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D-3-Hydroxyacyl-CoA Dehydratase/D-3-Hydroxyacyl-CoA Dehydrogenase Bifunctional Protein Deficiency: A Newly Identified Peroxisomal Disorder

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Summary

Peroxisomal β-oxidation proceeds from enoyl-CoA through D-3-hydroxyacyl-CoA to 3-ketoacyl-CoA by the D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxy-acyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein), and the oxidation of bile-acid precursors also has been suggested as being catalyzed by the D-bifunctional protein. Because of the important roles of this protein, we reinvestigated two Japanese patients previously diagnosed as having enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (L-bifunctional protein) deficiency, in complementation studies. We found that both the protein and the enzyme activity of the D-bifunctional protein were hardly detectable in these patients but that the active L-bifunctional protein was present. The mRNA level in patient 1 was very low, and, for patient 2, mRNA was of a smaller size. Sequencing analysis of the cDNA revealed a 52-bp deletion in patient 1 and a 237-bp deletion in patient 2. This seems to be the first report of D-bifunctional protein deficiency. Patients previously diagnosed as cases of L-bifunctional protein deficiency probably should be reexamined for a possible D-bifunctional protein deficiency.

Introduction

The peroxisomal fatty-acid β-oxidation cycle was considered to be catalyzed by acyl-CoA oxidase, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (L-bifunctional protein), and peroxisomal 3-ketoacyl-CoA thiolase (Hashimoto 1982). Increasing numbers of cases with deficiencies of peroxisomal β-oxidation enzymes, characterized by severe psychomotor delay and an accumulation of very-long-chain fatty acids (VLCFAs), have been reported (Schram et al. 1987; Poll-The et al. 1988; Watkins et al. 1989, 1995; Wanders et al. 1990, 1992; Suzuki et al. 1994). It seems that only VLCFAs accumulate in patients with acyl-CoA oxidase deficiency (Poll-The et al. 1988), whereas both VLCFAs and bile-acid intermediates accumulate in patients with bifunctional protein deficiency (Clayton et al. 1988; Watkins et al. 1989).

Recently, we purified D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein), which converts enoyl-CoAs to 3-ketoacyl-CoAs via D-3-hydroxyacyl-CoAs, in peroxisomes (Jiang et al. 1996*a*, 1996*b*). In other studies, we suggested that the peroxisomal β-oxidation in human skin fibroblasts proceeds from enoyl-CoA through D-3-hydroxyacyl-CoA to 3-ketoacyl-CoA by D-bifunctional protein but not by L-bifunctional protein, since the content and the activity of L-bifunctional protein are very low in cultured human skin fibroblasts, as compared with those of D-bifunctional protein (Jiang et al. 1997*a*). Furthermore, oxidation of bile-acid precursors and 2-methyl-branched fatty acids also has been suggested as being catalyzed by D-bifunctional protein but not by L-bifunctional protein (Jiang et al. 1997*a*).

Because of the important roles of D-bifunctional protein, we reinvestigated the two Japanese patients who were diagnosed as having L-bifunctional protein deficiency, by means of a complementation study using an established cell line with L-bifunctional protein deficiency (Suzuki et al. 1994). Measurement of the specific activity of L-bifunctional protein was impossible at that time, and D-bifunctional protein had not been identified yet. We now have clarified the deficiency of the protein and the enzyme activity of D-bifunctional protein, and molecular analyses have revealed mutations in the cDNA.

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Patients and Methods

Patients

Patient 1 was a female child of consanguineous Japanese parents (second cousins). The mother was gravida I, para I, and had not experienced a miscarriage. Hypotonia, mild craniofacial dysmorphism (scaphocephaly, frontal bossing, micrognathia, and high-arched palate), hepatic dysfunction, multifocal tonic-clonic convulsions, and calcific stippling of the shoulder and knee joints were present in patient 1. She could smile and follow a person, at 3 mo of age, then she regressed. She manifested adrenocortical insufficiency from 11 mo of age and died of airway obstruction at 21 mo of age.

Patient 2 was a female child of nonconsanguineous parents of Japanese origin. The mother was gravida I, para I, and had not experienced a miscarriage. Profound hypotonia, psychomotor delay, intractable convulsions, craniofacial dysmorphism (large fontanelle, frontal bossing, low nasal bridge, and upward slanting of palpebral fissures), funnel chest, talipes equinovarus, calcific stippling of the patella, and hepatomegaly were present in patient 2 soon after obstetric delivery. She suffered a subdural hematoma due to vitamin K deficiency, on the 49th postnatal day, and died of pneumonia at 12 mo of age (Nakada et al. 1993).

Accumulation of VLCFAs and bile-acid intermediates, such as $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanic acid, and decreased activity of lignoceric acid oxidation, in fibroblasts, were evident. The enzyme protein of peroxisomal acyl-CoA oxidase, L-bifunctional protein, and 3-ketoacyl-CoA thiolase were detected. Cell-fusion study revealed that both patients 1 and 2 belonged to the same complementation group as the cell line from a patient previously diagnosed with L-bifunctional protein deficiency with negative cross-reactive material (Suzuki et al. 1994). Control specimens (fibroblasts and autopsied liver tissues) were obtained, with permission, from patients with other disorders.

Enzyme Assay

Enoyl-CoA hydratase activity was assayed by a decrease in absorbance at 280 nm in the presence of 0.1 mM crotonyl-CoA or octenoyl-CoA, as described elsewhere (Jiang et al. 1996*a,* 1996*b*). D-3-Hydroxyacyl-CoA dehydratase activity was assayed with 0.1 mM D-3-hydroxyoctanoyl-CoA. 3-Hydroxyacyl-CoA dehydrogenase activity was assayed with 20 μM acetoacetyl-CoA and 0.1 mM NAD^+, as described elsewhere (Kobayashi et al. 1996). The enzyme activities were assayed at 30°C, and one enzyme unit was defined as the amount of enzyme converting 1 μmol substrate/min, under the assay conditions. [1-^{14}C]-Palmitate and [1-^{14}C]-lignocerate oxidation activities were assayed as described elsewhere (Suzuki et al. 1991).

Procedures for Protein Analysis

Preparation II of D-bifunctional protein and L-bifunctional protein were purified from human liver (Jiang et al. 1996*a*), and antibodies against these enzymes were prepared. Antibodies against crotonase (Furuta et al. 1980), acyl-CoA oxidase (Osumi et al. 1980), and peroxisomal 3-ketoacyl-CoA thiolase (Miyazawa et al. 1980) were developed with rat enzymes. Protein concentration was determined by a modification (Markwell et al. 1978) of a procedure described by Lowry et al. (1951). SDS-PAGE and western blotting were performed as described elsewhere (Laemmli 1970; Towbin et al. 1979). Color development was done by use of alkaline phosphatase–conjugated goat anti–rabbit IgG antibody (KPL Laboratories) and the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate kit (Pierce).

Skin Fibroblasts and Preparation of Extract

Fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS. The cells were trypsinized, washed with PBS, and spun down. The cell pellet was suspended in 50 mM potassium phosphate, pH 7.5, 0.5% Tween 20, 0.2 M NaCl, 2 mM mercaptoethanol, and 1 mM EDTA. After standing on ice for 30 min, the suspension was centrifuged at 10,000 *g* for 10 min, and the supernatant was preserved.

Immunofluorescence Examination of Fibroblasts

Cultured fibroblasts were fixed with 4% paraformaldehyde and 0.1 M potassium phosphate, pH 7.4, were permeabilized with 0.1% Triton X-100 and PBS, and were blocked with 4% FCS, 0.1% Triton X-100, and PBS. The cells were treated with anti–D-bifunctional protein rabbit antibody and anti–human catalase guinea pig antibody, kindly provided by Dr. S. Yokota of the Yamanashi Medical College, and then were doubly stained with FluoroLink Cy3-labeled goat anti–rabbit IgG and FluoroLink Cy3-labeled goat anti–mouse IgG (Amersham).

Pulse-Labeling Experiment

Fibroblasts were grown in 28-cm^2 dishes, were preincubated with methionine-depleted Eagle's minimum essential medium containing 5% dialyzed FCS, and then were incubated with 18.5 MBq ^{35}S-methionine, as described elsewhere (Yamaguchi et al. 1988). After 3 h, the cells were extracted with 50 mM potassium phosphate, pH 7.5, 0.2 M NaCl, 2 mM mercaptoethanol,

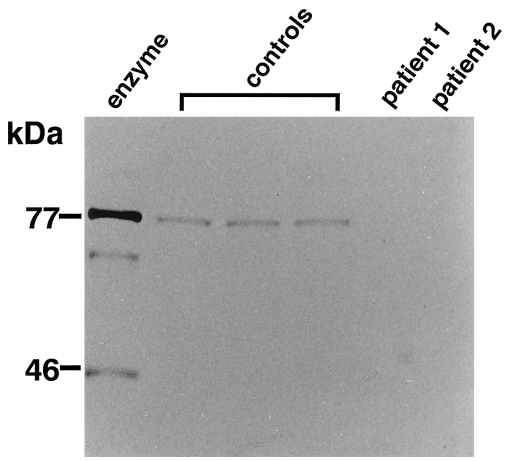

Figure 1 Immunoblot analysis of D-bifunctional protein in fibroblasts. Lane 1, Enzyme (20 ng). Lanes 2–4, Controls; Lane 5, Patient 1; Lane 6, Patient 2 (15 μg protein).

0.5 mM EDTA, 0.5% (v/v) Tween 20, 1 mg BSA/ml, 10 μg protease inhibitors/ml, and 10 mM methionine. The extract was treated with the anti–D-bifunctional protein, and the immune complex was recovered with 50 μl of 20% fixed *Staphylococcus aureus* cells (w/v). The *S. aureus* cells were washed twice with PBS containing 0.1% Tween 20 and once with saline containing 0.1% Tween 20 and 0.05% SDS and then were subjected to SDS-PAGE and fluorography. Competition experiments were performed by use of an excess amount of the purified D-bifunctional protein, during immunoprecipitation.

Northern Blot Analysis

cDNA for human D-bifunctional protein had been isolated previously (Jiang et al. 1997*b*). The region of the cDNA ([−37]–2350) of D-bifunctional protein was used as a probe in northern blot analysis. This region was completely matched to that of the human 17 β-hydroxysteroid dehydrogenase IV cDNA (Adamski et al. 1995). Digoxigenin (DIG) RNA-labeling and DIG luminescent-detection kits were from Boehringer Mannheim. Full-length cDNA of human L-bifunctional protein (Fukuda et al., in press) and β-actin gene fragments (Iijima et al. 1985) also were used as probes. Total RNAs were isolated from skin fibroblasts by means of the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). The RNAs were denatured, electrophoresed in a 1% agarose gel containing formaldehyde, and transferred to a Hybond N^+ membrane (Amersham). Hybridization, preparation of the DIG-labeled antisense RNAs, and detection were performed in accordance with the manufacturer's protocols.

PCR Amplification and Sequencing

D-Bifunctional protein cDNA was synthesized from fibroblast RNA by a reverse-transcriptase (RT) reaction. The coding region of the cDNA ([−20]–2230) was amplified by PCR, by use of an LA PCR Kit (Takara), with the primer set 5′-TGTCGTTGCAGGCCTTATTC-3′ and 5′-TAATAGTGTAGTGTGCCCTTC-3′. The 25 cycles of PCR reaction were performed by use of a DNA Thermal Cycler 480 (Perkin Elmer Japan, Applied Biosystems), under the following conditions: 96°C for 30 s, 60°C for 1 min, and 72°C for 10 min. The PCR products were separated, by electrophoresis, on a 1% (w/v) agarose gel and were extracted from the gel by use of a GENECLEAN II kit (BIO 101). Subcloning of PCR-amplified cDNA was performed by use of a pT7Blue T-vector (Novagen). Sequencing was performed by the dideoxy-sequencing method (Sanger et al. 1977) by use of a Dye Terminator Cycle Sequencing kit (Perkin Elmer Japan, Applied Biosystems).

Results

Absence of D-Bifunctional Protein in the Patients

With immunoblot analysis of D-bifunctional protein, enzyme-preparation II gave signal bands with masses of 77 kD, 68 kD, and 46 kD (fig. 1, lane 1). Native D-bifunctional protein was suggested to be a homodimer of a 77-kD polypeptide. Bands of 68 kD and 46 kD were considered to be degradation products (Jiang et al. 1996*a*). The fibroblasts from three control subjects gave only one band, with a mass of 77 kD (fig. 1, lanes 2, 3, and 4). However, neither the 77-kD subunit nor its proteolytically processed polypeptide was detected for either patient (fig. 1, lanes 5 and 6).

A band for L-bifunctional protein was found in the fibroblasts both of controls and of the patients (fig. 2). This band previously had been confirmed to be the L-bifunctional protein (Jiang et al. 1997*a*). The amount of L-bifunctional protein was greatly reduced in fibroblasts from patient 1 and was moderately reduced in those from patient 2. Acyl-CoA oxidase and peroxisomal 3-ketoacyl-CoA thiolase also were detected in fibroblasts from the patients (data not shown).

D-Bifunctional protein also was defective in the patients' liver tissue (fig. 3*A*), even when a large amount of sample was loaded. The content of D-bifunctional protein in the patients' livers was estimated to be < 1/50 of that found in the controls. L-Bifunctional protein was clearly recognized in the patients (fig. 3*B*).

Immunocytochemical examination of the fibroblasts revealed a typical punctate fluorescence pattern of peroxisomes, in the control fibroblasts, by use of the antibody against D-bifunctional protein (fig. 4*A*), and this

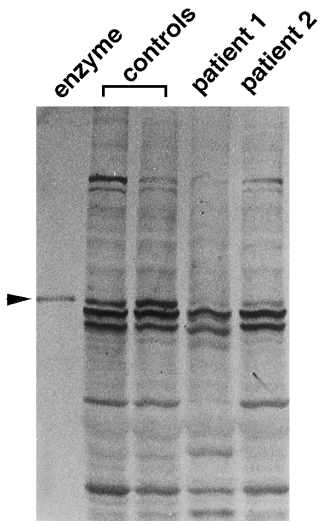

Figure 2 Immunoblot analysis of L-bifunctional protein in fibroblasts. Lane 1, Enzyme (1 ng). Lanes 2 and 3, Controls; Lane 4, Patient 1; Lane 5, Patient 2 (30 μg protein). The arrowhead indicates the position of the L-bifunctional protein.

pattern was superimposable on that of catalase (fig. 4*D*). However, only a small number of fluorescence dots were observed in the patient samples (fig. 4*B* and *C*), which is considered to be nonspecific fluorescence of the second antibodies. The large punctate fluorescence pattern of catalase was observed (fig. 4*E* and *F*), as described elsewhere (Suzuki et al. 1994). Similar punctate patterns for acyl-CoA oxidase and 3-ketoacyl-CoA thiolase were seen (data not shown). The pattern for the L-bifunctional protein was not evident, because content was low.

Defect of the Synthesis of the Enzyme Protein

A fluorographic band at a position of 77 kD was seen in the pulse-labeling experiment for the control cells (fig. 5). This band disappeared in a competition experiment with an excess amount of the purified enzyme. No band was detected for the patients' cells. Biosynthesis of acyl-CoA oxidase, L-bifunctional protein, and 3-ketoacyl-CoA thiolase was normal for both patients and controls (Suzuki et al. 1994).

Absence of Catalytic Activities of D-Bifunctional Protein

In control fibroblasts, treatment with anti–D-bifunctional protein antibody resulted in a decrease in the dehydratase activities with medium-chain enoyl-CoA. The titrated octenoyl-CoA hydratase activity was ~160 milliunits/mg (fig. 6*A*), a value similar to that for D-3-hydroxyoctanoyl-CoA dehydratase activity (data not shown). On the other hand, no significant reduction in octenoyl-CoA hydratase activity was observed after treatment with the anti–D-bifunctional protein antibody, for patients 1 and 2 (fig. 6*B* and *C*). Residual activities after treatment with both anticrotonase and anti–D-bifunctional protein may be attributed to the trifunctional protein (Jiang et al. 1996*a*, 1996*b*).

D-3-Hydroxyoctanoyl-CoA dehydratase activity in a control liver was 0.56 ± 0.10 unit/mg protein of the extract ($n = 5$). The activity in samples from patients 1 and 2 was estimated to be <0.01 unit/mg protein.

Figure 7*A* shows results of the separation of D-bifunctional and L-bifunctional proteins, by phosphocellulose column chromatography, in a control liver. Two octenoyl-CoA hydratase-activity peaks were observed; the first peak exhibited D-3-hydroxy-octanoyl-CoA dehydratase activity, but the second peak showed no dehydratase activity. The substrate specificity of hydratase activities in the first and second peaks was similar to that of the purified D-bifunctional and L-bifunctional proteins, indicating that the first peak is the D-bifunctional protein and the second is the L-bifunctional protein. On the other hand, only the second peak was found for patient 1 (fig. 7*B*). The L-3-hydroxyoctanoyl-CoA dehydratase activity was identified in the second peak, but the D-3-hydroxyoctanoyl-CoA dehydratase activity was hardly detectable.

When the dehydrogenase activity was monitored by acetoacetyl-CoA-dependent NADH oxidation, the first peak, due to mitochondrial 3-hydroxyacyl-CoA dehydrogenase, and the second peak, due to the L-bifunctional protein, were observed both in a control and in patient 1 (fig. 7). The D-bifunctional protein fraction did not exhibit dehydrogenase activity with acetoacetyl-CoA but did so with 3-keto-octanoyl-CoA. In contrast, the L-bifunctional protein fraction exhibited the activity

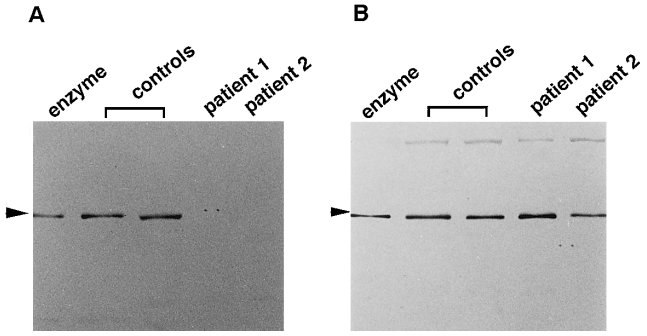

Figure 3 Immunoblot analyses of liver tissue. *A*, Results for D-bifunctional protein. Lane 1, Enzyme (10 ng). Lanes 2 and 3, Controls; Lane 4, Patient 1; Lane 5, Patient 2 (5 μg protein). *B*, Results for L-bifunctional protein. Lane 1, Enzyme (5 ng). Lanes 2 and 3, Controls; Lane 4, Patient 1; Lane 5, Patient 2 (5 μg protein).

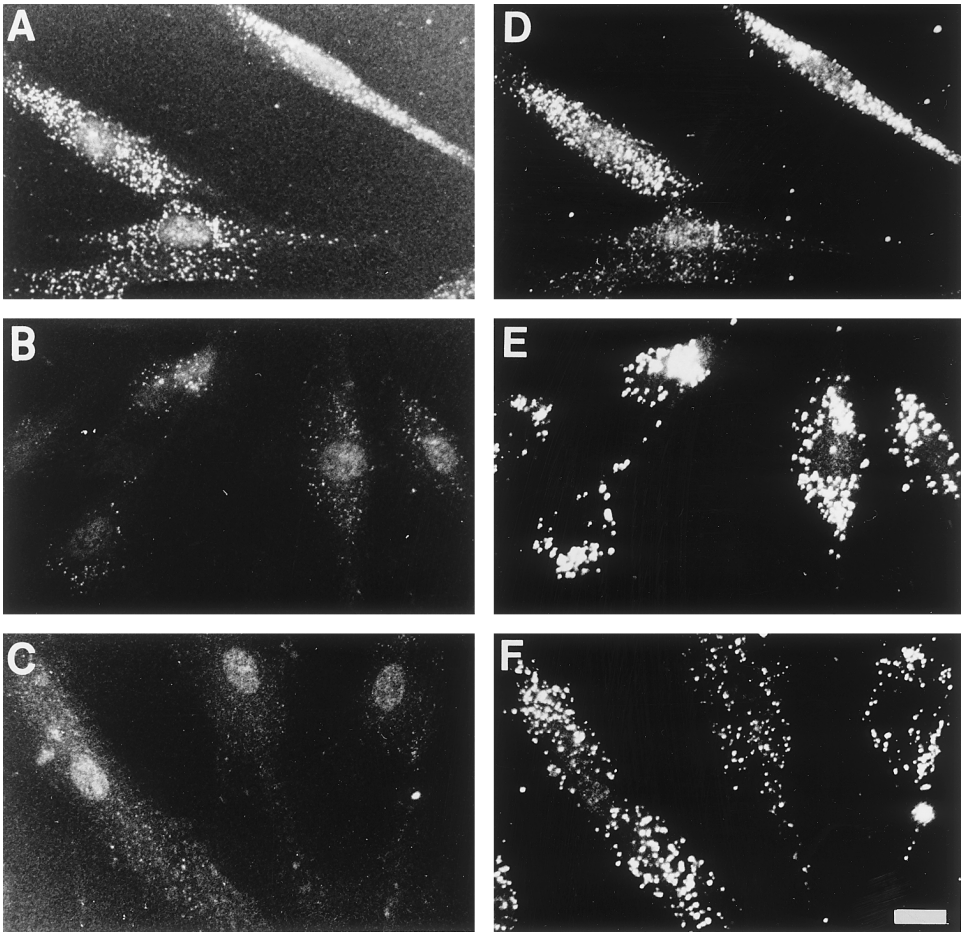

Figure 4 Immunofluorescence examination of the D-bifunctional protein and the catalase. *A*, Results for control (D-bifunctional protein). *B*, Results for patient 1 (D-bifunctional protein). *C*, Results for patient 2 (D-bifunctional protein). *D*, Results for control (catalase). *E*, Results for patient 1 (catalase). *F*, Results for patient 2 (catalase). Bar = 10 μm.

with both 3-keto-octanoyl-CoA and acetoacetyl-CoA. These substrate specificities were similar to those of the purified enzyme preparations. The ratios of hydratase and dehydrogenase activities in the first and second peaks also were similar to those of the purified D-bifunctional and L-bifunctional proteins.

Oxidation of Palmitate and Lignocerate in Fibroblasts

Table 1 summarizes fatty-acid–oxidation activities in the presence or the absence of sodium 2-(5-[4-chlorophenyl]pentyl)oxirane-2-carboxylate (POCA), a potent inhibitor of carnitine palmitoyltransferase I (Suzuki et

Table 1

Fatty-Acid Oxidation in Human Skin Fibroblasts

	Palmitate Oxidation (pmol/h/mg protein)		Lignocerate Oxidation (pmol/h/mg protein)	
Subject (s)	POCA Absent	POCA Present	POCA Absent	POCA Present
Controls (n = 5)	799 ± 299	62 ± 19	283 ± 98	280 ± 35
X-linked ALD patients (n = 3)[a]	566 ± 77	66 ± 12	77 ± 10	63[b]
Patient 1	785	14	24	15
Patient 2	763	32	25	15
Zellweger syndrome patients (n = 5)	683 ± 206	12 ± 9	19 ± 14	7 ± 11

[a] ALD = adrenoleukodystrophy.
[b] n = 1.

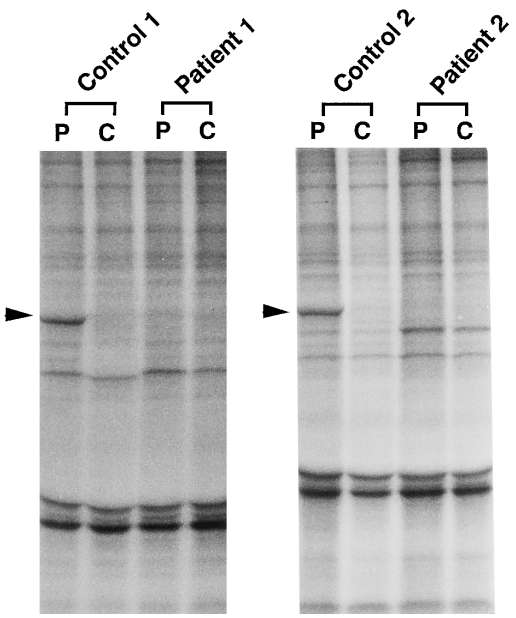

Figure 5 Results of pulse labeling of the D-bifunctional protein in fibroblasts. Lane P, Pulse labeling for 3 h. Lane C, Competition experiment using an excess amount of the purified enzyme. Arrowheads indicate the position of the 77-kD polypeptide of the D-bifunctional protein.

al. 1991). Palmitate oxidation for patients 1 and 2 was normal in the absence of POCA, thereby indicating the normal mitochondrial β-oxidation of palmitate. In the presence of POCA, palmitate oxidation for the patients was lower, as has been observed for Zellweger syndrome, a defect of functional peroxisomes. Lignocerate oxidation remained unvaried with the addition of POCA, suggesting that this activity reflects peroxisomal β-oxidation. These activities in the patients also were reduced markedly. We propose that D-bifunctional protein plays a key role in the oxidation of long-chain fatty acids and of VLCFAs, in human fibroblasts.

Northern Blot Analysis

The D-bifunctional protein mRNA from control fibroblasts was ~2.7 kb. mRNA was not detected for patient 1 but was detected for patient 2. The content and size of the L-bifunctional protein and the β-actin mRNAs in the patients' fibroblasts were similar to those in the control fibroblasts (fig. 8).

Mutation Analysis

The coding region of D-bifunctional protein cDNA was amplified by RT-PCR, to search for mutations (fig. 9). Although D-bifunctional protein mRNA was not detected for patient 1, a small amount of cDNA was amplified. Two types of PCR products were identified for patient 1. One allele had a 52-bp deletion encompassing bases at nucleotide positions 1210–1261, which led to a 17-amino-acid deletion at 404V–420A and which in-

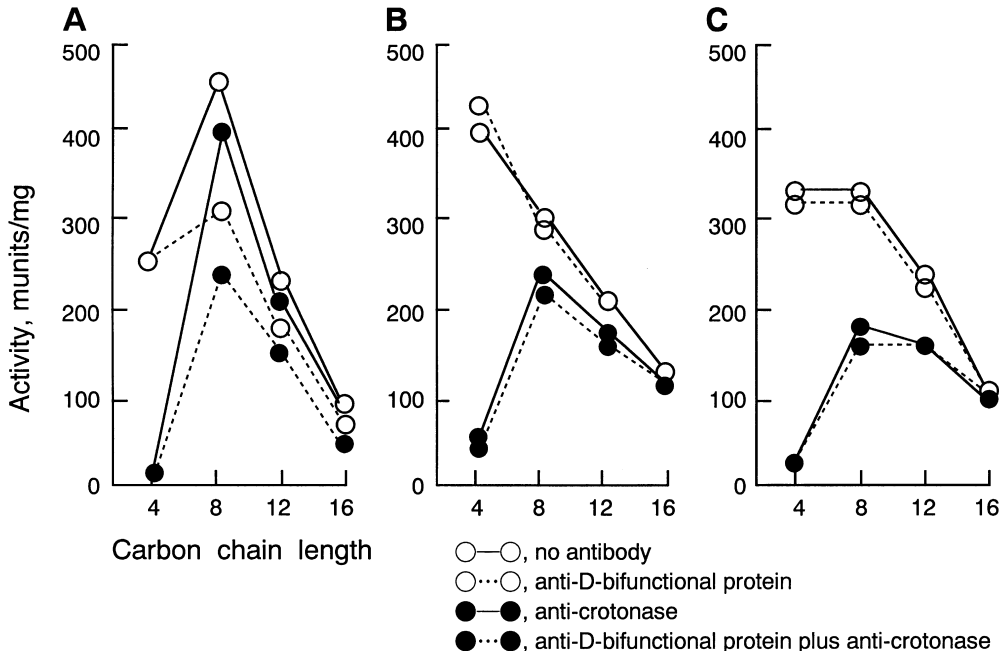

Figure 6 Enoyl-CoA hydratase activities in fibroblasts. The activities were measured before and after immunoprecipitation. *A*, Results for control. *B*, Results for patient 1. *C*, Results for patient 2.

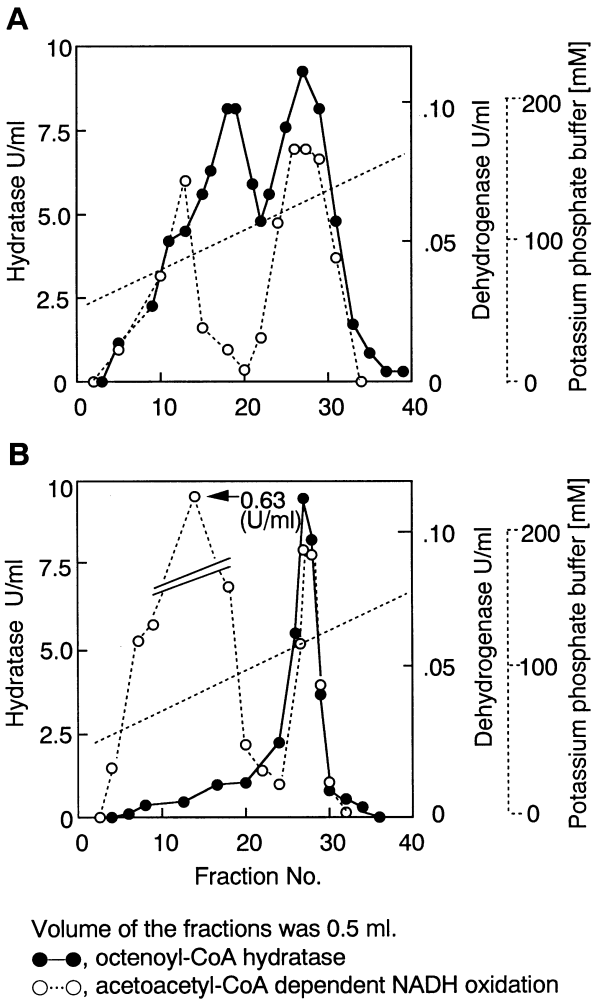

Figure 7 Phosphocellulose column chromatography of liver extracts. *A*, Results for control. *B*, Results for Patient 1. The liver (0.25 g) was extracted with the same extraction buffer used for the fibroblasts. After the extract was desalted by passage through a column of Sephadex G-25 with 25 mM potassium phosphate, pH 7.5, 0.5% Tween 20, 2 mM mercaptoethanol, 1 mM EDTA, and 10% (v/v) ethylene glycol, the sample was applied onto a phosphocellulose column (2 ml, equilibrated with the desalting buffer). Elution was performed after washing with 2 ml of 50 mM potassium-phosphate buffer, by use of a linear-gradient concentration of potassium phosphate, pH 7.5, within a range of 50–200 mM and containing the same ingredients as the desalting buffer, in a total volume of 25 ml.

troduced a premature termination codon at amino acid position 423. An A1269→G substitution, with no amino acid change, also was identified in both types of PCR products. When PCR amplification was performed by use of a set of the sense (976–996) and antisense (1377–1357) primers, a 402-bp fragment was amplified in the controls, whereas a 52-bp deleted fragment was a major product for patient 1 (fig. 9*B*).

The PCR-amplified cDNA of patient 2 was smaller in size than that of the controls, with a 237-bp deletion encompassing bases at nucleotide positions 973–1209, an event that led to an in-frame 79-amino-acid deletion beginning at amino acid position 325 (fig. 9*A*). PCR using a set of the sense (934–964) and the antisense (1239–1219) primers produced a 306-bp fragment in the controls, whereas only a 69-bp fragment was detected for patient 2 (fig. 9*C*).

Discussion

The two patients investigated in this study were diagnosed previously as cases of L-bifunctional protein deficiency, by means of a complementation study (Suzuki et al. 1994). In the present study, examination of fibroblasts and of the autopsied livers from these patients revealed that both the activity and the immunoreactive material of D-bifunctional protein were absent. Mutation analyses of the cDNA revealed the presence of a short deletion in each patient, which produced a frameshift and a premature termination in patient 1 and an in-frame 79-amino-acid deletion in patient 2. Mutation analyses of family members could not be performed, since permissions for skin biopsies were not obtained. It is possible that other mutations, yet to be identified, may have resulted in the absence of mRNA production from the second allele, in both patients. The structure of genomic DNA for human D-bifunctional protein and mutations at the genomic level remain to be elucidated. Since ~46 kD of the C-terminal of the D-bifunctional protein showed only dehydratase activity (Jiang et al. 1997*b*), deleted portions seem to belong to the dehydratase domain. Results of northern blot analysis and of PCR amplification of the cDNA indicate that the

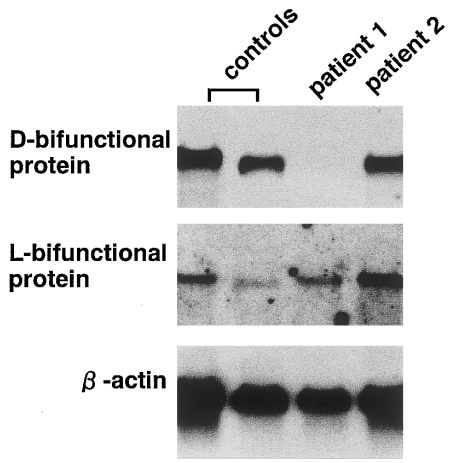

Figure 8 Results of northern blot analyses of mRNAs from fibroblasts. Four milligrams of total RNA were used. The exposure period to x-ray film was 7 min, 15 min, and 5 s, for detection of mRNAs of D-bifunctional protein, L-bifunctional protein, and β-actin, respectively. Lanes 1 and 2, Controls. Lane 3, Patient 1. Lane 4, Patient 2.

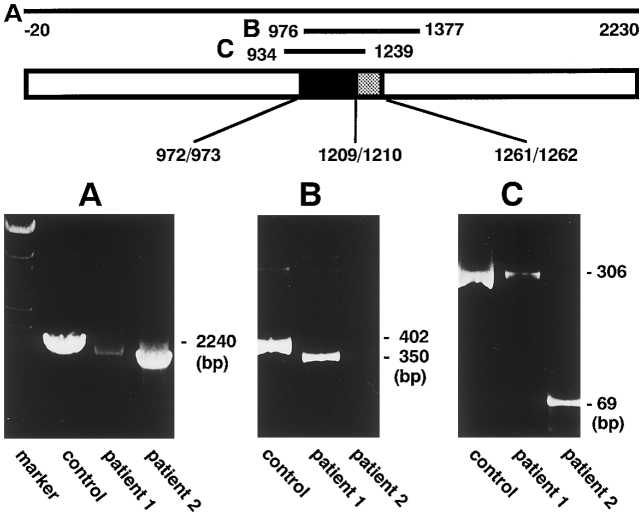

Figure 9 Results of PCR amplification of D-bifunctional protein cDNA, and mutations in the patients. The entire coding region of the D-bifunctional protein cDNA is indicated by the unblackened bar. Lines A–C indicate the sections of PCR amplification, and the numbers are the nucleotide positions of the cDNA corresponding to the 5′ ends of the primers. The lane labeled "marker" indicates the size marker. All lanes contain *Sty*I-digested λ-DNA as a size marker. The shaded (1210–1261) and blackened (973–1209) areas of the bar indicate deletions in patients 1 and 2, respectively.

mRNA of patient 1 would be unstable or poorly transcribed. On the other hand, the amount of mRNA in patient 2 was almost normal, suggesting that the truncated D-bifunctional protein with the in-frame 79-amino-acid deletion would be degraded so rapidly that it would not be detected in pulse-labeling experiments. The content and the two activities of L-bifunctional protein were within a normal range, in liver tissues from these patients, although the amount of L-bifunctional protein in fibroblasts was reduced. Therefore, the actual disease was D-bifunctional protein deficiency, not L-bifunctional protein deficiency.

Several acyl-CoA compounds other than VLCFAs and long-chain fatty acids are known to be oxidized predominantly in peroxisomes (Singh et al. 1990; Reddy and Mannaerts 1994; Kunau et al. 1995). In a previous study, we found distinct differences between functions of the D-bifunctional and the L-bifunctional proteins (Jiang et al. 1997*a*): D-bifunctional protein reacted with 2-methylhexadecenoyl-CoA, a branched fatty acid, and with 3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA, an intermediate metabolite of bile-acid synthesis. The deficiency of peroxisomal acyl-CoA oxidase resulted in accumulation of VLCFAs but not of pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) (ten Brink et al. 1991*a*), whereas the pristanic acid concentration was greatly elevated in patients with L-bifunctional protein deficiency, as has been reported elsewhere (ten Brink et al. 1991*b;* Van Veldhoven et al. 1993). Another characteristic feature of peroxisomal β-oxidation is the bile-acid formation from di- and trihydroxy-5β-cholestanic acid to chenodeoxycholic acid and cholic acid, respectively. Accumulation of bile-acid intermediates with an uncleaved side chain, such as 3α,7α,12α-trihydroxy-5β-cholestanic acid, occurs in peroxisome-biogenesis disorders and in cases of L-bifunctional protein deficiency (Watkins et al. 1989; Pedersen 1993; Natowicz et al. 1996).

In our previous study, the significance of D-bifunctional protein in the metabolism of VLCFAs and of bile acids, in peroxisomes, was clarified (Jiang et al. 1997*a*). Both D-bifunctional and L-bifunctional protein were highly expressed in hepatocytes. On the other hand, expression of L-bifunctional protein in fibroblasts was very low, and D-bifunctional protein was considered to play a major role in fibroblasts. Furthermore, β-oxidation of branched-chain fatty acids and of bile-acid intermediates was catalyzed exclusively by D-bifunctional protein, not by L-bifunctional protein. Defects in the D-bifunctional protein could explain not only the accumulation of VLCFAs but also the accumulation of bile-acid intermediates and of pristanic acid. We propose that patients previously diagnosed as cases of L-bifunctional protein deficiency probably should be reexamined.

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References

Adamski J, Normand T, Leenders F, Monte D, Begue A, Stehelin D, Jungblut PW, et al (1995) Molecular cloning of a widely expressed human 80 kDa 17β-hydroxysteroid dehydrogenase IV. Biochem J 311:437–443

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Clayton PT, Lake BD, Hjelm M, Stephenson JB, Besley GTN, Wanders RJA, Schram AW, et al (1988) Bile acid analyses in "pseudo-Zellweger" syndrome: clues to the defect in peroxisomal β-oxidation. J Inherit Metab Dis Suppl 11: 165–168

Fukuda S, Suzuki Y, Shimozawa N, Zhang Z, Orii T, Aoyama T, Hashimoto T, et al. Amino acid and nucleotide sequences of human peroxisomal enoyl-CoA hydratase: 3-hydroxyacyl-CoA dehydrogenase cDNA. J Inherit Metab Dis (in press)

Furuta S, Miyazawa S, Osumi T, Hashimoto T, Ui N (1980) Properties of mitochondrial and peroxisomal enoyl-CoA hydratases from rat liver. J Biochem (Tokyo) 88: 1059–1070

Hashimoto T (1982) Individual peroxisomal β-oxidation enzymes. Ann N Y Acad Sci 386:5–12

Iijima S, Hamada H, Reddy P, Kakunaga T (1985) Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. Proc Natl Acad Sci USA 82:6133–6137

Jiang LL, Kobayashi A, Matsuura H, Fukushima H, Hashimoto T (1996*a*) Purification and properties of human D-3-hydroxyacyl-CoA dehydratase: medium-chain enoyl-CoA hydratase is D-3-hydroxyacyl-CoA dehydratase. J Biochem (Tokyo) 120:624–632

Jiang LL, Kurosawa T, Sato M, Suzuki Y, Hashimoto T (1997*a*) Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. J Biochem (Tokyo) 121:506–513

Jiang LL, Miyazawa S, Hashimoto T (1996*b*) Purification and properties of rat D-3-hydroxyacyl-CoA dehydratase: D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. J Biochem (Tokyo) 120: 633–641

Jiang LL, Miyazawa S, Souri M, Hashimoto T (1997*b*) Structure of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. J Biochem (Tokyo) 121:354–369

Kobayashi A, Jiang LL, Hashimoto T (1996) Two mitochondrial 3-hydroxyacyl-CoA dehydrogenases in bovine liver. J Biochem (Tokyo) 119:775–782

Kunau W-H, Dommes V, Schulz H (1995) β-Oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. Prog Lipid Res 34:267–342

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Markwell MAK, Haas SM, Bieber LL, Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87:206–210

Miyazawa S, Osumi T, Hashimoto T (1980) The presence of a new 3-oxoacyl-CoA thiolase in rat liver peroxisomes. Eur J Biochem 103:589–596

Nakada Y, Hyakuna N, Suzuki Y, Shimozawa N, Takaesu E, Ikema R, Hirayama K (1993) A case of pseudo-Zellweger syndrome with a possible bifunctional enzyme deficiency but detectable enzyme protein. Brain Dev 15:453–456

Natowicz MR, Evans JE, Kelley RI, Moser AB, Watkins PA, Moser HW (1996) Urinary bile acids and peroxisomal bifunctional enzyme deficiency. Am J Med Genet 63:356–362

Osumi T, Hashimoto T, Ui N (1980) Purification and properties of acyl-CoA oxidase from rat liver. J Biochem (Tokyo) 87:1735–1746

Pedersen JI (1993) Peroxisomal oxidation of the steroid side chain in bile acid formation. Biochimie 75:159–165

Poll-The BT, Roels F, Ogier H, Scotto J, Vamecq J, Schutgens RBH, Wanders RJA, et al (1988) A new peroxisomal disorder with enlarged peroxisomes and a specific deficiency of acyl-CoA oxidase (pseudo–neonatal adrenoleukodystrophy). Am J Hum Genet 42:422–434

Reddy JK, Mannaerts GP (1994) Peroxisomal lipid metabolism. Annu Rev Nutr 14:343–370

Sanger F, Nickelen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schram AW, Goldfischer S, van Roermund CWT, Brouwer-Kelder EM, Collins J, Hashimoto T, Heymans HSA, et al (1987) Human peroxisomal 3-oxoacyl-coenzyme A thiolase deficiency. Proc Natl Acad Sci USA 84:2494–2496

Singh H, Usher S, Johnson D, Poulos A (1990) A comparable study of straight chain and branched chain fatty acid oxidation in skin fibroblasts from patients with peroxisomal disorders. J Lipid Res 31:217–225

Suzuki Y, Shimozawa N, Yajima S, Tomatsu S, Kondo N, Nakada Y, Akaboshi S, et al (1994) Novel subtype of peroxisomal acyl-CoA oxidase deficiency and bifunctional enzyme deficiency with detectable enzyme protein: identification by means of complementation analysis. Am J Hum Genet 54:36–43

Suzuki Y, Shimozawa N, Yajima S, Yamaguchi S, Orii T, Hashimoto T (1991) Effect of sodium 2-[5-(4-chlorophenyl) pentyl]oxirane-2-carboxylate (POCA) on fatty acid oxidation in fibroblasts from patients with peroxisomal diseases. Biochem Pharmacol 44:453–456

ten Brink HJ, Poll-The BT, Saudubray JM, Wanders RJA, Jakobs C (1991*a*) Pristanic acid does not accumulate in peroxisomal acyl-CoA oxidase deficiency: evidence for a distinct peroxisomal pristanoyl-CoA oxidase. J Inherit Metab Dis 14:681–684

ten Brink HJ, Wanders RJA, Stellaard F, Schutgens RBH, Jakobs C (1991*b*) Pristanic acid and phytanic acid in plasma from patients with single peroxisomal enzyme deficiency. J Inherit Metab Dis 14:345–348

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of protein from acrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Van Veldhoven PP, Huang S, Eyssen HJ, Mannaerts GP (1993) The deficient degradation of synthetic 2- and 3-methyl-branched fatty acids in fibroblasts from patients with peroxisomal disorders. J Inherit Metab Dis 16:381–391

Wanders RJA, van Roermund CWT, Brul S, Schutgens RBH, Tager JM (1992) Bifunctional enzyme deficiency: identification of a new type of peroxisomal disorder in a patient with an impairment in peroxisomal β-oxidation of unknown aetiology by means of complementation analysis. J Inherit Metab Dis 15:385–388

Wanders RJA, van Roermund CWT, Schelen A, Schutgens RBH, Tager JM, Stephenson JBP, Clayton PT (1990) A bifunctional protein with deficient enzymic activity: identification of a new peroxisomal disorder using novel methods to measure the peroxisomal β-oxidation enzyme activities. J Inherit Metab Dis 13:375–379

Watkins PA, Chen WW, Harris CJ, Hoefler G, Hoefler S, Blake DC Jr, Balfe A, et al (1989) Peroxisomal bifunctional enzyme deficiency. J Clin Invest 83:771–777

Watkins PA, McGuinness MC, Raymond GV, Hicks BA, Sisk JM, Moser AB, Moser HW (1995) Distinction between peroxisomal bifunctional enzyme and acyl-CoA oxidase deficiencies. Ann Neurol 38:472–477

Yamaguchi S, Orii T, Sakura N, Miyazawa S, Hashimoto T (1988) Defect in biosynthesis of mitochondrial acetoacetyl-CoA thiolase in cultured fibroblasts from a boy with 3-ketothiolase deficiency. J Clin Invest 81:813–817