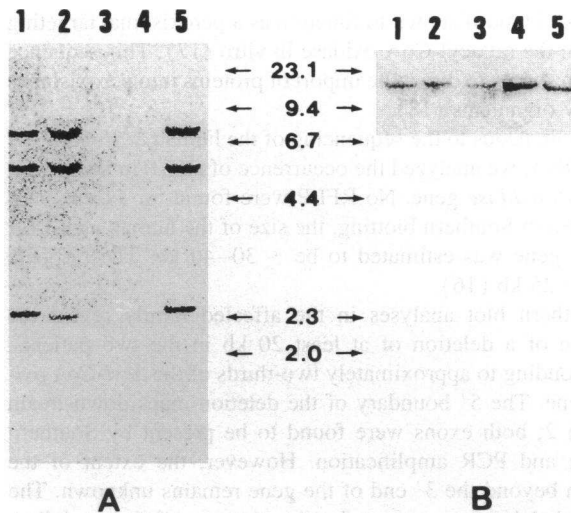


Figure 4. Southern blot analysis of the affected family. DNA from control (lane 1), the mother (lane 2), the two patients (lanes 3 and 4), and a Zellweger patient (lane 5) was digested with the restriction enzyme *Eco*RI and probed with a 1827-bp cDNA fragment (nucleotides 265–2092) (A) or a 264-bp cDNA fragment (nucleotides 0–264) which covers exons 1 and 2 (B). 8.0-, 5.5-, and 2.4-kb fragments were found to be missing in patients' DNA (A), whereas a 16-kb fragment was present (B). The pattern observed for the Zellweger patient DNA was identical to control DNA. λ -DNA digested with *Hind*III is shown as a marker. Inspection of the ethidium bromide-stained gel revealed that comparable amounts of DNA were loaded in each lane (not shown). Similar results were obtained with a *Bgl*II digest (not shown).

Discussion

The human *acyl-CoA oxidase* gene has not been characterized previously. However, the rat *acyl-CoA oxidase* gene (13) and the yeast *Candida tropicalis* POX 4 and POX 5 genes (15) have been characterized. Significant similarities between the amino acid sequences of the rat *acyl-CoA oxidase* and the yeast POX 4 and POX 5 enzymes are reported. Particularly, the COOH-terminal end of these enzymes (amino acid position 622–634 in the rat enzyme) displayed a high degree of homology. Based on these observations, exon 13 of the rat *acyl-CoA oxidase* was amplified and used as a probe to screen human cDNA libraries. Subsequent PCR cloning and cDNA library screens eventually allowed us to isolate a full-length *acyl-CoA oxidase* cDNA.

Sequence analysis of the human *acyl-CoA oxidase* cDNA revealed an 85% homology with the rat nucleotide sequence. Interestingly, we have observed that one codon of the rat exon 14 (nucleotides 1951–1953) is absent in the human cDNA sequence. We have also observed the presence of two cDNAs in the liver differing in the region of nucleotides 270–429, a region which corresponds to an alternative usage of rat exon 3 (16). The presence of both types of mRNA in the liver but not in lymphocytes may be related to the essential role of liver peroxisomes in β -oxidation of VLCFA.

An 89% homology was found between the deduced amino acid sequences of rat and human *acyl-CoA oxidase* genes. The carboxy termini of the human and rat *acyl-CoA oxidase* enzyme are highly homologous (88% homology in the last 50 amino acids). Like the rat homologue, the human sequence also contains the carboxy-terminal Ser-Lys-Leu (SKL) (residues 656–

660, Fig. 1) motif shown to function as a peroxisomal targeting signal in the rat acyl-CoA oxidase in vitro (17). This sequence has been shown to direct the import of proteins into peroxisomes in many organisms (18).

Simultaneous to the sequencing of the human *acyl-CoA oxidase* cDNA, we analyzed the occurrence of RFLP in the human *acyl-CoA oxidase* gene. No RFLP were found for 11 enzymes tested. From Southern blotting, the size of the human *acyl-CoA oxidase* gene was estimated to be ~ 30–40 kb. The rat gene spans ~ 25 kb (16).

Southern blot analyses in the affected family reveal the presence of a deletion of at least 20 kb in the two patients, corresponding to approximately two-thirds of the *acyl-CoA oxidase* gene. The 5' boundary of the deletion maps downstream of exon 2; both exons were found to be present by Southern blotting and PCR amplification. However, the extent of the deletion beyond the 3' end of the gene remains unknown. The observed deletion accounts for the absence of the acyl-CoA oxidase protein shown by immunoblotting experiments on liver tissues from one of the patients (11). The detection of 25% residual enzymatic activity in patient's liver, however, is likely due to the activity of trihydroxycoprostanoyl-CoA acid oxidase (19). The normal plasma values of di- and trihydroxycoprostanoic acids are indirect evidence of the normal activity of the trihydroxycoprostanoyl-CoA oxidase enzyme in the patients.

Very little is known about the molecular basis of peroxisomal diseases, as peroxisome research is a relatively new field. The precise cause of the defects remains unknown in many cases of generalized peroxisomal deficiencies. However, the specific genes involved and the molecular defects are being elucidated gradually and, as expected, include both DNA rearrangements and point mutations.

Disorders with multiple peroxisomal dysfunction (Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease) can be classified in at least eight complementation groups (20), suggesting a multigenic origin of the disease. A microdeletion of the proximal long arm (21) and a pericentric inversion of chromosome 7 (22) have been observed in two unrelated patients affected by Zellweger syndrome, a disorder of peroxisome assembly. These findings have led to the tentative assignment of one of the genes responsible for Zellweger syndrome to 7q11.23.

Recently, point mutations were found in two peroxisomal membrane proteins in Zellweger syndrome patients. One patient had a point mutation in the 35-kD membrane protein-coding sequence (23). Two other patients had a splice site or missense mutation in the gene of the 70-kD membrane protein (24).

A patient suffering from a single enzyme defect in the peroxisomal β -oxidation did not display a detectable DNA rearrangement of the human *peroxisomal β -ketothiolase* gene in genomic DNA (9). This patient had more serious clinical symptoms than our patients which may be due to the peroxisomal thiolase involvement in the β -oxidation of di- and trihydroxycoprostanoic acids as well as of VLCFA.

Recently, a rearrangement altering the color pigment genes in a patient with X-linked adrenoleukodystrophy was shown to include two deletions apparently separated by a large inversion on chromosome Xq28 (25). Further investigation of this region led to the cloning of a gene with significant homology to a peroxisomal 70-kD membrane protein which was partially deleted in 7% of 85 X-linked adrenoleukodystrophy patients (26). X-linked adrenoleukodystrophy is now thought to be associated

with a defect in the transport of the peroxisomal acyl-CoA synthetase, the enzyme responsible for the activation of VLCFA before their β -oxidation in the peroxisome (27).

The present study reports the existence of a large deletion in two related patients with a deficiency in the peroxisomal acyl-CoA oxidase. This is the first molecular defect found to be associated with this disease. Additional patients, unrelated to the probands of this study, have been identified by using complementation analysis between cell lines of unknown patients with those of patients with known specific enzymatic defects in the VLCFA β -oxidation (28). The available human *acyl-CoA oxidase* cDNA sequence will allow the determination of additional molecular defects resulting in this disease.

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