

ORIGINAL ARTICLE

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The peroxin *Pex6p* gene is impaired in peroxisomal biogenesis disorders of complementation group 6

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Abstract Human genetic peroxisomal biogenesis disorders (PBDs), such as Zellweger syndrome, comprise 13 different complementation groups (CGs). Eleven peroxin genes, termed *PEX*s, responsible for PBDs have been identified, whereas pathogenic genes for PBDs of 2 CGs, CG-A (the same CG as CG8 in the United States and Europe) and CG6, remained unidentified. We herein provide several lines of novel evidence indicating that *PEX6*, the pathogenic gene for CG4, is impaired in PBD of CG6. Expression of *PEX6* restored peroxisome assembly in fibroblasts from a CG6 PBD patient. This patient was a compound heterozygote for *PEX6* gene alleles. Accordingly, by merging CG6 with CG4, human PBDs are now classified into 12 CGs.

Key words Peroxisomal biogenesis disorders · Complementation groups · Peroxin · *PEX6* · AAA ATPase · Protein import

Introduction

Human fatal genetic peroxisomal biogenesis disorders (PBDs; MIM 601539) include Zellweger syndrome (ZS; MIM 214100), neonatal adrenoleukodystrophy (NALD;

MIM 202370), infantile Refsum disease (IRD; MIM 266510), and rhizomelic chondrodysplasia punctata (RCDP; MIM 215100) (Lazarow and Moser 1995). Genetic heterogeneity, comprising 16 different complementation groups (CGs), has been identified in mammals, including 13 CGs of PBDs and 3 distinct CGs of mutant Chinese hamster ovary (CHO) cell lines, as determined by cell-fusion and/or peroxin gene (*PEX*) transfection, using fibroblasts from patients with PBDs and peroxisome-deficient CHO cell mutants (Ghaedi et al. 1999; Fujiki 2000) (see Table 1). The primary cause responsible for the peroxisome deficiency in the 13 CGs of PBDs is a failure in peroxisome biogenesis (Fujiki 2000; Gould and Valle 2000). Eleven *PEX* genes involved in peroxisome biogenesis — *PEX1*, *PEX2*, *PEX3*, *PEX5*, *PEX6*, *PEX7*, *PEX10*, *PEX12*, *PEX13*, *PEX16*, and *PEX19* — have been identified as the genetic-related cause responsible for PBD groups E in Japan (CG1 in the United States and Europe), F (CG10), G (CG12), CG2, C (CG4), R (CG11), B (CG7), CG3, H (CG13), D (CG9), and J (CG14), respectively (Fujiki 2000; Gould and Valle 2000; Ghaedi et al. 2000; Shimozawa et al. 2000; Muntau et al. 2000; South et al. 2000). Thus, pathogenic genes responsible for two CGs, CG-A (CG8) and CG6, remain to be isolated (Fujiki 2000; Ghaedi et al. 2000).

The fibroblast cell line from a CG6 patient (PBD6-01) with NALD (patient 6 in the report of Kelly et al. (1986)) was difficult to study because of poor growth. PBD6-01 cells were previously shown to complement CG1 cells in cell-fusion analysis (Moser et al. 1995). In regard to complementation with CG4 cell lines, it was concluded that CG6 was distinct from CG4, after repeated analyses with borderline results (Moser et al. 1995). PBD6-01 is the only patient whose CG assignment had remained in doubt until now. In an earlier publication (Moser et al. 1995), three patients were assigned to CG6. Subsequently, two of these patients were found to have mutations in *PEX6* (Yahraus et al. 1996), and they were therefore reassigned to CG4. Earlier efforts to demonstrate a mutation in patient PBD6-01 had been unsuccessful. This, combined with the equivocal results of biochemical studies referred to earlier, had made

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it impossible to exclude the existence of CG6, prior to the new mutation analysis presented here. In this report, we provide several lines of novel evidence at the molecular level, indicating that CG6 is the same CG as CG4.

Materials and methods

Cell lines

Skin fibroblast cell lines from patients, including fibroblasts from a CG6 patient (PBD6-01) with NALD (Kelly et al. 1986), were cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM) supplemented with 10% fetal bovine serum, as described (Shimozawa et al. 1992). CHO cell mutants, including *pex6* ZP92 (Shimozawa et al. 1992; Tsukamoto et al. 1995) and *pex1* ZP107 (Tamura et al. 1998), were cultured as described.

Cell fusion and DNA transfection

Parent fibroblasts and cells to be fused were cocultured for 1 day and then fused using polyethylene glycol, as described (Shimozawa et al. 1992; Tsukamoto et al. 1990). Cells were stained using specific antibodies 3 days after cell fusion. DNA transfection was performed by electroporation, using a Gene Pulser II electroporator (Bio-Rad, Hercules, CA, USA) with the setting of 320V/500 μ F and by lipofection, using Lipofectamine (Life Technologies, Tokyo, Japan) as described (Tamura et al. 1998).

Morphological analysis

Peroxisomes in human fibroblasts and CHO cells were visualized by indirect immunofluorescence microscopy, as described (Shimozawa et al. 1992). Rabbit antibody to human catalase (Sigma, St. Louis, MO, USA) was raised by conventional subcutaneous injection. Antigen-antibody complexes were detected by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (IgG) antibody (Cappel, Durham, NC, USA), under an Axioskop FL microscope (Carl Zeiss, Oberkochen, Germany).

Isolation of *PEX6*

Human *PEX6* cDNA was cloned by colony hybridization of a human liver cDNA library (Tamura et al. 1998), using as a probe a partial sequence of *PEX6* (Fukuda et al. 1996) amplified by polymerase chain reaction (PCR) with a set of primers comprising nucleotide residues at 2375–2393 and 2913–2943. Subsequent sequencing of a cDNA clone was done by the dideoxy-chain termination method, using a Dye-terminator DNA sequence kit (Applied Biosystems, Foster City, CA, USA), as described (Tamura et al. 1998).

Mutation analysis

Poly(A)⁺ RNA was obtained from cultured patient's fibroblasts, using a QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech, Tokyo, Japan). Reverse transcription (RT)-PCR was done as described (Honsho et al. 1998), using poly(A)⁺ RNA and a pair of *HsPEX6*-specific PCR primers: a sense forward (F)1: 5'-GTCCTG TCACCATGGGCGC-3' (initiation codon, underlined) and an antisense reverse (R)1: 5'-(CTGGGTCTAGA)CTA GCAGGCAGCAAACCTTGCGCTGGATGCGC-3' (*Xba*I site, in parenthesis; termination codon, underlined), containing nucleotide residues at positions –11 to 7 (A starting in the initiation codon as 1) and 2913–2943, to cover a full length of the *PEX6* open reading frame. Amplification was done using the RT product as a template, with a forward primer F1 and a reverse XN.R: 5'-GCTAGCTG(ATGCAT)GTTCACCTCCTGGCCCCAG-3' (*Nsi*I site, in parenthesis; residues at 1840–1871). Amplification was, likewise, performed by the use of a set of primers: a forward F4, 5'-AGATGGACCAGCCAGTGC-3' (residues at 1179–1196), and a reverse R1, as well as another set: a forward F2-1, 5'-GGTTGGGCACTG CTTGGCAC-3' (residues at 310–329), and R2, 5'-GCCAAAGAGTCCAGTTCATC-3' (residues at 2407–2426). Respective cDNA clones were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and were sequenced.

Genomic DNA was prepared from cultured fibroblasts, as described (Laird et al. 1991). To determine the zygosity of the mutant *PEX6* alleles (see Fig. 2) in patient PBD6-01, PCR was performed to amplify the sequence encompassing nucleotide residues 619–882 in the human *PEX6* gene (GenBank accession number, AF108095-AF108098) (Zhang et al. 1999), using a pair of *PEX6*-specific primers, a forward F3-1: 5'-CTTTTAATCTTGGCTGTGAC-3' (nucleotide residues at 833–852 in exon 1) and a reverse 42.R: 5'-CCGTCATAATTTCCATTAGTG-3' (residues at 996–1016 in exon 2), and another set of primers, a forward F2: 5'-GGGCTCGGACCGCGAGTC-3' (residues at 343–360 in exon 1) and a reverse IVS1-R: 5'-CGGCTAGAA ACATTATGTTCA-3' (residues at 109–129 in intron 1). Next, to amplify the sequence between nucleotide residues at 2095–2362, PCR was, likewise, done using a set of primers, a forward F6: 5'-TGGCAGGTGGCTTGACTG-3' (nucleotide residues at 1964–1981 in exon 10) and a reverse R2 (see above). PCR products were cloned into pGEM-T Easy vector and sequenced.

Generation of mutant constructs

An expression vector carrying the patient PBD6-01-derived *PEX6* was constructed as follows. pCMVSPORT-*HsPEX6* was first digested with *Xba*I and *Hind*III, blunted, and then ligated. A 264-bp deleted form of *PEX6*, termed *PEX6del619–882* (see Results), in pCMVSPORT was formed by replacing a *Bss*HII-*Xho*I fragment of the pCMVSPORT-*HsPEX6* with a *Bss*HII-*Xho*I fragment of

the patient PBD6-01-derived *PEX6del619–882* in pGEM-T Easy vector. The other mutant type, termed *PEX6del2095–2362/21ins*, with a 268-bp deletion at positions from 2095 to 2362, plus a following 21-bp insertion, was constructed by replacing a *XhoI-NotI* fragment of the pCMVSPORT-*HsPEX6* with a *XhoI-NotI* fragment of the patient-derived *PEX6del2095–2362/21ins* in pGEM-T Easy vector. Both constructs were confirmed by nucleotide sequence analysis.

Results and discussion

CG analysis

In the present work, as a step toward searching for a complementing cDNA for CG6, we first re-evaluated CG analysis of PBD6-01 fibroblasts, basically by cell fusion and *PEX* gene transfection. In the patient's fibroblasts, peroxisomes were not detected when the cells were immunostained with an anti-catalase antibody (Fig. 1*a*). Peroxisome-positive cells were occasionally discernible by cell staining, using antibody to peroxisomal targeting signal type 1 (called PTS1) (data not shown). We performed cell fusion between CG6 PBD6-01 fibroblasts and fibroblasts from a *PEX6*-defective CG4 patient (PBDC-03) with ZS (Fukuda et al. 1996), as well as a *PEX1*-defective CG1 ZS patient (PBDE-14) (Imamura et al. 1998). Numerous peroxisomes were detected 3 days after the fusion of PBD6-01 fibroblasts with CG1 cells, as assessed by staining with anti-catalase antibody (Fig. 1*d*), whereas no peroxisome was visible in cells fused with CG4 fibroblasts (Fig. 1*b*). Moreover, cell fusion of PBD6-01 fibroblasts with a CHO *pex6* mutant ZP92 did not restore peroxisomal protein import, while a cell hybrid with a *pex1* ZP107 showed numerous catalase-positive structures, i.e., peroxisomes (Fig. 1, *c* and

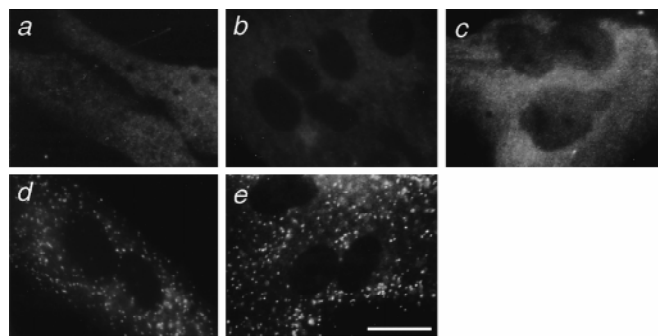


Fig. 1. Cell fusion analysis. Fibroblasts from a complementation group (CG)6 neonatal adrenoleukodystrophy (NALD) patient (PBD6-01) were fused with peroxisome-deficient cells of other CGs. *a*, CG6 PBD6-01 fibroblasts; *b* and *c*, cell fusion was done with fibroblasts from a *PEX6*-defective CG4 Zellweger syndrome (ZS) patient and Chinese hamster ovary (CHO) *pex6* mutant cells, respectively; *d* and *e*, PBD6-01 fibroblasts fused with *PEX1*-deficient CG1 patient's fibroblasts and CHO *pex1* mutant ZP107, respectively. Cells were stained with anti-human catalase antibody. Note that peroxisomes were restored in *d* and *e*, but not in *b* and *c*. Bar 30 μ m

e). These observations suggested that CG6 is the same CG as *PEX6*-defective CG4. The formation of peroxisomes was, likewise, observed after cell fusion between PBD6-01 fibroblasts and fibroblasts from the other 10CGs of PBDs (data not shown). We have observed that PBD6-01 fibroblasts are unstable with respect to peroxisome deficiency, perhaps representing a mosaicism of peroxisomes in this NALD patient, and having led us to the previous conclusion, i.e., assignment of PBD6-01 to CG6. It is of interest to note that the very early development of the patient PBD6-01 was not considered abnormal (Kelly et al. 1986), suggesting that, in vivo, the peroxisomal protein import deficit was not complete.

We cloned human *PEX6* cDNA, encoding a 980-amino-acid AAA ATPase family peroxin, by colony hybridization of a human liver cDNA library (Tamura et al. 1998). Sequencing of one full-length cDNA out of several clones showed a single base-change in a codon for Pro⁹³⁹ (CCA) (Fukuda et al. 1996; Yahraus et al. 1996) to Gln⁹³⁹ (CAA), implying a single nucleotide polymorphism in *PEX6* (GenBank accession number, AB051076). We then introduced this *PEX6* cDNA into PBD CG6 fibroblasts by electroporation. Numerous peroxisomes were detected 3 days post-transfection (Fig. 2*A, a*). Expression of Pex6p possessing Pro⁹³⁹, likewise, complemented peroxisome biogenesis (data not shown). PBD6 fibroblasts were also transfected with human *PEX1* cDNA (Tamura et al. 1998) encoding another AAA-family peroxin with a mass of 143 kDa. Peroxisomes were not evident in the transfectants (data not shown). Furthermore, no complementation was evident upon transfection of PBD6 fibroblasts with other human *PEX* cDNAs (data not shown). Therefore, *PEX6* was most likely to be impaired in CG6 PBDs.

Mutation analysis

To determine the dysfunction of *PEX6* in PBD6-01 fibroblasts, we performed reverse transcription (RT)-PCR, using poly(A)⁺ RNA and a pair of *HsPEX6*-specific PCR primers: a sense F1 and an antisense R1. Amplification was done using the RT product as a template with a forward primer F1 and a reverse XN.R. Amplification was, likewise, performed by the use of a set of primers: a forward F4 and a reverse R1, as well as another set: a forward F2-1 and R2. Subsequent sequencing of the respective cDNA clones revealed that 7 of 12 clones had a 264-bp deletion of nucleotide residues at positions from 619 to 882, termed *PEX6del619–882*, resulting in an 88-amino-acid truncation in one allele (Fig. 2*B*, upper panel) (GenBank accession number, AB051077). Of note, these clones showed Pro⁹³⁹ (see above). The truncated sequence corresponded to about one-third of the 3'-side of exon 1 (Zhang et al. 1999). Another type of mutation was identified in 6 of 10 clones: a 268-bp deletion at positions from 2095 to 2362, plus a following 21-bp insertion, termed *PEX6del2095–2362/21ins*, resulting in the amino-acid sequence from the N-terminus to the residue at 698 of the normal Pex6p, followed by a distinct 40-amino-acid sequence and a termination (Fig. 2*B*,

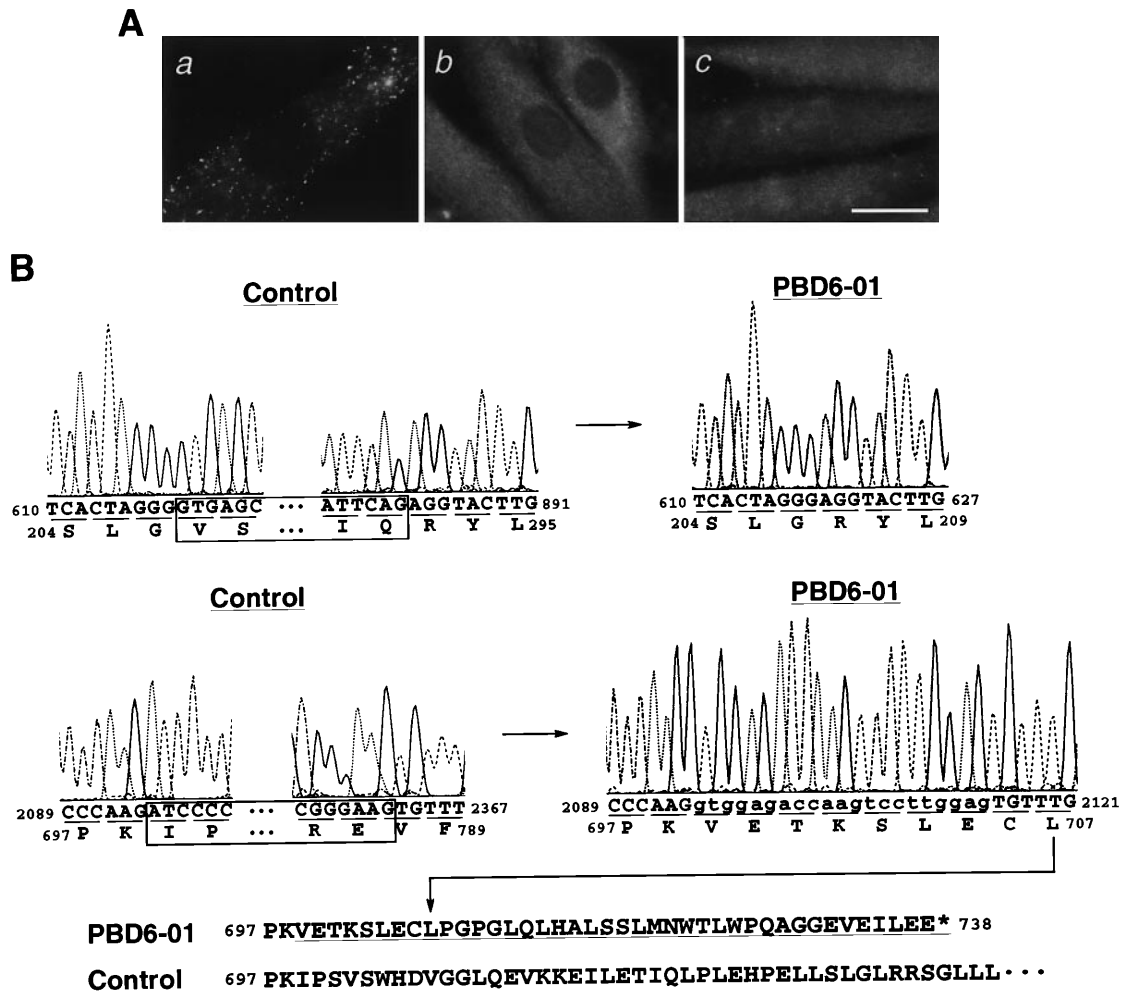


Fig. 2A,B. *PEX6* is impaired in peroxisomal biogenesis disorders (PBDs) of CG6. **A** Expression of *HsPEX6* restores peroxisome assembly. *HsPEX6* from a normal control (*a*) and CG6 patient (PBD6-01)-derived *PEX6del619–882* (*b*) and *PEX6del2095–2362/21ins* (*c*) (see **B**) were separately expressed in PBD6-01's fibroblasts. Cell staining was done with anti-catalase antibody. Note that peroxisomes were restored in *a*, but not in *b* and *c*. Bar 30 µm. **B** Mutation analysis of *PEX6* from the patient PBD6-01. Partial nucleotide sequence and deduced amino

acid sequence of *PEX6* cDNA isolated from a normal control (*left panel*) and the patient PBD6-01 (*right panel*) are shown. A 264-bp deletion of nucleotide residues from 619 to 882 (*upper panel, boxed*), termed *PEX6del619–882*, and a 268-bp deletion from 2095–2362, plus a 21-bp insertion (*lower panel*) inducing a 40-amino acid sequence entirely distinct from normal Pex6p (*bottom, underlined*), termed *PEX6del2095–2362/21ins*, were identified

lower panel) (GenBank accession number, AB051078). These mutated forms of *PEX6* possessed Gln⁹³⁹ (see above). The deleted sequence 2095 to 2362 encompasses exons 11 and 12 (Zhang et al. 1999). These mutations inactivated the function of *PEX6*, as verified by the expression of *PEX6del619–882* and *PEX6del2095–2362/21ins* in PBD6-01 fibroblasts (Fig. 2A, *b* and *c*), as well as in CHO *pex6* ZP92 cells (data not shown), where peroxisomes were not re-established. The data collectively indicate that mutation of *PEX6* is the genetic-related cause of CG6 PBDs.

To confirm the mutation sites and zygosity of the mutant alleles, genomic PCR was done to amplify the sequence encompassing nucleotide residues 619–882 in the human *PEX6* gene (Zhang et al. 1999). In 8 of 11 independent clones isolated from PCR products in the search for a deletion of residues at 619–882, we identified a single nucleotide substitution at 399, T → G (a silent mutation in a codon for

Gln¹³³V), and a 14-bp deletion at 800–813 in exon 1, comprising residues 1–882, which, presumably, gave rise to the deletion of residues 619–882 (data not shown). Three of the 11 clones showed the normal *PEX6* sequence. Next, to amplify the sequence between nucleotide residues at 2095–2362, PCR was, likewise, done. In 5 of 6 independent clones, a point mutation, G → A, at the 3'-terminus of exon 12 was identified, which apparently caused a deletion of exons 11 and 12. One of the 6 clones contained the normal *PEX6* gene sequence. We interpreted these results to mean that this NALD patient, PBD6-01, was a compound heterozygote for the mutations. Each of the two alleles, the *PEX6del619–882* allele, apparently encoding for the Pro⁹³⁹-Pex6p truncated at 207–294, and the other allele, *PEX6del2095–2362/21ins*, apparently encoding for the Gln⁹³⁹-Pex6p with a deletion in the C-terminal part encoded by exons 11 and 12 plus a frameshift/termination, was, most likely, derived from each parent.

Table 1. Complementation groups of patients with PBDs, and of CHO cell mutants

CG Designation of patients with PBDs		Phenotype	CHO mutant ^a	Gene
Japanese	US/European			
A	8	ZS, NALD, IRD	ZP124	
B	7(5) ^b	ZS, NALD		<i>PEX10</i>
C	4(6)	ZS, NALD	ZP92	<i>PEX6</i>
D	9	ZS		<i>PEX16</i>
E	1	ZS, NALD, IRD	Z24/ZP107	<i>PEX1</i>
F	10	ZS, IRD	Z65	<i>PEX2</i>
G	12 ^c	ZS	ZPG208	<i>PEX3</i>
H	13 ^c	ZS, NALD	ZP128	<i>PEX13</i>
J	14 ^c	ZS	ZP119	<i>PEX19</i>
	2	ZS, NALD	ZP105/ZP139	<i>PEX5</i>
	3	ZS, NALD, IRD	ZP109	<i>PEX12</i>
R	11	RCDP	ZPG207	<i>PEX7</i>
			ZP110	<i>PEX14</i>
			ZP114	
			ZP126	

Numbers in parentheses represent the previously assigned, distinct CGs

PBD, Peroxisomal biogenesis disorders; CHO, Chinese hamster ovary; CG, complementation groups; ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; IRD, infantile Refsum disease; RCDP, rhizomelic chondrodysplasia punctata

^aFrom (Fujiki 2000; Ghaedi et al. 2000)

^bFrom (Moser et al. 1995; Shimozawa et al. 1998)

^cFrom (Gould and Valle 2000)

Accordingly, by merging CG6 with CG4, human PBDs are now classified into 11 CGs of peroxisome-deficient PBDs such as ZS, NALD, and IRD, and one CG of *PEX7*-defective RCDP, impaired solely in the PTS2 import (Table 1).

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