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Report

Defective Peroxisome Membrane Synthesis Due To Mutations in Human *PEX3* Causes Zellweger Syndrome, Complementation Group G

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Zellweger cerebro-hepato-renal syndrome is a severe congenital disorder associated with defective peroxisomal biogenesis. At least 23 *PEX* genes have been reported to be essential for peroxisome biogenesis in various species, indicating the complexity of peroxisomal assembly. Cells from patients with peroxisomal biogenesis disorders have previously been shown to segregate into ⩾12 complementation groups. Two patients assigned to complementation group G who had not been linked previously to a specific gene defect were confirmed as displaying a cellular phenotype characterized by a lack of even residual peroxisomal membrane structures. Here we demonstrate that this complementation group is associated with mutations in the *PEX3* gene, encoding an integral peroxisomal membrane protein. Homozygous *PEX3* mutations, each leading to C-terminal truncation of PEX3, were identified in the two patients, who both suffered from a severe Zellweger syndrome phenotype. One of the mutations involved a single-nucleotide insertion in exon 7, whereas the other was a single-nucleotide substitution eight nucleotides from the normal splice site in the 3′ acceptor site of intron 10. Expression of wild-type PEX3 in the mutant cell lines restored peroxisomal biogenesis, whereas transfection of mutated *PEX3* cDNA did not. This confirmed that the causative gene had been identified. The observation of peroxisomal formation in the absence of morphologically recognizable peroxisomal membranes challenges the theory that peroxisomes arise exclusively by growth and division from preexisting peroxisomes and establishes *PEX3* as a key factor in early human peroxisome synthesis.

The importance of peroxisomes in mammalian metabolism is illustrated by the existence of severe inherited metabolic diseases caused by the inability to assemble peroxisomes. Among these peroxisomal biogenesis disorders (PBDs), Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease represent a continuum of clinical phenotypes (PBD [MIM 601539]), whereas rhizomelic chondrodysplasia punctata (RCDP [MIM 215100]) is characterized by distinct clinical features (Wanders 1999). Peroxisomes have long been thought to arise exclusively by growth and division of preexisting peroxisomes (Lazarow and Fujiki 1985), and only recently have alternative hypotheses been proposed (South and Gould 1999). Proteins required for peroxisomal assembly are termed "peroxins" and are encoded by *PEX* genes. At least 23 peroxins have been identified in yeast, most of which are conserved among different eukaryotic organisms (Subramani 1997). In most cases, defects in *PEX* genes lead to a disruption of peroxisomal matrix protein import, whereas various peroxisomal membrane components are synthesized and accumulate in peroxisomal membrane remnants ("ghosts"). *PEX3* and *PEX19* mutants represent an exception to this rule, since the corresponding yeast mutants have been shown to lack even these peroxisomal ghosts (Baerends et al. 1996; Götte et al. 1998). We had previously cloned the human orthologue of *PEX3* (*PEX3* [MIM 603164]) (Kammerer et al. 1998). Evidence suggests that human PEX3 is a peroxisomal assembly protein displaying two transmembrane helices in the N-terminal half of the protein. Immunofluorescent microscopy studies indicate that it is an integral membrane protein, with its C terminus exposed to the cytosol and with its N terminus facing the intraorga-

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nellar space (Kammerer et al. 1998; Soukupova et al. 1999). Despite an extensive search, disease-causing mutations in the human *PEX3* gene have not been identified so far (Muntau et al. 2000).

In somatic cell–fusion experiments, fibroblasts from patients with PBDs have been shown to segregate into ⩾12 complementation groups (CGs [Shimozawa et al. 1998; Moser 1999]). In this study, we investigated fibroblasts from two patients (PBDG-01 and PBDG-02) previously assigned to CG-G (Gifu University nomenclature [Poulos et al. 1995]). CG-G fibroblasts lack peroxisomal ghosts, as evidenced by staining with an antibody directed against the human 70-kD peroxisomal membrane protein, PMP70 (Shimozawa et al. 1998; Poulos et al. 1995), and are therefore excellent candidates for a *PEX3* mutation. To our knowledge, CG-G is represented so far by only two patients worldwide, and cell lines from both individuals were available for this study.

The two male infants from unrelated families were born to consanguineous Dutch and Italian parents. Patient 1 (PBDG-01) showed a marked muscular hypotonia at birth. Dysmorphic features included hypertelorism, prominent epicanthic folds, and a high, broad forehead with a round face. Seizures developed on day 1 but were controlled with treatment. His condition deteriorated rapidly, and he died at age 4 mo. Patient 2 (PBDG-02) was cyanotic at birth, markedly hypotonic, and lacked deep tendon reflexes. He had a prominent midface and an antimongoloid slant of the palpebral fissures, ocular hypertelorism, small low-set ears, a prominent nose, and a high-arched palate. The liver was enlarged. Seizures developed during the first 20 h. The child required gavage feeding and died at age 19 d, of congestive heart failure. The patient's brother had been similarly affected and had died at age 15 d. The concentrations of very-long-chain fatty acids were elevated in the plasma and fibroblasts of patients 1 and 2. In fibroblasts from both patients, the percentage of sedimentable catalase, the rate of oxidation of pristanic acid, and the activities of both alkyl dihydroxyacetone phosphate synthase and dihydroxyacetone phosphate acyl transferase were all reduced (Poulos et al. 1995). In summary, the clinical and biochemical features of both patients correspond to a severe Zellweger syndrome phenotype.

By performing somatic cell–fusion experiments (fig. 1), we showed that fusion of cultured fibroblasts from patient PBDG-01 with cells from patient PBDG-02 did not rescue peroxisomal biogenesis. This confirms that the two cell lines were correctly assigned to the same complementation group and therefore would be expected to bear mutations in the same gene. By contrast, fusion of cultured fibroblasts from patients PBDG-01 and PBDG-02 with those from a patient (PBDJ-01) carrying a *PEX19* mutation (Matsuzono et al. 1999) restored peroxisomal biogenesis. These results indicate that CG-G and *PEX19* mutated cell lines can complement each other, despite the fact that both phenotypes are devoid of detectable preexisting peroxisomal membranes.

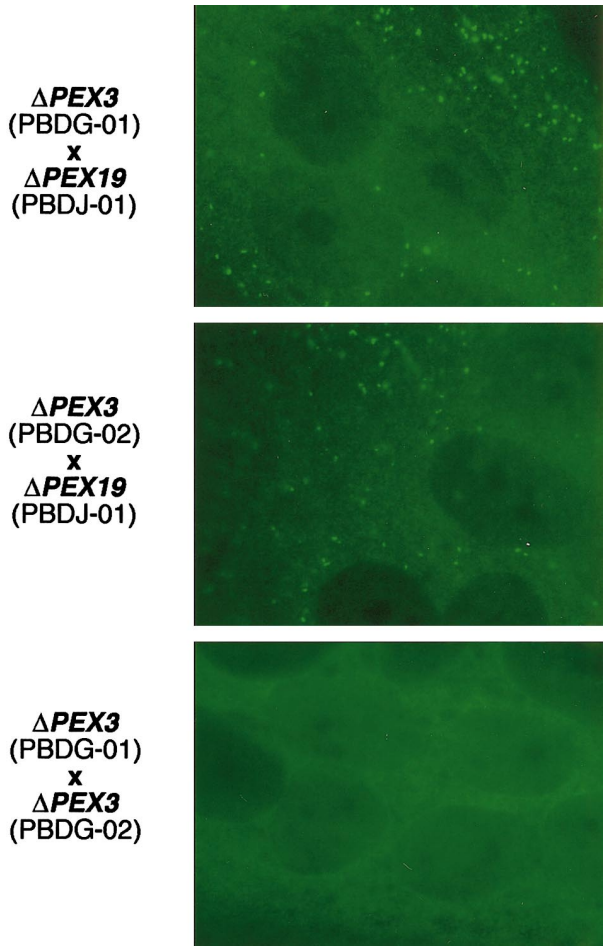

Figure 1 Somatic cell–fusion experiments. Fusion of cocultivated fibroblasts was performed using methods described elsewhere (Roscher et al. 1989). Fusion of fibroblasts from patients PBDG-01 and PBDG-02 with fibroblasts from a patient carrying a *PEX19* mutation (PBDJ-01 [Matsuzono et al. 1999]) restored peroxisomes in the majority of multinucleated cells, as demonstrated by a punctate immunofluorescent pattern after staining for catalase. Somatic cell fusion of fibroblasts from patient PBDG-01 with fibroblasts from patient PBDG-02 revealed only diffuse cytosolic staining with anti-catalase antibodies.

When analyzed by immunofluorescent microscopy, both patient cell lines lacked morphologically recognizable PMP-containing peroxisomal membranes (ghosts). This was demonstrated by the absence of punctate staining for the PMP adrenoleukodystrophy protein (ALDP; fig. 2*C* and *D*) and the membrane marker protein PMP70 (data not shown). However, these findings do

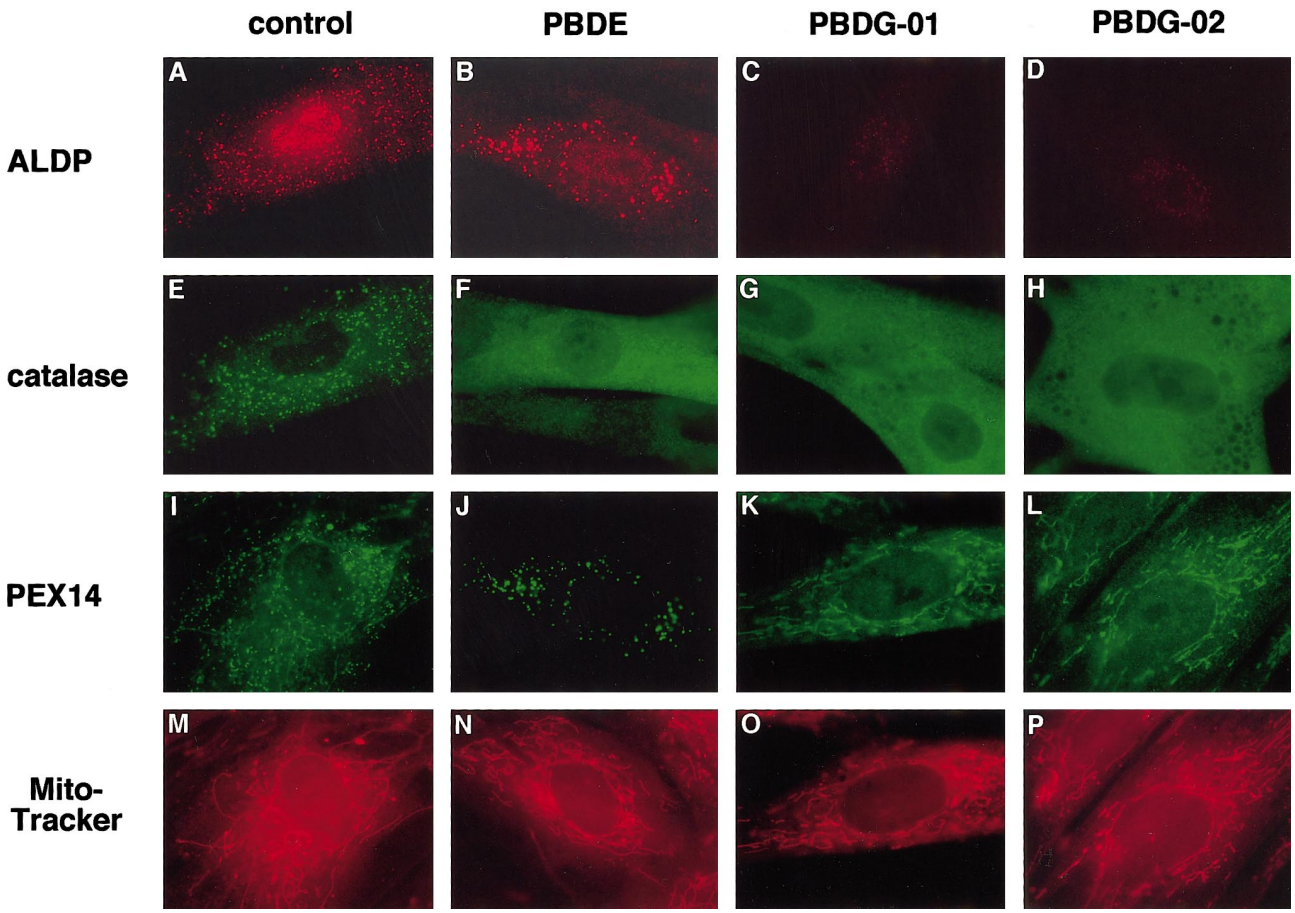

Figure 2 Intracellular localization of PMPs in CG-G cell lines. Indirect immunofluorescence microscopy was performed with fibroblasts from a healthy control (*A, E, I,* and *M*), from a patient with Zellweger syndrome who was assigned to CG-E (*B, F, J,* and *N*), and from the patients PBDG-01 (*C, G, K,* and *O*), and PBDG-02 (*D, H, L,* and *P*). The cells were stained using rhodamine-labeled antibodies to ALDP, a PMP (*A–D*); fluorescein-labeled antibodies to catalase, a peroxisomal matrix protein (*E–H*); fluorescein-labeled antibodies to PEX14, a PMP (*I–L*), or the mitochondrial marker MitoTracker (*M–P*). In normal fibroblasts, staining with antibodies to ALDP (*A*), catalase (*E*), and PEX14 (*I*) all yielded a punctate pattern, indicating the presence of peroxisomal membranes and intact peroxisomes. Fibroblasts from a patient with Zellweger syndrome who was assigned to CG-E (PBDE) were stained with antibodies to the PMPs ALDP (*B*) and PEX14 (*J*). Note the colocalization of the punctate patterns, indicating the presence of peroxisomal membranes. These membranes presumably are nonfunctional peroxisomal ghosts. Incubation with anti-catalase antibodies (*F*) resulted in a diffuse cytosolic localization, consistent with the absence of intact peroxisomes. In cells of PBDG-01 (*C*) and PBDG-02 (*D*), no ALDP positive particles were visible, indicating the complete absence of morphologically detectable peroxisomal membranes. Incubation with anti-catalase antibodies (*G* and *H*) exhibited a diffuse cytosolic pattern, consistent with the absence of intact peroxisomes. Staining the cells by use of an antibody specific for PEX14 (*K* and *L*) reveals a mitochondrial pattern, as demonstrated by colocalization of PEX14 with MitoTracker (*O* and *P*).

not exclude the possibility that some small peroxisomal vesicles may still exist in the patients' cells. The lack of peroxisomal staining was not accompanied by any detectable staining for these PMPs, in either the cytoplasm or any other cellular compartment. By contrast, fibroblasts from a patient with Zellweger syndrome who, because of the presence of a *PEX1* mutation, was assigned to CG-E (PBDE), displayed peroxisomal ghosts on immunofluorescence (fig. 2*B* and *J*). On western blot analysis, the PBDE cells contained an amount of ALDP similar to that in fibroblasts from a healthy control, whereas, in the fibroblasts from PBDG-01 and PBDG-02, ALDP was below the detection limit of our assay (data not shown). The lack of immunofluorescence for ALDP and PMP70, as well as the reduced amount of ALDP shown on western blot analysis, support the hypothesis that these PMPs are degraded rather than mislocalized. Analysis of the intracellular localization of another unrelated PMP, PEX14, revealed that the fate of this PMP in PBDG differs from that for ALDP and PMP70. Despite the lack of morphologically recognizable peroxisomal membrane staining, the patients' fibroblasts gave an unequivocal signal when antibodies to PEX14 were used, indicating that this protein was mis-

localized to mitochondria (fig. 2*K* and *L*). This finding is consistent with results from the western blots, in which the abundance of PEX14 in cells from PBDG-01 and PBDG-02 was not reduced when compared with that in cells from either a healthy control or from a patient with PBDE Zellweger syndrome (data not shown). Taken together, our data are consistent with the hypothesis that, in mutants lacking peroxisomal membranes, PMPs are either degraded, as proposed for ALDP and PMP70, or mislocalized, as shown for PEX14. Mitochondrial mislocalization of PEX14 has also been reported in *PEX19*-deficient fibroblasts (Sacksteder et al. 2000), and a similar mitochondrial mislocalization was observed when PEX3, PEX12, PEX13, PEX11β, or ALDP was overexpressed in these *PEX19*-deficient cells (Sacksteder et al. 2000). In our native *PEX3*-deficient cell lines, however, immunofluorescence studies using antibodies to ALDP did not lead to any detectable staining, neither in the peroxisomes nor in the mitochondria, cytoplasm, or any other cellular compartment.

Because the cell lines of the two patients described above were excellent candidates for a *PEX3* mutation, we performed *PEX3* mutation analysis by direct sequencing of genomic fragments, using a slight modification of the technique described elsewhere (Muntau et al. 2000). The primer sequences are listed in table 1. Patient PBDG-01 exhibited an insertion of a thymine in exon 7, at nucleotide 543 of the coding region (fig. 3*B*). This frameshift mutation is expected to result in a truncation of the C-terminal 190 amino acids of the protein (fig. 5*A*).

Sequence analysis of patient PBDG-02 did not reveal any exonic mutation. However, we identified a T→G transversion in the 3′ acceptor splice site of intron 10. Although the conserved polypyrimidine stretch in the wild type corresponds to the consensus sequence (Padgett et al. 1986), the thymine at −8 relative to exon 11 is replaced by a guanine in the patient (fig. 3*C*). To determine whether the point mutation interferes with correct splicing, reverse transcription–PCR (RT-PCR) was performed. A single PCR product was obtained that was missing the complete 97 bp of exon 11 (fig. 3*D* and *E*). This exon deletion causes a frameshift, with a premature termination after 3 amino acids, predicting a 56-amino-acid C-terminal truncation of the protein. Among the various types of mutations associated with splicing aberrations (Krawczak et al. 1992), 3′ splice-site mutations occur less frequently than 5′ splice-site mutations, and, in the majority of cases, the invariant AG dinucleotide is involved. Mutations at positions −3 to −14 of the splice-site consensus region have rarely been associated with splicing defects, but instances of mutations at position −8 that lead to aberrant splicing have been observed (Beldjord et al. 1988). Additional sequencing analysis confirmed that the *PEX3* intronic change was absent from 50 unrelated healthy individuals (data not shown). For these reasons, we conclude that the −8 mutation is the cause of the aberrant splicing in the patient described here.

Table 1

Primers for Mutation Analysis and RT-PCR of the Human *PEX3* Gene

Method and Exon	Primer	
	Name	Sequence (5′→3′)
Mutation analysis:		
1	5′F3	GAGAGCACAGAACGGGACGA
	1R	CTCTACATATCCCAGACTAGG
2	2FN	CTGTGTAGAATTTTTGGTACTC
	2RN	CAACCGTCAAACAACTTTAC
3	3F	GACTCTTGCTAGTTGCTAGC
	3R4	CATTGCCTTGAATGCTTTATGC
4	4FN	CTGCAGTTATGCTGTGTTGC
	4RN	GAGTCATCCTAATAGAATGC
5	5F	GGATGGTTCATGATTTTACAC
	5R	CATAGAAATTGTTATCAGG
6	6F	CCCGTCATTTCAGATCTTG
	6R2	GACAGTGATCAATTCTGTCAG
7	6F	CCCGTCATTTCAGATCTTG
	7R	GTCACTGGTTTCACTAGAAC
8	8F	GCAGTTACAGGTGTAAGCAG
	8R3	CTTACCTTTCAAGAGACTCAG
9	9FN	GTTTCATCATAACCCTGTGC
	9RN	CCGACCATGTTGTTCAAG
10	10F	CAAAGGTAACCACGTTATTACT
	10R2	GTTGTTATCTGGAACAAAAAG
11	11FN	GTCCTGTGGGGTCATTTCAG
	11RN	CCTCTCAGCAGAGAATGTAAC
12	12FN	CTTAGAGCTGAATTCATCGC
	1255R	GTATAGGTGATTTACCCAGTG
RT-PCR:		
5	404F	AGTACTGTGGCTGT
10	957R	GAACTCAGCCATATTGTCTAG
11	1062R	GATCTGTCCGTTTACTATAGG
12	1120R	GCTCCATTGTCAACAGATC

[a] F = forward; R = reverse.

The mutations identified in patients PBDG-01 and PBDG-02 each appeared to be present in a homozygous state, consistent with the consanguinity of both parental couples. Hemizygosity with a deletion of the second allele, however, cannot be excluded, since parental DNA was not available.

The functional impact of the identified mutations was subsequently investigated by complementation studies. Immunofluorescent microscopy analysis of fibroblasts from PBDG-01 (fig. 4*A–F*) and PBDG-02 (fig. 4*G–L*) indicated that both cell lines were lacking intact peroxisomes, as demonstrated by diffuse staining with antibodies to catalase (fig. 4*B* and *H*). Five days after transfection of PBDG-01 and PBDG-02 by wild-type *PEX3* cDNA, antibodies to ALDP (fig. 4*C* and *I*) and catalase (fig. 4*D* and *J*) showed a punctate pattern in both cell

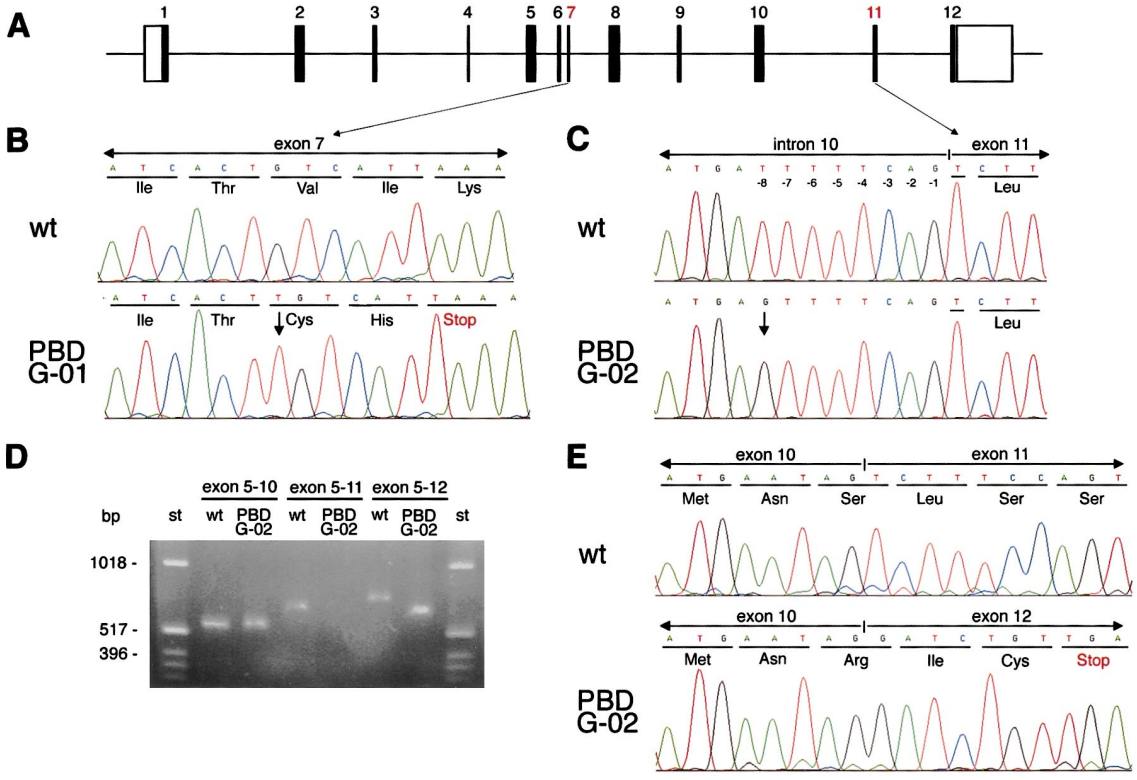

Figure 3 Mutation analyses. *A*, Genomic organization of the human *PEX3* gene. Exons are indicated as boxes. *B*, Exon 7 sequencing results and predicted protein sequences of a wild-type control (wt) and patient PBDG-01. Note the homozygous thymine insertion (*arrow*). The frameshift causes a premature stop two codons after the insertion. *C*, Genomic sequencing of the exon-intron boundary of a control individual (wt) and patient PBDG-02. Note the homozygous substitution of a thymine by a guanine in the 3′ splice site of intron 10, at position −8 (*arrow*). *D*, PCR amplification of *PEX3* fragments from total RNA of PBDG-02 after synthesis of first-strand cDNA by reverse transcription using primer 1255R (for primer sequences, see table 1). Amplification of a fragment containing exons 5–10 by primers 404F and 957R did not reveal any difference between the control individual (wt) and the patient (PBDG-02). The fragment containing exons 5–11 amplified by primer 404F and the exon 11–specific primer 1062R could not be amplified from the patient's cDNA. Amplification of exons 5–12 by primers 404F and 1120R produced an aberrantly sized PCR product from the patient's cDNA. *E*, Sequence analysis of the PCR fragment containing exons 5–12 show that the *PEX3* cDNA of patient PBDG-02 is missing 97 bp, which were present in wt *PEX3* cDNA. The 97-bp deletion corresponds to the sequence of exon 11 and leads to direct fusion of exons 10–12. Premature termination of the protein is predicted from the frameshift.

lines, demonstrating rescue of organelle formation. We also investigated whether the identified *PEX3* mutations affect the function of the PEX3 protein. This was particularly crucial for the splice-site mutation detected in patient PBDG-02. We generated *PEX3* expression vectors for each mutated *PEX3* cDNA. The resulting plasmids, pcDNA3-PBDG-01 and pcDNA3-PBDG-02, were transfected into cells from PBDG-01 and PBDG-02, respectively, and the cells were assayed 5 d later by indirect immunofluorescence. Transfection of the mutated *PEX3* constructs failed to restore peroxisomal biogenesis in the cells (fig. 4*E*, *F*, *K*, and *L*). In summary, these results demonstrate that the mutations identified in the *PEX3* gene disrupt the function of PEX3.

The observation of peroxisomal rescue despite the absence of preexisting peroxisomal membranes is at odds with the favored model of peroxisomal biogenesis, in which peroxisomes arise exclusively by budding from and/or fission of preexisting peroxisomal vesicles (Lazarow and Fujiki 1985). Instead, it lends support to the existence of an alternative pathway of peroxisome biogenesis. There is recent evidence from yeast and human studies that this pathway is likely to involve the interaction of three peroxins—namely, PEX3, PEX16, and PEX19 (South and Gould 1999; Sacksteder et al. 2000). In this regard, it is interesting to note that restoration of peroxisomes after expression of *PEX3* in the CG-G fibroblasts occurred very slowly, over several days. The stepwise nature of peroxisome synthesis in mutants deficient in peroxisomal membranes has been observed elsewhere, for both a *PEX16*-deficient cell line (South and Gould 1999) and a *PEX19*-deficient cell line (Matsuzono et al. 1999), with PMP-containing vesicles becoming visible first, followed by import of peroxisomal

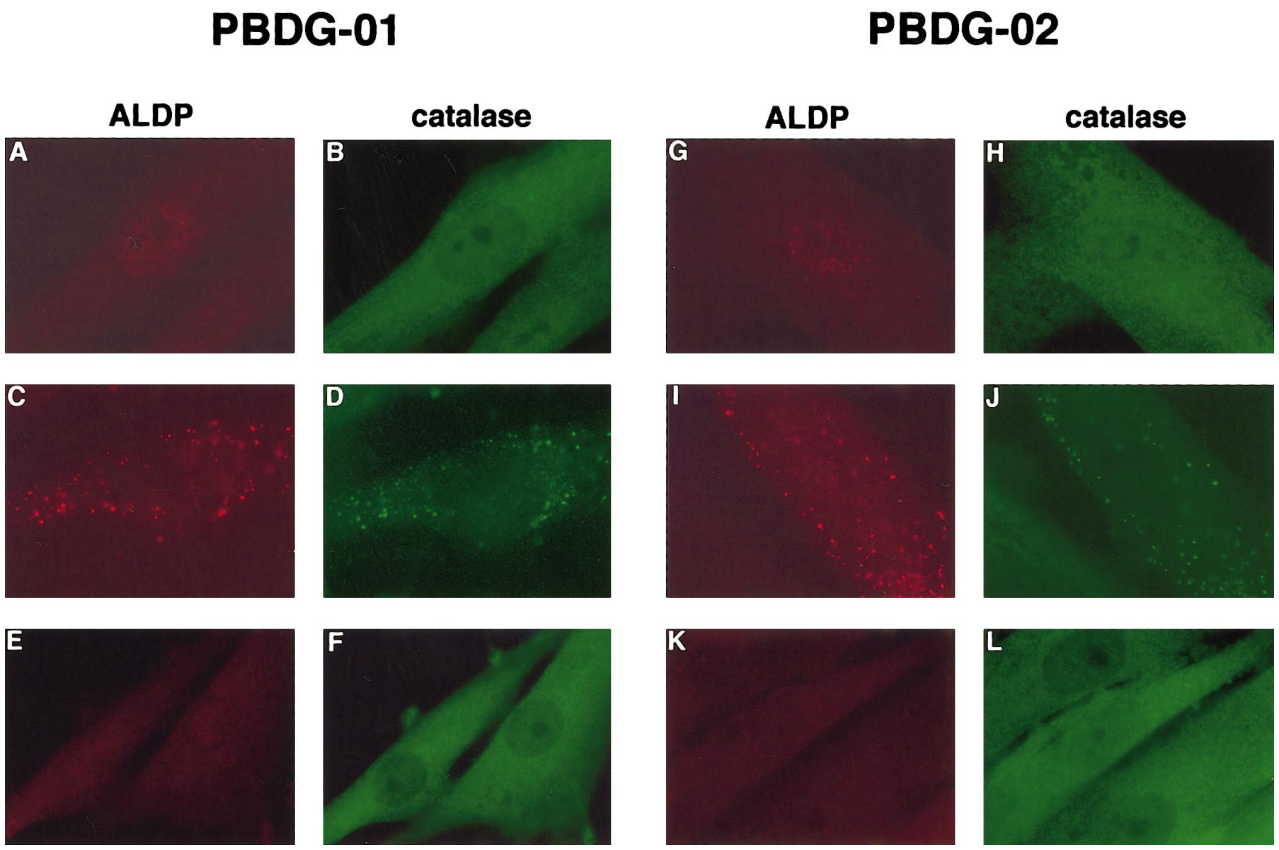

Figure 4 Functional complementation of patient fibroblasts. In untransfected cells from PBDG-01 (*A*, *B*) and PBDG-02 (*G* and *H*), no ALDP positive particles were visible (*A* and *G*). Incubation with anti-catalase antibodies (*B* and *H*) showed a diffuse cytosolic localization. Five days after transfection with a human *PEX3* cDNA vector (pcDNA3.1/Pex3-Myc-His [Kammerer et al. 1998]), punctate patterns for ALDP (*C* and *I*) and catalase (*D* and *J*) indicated that these proteins were colocalized either at or within restored peroxisomes. To generate the mutated *PEX3* expression vectors pcDNA3-PBDG-01 and pcDNA3-PBDG-02, RT-PCR was performed using the primers 5′F3 and 1255R, and the resulting fragments were cloned into pcDNA3. Transfection of the mutant *PEX3* cDNA constructs pcDNA3-PBDG-01 and pcDNA3-PBDG-02 into cells from PBDG-01 (*E* and *F*) and PBDG-02 (*K* and *L*), respectively, did not lead to restoration of a peroxisomal punctate pattern with either ALDP (*E* and *K*) or catalase (*F* and *L*) immunofluorescence. In an additional experiment, transfection efficiency was verified by cotransfection of pcDNA3-PBDG-01 and pcDNA3-PBDG-02 constructs, with the green fluorescent protein-expression vector pEGFP-N1 (Clontech) as a reporter plasmid (data not shown).

matrix proteins. These findings suggest that peroxisomal membrane vesicles form prior to the import of matrix proteins.

How might the *PEX3* mutations disrupt early peroxisomal biogenesis? The precise role of PEX3 in the complex processes leading to the assembly of peroxisomes remains to be elucidated. If not from preexisting vesicles, peroxisomes may arise from some other endomembranes of the cell (South and Gould 1999). Although not proved, it has been hypothesized that PEX3 may be sorted to the peroxisome via the endoplasmic reticulum (ER) (Baerends et al. 1996). It was therefore proposed that PEX3 mediates the early steps of peroxisome formation from the ER (Baerends et al. 1996; Kammerer et al. 1998; Kunau and Erdmann 1998; Titorenko and Rachubinski 1998), and mutations in the *PEX3* gene could potentially interfere with this process. PEX3 has been shown elsewhere to interact with PEX19 and PEX16 in yeast (Götte et al. 1998) and in humans (Soukupova et al. 1999; South and Gould 1999). A *PEX3* mutation might lead to disruption of the interaction between these early peroxins. In yeast, this protein-protein interplay appears to be a prerequisite for generation of preperoxisomal structures that can acquire and stabilize newly synthesized PMPs (Hettema et al. 2000). Thus, a lack of interaction due to a *PEX3* mutation could disturb peroxisomal assembly, by leading to degradation or mislocalization of other PMPs.

To address this question, we performed PEX3-interaction studies to see whether a *PEX3* mutation could

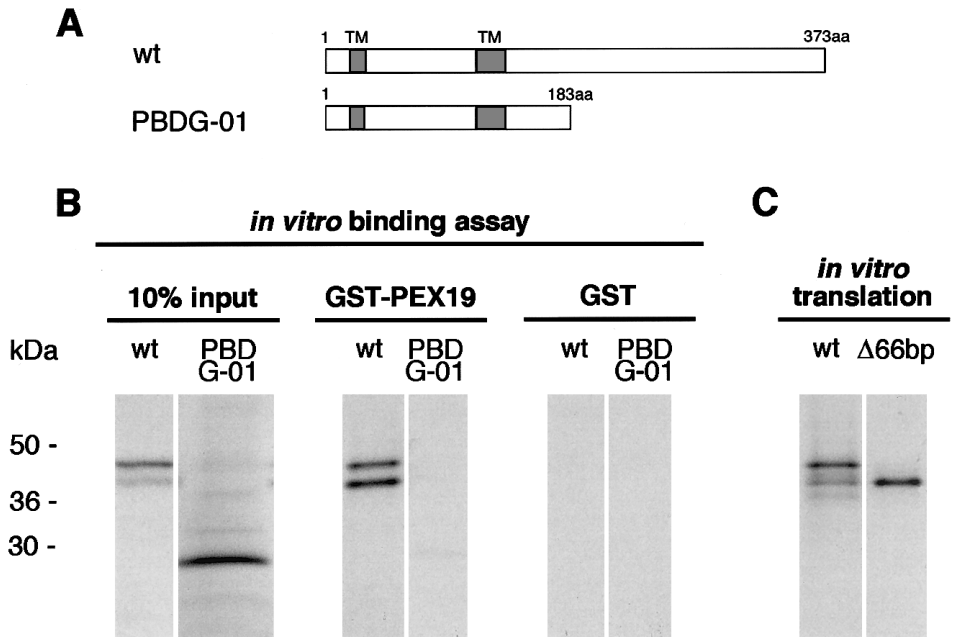

Figure 5 PEX3 in vitro binding assays. *A,* Schematic diagram of wild-type PEX3 and truncated PEX3 of patient PBDG-01. The mutation in PBDG-01 results in a C-terminally truncated PEX3 protein, whereas predicted transmembrane regions (TM) remain unaffected. *B,* Pull-down assays of in vitro–translated PEX3, with immobilized GST-PEX19, performed as described elsewhere (Glöckner et al. 2000). The left-hand gel was loaded with 10% of the [^{35}S]-methionine-labeled PEX3 translation products used as input for the binding assays. To ensure that even weak binding of the mutated PEX3 would be detected, the amount of mutated PEX3 was chosen such that it clearly exceeded the amount of wild-type PEX3 (wt), which was bound by GST-PEX19, whereas no detectable signal was obtained when the interaction of the mutated PEX3 protein (PBDG-01) with PEX19 was analyzed (*central gel*). GST alone did not bind to either protein (*right-hand gel*). Several repetitions of the assay yielded similar results. *C,* Construct lacking the first start codon and the following 63 bp of the *PEX3* gene (66 bp).We generated this construct to demonstrate that the molecular weight of the lower band in the PEX3 wild-type lanes (wt) is a result of the utilization of the internal start codon at position 199 of the coding region. In vitro translation of this construct by the internal start codon yielded a protein corresponding to the lower band of wild-type PEX3.

lead to a disruption of the interaction between PEX3 and PEX19. For this purpose, we performed a pull-down assay after in vitro translation of both wild-type *PEX3* cDNA and mutated *PEX3* cDNA from patient PBDG-01. In an in vitro binding assay, we investigated the ability of wild-type PEX3 and the truncated PEX3 protein from patient PBDG-01 to bind to an immobilized GST-PEX19 fusion protein (fig. 5*B*). Wild-type PEX3 eluted from the GST-PEX19 fusion protein yielded a strong signal, indicating that wild-type PEX3 binds PEX19. However, no signal was detected when the interaction of mutated PEX3 with PEX19 was analyzed, despite the use of an excess amount of mutated protein compared with the wild-type protein. In vitro translation of the wild-type sequence yielded two proteins of differing molecular weight (fig. 5*B* and *C*). To demonstrate that the molecular weight of the lower band in the PEX3 wild-type lanes was a result of the utilization of the internal start codon at position 199 of the coding region, we generated a construct lacking the first start codon and the following 63 bp of the *PEX3* gene (66 bp). In vitro translation of this construct by the internal start codon yields a protein corresponding to the lower band of wild-type PEX3 (fig. 5*C*). In summary, results of the in vitro binding assays reported here are consistent with the hypothesis that the interaction between mutated PEX3 and PEX19 is either markedly reduced or absent. Although this does not necessarily reflect the situation in vivo, the potential functional impairment might contribute to impaired peroxisomal membrane synthesis in cells from patient PBDG-01.

The molecular and biochemical evidence shown here leaves little doubt that *PEX3* mutations are responsible for Zellweger syndrome in CG-G patients. Both patients display a very severe Zellweger syndrome phenotype, with all tested peroxisomal functions being deficient. A correlation between the severity of the clinical phenotype and biochemical activities such as plasmalogen synthesis (Roscher et al. 1985; Lazarow and Moser 1995), as well as the extent of morphological changes such as the number of residual peroxisomes in tissues (Arias et al. 1985), has been described elsewhere for patients with PBD.

Therefore, it is not surprising that we observed a severe clinical phenotype associated with mutations in the *PEX3* gene, which encodes a protein considered to be important in the very early steps of peroxisomal biogenesis.

We have shown that, by expression of wild-type PEX3, intact peroxisomes are formed in the *PEX3* mutant cell lines, which had lacked detectable peroxisomal membrane structures. Similar observations have recently been made for human cells bearing mutations in either *PEX16* or *PEX19* (Matsuzono et al. 1999; South and Gould 1999). The combined data provide evidence for the essential role of these three early peroxins in a human peroxisome–formation pathway that does not require morphologically detectable preexisting peroxisomal structures. Besides supplying the basis for molecular diagnostics, the identification of *PEX3* mutations in humans provides an important model for elucidation of a fundamental mechanism in human organelle biosynthesis.

Note added in proof. While our manuscript was undergoing final revision, a report by South et al. (2000) was published that also describes new data relevant to the mechanisms of *PEX3*-mediated peroxisome synthesis.

Acknowledgments

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Electronic-Database Information

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

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for PBD [MIM 601539], RCDP [MIM 215100], and *PEX3* [MIM 603164])

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