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Molecular Mechanism of Detectable Catalase-Containing Particles, Peroxisomes, in Fibroblasts from a *PEX2*-Defective Patient

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Patients with peroxisome biogenesis disorders (PBD) can be identified by detection of peroxisomes in their fibroblasts, by means of immunocytochemical staining using an anti-catalase antibody. We report here data on three PBD patients with newly identified mutations (del550C and del642G) in the *PEX2* gene which encodes a 35-kDa peroxisomal membrane protein containing two membrane-spanning and a C-terminal cysteine-rich region. Some of the fibroblasts from the patient with the del642G mutation contained numerous catalase-containing particles, whereas no fibroblasts containing such particles were found in the patient with the del550C mutation. We confirmed that the del642G mutation caused a partial defect in peroxisome synthesis and import by expression of the mutated *PEX2* into *PEX2*-defective CHO mutant cells. We propose that the two putative membrane-spanning segments in Pex2p are important domains for peroxisome assembly and import and that a defect in one of these domains severely affects PBD patients. Furthermore, a defect in the C-terminal portion of Pex2p exposed to the cytosol containing a RING finger motif caused the mild phenotype, residual enzyme activities, and mosaic detectable peroxisomes in fibroblasts from the patient.

Key Words: **peroxisome biogenesis disorders; *PEX2*; catalase; membrane-spanning domain; RING finger motif.**

Abbreviations used: PBD, peroxisome biogenesis disorders; ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; IRD, infantile Refsum disease; RCDP, rhizomelic chondrodysplasia punctata; CHO, Chinese hamster ovary; *PEX*, genes of proteins involved in peroxisome biogenesis, peroxins; PTS, peroxisome targeting signal; VLCFA, very long chain fatty acid; DHAP-AT, dihydroxyacetone phosphate acyltransferase; PT, 3-ketoacyl-CoA thiolase.

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Peroxisome biogenesis disorders (PBD) which include Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and infantile Refsum's disease (IRD), are autosomal recessive diseases caused by a defective assembly of peroxisomes. Using somatic cell fusion, we have found that patients suffering from the different PBD can be classified into 12 complementation groups (CGs). The CGs include CG A-H and J of our group and CG 2, 3, and 6 of the Kennedy–Krieger Institute (1). There is no relationship between genotype and phenotype, and nine of these 12 different pathogenic genes of PBD have been identified.

The diagnosis of PBD patients is based on clinical features combined with a series of tests to assess peroxisomal function and structure. To this end, we analyze very long chain fatty acid (VLCFA) levels in plasma, and β-oxidation and dihydroxyacetone phosphate acyltransferase (DHAP-AT) activities in cultured skin fibroblasts, plus peroxisomes in liver biopsy specimens using electron microscopy or in fibroblasts by means of immunofluorescence microscopy (2). In most cases catalase immunofluorescence analysis reveals the absence of a punctate staining pattern. In some cases, however, especially in case of patients with IRD, a mosaic pattern is observed in fibroblasts with catalase-positive and negative cells. In such cases, it is difficult to diagnose PBD and to identify the complementation group by somatic cell fusion. We have now identified a mutation in the *PEX2* gene in a patient

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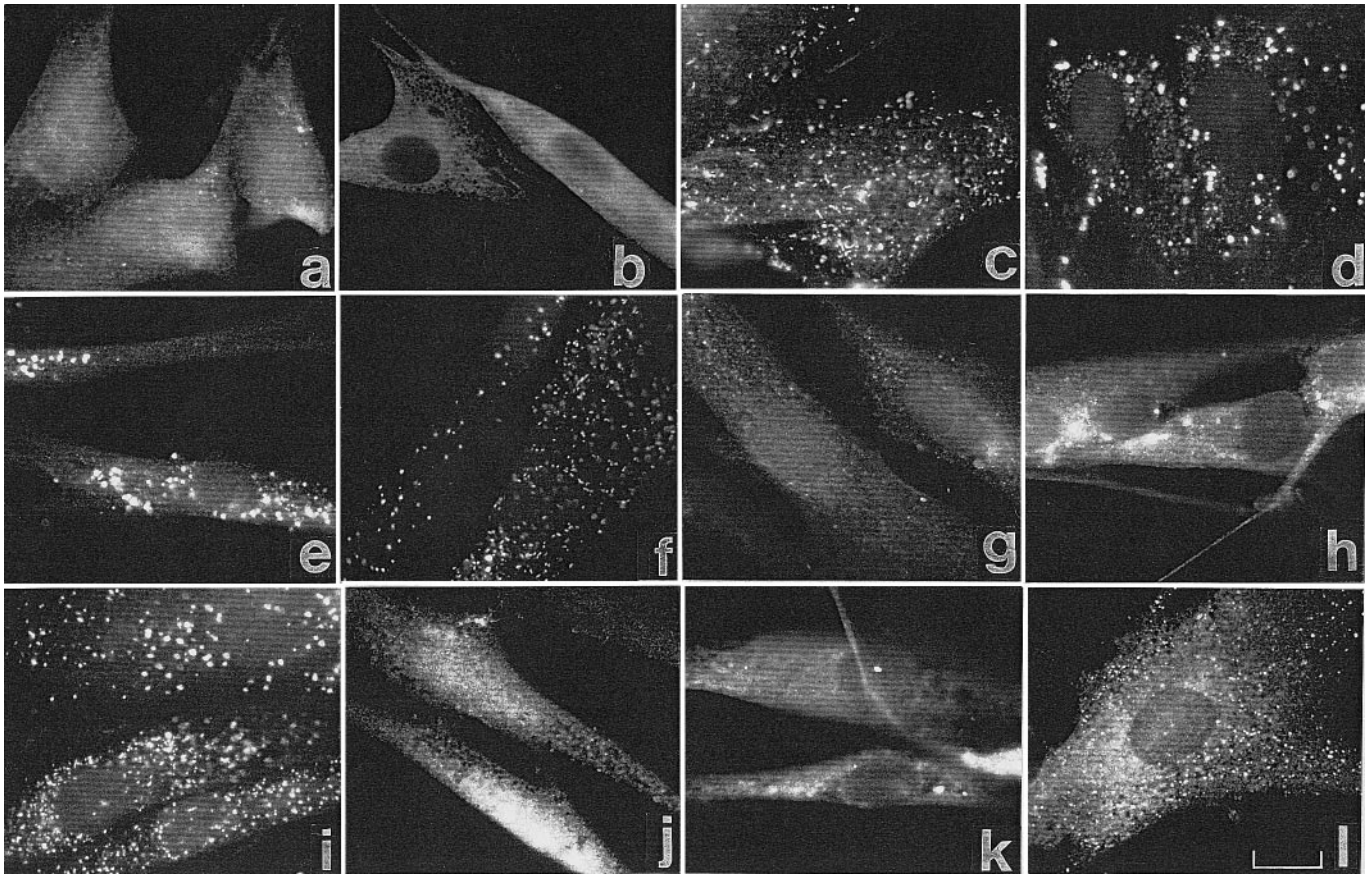

FIG. 1. Immunofluorescent staining of fibroblasts from PBD patients of complementation group 2. a, d, g and j, patient F-01. b, e, h, and k, patient F-08. c, f, i, and l, patient F-06. Cells were stained using antibodies to human catalase (a–c), human PMP70 (d–f), PTS1 (g–i), and rat PT (j–l). Bar = 12 μm.

with a mild form of PBD. The molecular mechanism underlying the phenomenon of peroxisome-positive and peroxisome-negative cells in relation to the topology and function of *PEX2*p is discussed.

PATIENTS AND METHODS

Patients. We studied 3 patients belonging to complementation group F, as determined by complementation studies using cell fusion and transfection with wild-type *PEX2* in the expression vector pUcD2SRαMCS (3) (data not shown). F-01 patient had the severe phenotype of ZS patient, the cause being a nonsense mutation, R119X in the *PEX2* gene (4). F-08 patient also had classical ZS and died at 2 months of age. F-06 patient had IRD, a relatively mild phenotypic expression. This female child of consanguineous parents was born after an uncomplicated pregnancy. At 12 months growth deficiency and mental retardation was identified. The diagnosis was only made at 19 months. At that time no facial dysmorphia, no hepatomegaly, retinitis pigmentosa, retarded myelination upon MRI and no adrenal insufficiency were present. At 4 years and 3 months she still had growth retardation and mental deficiency (IQ = 56), and she began to walk and spoke some words.

Cell lines. Fibroblasts from PBD patients and *PEX2* defective CHO mutants, Z65 (5) were cultured in MEM and Ham's F12 medium, respectively, supplemented with 10% fetal calf serum.

Morphological analysis. Peroxisomes in fibroblasts were visualized by indirect immunofluorescence light microscopy, as described (4), using rabbit antibodies to human and rat catalase, human peroxisomal 70-kDa integral membrane protein (PMP70) (6), PTS1 sequence (KHLKPLQSKL) (7) and rat 3-ketoacyl-CoA thiolase (PT), a PTS2-containing protein. For double immunofluorescence staining, guinea pig anti-rat catalase and rabbit anti-PTS1 antibodies, and rhodamine-conjugated goat anti-guinea pig and FITC-conjugated goat anti-rabbit secondary antibodies were used, as described elsewhere (8).

Biochemical analysis. Activities of DHAP-AT and peroxisomal β-oxidation system, as measured by oxidation of lignoceric acid relative to that of palmitic acid (C24:0/C16:0) were determined using documented methods (9, 10).

Mutation analysis of hsPEX2 in complementation group F patients. To identify mutations in the *PEX2* gene, genomic DNA was extracted from fibroblasts of patients F-08 and F-06. DNA fragments of 968 bp containing the entire coding region (1-915, hereafter, starting from the first nucleotide of the initiator methionine codon) of the *PEX2* gene were amplified by polymerase chain reaction (PCR), as described (11). Each amplified DNA fragment was directly sequenced, using an automated DNA sequencer.

PEX2 expression in the PEX2-defective CHO mutant. To assess the effect of *PEX2* mutations in patients F-08 and F-06, mutated *PEX2* gene fragments were inserted into the pUcD2SRαMCS (des-

ignated "pUcD2*PEX2*/del550C" and "pUcD2*PEX2*/del642G"). Z65, *PEX2*-defective CHO mutant cells were suspected in PBS (−) at 1×10^6 cells/ml. The cell suspension (400 μl) was mixed with 10 μg of these two mutated *PEX2* expression vectors, containing a neomycin-resistant gene (3) and electroporated by a Gene Pulser II electroporator (Bio-Rad) at 300 V and 400 μF. The stable transformants were produced in the presence of 400 μg/ml G418 (GIBCO BRL) and observed immunocytochemically.

RESULTS

Morphological analysis in fibroblasts from group F patients. Immunocytochemical studies of peroxisomes, using anti-human catalase antibody revealed the absence of punctate immunofluorescence in fibroblasts from patients F-01 and F-08 (Figs. 1a and 1b), whereas there were numerous catalase-containing particles in about 20% of F-06 fibroblasts (Fig. 1c). Immunofluorescence studies using an anti-human PMP70 antibody revealed the presence of larger and fewer PMP70-containing particles in fibroblasts from patients F-01 and F-08 (Figs. 1d and 1e). A different picture was found in patient F-06, in which fibroblasts few or many PMP70-positive particles were contained (Fig. 1f). Immunofluorescent particles were rare when an anti-PTS1 antibody was used in F-01 and F-08 fibroblasts (Figs. 1g and 1h), in contrast to the findings in fibroblasts from patient F-06 in which some or many particles were found (Fig. 1i). Particles stained with anti rat PT antibody were rare in F-01 and F-08 fibroblasts (Figs. 1j and 1k), whereas several particles staining positive with anti-PT were seen in F-06 fibroblasts (Fig. 1l), which implies that F-06 fibroblasts retain some function of PTS2 import as well as PTS1 import.

Biochemical analysis in fibroblasts from group F patients. The activity of DHAP-AT, a PTS2-containing protein with a major role in plasmalogen synthesis, was profoundly deficient in fibroblasts from patients F-01 and F-08 (both 0.2 nmol/120 min per mg protein), and fairly normal in patient F-06 fibroblasts (2.0 nmol/120 min per mg protein), when compared with control fibroblasts (2.1 nmol/120 min per milligram protein). The activity of the peroxisomal β-oxidation system was greatly decreased in both F-01 and F-08 fibroblasts (both 0.02), and slightly decreased in the F-06 (0.22), in comparison with findings in control cells (0.53).

Mutation analysis of hsPEX2 and expression of mutated hsPEX2 in the PEX2-defective CHO mutant. To determine the dysfunction of *PEX2* in patients F-08 and F-06, the coding region of the *HsPEX2* gene was amplified by PCR. Direct sequencing of the fragment from the F-08 indicated a homozygous 1-bp deletion at nucleotide 550C (designated "del550C") in codon 184Arg (CGT). This deletion disrupts the reading frame of the downstream sequence, completely changing the amino acid sequence starting from codon 184 and introducing a termination codon at 191 (Figs. 2 and 3). In the F-06 patient, direct sequencing of the *HsPEX2* gene indicated a homozygous 1-bp deletion at nucleotide 642G (designated "del642G") in codon 214Gln (CAG), resulting in a change to codon 215 and introducing a termination at codon 216 (Figs. 2 and 3). When pUcD2*PEX2*/del550C and pUcD2*PEX2*/del642G were transfected into the *PEX2*-defective CHO mutant, Z65, no particles were observed which stained positive with anti-catalase and -PTS1 antibodies in the *PEX2*/del550C transfectants (Figs. 4a and 4b), whereas different pictures were found in the *PEX2*/del642G transfectants. Many particles stained with anti-PTS1 antibodies were observed in all of the *PEX2*/del642G transfectants (Fig. 4d). On the other hand, some transfectants contained many catalase-containing particles, the rest contained no particle with catalase (Fig. 4c). To confirm this mosaic distribution of catalase-containing particles in the *PEX2*/del642G transfectants, we carried out double immunofluorescence staining, using guinea pig anti-rat catalase antibody and rabbit anti-

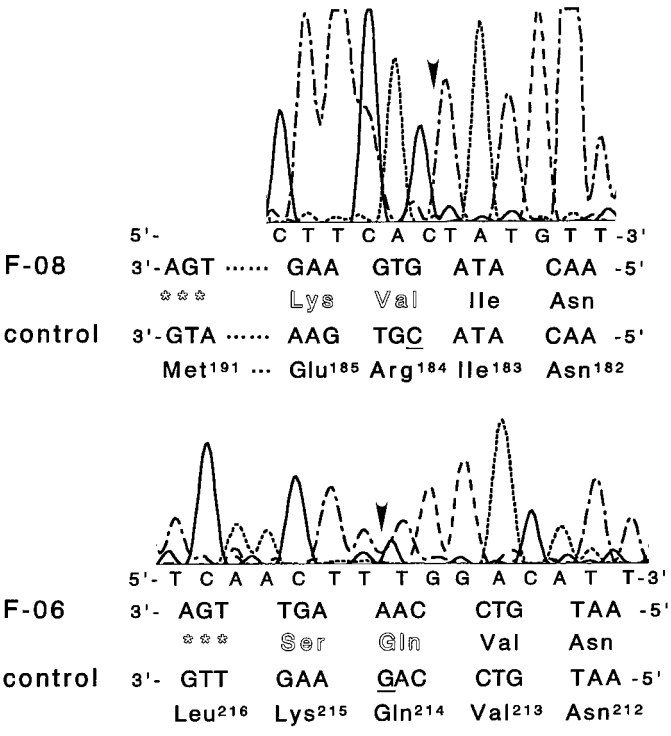

FIG. 2. Mutation analysis of *PEX2* from F-08 and F-06 patients. Partial nucleotide sequence and deduced amino acid sequence of *PEX2* gene from patients F-08 (upper) and F-06 (lower) are shown. F-08, 1-bp deletion at nucleotide 550 in a codon 184Arg (CGT), resulted in change of the amino acids codon (184–190) and introduced a termination codon (191). F-06, 1-bp deletion at nucleotide 642 in a codon 214Gln (CAG), resulted in change of codon (215) and introduced a termination codon (216).

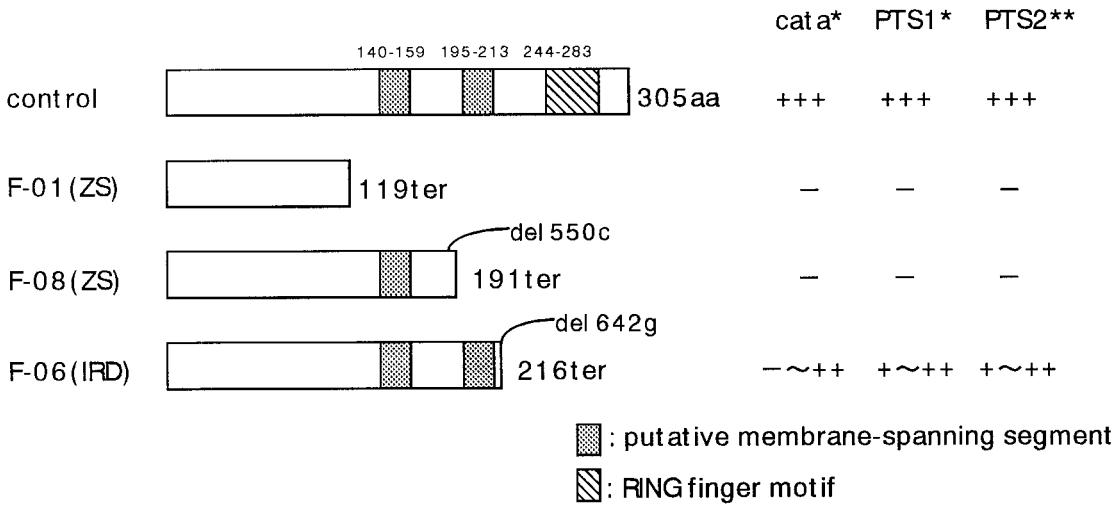

FIG. 3. Schematic illustration of the relation between peroxisomal protein import and mutated Pex2p in control and three PBD patients from complementation group F. *Immunofluorescent punctates with use of anti-human catalase (cata) and anti-PTS1, with reference to Figs. 1a–1c and 1g–1i. **Immunofluorescent punctates with use of anti-rat thiolase, with reference to Figs. 1j–1l.

PTS1 antibody. Some transfectants contained punctate structures stained with both anti-catalase and anti-PTS1 antibodies, and localization of these patterns was similar. Other transfectants contained punctate structures stained with only anti-PTS1 antibody (Figs. 4e and 4f). Therefore the *PEX2*/del642G mutation is directly responsible for the variable defect in the peroxisomal import of catalase.

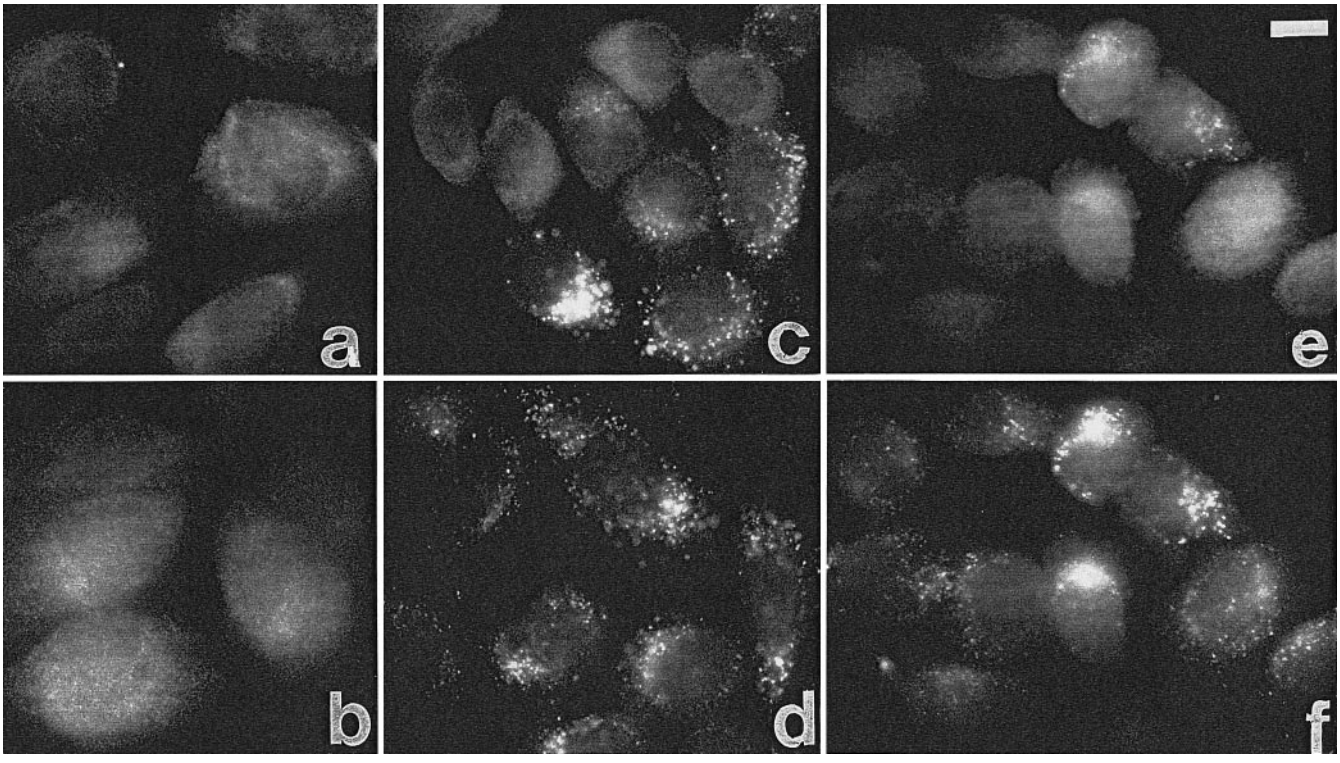

FIG. 4. Expression of mutated *PEX2* in Z65. a and b, pUcD2*PEX2*/del550C transformants. c–f, pUcD2*PEX2*/del642G transformants. Cells were stained using antibodies to human catalase (a and c) and PTS1 (b and d). A double immunofluorescence staining method was used as well as guinea pig anti-rat catalase and rhodamine-conjugated goat anti-guinea pig antibodies (e) and rabbit anti-PTS1 and FITC-conjugated goat anti-rabbit antibodies (f). Bar = 10 μm.

DISCUSSION

To search for a clue as to the molecular mechanism of detectable peroxisomes (catalase-positive particles) in fibroblasts from PBD patients, we carried out mutation analysis and immunocytochemical studies on fibroblasts from *PEX2*-defective patients. Patient F-08 has a 1 base pair deletion (del550C) introducing a termination codon at position 191 and fibroblasts from this patient contained no catalase-positive particles, whereas patient F-06 has a del642G mutation introducing a termination codon at position 216 and about 20% of the fibroblasts contained numerous catalase-positive particles.

Pex2p is an integral peroxisomal membrane protein, including two membrane spanning domains (140–159 and 195–213) and a RING finger motif in the C-terminal part (244–287). Both the N- and C-terminal parts are exposed to the cytosol (12–14) (Fig. 3). In a previous report, we suggested that the two membrane-spanning segments were important for topology and function of Pex2p. On the other hand, we thought the RING finger motif would be not necessarily essential for the peroxisome-restoring activity of Pex2p, as truncation studies, using anti-catalase antibodies (13). In this paper, we obtained evidence that in fibroblasts from patient F-06 with a del642G mutation in the *PEX2* gene, resulting in truncation of the C-terminal RING finger motif in Pex2p, some fibroblasts contained many particles staining positive for catalase, the rest contained no catalase positive particle. Interestingly, the clinical and biochemical findings in the patient are in line with these morphological mosaicism, since the patient is only mildly affected. On the other hand, patient F-08 with the del550C mutation in the *PEX2* gene with truncation of not only the RING finger motif but also the C-terminal membrane-spanning segment, was severely affected, both biochemically and clinically, according to the defect of peroxisomal proteins import. Further investigation of the functional domains of Pex2p is required to resolve the underlying basis of this remarkable phenomenon.

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