Mutations in PEX10 is the cause of Zellweger peroxisome deficiency syndrome of complementation group B

Kanji Okumoto1, Ryota Itoh1, Nobuyuki Shimozawa2, Yasuyuki Suzuki2, Shigehiko Tamura1, Naomi Kondo2 and Yukio Fujiki1,3,*

1Department of Biology, Faculty of Science, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan, 2Department of Pediatrics, Gifu University School of Medicine, Gifu 500-8076, Japan and 3CREST, Japan Science and Technology Corporation, Tokyo 170-0013, Japan

Received April 17, 1998; Revised and Accepted June 5, 1998

Peroxisome biogenesis disorders (PBD), such as Zellweger syndrome, are autosomal recessive diseases caused by a deficiency in peroxisome assembly as well as a malfunction of the peroxisomes, where at least 10 genotypes have been reported. We have isolated a human PEX10 cDNA (HsPEX10) by an expressed sequence tag homology search on a human DNA database using yeast PEX10 from Hansenula polymorpha, followed by screening of a human liver cDNA library. This cDNA encodes a peroxisomal protein (a peroxin Pex10p) comprising 326 amino acids, with two putative transmembrane segments and a C3 HC4 zinc finger RING motif. Both the N- and C-terminal regions of Pex10p are exposed to the cytosol, as assessed by an expression study of epitope-tagged Pex10p. HsPEX10 expression morphologically and biochemically restored peroxisome biogenesis in fibroblasts from Zellweger patients of complementation group B in Japan (complementation group VII in the USA). One patient (PBDB-01) possessed a homozygous, inactivating mutation, a 2 bp deletion immediately upstream of the RING motif, which resulted in a frameshift, altering 65 amino acids from the normal. This implies that the C-terminal part, including the RING finger, is required for biological function of Pex10p. PEX10 cDNA derived from patient PBDB-01 was defective in peroxisome-restoring activity when expressed in patient fibroblasts. These results demonstrate that mutation in PEX10 is the genetic cause of complementation group B PBD.

INTRODUCTION

Peroxisomes are present in a wide variety of eukaryotic cells, from yeast to humans, and function in various metabolic pathways, including β-oxidation of very long chain fatty acids and synthesis of ether lipids (1). Peroxisomal proteins, including membrane proteins, are encoded by nuclear genes and translated on free polyribosomes in the cytosol, mostly at their final sizes (2). Peroxisomes are formed by division of pre-existing peroxisomes after post-translational import of newly synthesized proteins (2). Genetic analysis of peroxisome-deficient mutants of yeast and mammalian cells have led to identification of a number of protein factors essential for peroxisome biogenesis (3,4). The primary cause of peroxisome deficiency in fatal genetic diseases, including Zellweger syndrome (ZS), neonatal adrenoleukodystrophy and infantile Refsum disease, was thought to be a failure in peroxisome assembly (4–6). Twelve complementation groups (CGs) have been identified in mammals: 10 of these were defined by analysis of fibroblasts from patients with peroxisome biogenesis disorders (PBD) (5,7,8) and peroxisome-deficient Chinese hamster ovary (CHO) cell mutants (5,9–13) and two additional ones by the use of CHO mutant cell lines only (14). Therefore, >12 genes are likely to be involved in mammalian peroxisome biogenesis. We have to date cloned PEX2 (formerly PAF-1) (15), PEX6 (formerly PAF-2) (16), PEX12 (17,18) and PEX1 (19) cDNAs by genetic phenotype complementation assay of CHO cell mutants Z65, ZP92, ZP109 and ZP107, respectively. Several human orthologs of yeast peroxins have been isolated by means of an expressed sequence tag (EST) search of a human database using yeast PEX genes (20).

A nonsense mutation of the PEX2 gene encoding a 35 kDa peroxisomal integral membrane protein was elucidated, for the first time, to be the primary cause of Zellweger syndrome CG-F (CG-X in the USA and CG-5 in Europe) (21). Dysfunction and mutations of PEX5, encoding the PTS1 receptor, were found in CG-II patients (22,23). PEX6, encoding a member of an ATPase family, was shown to be responsible for Zellweger syndrome CG-C (CG-IV in the USA) (24,25). PEX12 and PEX1 were recently shown to be mutated in PBD patients of CG-III (17,18,26) and CG-I (19,27,28), respectively.

We herein isolated human PEX10 cDNA (HsPEX10) encoding a peroxin, Pex10p, by an EST homology search using Hansenula polymorpha PEX10. Peroxisomes were complemented in peroxisome-deficient fibroblasts from patients with CG-B PBD by

*To whom correspondence should be addressed. Tel: +81 92 642 2635; Fax: +81 92 642 4214; Email: yfujiscb@mbox.nc.kyushu-u.ac.jp
Figure 1. Amino acid sequence alignment of human Pex10p protein and Pex10p from *H. polymorpha* and *P. pastoris*. The deduced amino acid sequence of human (Hs) Pex10p was compared with those of Pex10p from yeast *H. polymorpha* (Hp) and *P. pastoris* (Pp). –, a space. Identical amino acids between human and other species are shaded. Putative membrane-spanning segments are overlined. Conserved cysteine and histidine residues in the RING finger are designated by dots. The arrowhead indicates the position of the mutation in a CG-B Zellweger patient, PBDB-01. The database accession no. for the human *PEX10* cDNA is AB013818.

Figure 2. Complementation of peroxisomes in fibroblasts from a CG-B Zellweger patient. (A) Restoration of peroxisomal protein import. Transfection of fibroblasts from a CG-B patient (PBDB-01) with Zellweger syndrome with human *PEX10*. (a) Fibroblasts from a control; (b) PBDB-01 fibroblasts; (c) PBDB-01 fibroblasts transfected with pUcD2Hyg·*HsPEX10*. Cells were stained with anti-PTS1 antibody. Magnification ×630; bar 20 µm. (B) Biogenesis of peroxisomal protein. Cell lysates (∼1 × 10⁵ cells) were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membrane. Immunoblot analysis was done using rabbit anti-3-ketoacyl-CoA thiolase antibody (10). Lane 1, control fibroblasts; lane 2, PBDB-01 fibroblasts; lane 3, PBDB-01 fibroblasts transiently transfected with *HsPEX10*. Open and solid arrowheads indicate a larger precursor (P) and mature protein (M) of 3-ketoacyl-CoA thiolase, respectively. A dot indicates a non-specific band.

transfection with *HsPEX10*. In a CG-B patient we identified a homozygous mutation that inactivated *PEX10*.

RESULTS AND DISCUSSION

Cloning of a human *PEX10* cDNA

We used the EST homology search method as a cDNA cloning strategy. A BLAST search using a TBLASTN program (29) for mammalian orthologs of a conserved zinc finger, the RING region, of *H. polymorpha* *PEX10* (*HpPEX10*) (30) identified several cDNA clones, a mouse EST (AA27050) and human ESTs (AA368416, AA173132 and AA357702). We isolated two positive clones by screening a human liver cDNA library with AA368416-derived probes; one longer plasmid F7-15-42 contained a 208 bp cDNA with an open reading frame (ORF) encoding a 326 amino acid protein of 37.069 kDa (Fig. 1). Homology analysis suggested that this ORF was most likely to encode the human ortholog of *HpPEX10* and *PEX10* from *Pichia pastoris* (*PpPEX10*) (31). Therefore, we termed this cDNA *HsPEX10*. The *HsPEX10* protein, HsPex10p, was longer in the primary sequence by 31 amino acids than *HpPex10*, but shorter by 93 amino acids than *PpPex10*. The average amino acid identities to *HpPex10* and *PpPex10* were 27 and 25%, respectively. Moreover, -50% identity was found in the RING finger motif region.
Figure 3. Mutation analysis of PEX10 from a CG-B Zellweger patient. (A) Nucleotide sequence analysis of PEX10 from patient PBDB-01. Partial sequence and deduced amino acid sequence of PEX10 cDNA isolated from a normal control (left) and ZS patient PBDB-01 (right) are shown. A 2 bp deletion at nt 814–815 (shaded) causes a frameshift in the PBDB-01 PEX10 sequence (right). (B) Sequence comparison of the C-terminus of Pex10p from a control and patient PBDB-01. The C-terminal amino acid sequence resulting from the frameshift due to the 2 bp deletion in PBDB-01 is underlined. The arrowhead indicates the position of mutation in PBDB-01. Conserved cysteine and histidine residues in the RING finger are shaded. (C) PEX10-transfection of patient fibroblasts. Mutant PEX10 derived from PBDB-01, PEX10Δ814/815, was transfected into PBDB-01 fibroblasts (a). Fibroblasts from Zellweger patient PBDB-10 were transfected with HsPEX10 (b) and PEX10Δ814/815 (c), respectively. Immunofluorescence staining was as in Figure 2A. Note that peroxisomes were not restored in (a) and (c). Magnification ×630; bar 20 µm.

Table 1. Complementation analysis of patient fibroblasts, and CHO cell mutants with HsPEX10

<table>
<thead>
<tr>
<th>Patient fibroblast</th>
<th>Peroxisome-positive</th>
<th>CHO mutant</th>
<th>Peroxisome-positive</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>B (PBDB-01) (VII)</td>
<td>61/200</td>
<td>–</td>
<td>–</td>
<td>PEX10</td>
</tr>
<tr>
<td>B (PBDB-09) (VII)</td>
<td>51/200</td>
<td>–</td>
<td>–</td>
<td>PEX1</td>
</tr>
<tr>
<td>B (PBDB-10) (VII)</td>
<td>43/200</td>
<td>–</td>
<td>–</td>
<td>PEX5</td>
</tr>
<tr>
<td>E (I)</td>
<td>–</td>
<td>ZP107</td>
<td>–</td>
<td>PEX12</td>
</tr>
<tr>
<td>II</td>
<td>–</td>
<td>ZP105</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>–</td>
<td>ZP109</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>C (IV)</td>
<td>–</td>
<td>ZP92</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>A (VIII)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>D (IX)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>F (X)</td>
<td>–</td>
<td>Z65</td>
<td>–</td>
<td>PEX2</td>
</tr>
<tr>
<td>G</td>
<td>–</td>
<td>ZP110</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZP114</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Patient fibroblasts from 10 CGs of peroxisome deficiency diseases (4), i.e. CG-A, -B (patients PBDB-01, PBDB-09 and PBDB-10), -C, -D, -E, -F and -G of Gifu University (Gifu, Japan) and CG-II, -III and -VI of Kennedy-Krieger Institute (Baltimore, MD), and peroxisome-deficient CHO mutants (4,14), including PEX1-defective ZP107 (12,19), PEX5-impaired ZP105 (12,13), PEX12-defective ZP109 (12,17,18), PEX6-defective ZP92 (5,16) and PEX2-deficient Z65 (15,21,38), were transfected with pUCD2Hyg·HsPEX10 and examined for peroxisome assembly by immunostaining with antisera to PTS1 and rat catalase, respectively, at 3 days post-transfection. CGs in parentheses were not used in this experiment. For CG-B fibroblasts, peroxisome-positive cells were counted in 200 cells. In other cells: +, complemented; –, not complemented.
PEX10 restores peroxisome assembly in CG-B fibroblasts

Peroxisome targeting signal type 1 (PTS1) proteins in fibroblasts were visualized by immunostaining using anti-PTS1 antibody (13). Normal fibroblasts showed a punctate staining pattern typical for peroxisomes, whereas cells from a CG-B patient (PBDB-01) with ZS showed a diffuse cytosolic staining, indicative of peroxisome deficiency (Fig. 2A, parts a and b). HsPEX10 expression complemented peroxisomes in PBDB-01 fibroblasts, which were as numerous as those in normal cells (Fig. 2A, part c). Restoration of peroxisomes was also assessed by cell staining using antibodies to 3-ketoacyl-CoA thiolase (a PTS2 protein) and the 70 kDa integral membrane protein of peroxisomes (data not shown). These results demonstrate that Pex10p morphologically complemented peroxisome biogenesis in PBDB-01 fibroblasts. Peroxisomal 3-ketoacyl-CoA thiolase is synthesized as a larger precursor with an N-terminal, cleavable PTS2 (32,33) and is processed to its final size in peroxisomes (10). In normal fibroblasts, only the mature thiolase was detected (Fig. 2B, lane 1), thereby demonstrating rapid processing of the precursor form. In PBDB-01 cells, only the larger precursor was found (lane 2), implying a defect in import and processing activity. When PBDB-01 fibroblasts were transiently transfected with HsPEX10, both mature and unprocessed forms of the thiolase were discerned, presumably reflecting the efficiency of complementation at 3 days post-transfection (Fig. 2B, lane 3). Taken together, these results demonstrate that HsPEX10 can complement the abnormality in biogenesis of peroxisomal protein in PBDB-01 cells. Moreover, HsPEX10 was introduced into fibroblasts from two unrelated ZS patients of CG-B (PBDB-09 and PBDB-10) as well as those from nine other PBD CGs, i.e. A, C–F and G of Gifu University and II, III and VI of the Kennedy-Krieger Institute. As expected, peroxisome assembly was restored only in CG-B fibroblasts from PBDB-09 and PBDB-10, whereas none of the fibroblasts of other CGs was complemented, confirming that Pex10p is a peroxisome biogenesis factor for PBD of CG-B (CG-VII) (Table 1; Fig. 3C, part b). HsPEX10 expression did not restore peroxisomes in seven CGs of peroxisome-deficient CHO cell mutants thus far isolated (5,10,12–14), including ZP110 and ZP114 of two CGs distinct from CG-B (CG-VII) disorders. Five mammalian peroxin genes are responsible for peroxisome deficiency (5,10,12–14), including detection of C-terminally flagged protein after permeabilization with 25 µg/ml digitonin, which selectively permeabilizes plasma membranes and makes intra-peroxisomal proteins inaccessible to exogenous antibodies (17,18,34). Flag–Pex10p was observed in a punctate staining pattern, whereas there was nearly no staining of cells with anti-PTS1 antibody (Fig. 4A, parts d and e). When the cells were treated with Triton X-100, which solubilizes all cellular membranes, both PTS1 proteins and flag–Pex10p were detected in particulate fractions, and were similar in number (Fig. 4A, parts a–c; see above). Similar results, including detection of C terminally flagged protein after permeabilization with 25 µg/ml digitonin, were obtained when HsPEX10–flag was expressed in CHO-K1 cells (Fig. 4B, parts a and b).

Mutation analysis of CG-B patient PEX10

To determine the dysfunction of PEX10 in PBDB-01, we isolated PEX10 cDNA from PBDB-01 fibroblasts by means of RT–PCR. Subsequent sequencing of cDNA clones indicated a frameshift caused by deletion of 2 nt, C814T815, in the codon for Leu272, inducing a nucleotide sequence encoding a 65 amino acid peptide (Fig. 3A). This peptide sequence was longer by 10 amino acids and totally distinct from that of normal Pex10p (Fig. 3B). Five cDNA clones isolated, termed PEX10A814/815, all showed the same 2 bp deletion, implying that patient PBDB-01 was homozygous for the mutation. It is noteworthy that this mutated form of Pex10p entirely lacks the RING motif located immediately downstream of the mutated site. This mutation inactivated the function of PEX10, as assessed by back-transfection of PEX10A814/815 into PBDB-01 fibroblasts, where no peroxisomes were formed (Fig. 3C, part a). Fibroblasts from another, unrelated ZS patient, PBDB-10, were complemented in peroxisome biogenesis by transfection of HsPEX10, but not by PEX10A814/815, confirming PEX10A814/815 as impaired (Fig. 3C, parts b and c). Similar results were obtained using PBDB-09 fibroblasts (data not shown). These findings imply the importance of the C-terminal part, including the RING finger, of Pex10p to its biological activity.

Intracellular localization and topology of Pex10p

The subcellular localization of Pex10p was determined by immunofluorescent microscopy following ectopic expression of Pex10p tagged with a flag epitope at its N- or C-terminus. N-terminally flag-tagged HsPEX10 expression restored peroxisome assembly in PBDB-01 fibroblasts, indicating that N-terminal tagging did not interfere with Pex10p function (data not shown). In flag–HsPEX10-transfected fibroblasts from a normal subject as well as CG-B patients, Pex10p was barely detectable with an anti-flag antibody, implying a lower level of Pex10p expression per se (data not shown). When flag–HsPEX10 was expressed in CHO-K1 cells, Pex10p was detected in a punctate staining pattern (Fig. 4A, part a). The pattern was superimposable on that obtained using anti-PTS1 antibody (Fig. 4A, parts b and c), thereby suggesting that flag–Pex10p was localized in peroxisomes. Similar results were obtained when C-terminally epitope-tagged Pex10p–flag was expressed in CHO-K1 cells (Fig. 4B, parts a and b).

Given the findings demonstrating that Pex10p is a peroxisomal protein, the presence of two putative hydrophobic membrane-spanning segments in Pex10p suggests that Pex10p is localized on peroxisomal membranes (Fig. 1). The membrane topology of Pex10p was determined by a differential permeabilization procedure. CHO-K1 cells expressing flag–Pex10p were permeabilized with 25 µg/ml digitonin, which selectively permeabilizes plasma membranes and makes intra-peroxisomal proteins inaccessible to exogenous antibodies (17,18,34). Flag–Pex10p was observed in a punctate staining pattern, whereas there was nearly no staining of cells with anti-PTS1 antibody (Fig. 4A, parts d and e). When the cells were treated with Triton X-100, which solubilizes all cellular membranes, both PTS1 proteins and flag–Pex10p were detected in particulate fractions, and were similar in number (Fig. 4A, parts a–c; see above). Similar results, including detection of C terminally flagged protein after permeabilization with 25 µg/ml digitonin, were obtained when HsPEX10–flag was expressed in CHO-K1 cells (Fig. 4B, parts a–d). Taken together, these results strongly suggest that both the N- and C-terminal parts of Pex10p are exposed to the cytosol, presumably anchored by two membrane-spanning segments (Fig. 1). Transmembrane topology of Pex10p was confirmed by expressing epitope-tagged HsPEX10 in COS-7 cells (data not shown). Hansenula polymorpha and P. pastoris Pex10p were shown to be integral peroxisomal membrane proteins (30,31). It is noteworthy that P. pastoris Pex10p, possessing two putative transmembrane segments, was suggested to expose the C-terminal part to the luminal side of peroxisomes, assessed by its resistance to an externally added protease (31).

Collectively, the data in the present work demonstrate that dysfunction of PEX10 is responsible for peroxisome deficiency in CG-B (CG-VII) disorders. Five mammalian peroxin genes involved in peroxisome assembly have been isolated, either by a homology search on a human EST database or genetic functional
that Pex12p exposes the RING finger to the cytosol (17,18,26), as in mammalian Pex10p (this study). Pex10p was suggested to be involved in proliferation of peroxisomes in the yeast H. polymorpha (30), but not in Ppastoris (31). It is possible that Pex10p functions as a peroxisomal biogenesis factor in the peroxisomal protein import machinery by interacting with other PEX proteins (3,4,19,20), including RING motif peroxins Pex2p (15,21,38) and Pex12p (17,18,26).

MATERIALS AND METHODS

Cell lines
Skin fibroblast cell lines from patients, including several patient fibroblasts transformed with SV40, were cultured in complete medium (high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) as described (18,24). Wild-type and mutant CHO cells were cultured as described (10,15).

Cloning of human PEX10
A human liver cDNA library constructed in vector pSPORT I (Gibco BRL) (19) was screened using as probes the nucleotide oligomers sense HsP10.FL (5'-GTCTCTGTTGGAGTGCG-ATCACCCGCTGGTGCAAGCAGAGGGAAGGTGAGGT GGAGCCCTG-3') and antisense HsP10.RL (5'-CTCCACAGGAACTTCCCTCTCTCAAGGGAGGAGTCTTCAGTAGAT GGAAGCCGTG-3'), corresponding to nucleotide residues 34–89 and 67–122, respectively, of human EST AA368416, which showed high homology (40% identical) in the region encoding the RING finger of HpP EX10 (formerly PER8) (30). A single clone, HsPEX10-A, hybridized to both probes, comprising a 1.2 kb insert with a 100 bp extension upstream of the full sequence of AA368416 and containing a 250 bp ORF, apparently for the C-terminal part of Pex10p. Primers HsP10.FES (5'-GCCAGGAAGGAGTGGAGGC-3') and HsP10.ROK (5'-AAAGGGCAAGGAGGGACAG-3') were used to PCR amplify the C-terminal region in the human cDNA library. Two positive clones, F7-13 and F7-15-42, were isolated from a subpool F7 and their nucleotide sequences determined; F7-15-42 comprised 1992 bp containing a 981 bp ORF encoding a 326 amino acid polypeptide.

Transfection of HsPEX10
Plasmid pUcDHyg·HsPEX10 was generated by inserting the Smal fragment (nt –69 to 991) of F7-15-42 into vector pUcD10 (16,18). Fibroblasts (1 × 10⁶ cells) derived from peroxisome-deficient patients were transfected with 20 µg pUcD2Hyg·HsPEX10 using a Gene Pulser II electroporator (Bio-Rad) on settings 320 V and 500 µF. Transfection of CHO cell mutants with pUcD2Hyg·HsPEX10 was done using Lipofectamine (Gibco BRL), as recommended by the manufacturer.

Morphological analysis
Peroxisomes in human fibroblasts and CHO cells were visualized by indirect immunofluorescence light microscopy as described (5). We used rabbit antibodies to PTS1 peptide (13) and rat liver catalase (10). Antigen–antibody complexes were detected with

Figure 4. Intracellular localization and topology of Pex10p. (A) Human Pex10p tagged at the N-terminus was expressed in CHO-K1 cells. Cells were treated with 1% Triton X-100 (a–c) or 25 µg/ml digitonin, which permeabilized the plasma membrane (17,18,34) (d and e). Cells were stained with mouse anti-flag antibody (a and d) and rabbit anti-PTS1 peptide antibody (b and e). (c) A merged view of (a) and (b). Rabbit IgG was detected with rhodamine-labeled goat anti-rabbit IgG antibody (Bio Source International). Note that punctate structures, peroxisomes, are superimposable in (a) and (b). Large spots found in (b) are apparently unwashed lipofectamine or its debris. (B) CHO-K1 cells transfected with C-terminally flag-tagged human PEX10 were treated with 1% Triton X-100 (a and b) or with 25 µg/ml digitonin (c and d). Cells were stained with antibodies to flag (a and c) and PTS1 (b and d), respectively. Magnification 630; bar 20 µm. Note that flag–Pex10p and Pex10p–flag were detected after both types of treatments.

Complementation of CHO cell mutants: PEX1 for CG-I (CG-E in Japan) (19,27,28), PEX2 for CG-F in Japan (CG-X) (21), PEX3 for CG-II (13,22,23), PEX6 for CG-C in Japan (CG-IV) (16,24,25) and PEX12 for CG-III (17,18,26; Table 1). Thus, PEX10 is the sixth gene identified as responsible for peroxisome deficiency diseases. PEX7, encoding the PTS2 receptor, was defective in import of PTS2 protein (35–37). Three peroxins localized in peroxisomes, i.e. peroxisomal integral membrane proteins Pex2p (15,21,38), Pex12p (17,18,26) and Pex10p (this study), all contain a RING finger motif, inferring a pivotal role of the RING finger in peroxisome assembly, although they are not mutually complementary in three distinct CGs. It is noteworthy
FITC-labeled sheep anti-rabbit immunoglobulin G antibody (Cappel) under a Carl Zeiss Axioskop FL microscope.

**Mutation analysis**

Poly(A)^+ RNA was obtained from cultured fibroblasts from a normal control and a CG-B patient (PBDB-01) using a QuickPrep mRNA purification kit (Pharmacia Biotech). RT–PCR using poly(A)^+ RNA was performed with a pair of human PEX10-specific PCR primers, sense RTF (5'-GGCGGATGCCCATGGCGCCCG-GCCGCCGGCCAG-3'; initiation codon underlined) and antisense RTR (5'-GGCGGTTCGACATCGGCCGTTGCCGAAGTAG-3'; termination codon underlined), to cover the full-length PEX10 ORF. PCR products were cloned into pBluescript II SK(−) and sequenced. Patient PEX10 cDNA was ligated with the 3'-non-coding region of HsPEX10 and cloned into pUCD2SRTMCSHyg. Transfection of fibroblasts was performed by electroporation.

**Expression of epitope-tagged Pex10p**

Epitope-flagging of the N-terminus of Pex10p was done by a PCR-based technique, using forward primer HsP10.RTF and reverse primer HsP10.R4 (5'-GGGTTGCGCCACCTGTCG-3') and Flagging of Pex10p, flag–HsP10, was constructed in vector pUCD2SRTMCSHyg by inserting a BamHI–SpHL fragment of the PCR product and a SpHL–Small fragment of F7-15-42 into a BamHI–Apal (blunted) vector fragment containing the flag sequence, originating from pUCD2Hyg-flag–HsPEX12 (17,18). C-terminal flagging of Pex10p was likewise done using forward primer HsP10.F1 (5'-CCCTGAGACAGGAGCTG-3') and reverse primer HsP10.flagR (5'-CGGTTGCGCCACCTGTCG-3') and Flagging of Pex10p, flag–HsP10, was constructed in vector pUCD2SRTMCSHyg by replacing an Apal–KpnI fragment of pUCD2Hyg-HsPEX10 with an Apal–KpnI fragment of the PCR product. Flagged Pex10p was detected using a mouse monoclonal antibody to the flag (M2; Scientific Imaging Systems) and FITC-labeled sheep anti-mouse IgG second antibody (Amersham) in cells that had been permeabilized with 25 µg/ml digitonin (17,34) or 1% Triton X-100.

**Other methods**

Nucleotide sequence was determined by the dideoxy chain termination method using a Dye-terminator DNA Sequence Kit (Applied Biosystems). Alignment was done using the GENE-TYX-Mac program (SDC, Tokyo).

**ABBREVIATIONS**

CG, complementation group; CHO, Chinese hamster ovary; EST, expressed sequence tag; PBD, peroxisome biogenesis disorders; PEX10, cDNA encoding the peroxin Pex10p; PTS1 and PTS2, peroxisome targeting signal types 1 and 2; ZS, Zellweger syndrome.

**ACKNOWLEDGEMENTS**

We thank T. Sakaguchi and N. Matsumoto for technical assistance and the members of the Fujiki laboratory for comments. This work was supported in part by a CREST grant to Y.F. from the Japan Science and Technology Corporation and by Grants-in-Aid for Scientific Research (07408016, 08249232 and 08557011) to Y.F. from the Ministry of Education, Science, Sports and Culture.

**REFERENCES**

12. Okumoto, K., Bogaki, A., Taiteshi, K., Tsukamoto, T., Osumi, T., Shimoza-


