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Peroxisomal Bifunctional Protein Deficiency Revisited: Resolution of Its True Enzymatic and Molecular Basis

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Summary

In the past few years, many patients have been described who have a defect of unknown origin in the peroxisomal β-oxidation pathway. Complementation analysis has been done by various groups to establish the extent of the genetic heterogeneity among the patients. These studies were based on the use of two established cell lines, one with a deficiency of acyl-CoA oxidase and one with a deficiency of L–bifunctional protein (L-BP), and they showed that most patients belong to the L-BP–deficient group. However, molecular analysis of the cDNA encoding L-BP in patients failed to show any mutations. The recent identification of a new D-specific bifunctional protein (D-BP) prompted us to reinvestigate the original patient with presumed L-BP deficiency. In a collaborative effort, we have now found that the true defect in this patient is at the level of the D-BP and not at the level of the L-BP. Our results suggest that most, if not all, patients whose condition has been diagnosed as L-BP are, in fact, D-BP deficient. We tested this hypothesis in nine patients whose condition was diagnosed as L-BP deficiency on the basis of complementation analysis and found clear-cut mutations in the D-BP cDNA from all patients.

Introduction

β-Oxidation of fatty acids is a vital function of eukaryotic cells. Fatty acid oxidation can proceed both in mitochondria and in peroxisomes via a four-step pathway involving dehydrogenation, hydratation, dehydrogenation again, and thiolytic cleavage. There are major differences between the mitochondrial and peroxisomal β-oxidation systems in regard to both their enzymatic organization and their precise role in cellular fatty acid oxidation. Mitochondria are involved in the β-oxidation of the bulk of fatty acids derived from our daily diet, whereas peroxisomes play an indispensable role in the oxidation of very-long–chain fatty acids, branched-chain fatty acids such as pristanic acid, and bile acid intermediates such as dihydroxycholestanoic and trihydroxycholestanoic acid (THCA).

It has been shown that multiple enzymes are involved in each step of the peroxisomal β-oxidation pathway. In humans two acyl-CoA oxidases with different substrate specificities have been identified, one for straight-chain fatty acids and one for 2-methyl branched-chain fatty acids (Vanhove et al. 1993). Furthermore, two different thiolases are involved in the last step of the β-oxidation spiral: Straight-chain fatty acids are thiolytically cleaved by the conventional thiolase (Miyazawa et al. 1981; Antonenkov et al. 1997), whereas 2-methyl branched-chain fatty acids are thiolytically cleaved by sterol-carrier protein 2/3-oxoacyl-CoA thiolase (Antonenkov et al. 1997; Wanders et al. 1997; Bunya et al. 1998), which contains both thiolase activity and sterol-carrier protein activity. In addition, a new bifunctional protein with a substrate specificity quite different from that of the original bifunctional protein was recently described. Unlike the other bifunctional protein, which produces an L-3-hydroxyacyl-CoA as intermediate, this newly identified enzyme generates a D-hydroxyacyl-CoA intermediate (Dieuaide-Noubhani et al. 1996; Jiang et al. 1996; Jiang et al. 1997*a,* 1997*b;* Qin et al. 1997*b*). On the basis of these findings, the two proteins have been named "L–bifunctional protein" (L-BP) and "D–bifunctional protein" (D-BP) (Jiang et al. 1997*a,* 1997*b*). Both bifunctional proteins accept straight-chain enoyl-CoAs as substrates, but they differ with respect to other enoyl-

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CoAs such as the enoyl-CoA ester of THCA, which is accepted only by the D-specific enzyme (Jiang et al. 1997*a;* Qin et al. 1997*a,* 1997*b*).

In patients with L-BP deficiency one would expect elevated very-long–chain fatty acids with normal bile acid intermediates. Watkins and coworkers (1989), on the basis of immunoblot analysis performed in postmortem liver material and fibroblasts, concluded that the patient they analyzed lacked L–bifunctional enzyme. Nevertheless there was accumulation of both very-long–chain fatty acids and bile acid intermediates, which is hard to reconcile with an isolated deficiency of L-BP. Since fibroblasts from the patient of Watkins and coworkers (1989) are generally used as the reference cell line for complementation analysis (Wanders et al. 1992; McGuinness et al. 1993; Suzuki et al. 1994; Moser et al. 1995; Watkins et al. 1995; Paton et al. 1996; Wanders et al. 1996; van Grunsven et al. 1997; van Grunsven and Wanders 1997), it is important to establish the true genetic basis of bifunctional enzyme deficiency in this patient.

The results described in this paper provide evidence suggesting that the true genetic defect in the patient analyzed by Watkins et al. (1989) is in the gene coding for D-BP and not in the gene coding for L-BP. We have used this newly acquired knowledge to identify the molecular basis of the genetic defect in nine patients classified as L-BP deficient by means of complementation analysis (van Grunsven and Wanders 1997).

Patients and Methods

Patient T.C.

Patient T.C.'s clinical and biochemical characteristics have been described elsewhere (Watkins et al. 1989).

Fatty Acid Oxidation in Cultured Skin Fibroblasts

Pristanic acid β-oxidation in patient T.C. was measured as described by Wanders et al. (1995).

Cell Culture Conditions and Preparation of Extracts

Fibroblasts were cultured in HAM-F10 medium (Gibco BRL) supplemented with 10% FCS (Bio-Whittaker). The cells were trypsinized, washed with PBS, and spun down. Cell pellets were stored at −80°C until they were used. The cell pellets were resuspended in PBS, 0.25% Triton X-100, 1 μg/ml Pefabloc (Merck), and 10 μg/ml leupeptine (Boehringer Mannheim) and sonicated twice. Liver samples were homogenized in the same buffer and sonicated three times. The homogenates were centrifuged at 10,000 *g* for 5 min and the supernatants were preserved.

Immunoblot Analysis

SDS-PAGE was conducted as described by Laemmli (1970). For immunoblot analysis, proteins were transferred from 10% (w/v) acrylamide gels to nitrocellulose paper. After undergoing blocking with 5% Protifar (Nutricia) in PBS/0.1% Tween-20 for 1 h, the blots were incubated for 2 h with rabbit polyclonal antibodies raised against D-BP or L-BP diluted in 3 mg/ml BSA. The antibodies raised against human D-BP (preparation II) and L-BP have been characterized elsewhere (Jiang et al. 1996; Suzuki et al. 1997). After being washed five times with PBS/0.1% Tween-20, the blots were incubated for 1 h with goat anti–rabbit IgG (H+L) alkaline phosphatase (Biorad) in 5% Protifar in PBS/0.1% Tween-20. The blots were then washed five times with PBS/0.1% Tween-20 and stained with 0.33 mg/ml nitro blue tetrazolium (Biorad) and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Biorad) in 100 mM Tris-HCl buffer, at 9.5 pH, containing 100 mM NaCl and 5 mM $MgCl_2$.

Immunofluorescence Analysis

Cultured fibroblasts were washed twice with 10 mg/ml BSA in PBS and fixed for 20 min with 2% paraformaldehyde in PBS/0.1% Triton X-100. Cells were washed twice with PBS/0.1% Triton X-100, and free aldehyde groups were blocked by incubating for 10 min in 0.1 M NH_4Cl in PBS. Cells were washed three times with 10 mg/ml BSA in PBS and incubated for 45 min with anti–D-BP (see Immunoblot Analysis) or anticatalase (Heikoop et al. 1991). Cells were washed three times with 10 mg/ml BSA in PBS, incubated for 30 min with biotinylated donkey anti–rabbit Ig (Amersham), and stained with streptavidin-labeled fluorescein isothiocyanate (streptavidin-FITC) (Gibco BRL).

Measurement of D-BP

The combined activity of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase components of the D-BP was measured as described elsewhere (van Grunsven et al. 1998). Fibroblast homogenates were incubated for 60 min at 37°C in a medium containing 50 mM Tris-HCl at 8.5 pH, 1 mM NAD^+, 150 mM KCl, 0.1 mM (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA (24-ene-THC-CoA; prepared as described by Xu and Cuebas [1996]), 5 mM pyruvate, and 18 U/ml lactate dehydrogenase. The final protein concentration was 150 μg/ml. Reactions were terminated by addition of 2 M HCl to a final concentration of 0.18 M followed by neutralization to a pH of ~5.0 by addition of 0.6 M MES plus 2 M KOH. Resolution of the different CoA-esters was achieved essentially as described by Xu and Cuebas (1996).

RNA Isolation and cDNA Synthesis

We isolated total RNA from cultured skin fibroblasts (stored at −80°C) by following the acid guanidium thiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi (1987) and subsequently used to prepare cDNA (Ijlst et al. 1994).

PCR and Sequencing

The cDNAs encoding either L-BP or D-BP were amplified in three overlapping fragments by means of primers with −21M13 or universal M13 extensions (5′-tgt aaa acg acg gcc agt-3′ and 5′-cag gaa aca gct atg acc-3′, respectively).

L-BP.—(1) Fragment 1 (bases −57 to 737; ATG=1; Hoefler et al. 1994; Fukuda et al. 1998) was amplified with primers 5′-tgt aaa acg acg gcc agt GGA AAA GTG GTT TGC CTG (sense) and 5′-cag gaa aca gct atg acc TCA TAG GGA TAC TGC ACA GC-3′ (antisense). (2) Fragment 2 (bases 649 to 1,581) was amplified with primers 5′-tgt aaa acg acg gcc agt TCT TGA AGA TGC GGA GGC AGC-3′ (sense) and 5′-cag gaa aca gct atg acc CAA CCC AGC AAG ATC AGA CAC-3′ (antisense). (3) Fragment 3 (bases 1,408 to 2,212) was amplified with primers 5′-tgt aaa acg acg gcc agt GGA GTC GTT GTA GGC AAC TG-3′ (sense) and 5′-cag gaa aca gct atg acc ACC TGA TGC TAG CAT GTG AG-3′ (antisense).

D-BP.—(1) Fragment 1 (bases −48 to 806; ATG=1; Adamski et al. 1995) was amplified with primers 5′-tgt aaa acg acg gcc agt GGC CAG CGC GTC TGC TTG TTC-3′ (sense) and 5′-cag gaa aca gct atg acc ACT GCC TCA GGA GTC ATT GG-3′ (antisense). (2) Fragment 2 (bases 675 to 1,543) was amplified with primers 5′-tgt aaa acg acg gcc agt TTG TCA CGA GAG TTG TGA GG-3′ (sense) and 5′-cag gaa aca gct atg acc GTA AGG GAT TCC AGT CTC CAC-3′ (antisense). (3) Fragment 3 (bases 1,489 to 2,313) was amplified with primers 5′-tgt aaa acg acg gcc agt ACC TCT CTT AAT CAG GCT GC-3′ (sense) and 5′-cag gaa aca gct atg acc CCC TGC ATC TTA GTT CTA ATC AC-3′ (antisense).

PCR reactions (final volume, 25 μl) contained 2–5 μl cDNA, 10 mM Tris-HCl (at 8.4 pH), 50 mM KCl, 0.1 mg/ml BSA, oligonucleotide primers as indicated (12.5 pmol each), 1.5–2.0 mM $MgCl_2$, and 2.5 U *Taq* polymerase. Thermocycling conditions consisted of 120 s at 96°C from initial to cycling and 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72 °C, followed by 10 min of extension at 72°C.

We performed sequence analysis of the PCR fragments after we purified the fragments from the PCR reaction mixtures with the Prep-A-Gene DNA purification system (Biorad). We sequenced sense and antisense strands with −21M13 and universal M13 (M13rev) fluorescent primers, respectively, on an Applied Biosystems 377A automated DNA sequencer in keeping with standard protocols provided by the manufacturer.

Subcloning of PCR-Amplified Fragments and Sequencing

To separate the two cDNAs of L-BP, we amplified the cDNA encoding L-BP with primers 5′-tgt aaa acg acg gcc agt GGA GTC GTT GTA GGC AAC TG-3′ (sense) and 5′-cag gaa aca gct atg acc ACC TGA TGC TAG CAT GTG AG-3′ (antisense) and ligated the cDNA into a pGEM-T vector (pGEM-T Vector System; Promega). For sequencing, we amplified the inserts of the plasmids with the same primers and sequenced the PCR products as described above.

Results

Pristanic Acid β-Oxidation

Patient T.C. was previously described as the first patient with L-BP deficiency (Watkins et al. 1989). Abnormal levels of very-long–chain fatty acids were found in both plasma and fibroblasts, and elevated levels of THCA were found in plasma. In addition, C24:0 β-oxidation was deficient in fibroblasts from the patient (Watkins et al. 1989). We have now extended these studies and have found that oxidation of pristanic acid, a 2-methyl fatty acid, is also deficient in the patient's fibroblasts (table 1). These data are hard to reconcile with an isolated deficiency of L-BP, since it is now known that the enoyl-CoA ester of pristanic acid is primarily if not exclusively handled by the newly recognized D-BP (Jiang et al. 1997*a*).

Table 1

Activity Measurements of Pristanic Acid β-Oxidation and D-BP in Fibroblasts

Parameter Measured	Patient T.C.	Controls
Peroxisomal β-oxidation (nmol/h/mg protein):		
Pristanic acid as substrate	13[a] 47[a] 97[a] 4[a]	1,030 ± 475 (74)[b]
D-BP activity (nmol/min/mg protein):		
Enoyl-CoA hydratase[c]	.05	.28 ± .02 (10)[b]
D-hydroxyacyl-CoA dehydrogenase[d]	ND[e]	.11 ± .02 (10)[b]

[a] Four separate experiments were conducted.
[b] Values listed represent mean ± SD ($n - 1$).
[c] Production of 24-hydroxy-THC-CoA.
[d] Production of 24-keto-THC-CoA.
[e] ND = not detectable.

D-BP Measurements

The recent identification of D-BP, which reacts with the enoyl-CoA esters of straight-chain fatty acids, 2-methyl branched-chain fatty acids, and bile acid intermediates, prompted us to focus on this enzyme as the potential primary defect in this patient. We incubated fibroblast homogenates with the enoyl-CoA ester of THCA, which is handled by D-BP but not by L-BP, and measured the formation of 24-hydroxy-THC-CoA and 24-keto-THC-CoA by high-performance liquid chromatography. As shown in table 1, formation of both 24-hydroxy-THC-CoA and 24-keto-THC-CoA was strongly deficient in fibroblast homogenates from the patient.

Analysis of D-BP by Immunoblot and Immunofluorescence Analyses

The recent generation of a good antibody against D-BP (see Jiang et al. 1996 for details) allowed studies at the protein level. As shown in figure 1, a punctate fluorescence pattern of peroxisomes was found in control fibroblasts (fig. 1*a*), whereas a faint but diffuse pattern of immunofluorescence in the cytosol was detected in the patient's fibroblasts (fig. 1*b*). We observed that the nuclei in the patient's fibroblasts were highly fluorescent and irregularly shaped. When we used antibodies against catalase, we observed a punctate fluorescence pattern of peroxisomes in the patient's fibroblasts (fig. 1*e*). The immunofluorescent particles were enlarged and reduced in number in comparison to those in control fibroblasts (fig. 1*d*). This observation is in accordance with observations based on fibroblasts from other patients with a defect in peroxisomal β-oxidation (Suzuki et al. 1994). In tissue samples from patients with Zellweger syndrome, whose fibroblasts lack peroxisomes because of a defect in peroxisome biogenesis, we found a diffuse immunofluorescence pattern in the cytosol when we performed immunofluorescence, both with anti–D–bifunctional enzyme and with anticatalase (fig. 1*c* and *f*). These data show that peroxisomes were normally present in the patient's fibroblasts but that D–bifunctional enzyme was strongly deficient. We were not able to perform immunofluorescence with the antibody against L-BP (see also Suzuki et al. 1997).

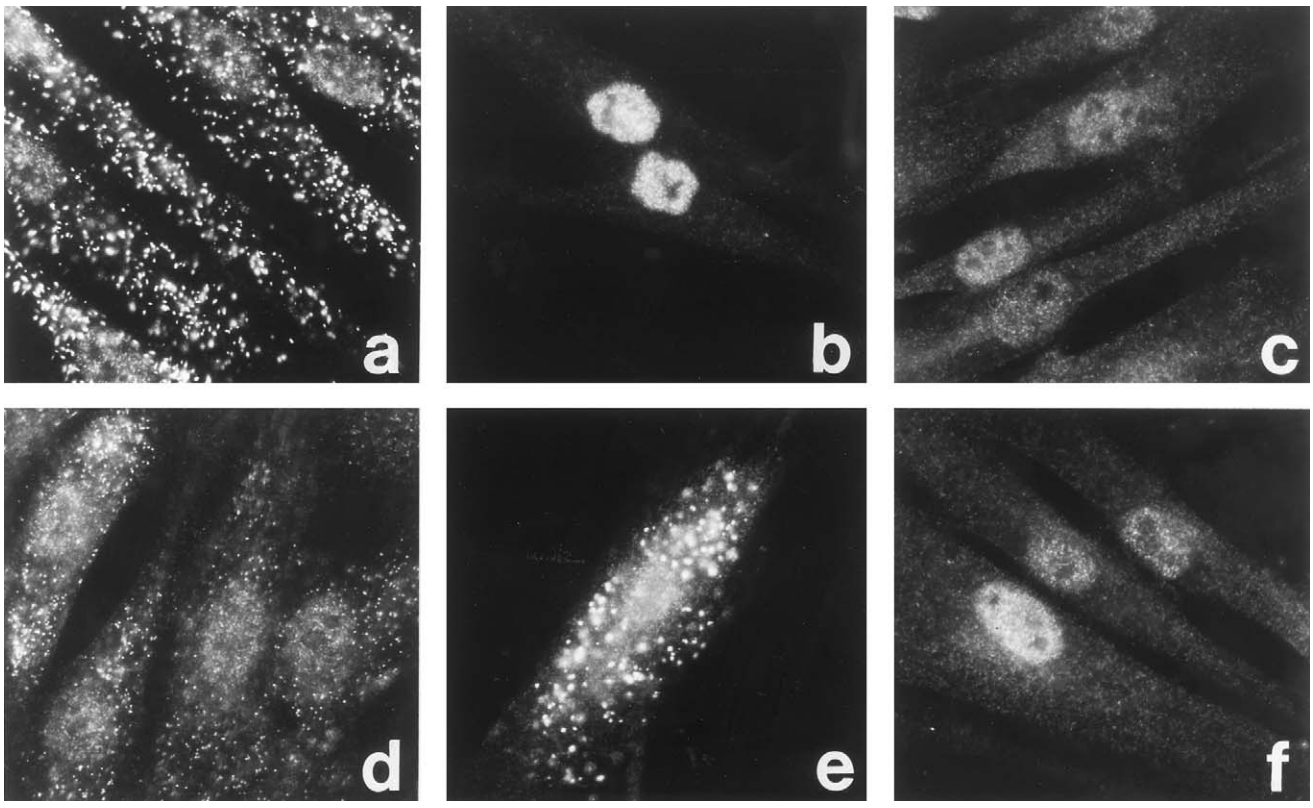

Figure 1 Immunofluorescence analysis of fibroblasts with anti–D-BP and anticatalase. *a,* Control (C1) fibroblasts, stained with anti–D-BP. *b,* Fibroblasts from patient T.C., stained with anti–D-BP. *c,* Fibroblasts from a Zellweger patient (Z1), stained with anti–D-BP. *d,* Control (C1) fibroblasts, stained with anticatalase. *e,* Fibroblasts from patient T.C., stained with anticatalase. *f,* Fibroblasts from a Zellweger patient (Z1), stained with anticatalase.

Immunoblot analysis confirmed the finding that D-BP was absent in the liver and fibroblasts of the patient (fig. 2). In control livers and fibroblasts, not only the full-length protein of 79 kD but also the two proteolytically processed polypeptides were present: the 45-kD band, corresponding to the enoyl-CoA hydratase component of D-BP, and the 35-kD band, corresponding to the 3-hydroxyacyl-CoA dehydrogenase component of D-BP (Leenders et al. 1994*a*, 1994*b*; Dieuaide-Noubhani et al. 1996; Jiang et al. 1996; Jiang et al. 1997*b*; Qin et al. 1997*b*). None of these bands were present in liver tissue or fibroblasts from the index patient. In Zellweger patients, minor amounts of the full-length protein were present in liver tissue, whereas about half the normal amount of full-length protein was present in fibroblasts. Only trace amounts of the processed components were present in liver samples from Zellweger patients, whereas these components could not be detected at all in fibroblasts from the Zellweger patients.

Using newly generated antibodies raised against L-BP, we found that L-BP was normally present in both liver tissue and fibroblasts of the patient (fig. 2). In fibroblasts many aspecific bands were present, but all bands were present in fibroblasts of the index patient. However, the suspected band for L-BP is specifically abolished by immunoprecipitation (see Jiang et al. 1997*a*), and this band was not detectable in fibroblasts from Zellweger patients. Furthermore, trace amounts of L-BP could be detected in liver tissue from Zellweger patients.

Resolution of the Molecular Basis of BP Deficiency

To identify the molecular basis of the deficiency of D-BP, we amplified the cDNA encoding D–bifunctional enzyme by PCR via three overlapping primer sets and subsequently sequenced the PCR products. Figure 3 shows that the cDNA of the patient had deletions at bp 422 and bp 423 of the cDNA, which causes a frameshift resulting in a truncated protein.

To determine whether the patient was homozygous for this deletion, we amplified the exon in which the deletion is located and parts of the surrounding introns, following the intron sequences worked out by Leenders and coworkers (in press). Sequence analysis revealed only the deleted sequence, which suggests that the patient might indeed be homozygous for the mutation (results not shown). Unfortunately, no material from the parents was available for analysis.

We also analyzed the cDNA encoding L-BP in the patient's cells to be sure that L-BP was not defective in the patient. Two cDNAs were found that most likely corresponded to the maternal and paternal alleles. Sequencing of these cDNAs revealed that the sequence of one cDNA was indistinguishable from the wild-type sequence, whereas in the second cDNA two nucleotide alterations were found (bp 2050 and bp 2066), leading to Arg→Gly and Ile→Thr amino acid substitutions at positions 684 and 689, respectively (see Discussion). The two (heterozygous) mutations were not present in the cDNA encoding L-BP of 24 control individuals. However, the presence of (at least) one normal allele for L-BP and the finding of normal L-BP levels via immunoblot analysis in both liver tissue and fibroblasts from the patient (fig. 2) argue against L-BP deficiency as the underlying basis for the deficient peroxisomal β-oxidation in this patient.

Analysis of D-BP cDNA in Additional Patients Classified as L-BP Deficient

Many patients have been classified as L-BP deficient on the basis of complementation studies that use cells from patient T.C. as the reference cell line (Wanders et al. 1992; McGuinness et al. 1993; Suzuki et al. 1994; Moser et al. 1995; Watkins et al. 1995; Paton et al. 1996; Wanders et al. 1996; van Grunsven et al. 1997; van Grunsven and Wanders 1997). To ascertain whether these patients were also affected in the gene coding for D-BP, we performed sequence analysis of the cDNA coding for D-BP in a series of nine patients identified elsewhere by two of us (van Grunsven and Wanders 1997). The results, presented in table 2, show a series of different mutations in the D-BP cDNA, including two missense mutations (Gly16Ser and Leu21Phe), a 66-bp deletion (1438-1503del), a 342-bp deletion (281-622del), and a 13-bp deletion (869-881del). All mutations were found to be homozygous at the cDNA level. The parents of patient 1, patient 2, and patient 9 are consanguineous, suggesting that these patients are indeed homozygous for the mutations found. Whether the other patients are truly homozygous for the mutations identified or heterozygous, with the other allele being a null allele, remains to be established. Studies at the genomic level are under way to resolve this.

We also performed sequence analysis of the L-BP cDNA, and we found no mutations in our series of nine patients.

Discussion

The patient studied in this paper is known in the literature as the only patient with a documented deficiency of the L-BP, as established by Watkins and coworkers (1989). L-BP deficiency was confirmed from the fact that immunoblot analysis detected no L-BP in postmortem liver and fibroblast fractions, whereas its corresponding mRNA was found to be normally present. Earlier studies of plasma from the patient had shown elevated levels of very-long–chain fatty acids and of THCA. Until recently these data were generally explained on the basis of the

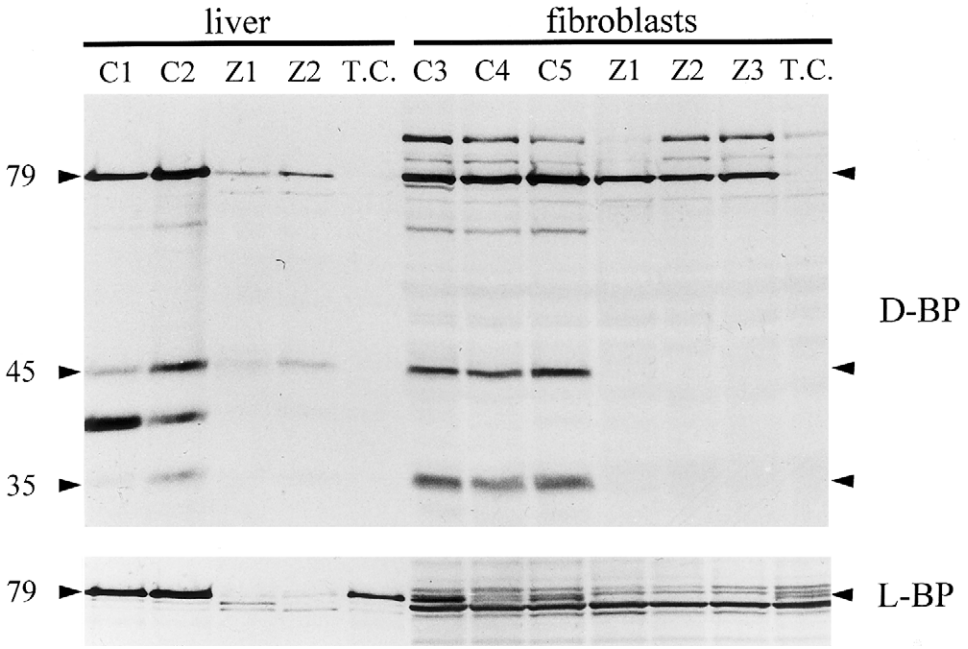

Figure 2 Immunoblot analysis of D-BP and L-BP in liver tissue (5 μg protein) and fibroblasts (50 μg protein) of five controls (C1–C5), three Zellweger patients (Z1–Z3), and patient T.C. Arrowheads indicate the D-BP and L-BP.

belief that L-BP catalyzes the hydration and dehydrogenation of all enoyl-CoA esters, including the enoyl-CoAs of straight-chain fatty acids, of 2-methyl branched-chain fatty acids such as pristanic acids, and of bile acid intermediates such as THCA. The recent discovery that L-BP is not able to convert the enoyl-CoA esters of THCA (Xu and Cuebas 1996; Jiang et al. 1997*a*) and pristanic acid (Dieuaide-Noubhani et al. 1997; Jiang et al. 1997*a*) to their β-keto-acyl-CoAs was hard to reconcile with the conclusion that the index patient's condition was truly L-BP deficiency.

Our present data show that the defect in this patient is indeed D-BP deficiency. We conclude this on the basis of the following findings: (1) deficient activity of D-BP as shown enzymatically in fibroblast homogenates (table 1); (2) absence of D-BP as shown by immunofluorescence analysis (fig. 1) and immunoblot analysis (fig. 2); and (3) the identification of a homozygous deletion of 2 bp on the basis of studies at the cDNA (fig. 3) and genomic levels. The deletion of these 2 bp at position 422 and 423 of the cDNA causes a frameshift leading to a premature stop codon at position 490, which results in a shortened product of 163 amino acids (±17.5 kD). This protein lacking 573 amino acids is probably fully inactive since it lacks the catalytic amino acids tyrosine and lysine in the NYSAAK-motif characteristics of the short-chain–alcohol dehydrogenase gene family (Krozowski 1992). In addition, the truncated protein also lacks the C-terminus required for correct targeting of the protein to the peroxisomes. Indeed, D-BP has a typical PTS1-signal involving AKL as the terminal three amino acids. This signal has been shown to be both necessary and sufficient to target peroxisomal-matrix proteins to peroxisomes (Subramani 1996; Erdmann et al. 1997). The mislocalized truncated protein is probably unstable in the cytosol as reflected in the absence of any cross-reactive immunological material of the expected size as shown by immunoblot analysis. However, although it is

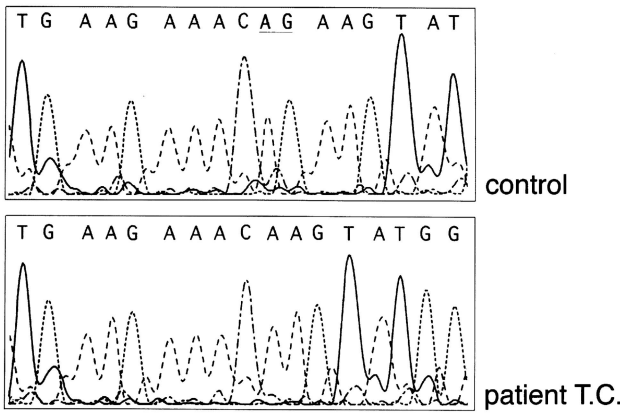

Figure 3 Mutation analysis of D-BP. Electropherograms show that patient T.C. is homozygous (on cDNA) for a deletion of 2 bp. The deleted base pairs are underlined in the electropherogram of the control.

Table 2

Analysis of the D-BP Encoding cDNA in Nine Patients Previously Classified as L-BP Deficient

cDNA Source[a]	Mutation	Coding Effect
Patient 1	1438-1503del	del480-501
Patient 2	1438-1503del	del480-501
Patient 3	46G→A[b]	Gly16Ser
Patient 4	46G→A[b]	Gly16Ser
Patient 5	46G→A[b]	Gly16Ser
Patient 6	281-622del	del94-208
Patient 7	46G→A[b]	Gly16Ser
Patient 8	63G→T	Leu21Phe
Patient 9	869-881del	Frameshift

[a] The patients were identified by complementation analysis as described elsewhere by van Grunsven and Wanders (1997).

[b] This mutation was found in a patient described elsewhere by van Grunsven et al. (1998).

an unlikely hypothesis, it cannot be excluded that the truncated protein lacks the epitope for recognition by the antibody used. In fibroblasts from Zellweger patients, the full-length protein is present in about half the normal amounts, but the processed components are completely absent. We hypothesize that the full-length D-BP is processed after transport into the peroxisome. Therefore, the full-length D-BP is present in the cytosol, which explains why the cytosolic labeling in Zellweger patients is more pronounced than that in cells from the index patient T.C. (fig. 1*b* and *c*). Since D-BP cannot be transported into peroxisomes in Zellweger patients, D-BP will not be processed, which explains why only the full-length protein is detected.

The data we obtained with L-BP suggest that L-BP is normally expressed in the patient's fibroblasts and liver tissue and show that L-BP deficiency is definitely not the cause of the defect in peroxisomal β-oxidation in this patient. We conclude this from the normal presence of L-BP as shown by immunoblot analysis (fig. 1*b*) and the identification of one mRNA species without mutations. The second mRNA was found to contain two mutations causing amino acid substitutions at positions 684 (Arg→Gly) and 689 (Ile→Thr). The consequences of these mutations for the L-BP protein generated have not been studied, since the product of the other allele will be completely normal. This means that the patient is either homozygously normal or heterozygously normal for L-BP.

The data presented in this paper that show the normal presence of L-BP when immunoblot analysis is performed are difficult to reconcile with the earlier data from Watkins et al. (1989). The most likely explanation for these discrepant results would be that the previously used antibody (Furuta et al. 1980) was directed primarily against the D-BP. It should be mentioned that the two bifunctional proteins have almost identical molecular weights and show comparable behavior as shown by chromatography. Unfortunately, this possibility cannot be investigated, since the antibody used in these earlier studies is no longer available.

Taken together, the data presented here show that the actual defect in the patient described by Watkins et al. (1989) is not at the level of L-BP but at the level of D-BP. This finding is of importance, especially since cells from this patient with presumed L-BP deficiency have been used in several complementation studies as a reference cell line to classify patients with an unknown defect in peroxisomal β-oxidation (Wanders et al. 1992; McGuinness et al. 1993; Suzuki et al. 1994; Moser et al. 1995; Watkins et al. 1995; Paton et al. 1996; Wanders et al. 1996; van Grunsven et al. 1997; van Grunsven and Wanders 1997). In one of our earlier studies (van Grunsven and Wanders 1997) we identified nine patients with presumed L-BP deficiency on the basis of complementation analysis. Mutation analysis of L-BP cDNA in these patients revealed no abnormalities. However, in all patients studied, clear mutations in the D-BP cDNAs were found (table 2). We found four patients with homozygous deletions: a 66-bp deletion leading to an in-frame deletion of 22 amino acids (two patients); a 342-bp deletion leading to an in-frame deletion of 114 amino acids; and a 13-bp deletion that caused a frameshift resulting in a truncated protein. Four patients were homozygous for a Gly16Ser mutation, which is in the dehydrogenase-coding part of the gene. The glycine at position 16 is in the Rossman fold forming the NAD^+–binding site (see van Grunsven et al. 1998). One patient is homozygous for the Leu21Phe mutation. The leucine at position 21 is also in the Rossman fold forming the NAD^+–binding site (McKie and Douglas 1991; Leenders et al. 1994*b;* Ghosh et al. 1995; Lin et al. 1996).

Recently, we identified a patient with D-BP deficiency who has the same Gly16Ser mutation as now found in four additional patients (table 2) (van Grunsven et al. 1998). We have performed complementation analysis and have found that cells from this particular patient and the index patient T.C., studied in the present article, do not show complementation, which is in line with the view that the molecular defect in both patients is in the gene coding for D-BP. Our data also provide an explanation for earlier results of Suzuki et al. (1997), who recently studied two patients whose condition was previously diagnosed as L-BP deficiency on the basis of complementation analysis. Both patients were indeed found to be D-BP deficient (Suzuki et al. 1997).

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