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Report

PEX3 Is the Causal Gene Responsible for Peroxisome Membrane Assembly–Defective Zellweger Syndrome of Complementation Group G

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Peroxisome biogenesis disorders (PBDs) such as Zellweger syndrome (ZS) and neonatal adrenoleukodystrophy are autosomal recessive diseases caused by defects in peroxisome assembly, for which 13 genotypes have been identified. Expression of the human peroxin Pex3p cDNA encoding a 373-amino-acid peroxisomal membrane protein morphologically and biochemically restored peroxisome biogenesis, including peroxisomal membrane assembly, in fibroblasts from PBDG-02, a patient with complementation group G (CG-G) ZS. Patient PBDG-02 carried a homozygous, inactivating mutation—a 97-bp deletion of nucleotide residues at positions 942–1038—resulting in a 32-amino-acid truncation and in a frameshift inducing both a 3-amino-acid substitution and a termination codon. Genomic PCR analysis revealed mutation of T→G at eight bases upstream of the splicing site at the boundary of intron 10 and exon 11 of *PEX3* gene, giving rise to a deletion of all of exon 11. When assessed by expression in a *pex3* mutant of Chinese hamster ovary cells and the patient's fibroblasts, PBDG-02–derived *PEX3* cDNA was found to be defective in peroxisome-restoring activity. These results provide evidence that *PEX3* is a novel, pathogenic gene responsible for CG-G PBDs.

Peroxisomes function in various metabolic pathways, including β-oxidation of very-long-chain fatty acids and synthesis of ether lipids (van den Bosch et al. 1992). Human fatal genetic peroxisomal biogenesis disorders (PBDs) include Zellweger syndrome (ZS [MIM 214100]), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD), and rhizomelic chondrodysplasia punctata (RCDP) (Lazarow and Moser 1995). The primary cause of the peroxisome deficiency in PBDs, comprised of 13 complementation groups (CGs) (Kinoshita et al. 1998; Shimozawa et al. 1998; Ghaedi et al. 1999), is a failure in peroxisome biogenesis (Braverman et al. 1995; Subramani 1997; Fujiki 2000). Genetic heterogeneities containing >15 CGs have been identified in mammals, having been determined by use of fibroblasts from patients with PBDs and of peroxisome-deficient Chinese hamster ovary (CHO) cell mutants (Ghaedi et al. 1999; Fujiki 2000) (see table 1). To date, 10 *PEX* genes have been shown to be pathogenic for 10 CGs of PBDs; several genes have yet to be identified (Fujiki 2000). Much knowledge of peroxisome biogenesis has been gained on the basis of findings of topogenic signals and peroxins required for peroxisomal protein import. However, thoroughly molecular mechanisms involved in assembly of peroxisomal membrane vesicles have yet to be defined. In most CGs, fibroblasts from patients with PBDs contain morphologically recognizable peroxisomal membrane remnants, which implies that membrane biogenesis is normal, despite the impaired import of matrix proteins (Santos et al. 1992; Wendland and Subramani 1993; Shimozawa et al. 1998). In contrast, in three CGs—CG-D (CG9 in the United States), CG-G, and CG-J—fibroblasts from patients with PBDs are absent from peroxisomal remnants, and are thus indicative of apparent defects in peroxisome membrane assembly (Kinoshita et al. 1998; Shimozawa et al. 1998). *PEX16* (Honsho et al. 1998; South and Gould 1999) and *PEX19* (Matsuzono et al. 1999) were shown to be responsible for PBDs of CG-D and CG-J, respectively. Using ZPG208, a CHO cell mutant deficient

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Table 1

Complementation Groups of Patients with PBDs and CHO Cell Mutants

CG Designation of Patients with PBDs					
Japanese	U.S./European	Phenotype	CHO Mutant	Peroxisomal Ghosts[a]	Gene
G		ZS	ZPG208	−	*PEX3*
A	8	ZS, NALD, IRD	ZP124	+	
B	7(5)[b]	ZS, NALD		+	*PEX10*
C	4	ZS, NALD	ZP92	+	*PEX6*
D	9	ZS		−	*PEX16*
E	1	ZS, NALD, IRD	Z24/ZP107	+	*PEX1*
F	10	ZS, IRD	Z65	+	*PEX2*
H	13	ZS, NALD	ZP128	+	*PEX13*
J		ZS	ZP119	−	*PEX19*
	2	ZS, NALD	ZP105/ZP139	+	*PEX5*
	3	ZS, NALD, IRD	ZP109	+	*PEX12*
	6	ZS, NALD		+	
R	11	RCDP	ZPG207	+	*PEX7*
			ZP110	+	*PEX14*
			ZP114	+	
			ZP126	+	

[a] A plus sign (+) denotes that peroxisomal remnants (ghosts) are present; a minus sign (−) denotes that they are absent.

[b] From the study by Shimozawa et al. (1998)

in peroxisome assembly, we cloned rat *PEX3* cDNA by genetic phenotype-complementation assay (Ghaedi et al. 2000). We now report that human *PEX3* (*HsPEX3* [MIM 603164]) complements the impaired peroxisome biogenesis in fibroblasts devoid of peroxisomal membrane vesicles from a patient with CG-G PBD.

Fibroblasts from PBDG-02, a patient with CG-G ZS, showed a diffused, cytosolic localization of catalase, which is indicative of peroxisome deficiency, as verified by cell staining with an anticatalase antibody (Shimozawa et al. 1992) (see fig. 1*A*, subpanel *b*). When PBDG-02's fibroblasts were fused with the CHO *pex3* mutant ZPG208 (Ghaedi et al. 2000), the impaired protein import was not restored (data not shown), which demonstrates that PBDG-02's cells are in the same CG as is ZPG208 (table 1). Peroxisomal remnants, so-called ghosts, were not discernible in PBDG-02's fibroblasts (table 1), as assessed by cell staining with antibody to 70-kD peroxisomal membrane protein (PMP70) (not shown), thereby confirming a defect in peroxisome membrane biogenesis. These results collectively show that PBDG-02's cells are impaired in biogenesis of not only soluble proteins but also membrane polypeptides, an impairment most likely caused by a defect in *PEX3*. *HsPEX3* expression indeed complemented impaired import of catalase (fig. 1*A*, subpanel *a*) and of peroxisome-targeting signal type I (PTS1) proteins, as well as 3-ketoacyl-CoA thiolase, a PTS2-protein (data not shown), in PBDG-02's fibroblasts. PMP70-positive peroxisomes were also observed, in a superimposable manner, with peroxisomes stained by use of anticatalase antibody (not shown). Therefore, Pex3p could morphologically restore peroxisome assembly in PBDG-02's fibroblasts.

We next verified the biogenesis of peroxisomal proteins in CG-G fibroblasts. PMP70 was not discernible in PBDG-02's fibroblasts (fig. 2, top panel, lane 2), presumably because of a rapid degradation, as seen in the CHO *pex19* mutant ZP119 (Kinoshita et al. 1998), although PMP70 was detectable in normal fibroblasts (lane 1). When PBDG-02's fibroblasts were transiently transfected with *HsPEX3,* PMP70 was detected, as in normal cells (lane 3), indicating that the impaired biogenesis of PMP70 was restored. On transfection of *HsPEX3,* Pex3p was also detectable, albeit at a low level, when anti-C-terminal Pex3p peptide antibody was used (Ghaedi et al. 2000), thereby confirming the expression of HsPex3p, whereas Pex3p was not discernible in PBDG-02's fibroblasts (data not shown). Peroxisomal 3-ketoacyl-CoA thiolase of fatty acid β-oxidation system is synthesized as a larger, 44-kD precursor with an amino-terminal, cleavable PTS2 and is processed to 41-kD mature form in peroxisomes (Tsukamoto et al. 1990). In normal fibroblasts, only the matured thiolase was detected (fig. 2, middle panel, lane 1), thereby providing evidence for a rapid processing of the precursor form. In PBDG-02's cells, the larger precursor was not detectable (fig. 2, middle panel, lane 2), presumably because of degradation. When PBDG-02's fibroblasts were transiently transfected with *HsPEX3,* the mature thiolase was clearly discerned (fig. 2, middle panel, lane 3). A PTS1 protein, acyl-CoA oxidase (AOx), is synthesized as a 75-kD polypeptide (A component) and is proteo-

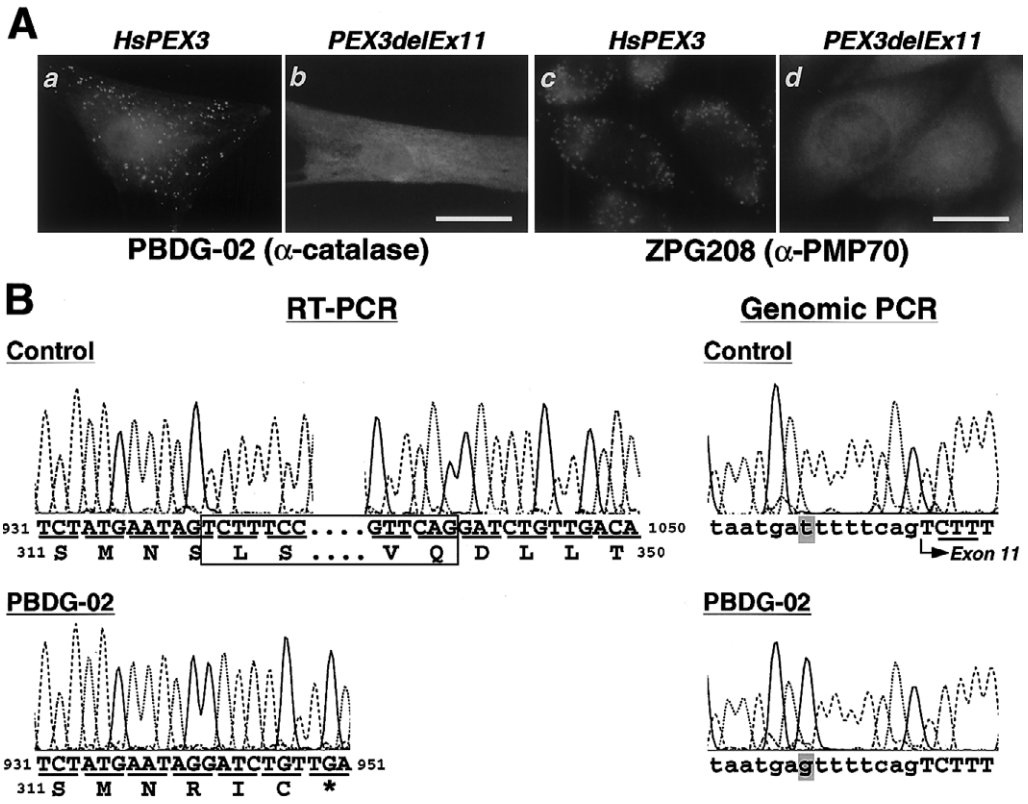

Figure 1 Impaired *PEX3* in a patient with CG-G ZS. *A,* Expression of *HsPEX3,* which restores peroxisome assembly. *HsPEX3* and *flag*-tagged *PEX3delEx11* derived from PBDG-02 (see fig. 1*B*) were separately expressed in PBDG-02's fibroblasts (*a* and *b*); *flag*-tagged *HsPEX3* and *flag*-tagged *PEX3delEx11* were transfected into *pex3* ZPG208 cells (*c* and *d*). Cell staining was done by use of primary rabbit antibodies to catalase (*a* and *b*) and PMP70 (*c* and *d*) and of a secondary Texas Red–labeled sheep anti-rabbit immunoglobulin G antibody. In *b,* the scale bar (which also applies to *a*) = 40 μm; in *d,* the scale bar (which also applies to *c*) = 20 μm. Note that peroxisomes were restored in (*a* and *c*) but not in (*b* and *d*). *B,* Mutation analysis of *PEX3* from a patient with CG-G ZS, PBDG-02. Partial nucleotide sequence and the deduced amino acid sequence of *PEX3* cDNA isolated from a normal control (*upper-left panel*) and PBDG−02 (*lower-left panel*) are shown. A 97-bp deletion of nucleotide residues 942–1038 (*boxed*) was identified. PCR was also done for DNA from a normal control (*upper-right panel*) and from PBDG-02's fibroblasts (*lower-right panel*); the nucleotide sequence of PCR products was determined. Only partial sequence at the boundary of intron 10 and exon 11 is shown; a codon for Leu^{315} is underlined in the sequence of the normal control.

lytically converted into 53- and 22-kD polypeptides (B and C components, respectively) in peroxisomes (Tsukamoto et al. 1990). All three polypeptide components were evident in normal fibroblasts from a control (fig. 2, bottom panel, lane 1), whereas they were barely detectable in PBDG-02's cells (fig. 2, bottom panel, lane 2), possibly reflecting the occasional peroxisome-positive cell and degradation of most of AOx-A (Tsukamoto et al. 1990; Ghaedi et al. 1999). When PBDG-02's cells were transfected with *HsPEX3,* the three components of AOx were detected at a distinct level, indicative of proper import and proteolytic conversion of AOx (fig. 2, bottom panel, lane 3). These results demonstrate that *HsPEX3* can complement the impaired biogenesis of peroxisomal proteins in PBDG-02's cells. *HsPEX3* expression restored peroxisome biogenesis only in the CHO *pex3* mutants (Ghaedi et al. 2000) (see fig. 1*A,* subpanel *c*) in 13 CGs of peroxisome-deficient CHO cell mutants, where the CHO mutants of 10 CGs represent PBD CGs (Tsukamoto et al. 1990; Shimozawa et al. 1992; Kinoshita et al. 1998; Ghaedi et al. 1999; Toyama et al. 1999) (table 1). Taken together, these observations confirm that Pex3p is a peroxisome biogenesis factor for CG-G PBD. Therefore, *PEX3* is the 11th gene identified as being responsible for peroxisome-deficiency diseases.

Both to isolate *PEX3* cDNA from PBDG-02's fibroblasts and to determine the *PEX3* dysfunction, we conducted reverse transcription–PCR (RT-PCR), using poly(A)$^+$ RNA and a pair of *HsPEX3*-specific PCR primers: a sense *HsPEX3-Bam*F (5′-TAAGCTTGAGATCTTGAGGTCTGTATGGA-3′) and an antisense *HsPEX3-Apa*R (5′-GGGTACCGGGCCCTCATTTCTCCAGTTGC-3′) (where the termination codon is underlined), as described elsewhere (Honsho et al. 1998). Subsequent sequencing of cDNA clones by the dideoxy-chain termination method indicated a 97-bp deletion of nucle-

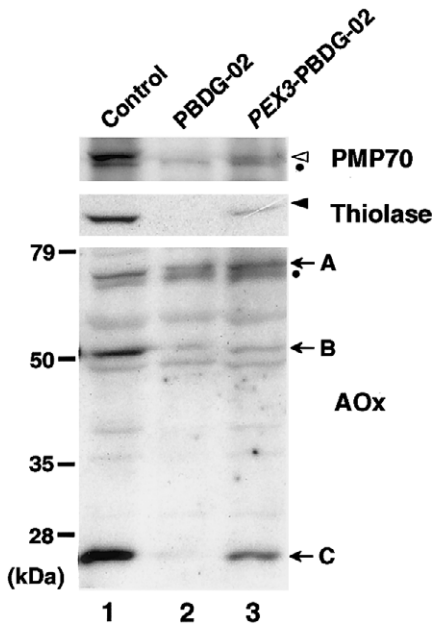

Figure 2 Complementation of biogenesis of peroxisomal proteins. Cell lysates (~ 2 × 10^5 cells) were subjected to SDS-PAGE and were transferred to polyvinylidene difluoride membrane. Immunoblot analysis was done by use of rabbit antibodies to PMP70 (*top*), thiolase (*middle*), and AOx (*bottom*) and an ECL western-blotting detection reagent. *Lane 1,* Normal control fibroblasts. *Lane 2,* PBDG-02's fibroblasts. *Lane 3,* PBDG-02's fibroblasts transiently transfected with *HsPEX3*. Unblackened and blackened arrowheads indicate PMP70 and a mature form of thiolase, respectively; arrows indicate AOx components A–C. Dots indicate nonspecific bands (Kinoshita et al. 1998; Tamura et al. 1998).

otide residues at positions 942–1038, resulting in a 32-amino-acid truncation and a frameshift inducing a 3-amino-acid substitution and a termination codon (fig. 1*B,* left). This truncated part corresponded exactly to exon 11 of the *HsPEX3* gene (Muntau et al. 2000). Fifteen of the isolated *PEX3* cDNA clones, termed "*PEX3delEx11,*" all carried the same mutation, thereby suggesting a homozygous event causing a defective splicing. To search for both a mutation site in the intron and zygosity of a *PEX3delEx11* mutant allele, genomic PCR with a pair of *PEX3*-specific primers—forward primer *Ex11*-104–133F (5′-TCACAGCTAGAGGACAGATTACTGTATAGT-3′) and reverse primer *Ex11*-711–737R (5′-TGCTATGGTCCGTTATTACTCTCATGG-3′)—was used to amplify the sequence between residues 104 and 737, encompassing 361 nucleotide residues on the 3′ side of intron 10, the 97 nucleotides of exon 11, and 176 nucleotides on the 5′ side of intron 11 in the *HsPEX3* gene (GenBank accession number AJ009873) (Muntau et al. 2000). In seven independent clones isolated from PCR products, we identified a single type of nucleotide sequence, which, apparently, gave rise to a deletion of exon 11: T was mutated to G at eight bases upstream of the splicing site at the boundary of intron 10 and exon 11 (fig. 1*B,* right). These observations were interpreted to mean that patient PBDG-02, who has ZS, was a homozygote for the T→G mutation, causing the deletion of exon 11. This homozygosity may be explained by this patient's history, since PBDG-02 was the third child of consanguineous Italian parents (Poulos et al. 1995). This mutation inactivated the function of *PEX3,* as verified by expression of *flag-PEX3delEx11* in PBDG-02's fibroblasts and in *pex3* ZPG208 cells of the same CG, where peroxisomes were not formed, as assessed by staining of catalase and PMP70, respectively (fig. 1*A,* subpanels *b* and *d*). Normal *flag-HsPEX3* restored peroxisome assembly in ZPG208 (fig. 1*A,* subpanel *c*), as well as in PBDG-02's fibroblasts (not shown), indicating that flag tagging did not interfere with Pex3p function. The data collectively indicate that mutation of *PEX3* is the genetic-related cause of CG-G PBDs. Moreover, these findings show the importance that the C-terminal area of Pex3p has for biological activity.

We also investigated the kinetics of peroxisome assembly, with respect to both membrane vesicle formation and soluble-protein import. PBDG-02's fibroblasts were transfected with *HsPEX3* and were monitored for peroxisome biogenesis, at various time points, by our visualizing of several endogenous peroxisomal proteins, including the membrane peroxin Pex14p—a convergent component of a potential import machinery (Shimizu et al. 1999; Otera et al. 2000)—and PMP70, as well as matrix proteins PTS1 and catalase. We observed that peroxisomal membrane vesicles were apparently formed prior to the import of matrix proteins (data not shown), in agreement with the findings for the CHO *pex3* mutant ZPG208 (Ghaedi et al. 2000). Therefore, Pex3p is most likely to be involved in peroxisome assembly at the early stage of peroxisomal membrane vesicle formation. Mammalian Pex3p interacts with Pex19p (Soukupova et al. 1999; Ghaedi et al. 2000; Sacksteder et al. 2000). It is likely that Pex3p functions as a peroxisome biogenesis factor, possibly by interacting with other *PEX* proteins as well (Soukupova et al. 1999; Ghaedi et al. 2000; Sacksteder et al. 2000). The precise mechanisms by which Pex3p functions in peroxisome membrane assembly remain to be defined.

We had previously cloned mammalian *PEX3* cDNAs by functional phenotype-complementation assay, using CHO cell mutants (Ghaedi et al. 2000). In that report, we had concluded that *PEX3* expression could not complement fibroblasts from PBDG-02 (who, in that report, was designated "CG-G patient 2") (Ghaedi et al. 2000). We observed that PBDG-02's fibroblasts are unstable with respect to peroxisome deficiency: peroxisomes, including peroxisomal remnants, emerge either occasionally or readily, depending on cell-culture conditions such as consecutive cell-culture passages. Moreover, cell growth rate

varies significantly. The main reason for the conclusion in our earlier report appears to have been the following: cell-doubling time was >4 d, and phenotype complementation was verified 4 d after *PEX3* transfection (Ghaedi et al. 2000)—whereas the cell-doubling time in the present study was 1.5 d, and peroxisome restoration was mostly assessed 5 d after *HsPEX3* transfection. In the present work, we selected, from several different lots of stocks that had been prepared at various times of cell passage, one cell stock showing a better, typical mutant phenotype. For a more prolonged time after transfection of *HsPEX3,* we observed cells for phenotype complementation; for cell mutants, including patients' fibroblasts, we did not find that cell-culture conditions resulted in such a difference in morphological phenotype. Possibly because of differences in cell growth rate, the kinetics of peroxisome assembly apparently vary, depending on the genotype of >15 CGs of impaired peroxisome biogenesis in mammals. To what extent such differentiated peroxisome assembly is relevant to the severity of PBDs is, at present, unclear.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for human *PEX3* cDNA sequence [accession number AB035307])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for ZS [MIM 214100] and for *PEX3* [MIM 603164])

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