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A Novel Aberrant Splicing Mutation of the PEX16 Gene in Two Patients with Zellweger Syndrome

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Human Pex16p, a peroxisomal membrane protein composed of 336 amino acids, plays a central role in peroxisomal membrane biogenesis. A nonsense mutation (R176ter) in the *PEX16* gene has been reported in the case of only one patient (D-01) belonging to complementation group D of the peroxisome biogenesis disorders. We have now identified two patients belonging to group D (D-02 and D-03) whose fibroblasts were found to contain no peroxisomal membrane structure ghosts. Molecular analysis of the PEX16 gene revealed aberrant cDNA species lacking 65 bp, corresponding to exon 10 skipping caused by a splice site mutation (IVS10 + 2T → C). Both patients, although unrelated, were homozygous for this mutation. This mutation changes the amino acid sequence starting from codon 298 and introduces a termination codon at codon 336. As a consequence, the cell's ability to membrane synthesis and protein import is disrupted, which implies that the changed C terminus of the Pex16p in these patients likely affects its function.

Key Words: **peroxisome biogenesis disorders; PEX16; Zellweger syndrome; peroxisomal membrane protein; splice site mutation.**

Peroxisome biogenesis disorders (PBDs), including Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and rhizomelic chondrodysplasia punctata (RCDP), can be classified into 12 genetic complementation groups (A–H, J, 2, 3, and R) (1, 2). The 8 *PEX* genes involved in peroxisome matrix protein import are *PEX 1, -2, -5, -6, -7, -10, -12,* and *-13,* identified as pathogenic genes for PBD groups E, F, 2, C, R, B, 3, and H, respectively. Fibroblasts from these groups of subjects could not import matrix proteins, but did contain empty peroxisomal membrane structures (peroxisomal ghosts). On the other hand, the *PEX3, -16* and *-19* genes involved in an early stage of peroxisomal membrane assembly, are those involved in PBD groups G, D, and J. Studies in fibroblasts from these patients' groups have shown an inability to import both matrix proteins and peroxisomal membrane proteins (PMPs). Therefore, these fibroblasts were shown to have no peroxisomal ghosts (1, 3). Human *PEX16* cDNA (Hs*PEX16*), encoding a 336-amino-acid integral peroxisomal membrane protein (PMP), Pex16p, was isolated in a homology search of the database of expressed sequence tags with *Yarrowia lipolytica PEX16* (4, 5). A ZS patient from PBD group D (D-01 or PBD061 of the Kennedy-Krieger Institute, USA) and in whom the fibroblasts manifested an inability to import PMPs, was found to have an inactivating nonsense mutation, introducing a stop codon at position 176 in Hs*PEX16* (4, 5). However, only one patient with the *PEX16* defect has been identified so far. We now report the identification of two more patients of group D (D-02 and -03) and an inactivating splice site mutation in the *PEX16* gene in these patients.

PATIENTS AND METHODS

Patients. The cell line of D-01 (GM06231) from a ZS patient with a typical clinical presentation and no peroxisomal ghost (1), was shown to have a homozygous, inactivating nonsense mutation, R176ter (4, 5). The unrelated patients of D-02 and D-03 also showed the typical phenotype of ZS.

Methods. Peroxisomes in fibroblasts were visualized by indirect immunofluorescence light microscopy, as described (6), using rabbit antibodies to human catalase and human peroxisomal 70-kDa integral membrane protein (PMP70) (7).

pCMVSPORT · Hs*PEX16* (4) was transfected into fibroblasts from D-02 and D-03 patients, by the calcium phosphate method (6).

Abbreviations used: PBDs, peroxisome biogenesis disorders; ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; IRD, infantile Refsum disease; RCDP, rhizomelic chondrodysplasia punctata; PMP, peroxisomal membrane protein; TMD, transmembrane domain.

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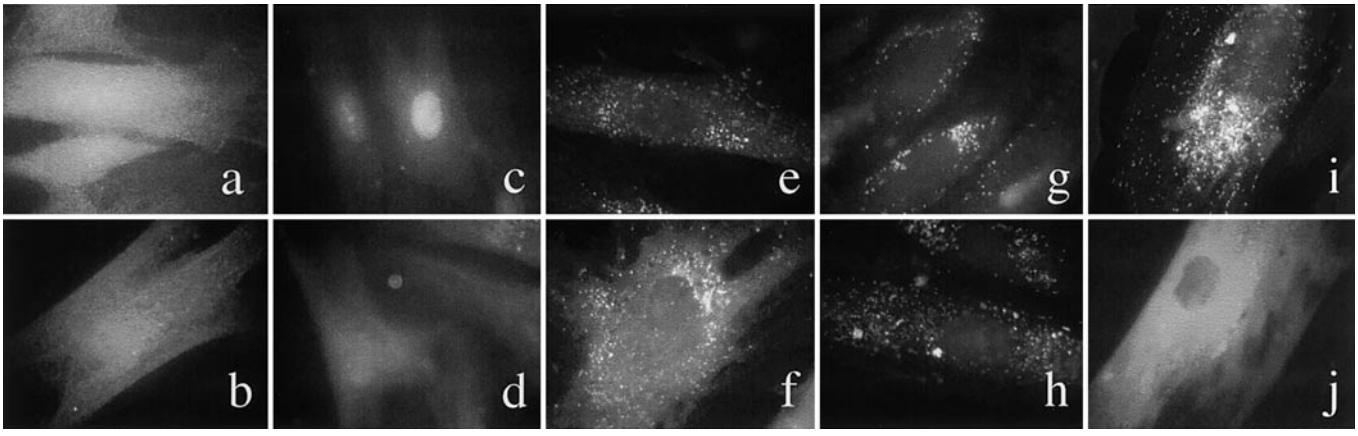

FIG. 1. Complementation of peroxisomes in fibroblasts from ZS patients. (a, c) Fibroblasts from patient D-02; (b, d) Fibroblasts from patient D-03; (e, g) D-02 fibroblasts transfected with PEX16; (f, h) D-03 fibroblasts transfected with *PEX16;* (i) D-01 fibroblasts transfected with PEX16; (j) D-01 fibroblasts transfected with PEX16/65 bp del. Cells were stained using antibodies to human catalase (a, b, e, f, i, j) or human PMP70 (c, d, g, h).

To search for mutations in the *PEX16* cDNA, we made use of RT/PCR to amplify *PEX16* cDNA (GenBank [accession numbers AB016531]) from fibroblasts RNA isolated from the 2 patients. We used primers 5′-TGTCGGTGCCGAGGGCAGGAT-3′ (forward) and 5′-AGCTGGATGAGGGCGATGACA-3′ (reverse) to obtain a 372 bp product, primers 5′-TGTTCATGGAGATGGGAGCT-3′ (forward) and 5′-AAGAGCCAGGGTTTCCACGA-3′ (reverse) to obtain a 463 bp product, and primers 5′-TTTGTACATTGCCCGGCCGCT-3′ (forward) and 5′-ATTAGGGAAGAGGGGCTCCCT-3′ (reverse) to obtain a 409 bp product. The amplified DNA fragments were directly sequenced using an automated DNA sequencer. To search for a possible 5′ side splice site mutation in intron 10, 156 bp genomic DNA fragments were obtained from the D-02 and -03 fibroblasts followed by PCR amplification using primers 5′-ATCCTCTTCCTGCTCCAGTTG-3′ (forward) and 5′-GCCAAGAATCAAATTGCAAG-3′ (reverse) and directly sequenced (Ensemble gene for *PEX16* [ENSG00000121680]). To assess the effect of mutations in the D-02 and D-03 patients, pCMVSPORT · Hs*PEX16*/65 bp-del was constructed using the 460-bp SacI-NotI fragment of the mutant *PEX16* cDNA from patient D-02, containing the 65-bp deletion, obtained by PCR amplification with primer set 5′-TGTTCATGGAGATGGGAGCT-3′ (forward) and 5′-TTTGCGGCCGCTGCCGGAGTCAGTTTTA-3′ (reverse), transfected into D-01 fibroblasts by the calcium phosphate transfection method (6).

RESULTS AND DISCUSSION

We transfected an expression vector containing the human *PEX16* cDNA (pCMVSPORT · Hs*PEX16*) into fibroblasts from patients of D-02 and -03, in which no particles stained positive with anti-catalase antibodies (Figs. 1a and 1b) and anti-PMP70 (Figs. 1c and 1d). After transfection, numerous punctate structures were seen in both D-02 and -03, determined using anti-catalase antibodies (Figs. 1e and 1f) and anti-PMP70 (Figs. 1g and 1h), which means that *PEX16* restored the peroxisomal membrane structures and matrix protein import in the D-02 and -03 fibroblasts.

To determine the dysfunction of *PEX16* in patients D-02 and -03, the coding region of the complete cDNA for human *PEX16* was amplified by RT-PCR. Direct sequencing of PCR amplified *PEX16* cDNA from both D-02 and -03 revealed a 65-bp deletion at position 888–952 (hereafter, starting from the first nucleotide of the initiator methionine codon), corresponding to exon 10 of the *PEX16* cDNA homozygously (Fig. 2a). This changes the amino acid sequence starting from codon 298 and introduces a termination codon at position 336 (Fig. 3). Only this deletion was observed in the patients' cDNA. Subsequent studies revealed a splice

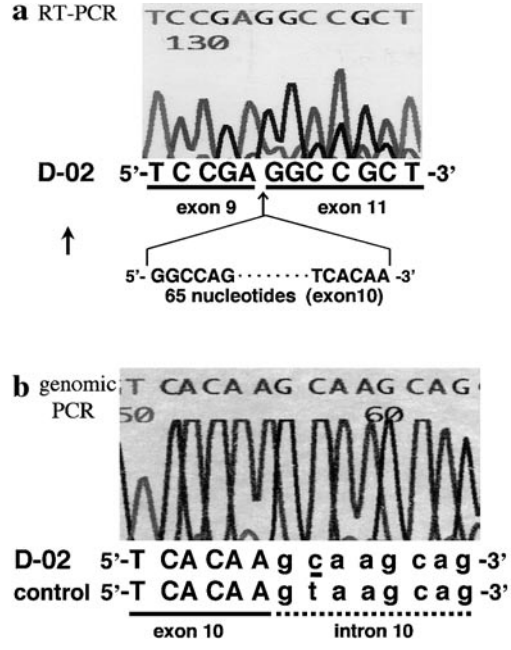

FIG. 2. Mutation analysis of *PEX16* from D-02 patient. (a) Nucleotide sequence of exon 10 skipping in D-02 patient's cDNA. (b) Nucleotide sequence of 5′ splice site of intron 3 in D-02 patient's genomic DNA. Splice site mutation of IVS10-2t → c (underline) caused exon 10 skip.

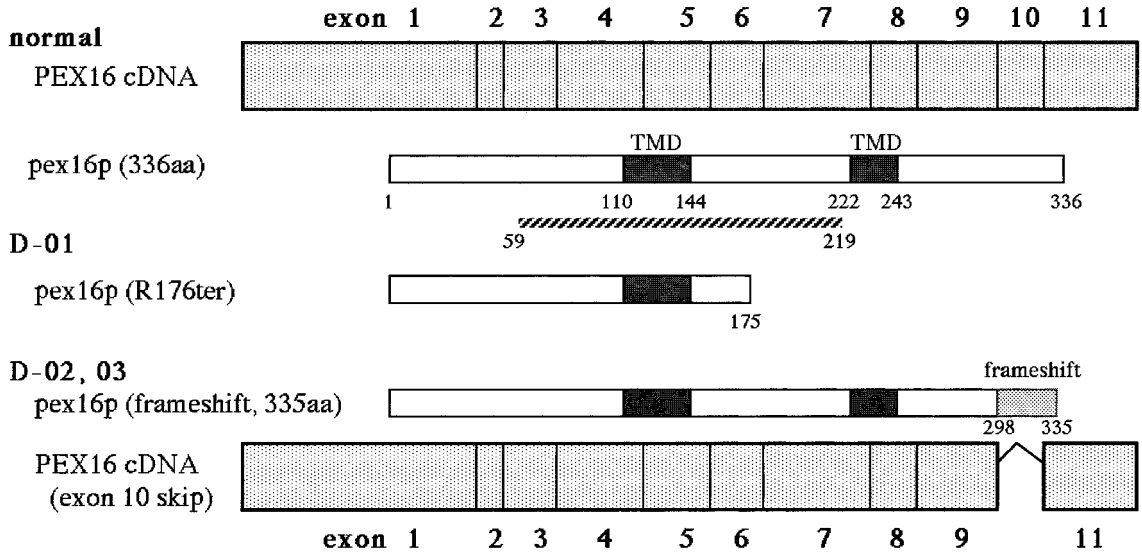

FIG. 3. Schematic diagram of *PEX16* cDNA and Pex16 protein from the normal control and patients D-01, D-02, and D-03. Two transmembrane domains (TMDs; amino acids 110–144 and 222–243) are shaded. The amino acid residues (59–219) required for sorting to peroxisomal membrane and for Pex19p binding are shown by oblique lines.

site mutation in genomic DNA from patients D-02 and -03. Both D-02 and -03 were shown to be homozygous for a splice site mutation of IVS10 + 2T → C (Fig. 2b). When pCMVSPORT · Hs*PEX16*/65 bp-del was transfected into D-01 fibroblasts, no particles stained with anti-catalase antibodies (Fig. 1j) and anti-PMP70 (data not shown) were evident, whereas numerous punctate structures stained with anti-catalase antibodies (Fig. 1i) and anti-PMP70 were seen in the D-01 fibroblasts transfected with pCMVSPORT · Hs*PEX16*, which means that the exon 10 skip mutation probably has a dramatic effect on the function of Pex16p.

PEX16 is responsible for PBD group D, whose fibroblasts are deficient in both matrix protein import and peroxisomal membrane ghosts, and is involved in the biogenesis of the peroxisome membrane, interacting with PEX19 (5, 8, 9).

The 336 amino acid-long Pex16p contains two transmembrane domains (TMDs) (amino acids 110–144 and 222–243) and located in the peroxisomal membrane with its NH2 and COOH termini extending into the cytoplasm and its intermembrane loop protected in the peroxisome lumen (5). Furthermore, Fransen *et al.* stated that amino acids 59–219 in the Pex16p are required for sorting to the peroxisome membrane and for binding to Pex19p, a predominantly cytosolic PMP-binding protein (9) (Fig. 3). The nonsense mutation of R176ter detected in the only reported case of Hs*PEX16* defect patient, D-01, affected PMP synthesis and matrix protein import, which means this mutated Pex16p was thought to be inactivated due to truncation of its intermembrane loop (Fig. 3). On the other hand, the exon 10 skip mutation of *PEX16* in patients D-02 and D-03 and which changes the amino acid sequence starting from codon 298 and introducing a termination codon at 336, also affected its potential for membrane synthesis and protein import, but two TMDs and a sufficient domain for sorting to the peroxisome membrane and Pex19p binding in this mutated Pex16p is thought to be left intact (Fig. 3). Therefore, we conclude that the changed C terminus of Pex16p may structurally affect its function and otherwise the C terminal 39 aa of Pex16p are essential for its function. The role of Pex16p in PMP import and peroxisome assembly, and its detailed functional domains are being addressed in ongoing studies.

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REFERENCES

1. Shimozawa, N., Suzuki, N., Zhang, Z., Imamura, A., Kondo, N., Kinoshita, N., Fujiki, Y., Tsukamoto, T., Osumi, T., Imanaka, T., Orii, T., Beemer, F., Mooijer, P., Dekker, C., and Wanders, R. J. A. (1998) Genetic basis of peroxisome-assembly mutants of humans, Chinese hamster ovary cells, and yeast: Identification of a new complementation group of peroxisome-biogenesis disorders apparently lacking peroxisomal-membrane ghosts. *Am. J. Hum. Genet.* **63,** 1898–1903.
2. Shimozawa, N., Suzuki, Y., Zhang, Z., Imamura, A., Ghaedi, K., Fujiki, Y., and Kondo, N. (2000) Identification of PEX3 as the gene mutated in a Zellweger syndrome patient lacking peroxisomal remnant structures. *Hum. Mol. Genet.* **9,** 1995–1999.
3. Gould, S. J., and Valle, D. (2000) Peroxisome biogenesis disorders: Genetics and cell biology. *Trends Genet.* **16,** 340–345.
4. Honsho, M., Tamura, S., Shimozawa, N., Suzuki, Y., Kondon, N., and Fujiki, Y. (1998) Mutation in PEX16 is causal in the

peroxisome-deficient Zellweger syndrome of complementation group D. *Am. J. Hum. Genet.* **63,** 1622–1630.

5. South, S. T., and Gould, S. J. (1999) Peroxisome synthesis in the absence of preexisting peroxisomes. *J. Cell. Biol.* **144,** 255–266.
6. Shimozawa, N., Tsukamoto, T., Suzuki, Y., Orii, T., Shirayoshi, Y., Mori, T., and Fujiki, Y. (1992) A human gene responsible for Zellweger syndrome that affects peroxisome assembly. *Science* **255,** 1132–1134.
7. Imanaka, T., Shiina, Y., Takano, T., Hashimoto, T., and Osumi, T. (1996) Insertion of the 70-kDa peroxisomal membrane protein into peroxisomal membranes in vivo and in vitro. *J. Biol. Chem.* **271,** 3706–3713.
8. Sacksteder, K. A., Jones, J. M., South, S. T., Li, X., Liu, Y., and Gould, S. J. (2000) PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *J. Cell. Biol.* **148,** 931–944.
9. Fransen, M., Wylin, T., Brees, C., Mannaerts, G. P., and Van Veldhoven, P. P. (2001) Human pex19p binds peroxisomal integral membrane proteins at regions distinct from their sorting sequences. *Mol. Cell. Biol.* **21,** 4413–4424.