Mutations in the Peroxin Pex26p Responsible for Peroxisome Biogenesis Disorders of Complementation Group 8 Impair Its Stability, Peroxisomal Localization, and Interaction with the Pex1p-Pex6p Complex

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The peroxisome is a single membrane-bound organelle present in eukaryotes, including humans, and functions in various metabolic pathways, such as β-oxidation of very-long-chain fatty acids and the synthesis of ether lipids (1). The functional significance of peroxisomes is highlighted by the human fatal genetic peroxisome biogenesis disorders (PBDs) including Zellweger syndrome (ZS), neonatal adrenoleukodystrophy, and infantile Refsum disease (IRD). We recently isolated PEX26 as the pathogenic gene for PBD of CG8. Pex26p functions in recruiting to peroxisomes the complexes of the AAA ATPase peroxins, Pex1p and Pex6p. In the present study, we identified four distinct mutations in PEX26 from five patients of CG8 PBD including 2 with ZS and 3 with IRD, in addition to 7 mutant alleles in 8 patients in the first report describing the pathogenic PEX26 gene for CG8 PBD. Phenotype-genotype analyses revealed that temperature-sensitive (ts) peroxisome assembly gave rise to a milder IRD in contrast to the non-ts phenotype of the cells from ZS patients. Furthermore, we present several lines of evidence that show that the instability, insufficient binding to Pex1p-Pex6p complexes, or mislocalization of patient-derived Pex26p mutants is most likely responsible for the CG8 PBDs.

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10% fetal calf serum (21). DNA transfection to CHO cells was performed by lipofection method with Lipofectamine (Invitrogen). Patient fibroblasts were transfected by electroporation using a Gene Pulser II electroporator (Bio-Rad) on setting at 320 V and 500 microfarads (10).

**Morphological Analysis**—Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Peroxisomes in human fibroblasts and CHO cells were visualized by indirect immunofluorescence light microscopy, as described (9). Rabbit antibodies used were antibodies to human catalase (5), peroxisome targeting signal type 1 (PTS1) peptide (22), rat 3-ketoyl-CoA thiolase (thiolase) (23), and rat Pex14p C-terminal peptide (24).

**Construction of Enhanced Green Fluorescent Protein (EGFP)-fused FLAG-PER26 and Its Variants**—pCMVSPORT-FLAG-EGFP-PER26X1C was constructed by replacing the BamHI-BglII fragment of pCMVSPORT-FLAG-PER26X1C with BamHI-BglII fragment of the PCR product amplified as a template using primers 97F (designed for translation starting after the second Met at position 96) and NotR. The BglII-NotI fragment of the PCR product was ligated into the BamHI-NotI site of pCMVSPORT-FLAG-PER26X1C. FLAG-PER26X1C expression did not restore the impaired protein import in pex26 ZP167 (see Table 4), whereas Pex26pM1T showed the complementing activity (9).

**Construction of Mutant Forms of FLAG-tagged PEX26**—FLAG-PER26 encoding N-terminally FLAG-tagged full-length Pex26p was as described (8). PEX26 was amplified using a pair of primers, 1F and NotR, digested with BglII and NotI, and ligated to the BamHI-NotI site in pCMVSPORT, termed pCMVSPORT-PEX26L53V/C861del. FLAG-PER26L53V/C861del were generated by blunting and ligation after digestion with PmaCI and NotI of pCMVSPORT-FLAG-PER26T3SinsC and pCMVSPORT-FLAG-PER26G25SinsT, respectively. pCMVSPORT-FLAG-PER26L53V/C861del was constructed by replacing the BssHII-NotI fragment of normal FLAG-PER26 with the BssHII-NotI fragment of pCMVSPORT-FLAG-PER26L53V/C861del. FLAG-PER26M1T was constructed as follows. PCR was done with pCMVSPORT-FLAG-PER26 as a template using primers 97F (designed for translation starting after the second Met at position 96) and NotR. The BglII-NotI fragment of the PCR product was ligated into the BamHI-NotI site of pCMVSPORT-FLAG-PER26X1C. FLAG-PER26M1T expression did not restore the impaired protein import in pex26 ZP167 (see Table 4), whereas Pex26pM1T showed the complementing activity (9).

**TABLE 1**

| Synthetic oligonucleotide primers used | Nucleotide sequence at: | Restriction site |
|---------------------------------------|------------------------|-----------------|
| 1F                                    | AAGCTTGCAGATCTCAAGAGCCGATTCCTCGAC | BglII site |
| -1F                                   | GCCCTGGACCCGCCGATTCGTTT | Sall site |
| 220F                                  | AGGCGCCGTCTGACTAACCGGATTCGTTG | NotI site |
| 1R                                   | CAGGAAAGATCCTGACAGATCGGAGTCGTG | BamHI site |
| -1R                                  | GCTGTGTACGGCGAGACC | NotI site |
| 2R                                   | GCTGTTGCAAAATCTCAC | BglII site |
| NotR                                 | GCCGGCGCGCTCGACTGACAGATCGGAGAG | BamHI site |
| 97F                                  | CTGGAGATACCTGATACGGTGCAAGAAGTC | NotI site |
| EGFP-Bam F                           | GCCCGGAGTCTGAGGAGAGGCGAG | BamHI site |
| EGFP-R                               | AGCAAGTAAAAACCTCTAC | NotI site |

* a F and R, forward and reverse primers, respectively.
* b Underlined sequence.
* c Italicized sequence.

**Mutation Analysis**—Poly(A)⁺ RNA was obtained from CG8 patient fibroblasts by a QuickPrep miRNA purification kit (Amersham Biosciences). Reverse transcription (RT)-PCR was performed using poly(A)⁺ RNA from GM17397, GM17399, and GM17400 with a pair of human PEX26 specific primers, a forward 1F (Table 1, nucleotide sequence at 42 – 41) and a reverse 1R (915 – 898), to cover the open reading frame of full-length PEX26. For GM17398 and GM16866, PCR was done with 1F (20 – 1) and 1R (938 – 929), and second PCR was also done for GM17398 with 1F and 1R; for GM16866 with a pair of 1F and 2R (815 – 798) or 220F (658 – 674) and 1R. The PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced using a Dye-terminator DNA sequence kit (Applied Biosystems).

**Construction of pCMVSPORT I vector by replacing BssHII-PmaCI fragment (nucleotide sequence at 75 – 487) of a normal PEX26 with that of patient PEX26 in pCMVSPORT-FLAG-PER26X1C with BamHI-NotI site Easy. PEX26L53V/C861del was constructed by replacing BssHII-NotI fragment of a normal PEX26 with PEX26L53V/C861del fragment of the PCR product amplified as a template using primers 97F (designed for translation starting after the second Met at position 96) and NotR, respectively. pCMVSPORT-FLAG-PER26L53V/C861del was constructed by replacing BssHII-NotI fragment of normal FLAG-PER26 with the BssHII-NotI fragment of pCMVSPORT-FLAG-PER26L53V/C861del. FLAG-PER26M1T was constructed as follows. PCR was done with pCMVSPORT-FLAG-PER26 as a template using primers 97F (designed for translation starting after the second Met at position 96) and NotR. The BglII-NotI fragment of the PCR product was ligated into the BamHI-NotI site of pCMVSPORT-FLAG-PER26X1C. FLAG-PER26M1T expression did not restore the impaired protein import in pex26 ZP167 (see Table 4), whereas Pex26pM1T showed the complementing activity (9).
FLAG antibody (M2, Sigma). The immunocomplexes were analyzed by SDS-PAGE and immunoblotting.

Other Methods—Western blot analysis was performed using primary antibodies to HA and a second antibody, donkey anti-rabbit immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Amer sham Biosciences), or using mouse anti-FLAG antibody and horseradish peroxidase-labeled donkey anti-mouse IgG antibody (Amer sham Biosciences). Antigen-antibody complexes were visualized with ECL Western blotting detection reagent (Amersham Biosciences).

RESULTS

Characterization of CG8 Fibroblasts

In fibroblasts derived from CG8 patients, ZS GM17398 and IRD GM17399, catalase was detected in a diffused staining pattern at 37 °C (Fig. 1A, panels a and c), indicative of cytosolic localization (8). When these cells were cultured at 30 °C for 3 days, catalase and a PTS2 thiolase were observed as punctate-staining structures only in IRD GM17399 fibroblasts (Fig. 1A, panels d and f), suggesting catalase and thiolase were imported to peroxisomes in a ts manner. In contrast, these two enzymes were not imported at 30 °C in ZS GM17398 fibroblasts (Fig. 1A, panels a, b, i, and j). Similar phenotypes were observed in cells from two patients with IRD and one ZS patient (Table 2), in good agreement with our earlier findings (9). Fibroblasts from a ZS patient GM16866, showed a milder ts-type import of PTS2 thiolase, but not catalase, hence implying that catalase more reliably reflected the severe ZS phenotype, as in CG1 pex1 ZS patient PBDE-04 (21). PTS1 proteins were detected in peroxisomes in fibroblasts derived from basically all of the CG8 patients at both temperatures, where PTS1-positive particles, peroxisomes, are distinctly less in number in fibroblasts from ZS patients than those in cells from IRD patients (Fig. 1A, panels e–h; Table 2). These findings suggested the less efficient import of PTS1 proteins in cells from the most severe ZS-type PBD patients, consistent with a recent report (18). Pex14p was observed in punctate structures representing membrane remnants at both temperatures in all of the CG8 fibroblasts, indicating that import of peroxisomal membrane proteins was normal (Fig. 1A, panels m–p). The cell phenotypes described here were in good agreement with our earlier findings (9).

PEX26 expression restored the impaired import of catalase and thiolase and also enhanced the import of PTS1 proteins in fibroblasts from ZS patient GM17398 (Fig. 1B, panels a–c) and other PBD patients (data not shown). These results suggested that PEX26 was responsible for the defects of peroxisomal matrix proteins in these CG8 patients.

Mutation Analysis of CG8 Patients

Patients with ZS/intG231T—To investigate the cause of the dysfunction of PEX26 in CG8 patients with ZS, we isolated PEX26 cDNA from fibroblasts of ZS GM17398 by means of RT-PCR. One point mutation of nucleotide G231 (the A of the initiator ATG being replacement of the A in the start codon) was found in CG8 patients with ZS, we isolated PEX26 cDNA from fibroblasts of ZS GM17398 by means of RT-PCR. One point mutation of nucleotide G231 (the A of the initiator ATG being replaced by T), which resulted in a frameshift in the translation of amino acids distinct from normal Pex26p. Therefore, only the N-terminal 77-amino acid sequence was identical with PEX26 expression restored the impaired import of catalase and thiolase and also enhanced the import of PTS1 proteins in fibroblasts from ZS patient GM17398 (Fig. 1B, panels a–c) and other PBD patients (data not shown). These results suggested that PEX26 was responsible for the defects of peroxisomal matrix proteins in these CG8 patients.

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| Patient | Sex | Disorder | Age of death or last follow-up | IT Catalase | PTS1 | Thiolα (PTS2) | Mutation | RT-PCR | AAα | Clones |
|---------|-----|----------|-------------------------------|-------------|------|--------------|----------|---------|-----|-------|
| GM16866 | NC  | ZS       | 14 f.w.i.                     | 0%          | 0    | 80%          | intG231T | C457G   | AA   |       |
| GM17398 | F   | ZS       | 7 m.i.                        | 0%          | 0    | 80%          | intG231T | C457G   | AA   |       |
| GM17397 | F   | IRD      | 27 y.i.                       | 0%          | 0    | 100%         | intG231T | C457G   | AA   |       |
| GM17399 | F   | IRD      | 3 y.i.                        | 0%          | 0    | 100%         | intG231T | C457G   | AA   |       |
| GM17400 | M   | IRD      | 5 m.i.                        | 0%          | 0    | 100%         | intG231T | C457G   | AA   |       |

* α, common to all of catalase, PTS1, and thiolase (PTS2).
* Thiolase.
* Nucleotide.
* Amino acid.
* Not checked.
* Fetal weeks gestation.
* PTS1-positive particles include ones stained more weakly than normal peroxisomes.
* α, RT-PCR product was under the detectable level.
* Month.
* Year.
* In/ter, frameshift resulting in termination.
* Siblings.

Figure 1. Characterization of fibroblasts from CG8 patients. A, patient-derived fibroblasts were cultured for 3 days at 37 or 30 °C and then stained with antibodies to catalase (panels a–d), PTS1 (panels e–h), a PTS2 protein (thiolase) (panels i–l), and Pex14p (panels m–p). Fibroblasts were derived from a patient with ZS (GM17398) (left panels) and IRD (GM17399) (right panels). Magnification, ×630; bar, 20 μm. B, PEX26 expression restores the impaired protein import to peroxisomes in CG8 PBD fibroblasts. GM17398-derived fibroblasts were transfected with PEX26. After 3 days of culture at 37 °C, cells were stained as in A; bar, 20 μm.
Pex26p. Of 8 clones sequenced, 6 were inserted with a 236-bp intron, and 2 clones were with 58-bp and 650-bp introns, respectively. We named it homozygous mutation PEX26intG231T.

Transfection of PEX26intG231T to a CHO pex26 mutant ZP167 defective in import of matrix proteins including those with PTS1 and PTS2 (8) did not restore matrix protein import at 37 and 30 °C (Fig. 3B, panels e and j and Table 3), basically reproducing the phenotype of GM17398 fibroblasts. In regard to the PTS1 protein import, the failure of PTS1 protein import in PEX26intG231T-transfected CHO ZP167 cells was consistent with the phenotype of GM17398 fibroblasts showing the reduced level of PTS1 import. Moreover, FLAG-Pex26p was not detectable at 37 °C in ZP167, but was detectable at a very low level when cultured at 30 °C (Fig. 3C, lane 6). These results suggested that the N-terminal 77-residue polypeptide of Pex26p was not functional; readily degraded at normal temperature. The RT-PCR product was under the detectable level with the first cycle of PCR of poly (A)/H11001 RNA from fibroblasts of a ZS patient GM16866, consistent with our earlier results (9). However, the second cycle of PCR using the first PCR products resulted in PEX26-related cDNAs. In 7 clones sequenced, any common mutation was not detected. We interpreted this to mean that PEX26 mRNA may be unstable and degraded or the transcription of PEX26 may be defective.

Patients with IRD:R98W/L44P—PEX26 cDNA was likewise isolated from fibroblasts of IRD patients, GM17399 and GM17400. Subsequent
sequencing revealed a point mutation of nucleotide C to T at position 292 in a codon (CGG) for Arg\textsuperscript{292}, resulting in a codon (TGG) for Trp, termed PEX26R98W (Fig. 2). Of 16 respective clones isolated, 6 clones from GM17399 and 9 from GM17400 showed the same mutation. The remaining 10 and 7 respective clones showed a different point mutation, nucleotide T to C at position 131 in a codon (CTC) for Leu, named PEX26L44P, in the fibroblasts of the patients, thereby representing the phenotype.

In our earlier report (8), we demonstrated that Pex26p interacts with Pex1p-Pex6p complexes in a Pex6p-dependent manner. To determine whether the mutations identified in PEX26 from CG8 BPD patients affect the Pex26p interaction with Pex1p-Pex6p complexes, we expressed FLAG-tagged Pex26p mutants together with Pex1p-HA and Pex6p-HA in ZP167 cells. FLAG-Pex26p mutants expressed here were all inactive when expressed in ZP167 cells (data not shown), consistent with the phenotype of fibroblasts from patients, GM07371 and GM16685. FLAG-Pex26p variants were immunoprecipitated from cell lysates using anti-FLAG antibody-conjugated agarose beads and were analyzed by SDS-PAGE and immunoblot with antibodies to FLAG and HA.

PEX26P117L expression restored the import of matrix proteins in a ts manner, as assayed by transfection to pex26 ZP167, where the import of PTS1 and PTS2 proteins was distinct at 30 °C (Table 3). Catalase was slightly imported at 30 °C, but not at 37 °C (Fig. 3A, panels a and f; Table 3). PEX26L153V/C861del was nearly as functional as normal Pex26p in import of catalase as well as PTS1 and PTS2 proteins at both temperatures (Fig. 3B, panels d and i; Table 3). FLAG-Pex26pL153V/C861del was expressed at a very low level at 37 °C and a little higher at 30 °C in ZP167 (Fig. 3C, lane 5). FLAG-Pex26pP117L was detected in a similar level to FLAG-Pex26pR98W, but less than normal Pex26p (Fig. 3C, lane 4). Taken together, these results suggest that the mutation of L153V/C861del is not responsible for the dysfunction of Pex26p, but rather the decrease in the protein stability. It is more likely that in GM17397 fibroblasts a mild form Pex26pL153V/C861del is expressed at a lower level than a severe form Pex26pP117L, together representing the phenotype.

**Patient-derived Pex26p Mutants Dysfunction in Interaction with Pex1p-Pex6p Complexes**

In our earlier report (8), we demonstrated that Pex26p interacts with Pex1p-Pex6p complexes in a Pex6p-dependent manner. To determine whether the mutations identified in PEX26 from CG8 BPD patients affect the Pex26p interaction with Pex1p-Pex6p complexes, we expressed FLAG-tagged Pex26p mutants together with Pex1p-HA and Pex6p-HA in ZP167 cells. FLAG-Pex26p mutants expressed here were all inactive when expressed in ZP167 cells (data not shown), consistent with the phenotype of fibroblasts from patients, GM07371 and GM16685. FLAG-Pex26p variants were immunoprecipitated from cell lysates using anti-FLAG antibody-conjugated agarose beads and were analyzed by SDS-PAGE and immunoblot with antibodies to FLAG and HA.

**TABLE 3**

| Genotype-Phenotype Correlation of PEX26 Dysfunction |
|---------------------------------|----------------|----------------|----------------|
| **Transfection of patient-derived PEX26 to CHO pex26 mutant ZP167** | | | |
| **PEX26 mutant** | **ts** | **catalase** | **PTS1** | **PTS2-EGFP** |
| | | **37 °C** | **30 °C** | **37 °C** | **30 °C** | **37 °C** | **30 °C** |
| Wild-type* | | | | | | | |
| | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| intG231T | 0 | 0 | 0 | 0 | 0 | 100 | 100 |
| R98W | 20 | 60 | 70 | 80 | 70 | 100 | 100 |
| L44P | 0 | 0 | 0 | 0 | 0 | 10 | 80 |
| P117L | 0 | 30 | 20 | 80 | 10 | 80 | 10 |
| L153V/C861del | 70 | 100 | 80 | 100 | 100 | 100 | 100 |
| **Co-expression** | | | | | | | |
| R98W + L44P | 0 | 5 | 5 | 30 | 50 | 60 | 60 |
| P117L + L153V/C861del | 0 | 50 | 90 | 100 | 100 | 100 | 100 |

*ts, common to all of catalase, PTS1, and thiolase (PTS2).

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3). Pex26p mutants, FLAG-Pex26pT35insC, FLAG-Pex26pG255insT, and FLAG-Pex26pG231T, encompassed 113-, 113-, and 159-amino acid long sequences that contained only 11, 85, and 77 authentic residues from the N terminus of Pex26p, respectively (Figs. 2 and 3A) (9). These mutants barely showed the complementing activity (Table 4), although they bound to Pex1p more efficiently than FLAG-Pex26pL44P and FLAG-Pex26pL45P.

Intracellular Localization of Patient-derived Pex26p Mutants

FLAG-EGFP-fused Pex26p mutants including Pex26pL45P, Pex26pT35insC, Pex26pG255insT, and Pex26pG231T, were expressed in CHO-K1 cells and their subcellular localization was determined by EGFP fluorescence. FLAG-EGFP-Pex26p and FLAG-EGFP-Pex26pl45P were detected in punctate staining pattern, in a manner superimposable on Pex14p-positive structures, peroxisomes (Fig. 5, panels a, b, f, and g). In contrast, FLAG-EGFP-Pex26pT35insC, FLAG-EGFP-Pex26pG255insT, and FLAG-EGFP-Pex26pG231T were discernible in a diffuse staining pattern, indicative of cytosolic localization (panels c–e and h–j), thereby suggesting that these mutants were defective in peroxisomal localization. Other FLAG-EGFP-Pex26p variants, FLAG-EGFP-Pex26pL44P, FLAG-EGFP-Pex26pP117L, FLAG-EGFP-Pex26pM1T, FLAG-EGFP-Pex26pG89R, FLAG-EGFP-Pex26pR98W, and FLAG-EGFP-Pex26pL153V/C861del, were also discernible on Pex14p-positive particles, peroxisomes (Table 4). FLAG-tagged forms of all of these Pex26p mutants were similarly expressed in CHO-K1 cells. Their subcellular localization was consistent with the results using FLAG-EGFP forms, except that FLAG-Pex26pG231T was apparently unstable, rather degraded, and undetectable (data not shown).

DISCUSSION

In this study, we further verified the genotype-phenotype relationship in fibroblasts derived from CG8 PBD patients. The ts import of catalase was observed only in fibroblasts from patients with the milder form of PBD, IRD, not in those from patients with severe PBD ZS. The assessment of catalase import at 30 °C is more reliable and predictive of the clinical severity between the most severe ZS and less severe NALD and IRD (Tables 2–4). These results support our earlier findings that the temperature dependence of peroxisome biogenesis reflects the clinical severity of CG8 patients (9). Such correlation was observed in fibroblasts from patients of several CGs including PEX1-deficient CG1 (10–12, 25). The most common mutant allele PEX1G843D identified in the fibroblasts from CG1 IRD was shown to cause the ts phenotype (20, 26).

| TABLE 4 |
| Summary of PBD patient-derived Pex26p |

FLAG-Pex26p mutants were assessed for the indicated activities. The activity levels were indicated as follows: ~, nearly the same as the wild-type Pex26p; ↓, 70–90% of the wild-type; ↓↓, 40–70%; ↓↓↓, less than 40%; +, localized to peroxisomes; –, not localized.

| FLAG-Pex26p mutant | Catalase import (30 °C) | Stability | Binding to 6p | Binding to 1p | Peroxisomal localization |
|---------------------|-------------------------|-----------|--------------|--------------|-------------------------|
| **Severe forms**    |                         |           |              |              |                         |
| L45P                | ↓                       | ↓         | ↓↓↓↓↓        | ↓↓↓↓↓        | +                       |
| L44P                | ↓                       | ↓         | ↓↓↓↓↓        | ↓↓↓↓↓        | +                       |
| P117L               | ↓                       | ↓         | ↓↓↓↓↓        | ↓↓↓↓↓        | +                       |
| M1T                 | ↓                       | ↓         | ↓↓↓↓↓        | ↓↓↓↓↓        | +                       |
| G89R                | ↓                       | ↓         | ↓↓↓↓↓        | ↓↓↓↓↓        | +                       |
| G255insT            | ↓                       | ↓         | ↓↓↓↓↓        | ↓↓↓↓↓        | +                       |
| T35insC             | ~                       | ↓         | ↓↓↓↓↓        | ↓↓↓↓↓        | –                       |
| **Mild forms**      |                         |           |              |              |                         |
| R98W                | ↓↓↓↓↓                   | ↓↓↓↓↓     | ↓↓↓↓↓        | ↓↓↓↓↓        | –                       |
| L153V/C861del       | ↓↓                      | ↓↓↓↓↓     | ↓↓↓↓↓        | ↓↓↓↓↓        | –                       |

FIGURE 4. Binding to Pex1p and Pex6p of patient-derived Pex26p mutants. ZP167 cells were transfected with plasmids encoding FLAG-Pex26p or mutant FLAG-Pex26p identified in CG8 PBD patients (see Fig. 3A), together with those for Pex6p-HA and Pex1p-HA. FLAG-Pex26p variants were immunoprecipitated from cell lysates using an antibody to FLAG. Immunoprecipitates (IP) were analyzed by SDS-PAGE and immunoblot with antibodies to HA and FLAG. Input, 5% input. Molecular markers are in the center. Upper panels, solid and open arrowheads indicate Pex1p-HA and Pex6p-HA, respectively; lower panels, solid arrow indicates the migration of FLAG-Pex26p with authentic size and upward and downward open arrowheads designate FLAG-Pex26p variants. Dot, a nonspecific band; asterisks, IgG heavy and light chains. In A, the IP panel of FLAG-Pex26p variants (lower right) was at one-third exposure time compared with the others.
Pex1pG843D was unstable at 37 °C, despite normal interaction with its binding partner Pex6p (21). In our earlier reports (8, 9), we analyzed 8 CG8-PBD patients, identifying 7 distinct mutant alleles. In the present work, we identified another 4 different mutated alleles from 5 CG8 patients (Table 2), including the patient GM17398 analyzed by PCR amplification of the RT-PCR product reported in Ref. 9. Very recently, these three mutations, L153V/C861del, P117L, and L44P, were also identified (17, 18). We found that the mutations R98W and P117L showed the ts phenotype in the matrix protein import when expressed in pex26 ZIP167. The mutations R98W and P117L are more likely responsible for the ts restoration of protein import in cells from 3 IRD patients. Moreover, it is plausible that the stability of another allele PEX26 product affects the phenotype of IRD patient-derived cells. A severe form Pex26pL44P, presumably co-expressed with a milder Pex26pR98W in the fibroblasts of patients, was more stable than Pex26pR98W in ZIP167. Likewise, a mild form Pex26pL153V/C861del co-expressed with a severe form Pex26pP117L in patient’s fibroblasts was less stable than Pex26pP117L. Such combinations of the mutants likely gave rise to the dysfunction of Pex26p in IRD cells of patients. PEX26intG231T, the mutation identified in a ZS patient GM17398, was completely inactive in transport of matrix proteins including catalase, thiolaese, and PTS1 proteins when expressed in ZIP167. Its product, Pex26pG231T, was extremely unstable, reflecting the severe ZS phenotype. In ZS GM16866 fibroblasts PEX26 transcript was difficult to detect by PCR. Therefore, we conclude that these 2 ZS patients have the severe phenotype because of the instability of PEX26 products.

Almost all of the mutations in Pex26p, including the 5 mutations identified here and 5 ones in our earlier report (9), were in the N-terminal part of Pex26p (see Fig. 3A), implying that the N-terminal region of Pex26p is crucial for its function. To address the molecular mechanisms underlying the impaired peroxisome biogenesis in CG8 PBDs, we verified the interaction of patient-derived Pex26p with Pex1p-Pex6p complexes by co-immunoprecipitation assays. Several mutants, including FLAG-Pex26pG89R, FLAG-Pex26pL45P, FLAG-Pex26pM1T, FLAG-Pex26pL44P, and FLAG-Pex26pP117L, showed a lower potency to form the ternary complexes with Pex1p and Pex6p, thereby recruiting the Pex6p-Pex1p complexes with very low efficiency. Although Pex6p-HA and a small amount of Pex1p-HA were co-immunoprecipitated with FLAG-Pex26pL45P, neither Pex6p-HA nor Pex1p-HA was detectable on peroxisomal membranes by cell staining with antibodies (data not shown). These results suggested that localization of Pex6p and Pex1p on peroxisomes requires the formation of ternary complexes. The relationship between the degree of clinical severity and binding ability to Pex1p implies that formation of the ternary complexes and their localizations on peroxisomes are very important for peroxisome biogenesis. Although FLAG-Pex26pT3SINS,FLAG-Pex26pG255INS, and FLAG-Pex26pG231T did not restore the impaired peroxisome biogenesis in ZIP167, they were co-immunoprecipitated with Pex1p-HA more efficiently than other FLAG-Pex26p mutants such as FLAG-Pex26pL44P and FLAG-Pex26pP117L. Contrary to this, FLAG-Pex26pG231T, FLAG-Pex26pG255INS, and FLAG-Pex26pG231T, with truncation in the C-terminal transmembrane segment, were not localized to peroxisomes. These findings raise several possibilities regarding the relation between Pex26p dysfunction and PBDs. Pex26p may interact with other peroxins and act as a scaffold protein to specify the target of ATPase activity of Pex1p and Pex6p. It is also possible that Pex26p may be involved in regulation of re-localization of Pex5p, including its export step from peroxisomes (15, 16).

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