Phenotype–genotype relationships in peroxisome biogenesis disorders of *PEX1*-defective complementation group 1 are defined by Pex1p–Pex6p interaction

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The peroxisome biogenesis disorders (PBDs), including Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD), are fatal autosomal recessive diseases caused by impaired peroxisome biogenesis, of which 12 genotypes have been reported. ZS patients manifest the severest clinical and biochemical abnormalities, whereas those with NALD and IRD show less severity and the mildest features respectively. We have reported previously that temperature-sensitive peroxisome assembly is responsible for the mildness of the clinical features of IRD. *PEX1* is the causative gene for PBDs of complementation group E (CG-E, CG1 in the U.S.A. and Europe), the PBDs of highest incidence, encoding the peroxin Pex1p of the AAA ATPase family. It has been also reported that Pex1p and Pex6p interact with each other. In the present study we investigated phenotype–genotype relationships of CG1 PBDs. Pex1p from IRD such as Pex1p with the most frequently identified mutation at G843D was largely degraded *in vivo* at 37°C, whereas a normal level of Pex1p was detectable at the permissive temperature. In contrast, *PEX1* proteins derived from ZS patients, including proteins with a mutation at L664P or the deletion of residues 634–690, were stably present at both temperatures. Pex1p-G843D interacted with Pex6p at approx. 50% of the level of normal Pex1p, whereas Pex1p from ZS patients mostly showed non-temperature-sensitive peroxisome biogenesis hardly bound to Pex6p. Taking these results together, we consider it most likely that the stability of Pex1p reflects temperature-sensitive peroxisome assembly in IRD fibroblasts. Failure in Pex1p–Pex6p interaction gives rise to more severe abnormalities, such as those manifested by patients with ZS.

Key words: AAA ATPase, infantile Refsum disease, temperature sensitivity, Zellweger syndrome.

INTRODUCTION

The peroxisome is a single-membrane-bounded ubiquitous intra-cellular organelle, catalysing various metabolic pathways such as the β-oxidation of very-long-chain fatty acids and the synthesis of plasmalogens [1]. Human fatal genetic peroxisome biogenesis disorders (PBDs) include Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and rhizomelic chondrodysplasia punctata [2]. Patients with ZS show severe neurological abnormalities and characteristic dysmorphism and hepatomegaly; they rarely survive, the average lifespan being only 6 months. NALD patients have symptoms similar to those of ZS patients but survive a little longer, into early childhood. In contrast, patients with IRD do not manifest significant abnormalities in the central nervous system and survive longest, with an average lifespan of 3–11 years [2]. The primary cause of the peroxisome deficiency in PBDs comprising 13 complementation groups (CGs) [3–5] is a failure in peroxisome biogenesis [6–9]. Genetic heterogeneity comprising more than 15 CGs has been identified in mammals, determined with fibroblasts from patients with PBDs and peroxisome-deficient Chinese hamster ovary (CHO) cell mutants [5,8]. Ten *PEX* genes (*PEX1, PEX2, PEX5, PEX6, PEX7, PEX10, PEX12, PEX13, PEX16 and PEX19*) involved in peroxisome biogenesis have been identified as the genetics-related cause of PBD CGs, CG-E (CG1 in the U.S.A. and Europe), CG-F (CG10), CG2, CG-C (CG4), CG-R (CG11), CG-B (CG7), CG3, CG-H (CG13), CG-D (CG9) and CG-J respectively [8,9]. *PEX3* has recently been identified as the eleventh causal gene responsible for PBDs of CG-G (CG12 in the U.S.A.) [10–14].

As a step towards addressing what represents at a molecular level the distinct differences in severity in clinical features from the severest, ZS, through NALD to the mildest, IRD, we found the restoration of peroxisome biogenesis in a temperature-dependent manner in fibroblasts from patients with the milder form of PBDs in several CGs, including CG-E, representing the highest incidence of PBDs [15,16]. Human *PEX1* (*H* *PEX1*) is the causative gene for CG-E (CG1) PBDs and encodes the peroxin Pex1p, which is a member of the AAA ATPase family. It has been demonstrated that human Pex1p and Pex6p interact with each other [17,18]. We also demonstrated that the mutation at G843D frequently identified in the *PEX1* allele of CG-E (CG1) IRD patients gives rise to the temperature-sensitive (ts) phenotype on peroxisome assembly [16].

However, the molecular mechanisms involved in the temperature sensitivity in peroxisome biogenesis remain unclear. It is of importance to investigate phenotype–genotype relationships in CG-E (CG1) PBDs, especially those linked to the ts peroxisome.

Abbreviations used: AOX, acyl-CoA oxidase; CG, complementation group; CHO, Chinese hamster ovary; IRD, infantile Refsum disease; NALD, neonatal adrenoleukodystrophy; PBDs, peroxisome biogenesis disorders; PMF70, 70 kDa peroxisomal membrane protein; PNS, post-nuclear supernatant; PTS, peroxisome targeting signal; RT–PCR, reverse-transcriptase-mediated PCR; thiolase, 3-ketoacyl-CoA thiolase; ts, temperature-sensitive; ZS, Zellweger syndrome.

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biogenesis. We found that the stability of Pex1p reflects ts peroxisome assembly in IRD fibroblasts. Failure of the Pex1p–Pex6p interaction most probably gives rise to more severe abnormalities such as those observed in ZS.

EXPERIMENTAL

Cell culture and DNA transfection

Skin fibroblast cell lines from a normal control and PBD patients were cultured at 37 or 30 °C in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum [19]. CHO cell mutant ZP107 [20] was cultured as described, at 37 or 30 °C. DNA transfection was performed with LIPOFECTAMINE (Life Technologies), as described [20].

Morphological analysis

Peroxisomes in human fibroblasts and CHO cells were revealed by indirect immunofluorescence microscopy, as described [19]. Antibodies used were rabbit antibodies against human catalase [21], peroxisome targeting signal type 1 (PTS1) peptide [22], 3-ketoacyl-CoA thiolase (thiolase) [23] and the 70 kDa integral membrane protein of peroxisomes (PMP70) of rat [3,23]. Antigen–antibody complexes were detected by FITC-labelled sheep anti-rabbit IgG antibody (Cappel), under a Carl Zeiss Axioskop FL microscope.

Mutation analysis

Poly(A)+ RNA was obtained from patients’ cultured fibroblasts with a QuickPrep mRNA purification kit (Amersham Pharmacia Biotech, Tokyo, Japan). Reverse-transcriptase-mediated PCR (RT-PCR) with poly(A)+ RNA was performed with a pair of human PEX1-specific PCR primers, RT1 (sense), 5’-CGCCGGGCCCCAGGACGCTCCGGGACG-3’ (nt −22 to −1, taking A as 1 of the initiation codon; Apol site, underlined) (GenBank accession number AB008112) [20], and RT2 (antisense), 5’-GGGGTACCGGGCCCCACTTACAGAACCACAACTC-3’ (nt 3868–3885; Apol site, underlined), to cover the full length of the PEX1 open reading frame. The PCR products were cloned into pGEM-T Easy Vector (Promega) and were determined for nucleotide sequence by the dideoxy-chain termination method using a Dye-terminator DNA sequence kit (Applied Biosystems).

Generation of mutant constructs

The wild-type construct, pCMVSPORT·HsPEX6, was as reported [20]. FLAG-tagged PEX1, pCMVSPORT·flag-HsPEX1, was cloned into pCMVSPORT I by replacing the N-terminal cDNA fragment of pCMVSPORT·HsPEX6 [20]. A FLAG-tagged PEX1 mutant PEX1Q261Ter in pCMVSPORT was generated by replacing the NspV–BglII fragment (nt 401–1327) of normal Flag-PEX1 with the NspV–BglII fragment of PBDE-14 derived PEX1Q261Ter. The other mutations identified in patients’ PEX1 cDNA were introduced into pCMVSPORT·flag-HsPEX1 by replacing the BglII–XhoI fragment (residues 1327–3192) of normal PEX1 with the BglII–XhoI fragment of the patient-derived PEX1 in pGEM-T Easy vector. All mutations were confirmed by nucleotide sequence analysis.

Subcellular fractionation

Patients’ fibroblasts grown to confluence were washed with PBS, removed with a scraper and collected by centrifugation at 500 g and 4 °C. Cells were then homogenized on ice by five strokes of an Elvehjem–Potter homogenizer in a homogenizing buffer [0.25 M sucrose/5 mM Hepes/KOH (pH 7.4)/25 μg/ml leupeptin/25 μg/ml antipain]. The homogenates were centrifuged at 750 g for 5 min at 4 °C; the resulting post-nuclear supernatants (PNS) were used. The PNS fraction was centrifuged at 100000 g for 1 h to separate organelles (heavy and light mitochondrial, microsomal fractions) from the cytosol.

Isopycnic centrifugation

Fibroblasts from a normal control and from CG1 IRD patient PBDE-06 (2 × 106 cells each) were homogenized in 1 ml of a homogenizing buffer [5 mM Hepes/KOH (pH 7.4)/0.25 M sucrose/1 mM EDTA/25 μg/ml leupeptin/25 μg/ml antipain/50 μl/ml aprotinin/1 mM PMSF]. To study the ts phenotype biochemically, PNS fractions were centrifuged into a linear sucrose-density gradient from 0.6 to 1.8 M in a Beckman SW41 rotor at 35000 rev./min (151000 g) for 16 h at 4 °C. The gradient was collected into 20 fractions: fractions 1–3 were 1 ml each; the others were 0.5 ml each. Each fraction was analysed by SDS/PAGE and immunoblotting with antisera against Pex1p [17], Pex6p [17] and PMP70 [23].

Pex1p–Pex6p interaction assay

pCMVSPORT·HsPEX6 was constructed by inserting a SalI/NorI fragment of pUCD25Rα·HsPEX6 [24] into the SalI/NorI site of pCMVSPORT. pCMVSPORT·flag-HsPEX1 or its mutant forms were co-transfected with pCMVSPORT·HsPEX6 into ZP107, a peroxisome-deficient CHO pex1 mutant cell line of CG-E, and cultured for 3 days at 30 °C, as described [20]. Cells were lysed with an immunoprecipitation buffer [50 mM Hepes/KOH (pH 7.4)/10% (v/v) glycerol/0.2% (v/v) Nonidet P40/150 mM NaCl/1 mM dithiothreitol/1 mM EDTA/25 μg/ml leupeptin/25 μg/ml antipain/50 μl/ml aprotinin/1 mM PMSF]. The lysates were subjected to centrifugation at 750 g for 5 min at 4 °C; the resulting supernatants were used as whole cell extracts. Immunoprecipitation of Flag-Pex1p was performed overnight at 4 °C with agarose beads conjugated with anti-FLAG antibody (Sigma). Pex1p and Pex6p were assessed by immunoblotting with antibodies against Pex1p and Pex6p respectively. Pex6p that co-immunoprecipitated with several types of mutant Flag-Pex1p was quantified and expressed relative to that of normal Pex1p as 100%.

Other methods

Western blot analysis on PVDF membrane (Bio-Rad) was done with primary rabbit antibodies against acyl-CoA oxidase (AOX) [23], thiolase [23], Pex1p [17] and Pex6p [17], and a second antibody, donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase, with ECL® (enhanced chemiluminescence) Western blotting detection reagent (Amersham Pharmacia Biotech). Quantification of protein bands in immunoblots was performed with an Epson ES-2000 scanner (Epson, Tokyo, Japan) and NIH Image software (National Institute of Mental Health).

RESULTS

Normal import of PTS1 and ts catalase import in CG1 IRD fibroblasts

Fibroblasts from a CG1 IRD patient (PBDE-06) were morphologically restored for peroxisome assembly with regard to import of catalase when cultured for 3 days at 30 °C but not at 37 °C.
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Figure 1 Temperature-sensitive phenotype of peroxisome deficiency in fibroblasts from PEX1-defective patients

Patient-derived fibroblasts were cultured for 3 days at 37 or 30 °C, then stained with antibodies against catalase (a–f), PTS1 (g–l), a PTS2 protein thiolase (m–r) and PMP70 (s–x). Fibroblasts were from an IRD patient (PBDE-06) (left panels) and from two ZS patients, PBDE-04 (middle panels) and PBDE-14 (right panels). Bar = 20 μm.

Table 1 Temperature sensitivity of peroxisome biogenesis and genotypes of CG1 PBD patients

Abbreviation: ts, temperature sensitivity of peroxisomal import of matrix proteins, including catalase, PTS1 and PTS2 proteins, unless specified otherwise.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Phenotype</th>
<th>Peroxisome-positive cells (%)</th>
<th>Age at death or last follow-up</th>
<th>PEX1 genotype</th>
<th>RT–PCR product clones analysed</th>
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<tbody>
<tr>
<td>E-05</td>
<td>IRD</td>
<td>0 90 (catalase, PTS2)</td>
<td>+</td>
<td>G843D/G843D</td>
<td>9</td>
</tr>
<tr>
<td>E-06</td>
<td>IRD</td>
<td>100 100 (PTS1)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-24</td>
<td>IRD</td>
<td>5 100 (catalase, PTS2)</td>
<td>+ 10 years</td>
<td>G843D/G843D*</td>
<td>10</td>
</tr>
<tr>
<td>E-13</td>
<td>NALD</td>
<td>1 1</td>
<td>-</td>
<td>R633Ter/abnormal splicing</td>
<td>7/2</td>
</tr>
<tr>
<td>E-04</td>
<td>ZS</td>
<td>0 1 (catalase)</td>
<td>-</td>
<td>L664P/634del690 (Δexon 12)†</td>
<td>6/6</td>
</tr>
<tr>
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<td>ZS</td>
<td>0 0</td>
<td>+ 4 months</td>
<td>Q261Ter/abnormal splicing</td>
<td>9/3</td>
</tr>
</tbody>
</table>

* From [16].
† From [20].

(Figures 1a and 1b), which is consistent with our earlier observation [16]. In contrast, PTS1 proteins were observed in numerous punctate structures in PBDE-06 fibroblasts, even at 37 °C (Figure 1g), suggesting normal import of these proteins into peroxisomes. PTS1-positive peroxisomes increased in number in the cells cultured at 30 °C (Figure 1h). Restoration of peroxisomes was also confirmed by immunofluorescent staining with antibody against thiolase, a PTS2 protein, whereas thiolase-positive particles were barely detectable at 37 °C, indicative of affected PTS2 import (Figures 1m and 1n). PMP70-positive particles were similarly seen at both temperatures (Figures 1s and 1t), suggesting normal import of peroxisomal membrane proteins. Similar morphological ts phenotypes, including not only the restoration of import of catalase [16] and PTS2 at 30 °C but also the import-competence of PTS1 at 37 °C, were observed in fibroblasts from two other CG1 patients (PBDE-05 and PBDE-24) with IRD (Table 1). In contrast, in fibroblasts from a patient PBDE-04 with ZS, catalase was seen in a cytoplasmically...
Diffused pattern, indicative of impaired biogenesis of peroxisomes, at both temperatures (Figures 1c and 1d), while restoration of PTS1 and PTS2 import was observed at 30 °C but not at 37 °C (Figure 1i, 1j, 1o and 1p). Import defect of all these proteins was observed in fibroblasts from PBDE-14 (ZS) (Figures 1e, 1f, 1k, 1l, 1q and 1r) and PBDE-13 (NALD) (Table 1). PMP70-positive membrane remnants were discernible at both temperatures in these fibroblasts derived from ZS and NALD patients, indicative of normal import of membrane proteins (Figures 1u–1x). In fibroblasts from a normal control, numerous peroxisomes were present at both temperatures (Table 1). Fibroblasts from patients PBDE-04, PBDE-05, PBDE-06, PBDE-13, PBDE-14 and PBDE-24 were morphologically complemented for peroxisome assembly at 37 °C, by transfection of human PEX1, HsPEX1 [20] (results not shown), indicating that dysfunction of PEX1 was responsible for peroxisome deficiency in CG-E (CG1) patients. Taken together, these results suggest strongly that the cellular phenotype of PEX1-defective CG1 IRD is ts, with respect to import of catalase (consistent with our earlier observation [16]) and of PTS2 protein. The ts phenotype is not distinct in import of PTS1. Instead, PTS1 proteins are transported into peroxisomes at normal temperature (37 °C).

**Figure 2 Biogenesis of peroxisomal proteins**

Fibroblasts (approx. 10^6 cells) from a normal control and from CG-E PBD patients were cultured for 3 days at 37 or 30 °C. Cell lysates were subjected to SDS/PAGE and transferred to a PVDF membrane. Immunoblot analysis was performed with rabbit antibodies against AOx (A) and thiolase (B). Lanes 1 and 2, a normal control (N.C.); lanes 3 and 4, IRD patient PBDE-05; lanes 5 and 6, IRD patient PBDE-06; lanes 7 and 8, IRD patient PBDE-24; lanes 9 and 10, ZS patient PBDE-4; lanes 11 and 12, NALD patient PBDE-13; lanes 13 and 14, ZS patient PBDE-14. Blots of lanes 1–8 and 9–14 are a composite of two separate analyses. A, B and C indicate 75, 52 and 23 kDa components of AOx respectively. The asterisk shows a non-specific band. Open and filled arrowheads indicate a larger precursor (P) and a mature protein (M) of thiolase respectively. The positions of molecular mass markers are indicated at the left of each panel.

Biogenesis of peroxisomal protein

In most PBD fibroblasts, peroxisomal proteins are mislocalized to the cytosol, rapidly degraded or not converted into mature forms, despite normal synthesis [19,20]. AOx is synthesized as a 75 kDa polypeptide (A component) and is converted proteolytically into 53 kDa B and 22 kDa C polypeptides in peroxisomes [19,25]. All three polypeptide components, A, B and C, were evident in normal fibroblasts cultured at 37 °C and at 30 °C (Figure 2A, lanes 1 and 2). AOx-A, B and C components were similarly detectable in fibroblasts from IRD patients PBDE-05, PBDE-06 and PBDE-24 and from ZS patient PBDE-04 after cell culturing for 3 days at 30 °C, whereas various levels of only the A component were seen in these patients' cells at 37 °C (Figure 2A, lanes 3–10). It is noteworthy that AOx-A was not converted.
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Figure 3 Mutation analysis of PEX1 in PBD patients of CG-E

(A) Nucleotide sequence of PEX1 from PBD patients of CG-E was determined: partial sequence and deduced amino acid sequence of PEX1 cDNA species from a normal control (upper panels) and from patients with IRD (PBDE-05, PBDE-06 and PBDE-24), NALD (PBDE-13) and ZS (PBDE-14) are shown. In IRD patients, one point mutation, G → A at position 2528 (shaded), in a codon (GGT) for Gly-843, resulted in a codon (GAT) for Asp-843; in PBDE-13, a C → T mutation at position 1897 (shaded) changed a codon (CGA) for Arg-633 to a codon (TGA) for termination; in PBDE-14, a mutation C → T at position 781 (shaded), in a codon (CAA) for Gln-261, resulted in a codon (TAA) for termination. (B) Other types of mutation identified. Only the mutated region, exons 1–13, of PEX1 comprising 24 exons is shown. In patient PBDE-13, 35 nt of intron 3 was inserted into one PEX1 allele. Deletion of exon 9 was identified in one allele of patients PBDE-13 and PBDE-14. Two more types of deletion, exon 6 and exons 6–9, were detected in PBDE-14 PEX1. Mutations identified in (A) and (B) were deposited in GenBank under data accession number AB008112 for human PEX1 cDNA [20].

into the B and C components at 37 °C in fibroblasts from the IRD patients, where PTS1 proteins were in punctate structures (presumably peroxisomal remnant-like particles; see Figure 1g). A protease required for the AOx conversion might not be imported or might not be functional in these cells. In contrast, the AOx-A component was not converted into B or C even at 30 °C in fibroblasts from NALD patient PBDE-13 and from ZS patient PBDE-14 (Figure 2A, lanes 11–14).

Peroxisomal thiolase is synthesized as a 44 kDa larger precursor with an N-terminal cleavable PTS2 [26,27] and processed to its final size (41 kDa) in peroxisomes [19,23]. In normal fibroblasts, only the matured thiolase was detected at both temperatures (Figure 2B, lanes 1 and 2), thereby demonstrating rapid processing of the precursor form. In fibroblasts from IRD patients and from ZS patient PBDE-04, only the larger precursor was found at 37 °C (Figure 2B, lanes 3, 5, 7 and 9), implying the defect of thiolase import and processing activity. When these fibroblasts were cultured for 3 days at 30 °C, only the matured form of thiolase was discerned, reflecting the complementation (Figure 2B, lanes 4, 6, 8 and 10). In contrast, only a 44 kDa larger precursor was detected in fibroblasts from PBDE-13 and PBDE-14, at both temperatures (Figure 2B, lanes 11–14).

Taken together, these results demonstrate that cell culturing at the permissive temperature can complement the abnormality in biogenesis of peroxisomal proteins in cells derived from IRD patients, confirming the ts phenotypic property characteristic of IRD cells.

Mutation analysis of CG1 patients

We previously investigated the genetic cause in two patients, PBDE-04 with ZS and PBDE-06 with IRD [16,20]. Determination of the entire sequence of PEX1 revealed that PBDE-04 carried compound heterozygous mutations: a mis-sense mutation, L664P, and a deletion of exon 12 encompassing the sequence at residues 634–690, which was probably caused by a splice-site mutation [20]. PBDE-06 was homozygous for the G843D mutation [16]. In the present study, we identified a single mutation (GGT → GAT) resulting in G843D in all of nine cDNA clones obtained by RT–PCR with cell RNA from IRD patients PBDE-05 and PBDE-24, suggesting that both patients were homozygotic for the G843D mutation (Figure 3A, left panel). However, heterozygous mutation was revealed by genomic PCR with these patients’ DNA [16]. It is possible that RNA from the other allele in these two IRD patients was not expressed owing to the defect at a level of transcription and/or splicing, or it was normally transcribed but rapidly degraded, all resulting in a single G843D-type allelic phenotype.
We also analysed PEX1 of two patients, PBDE-13 and PBDE-14, by RT-PCR and searched for the biochemical consequence of the dysfunction of impaired Pex1p. Seven cDNA clones isolated from PBDE-13 showed a nonsense mutation (CGA → TGA), R633Ter, named PEX1R633Ter, in one allele (Figure 3A, middle panel). Nine cDNA clones from PBDE-14 showed a nonsense mutation (CAA → TAA), Q261Ter, named PEX1Q261Ter, in one allele (Figure 3A, right panel). Interestingly, each of these two patients had several mutations, presumably owing to aberrant splicing in another allele (Figure 3B). Two distinct PEX1 cDNA clones were identified from another allele in each patient. In two cDNA clones, PBDE-13 had a 35 bp insertion into intron 3 as well as a complete deletion of exon 9. In three cDNA clones, PBDE-14 had a complete deletion of exons 6 and 9, as well as of exons 6–9. Other types of mutation were not found, besides the aberrant splicing site; all of these splicing mutations induced a frameshift resulting in the truncation of Pex1p.

The mutations identified in PBDE-13 and PBDE-14 inactivated Pex1p, as assessed by the transfection of PEX1R633Ter, PEX1Q261Ter and five types of aberrant splicing mutant into the PEX1-deficient CHO cell mutant ZP107 [20], and in none of the ZP107 cells was peroxisome biogenesis restored (Figure 4B, panels c and d). Similarly, the impaired peroxisomal protein import was not complemented by transfection of these PEX1 mutants back to the respective patient-derived fibroblasts (results not shown). Accordingly we conclude that these mis-sense, nonsense and aberrant splicing mutations are the genetic cause of PBDs in the CG-E (CG1) patients PBDE-13 and PBDE-14.

To assess the ts phenotypic property of Pex1p with mutation G843D (Figure 4A), ZP107 cells were transfected with HsPEX1G843D. Numerous PTS1-positive peroxisomes were detected by immunofluorescent staining at both temperatures (Figure 4B, panels a and b), as seen in IRD patients' fibroblasts (see Figures 1g and 1h). Peroxosomal import of catalase and thiolase was re-established only after 3 days of culture at 30 °C, but not at 37 °C (results not shown), which is consistent with the observation in patients' cells. Two types of mutant PEX1, PEX1L664P and PEX1–634del690 (Figure 4A), derived from ZS PBDE-04 fibroblasts were similarly expressed in ZP107 cells. PTS1-positive particles were discernible in a ts manner in ZP107 cells transfected with PEX1L664P but not with PEX1–634del690 (Figure 4B, panels e–h), suggesting that Pex1p-L664P was responsible for a ts phenotype in PTS1 import in PBDE-04 fibroblasts (see Figures 1i and 1j). Both the conversion of AOx- A to the B and C components and the processing of thiolase precursor were noted in a ts manner in ZP107 cells transfected with PEX1L664P but not with PEX1–634del690 (Figure 4C), confirming that the L664P mutation is responsible for this ts property (Figure 2, lanes 9 and 10). These findings imply a temperature-dependent configuration change, particularly involving the region encompassing the AAA family Walker motif, B-1, of Pex1p, which induces a functionally active form of Pex1p.

Expression level of mutated Pex1p in patients

To verify whether the identified mutant forms of PEX1 were expressed in each PBD patient's fibroblasts, a Western blot analysis was performed with cell lysates of patients' fibroblasts and anti-Pex1p C-terminal peptide) antibody. A Pex1p band with a mass of approx. 150 kDa was detected in a normal cells cultured at 37 °C (Figure 5, left panel, lane 1). In contrast, two bands were discernible in fibroblasts from ZS patient PBDE-04: one had the same size as normal Pex1p and the other was apparently of a slightly smaller size (Figure 5, left panel, lane 2), presumably representing Pex1p-L664P and Pex1p-634del690 respectively [20]. Pex1p of normal size was detectable at very low level in fibroblasts from PBDE-06 (Figure 5, left panel, lane 4), whereas Pex1p was barely detectable in IRD PBDE-05 and PBDE-24 (lanes 3 and 5). In fibroblasts from NALD patient PBDE-13 and ZS patient PBDE-14, no protein bands corresponding to several different types of mutant Pex1p were discernible at 37 °C (Figure 5, left panel, lanes 6 and 7), although the detection of Pex1p terminated at position 632 in PBDE-13 and at position 261 in PBDE-14 was not possible with this antibody against Pex1p C-terminal peptide. In the cells derived from several PBD patients, a protein band with an apparent mass of approx. 160 kDa, higher than 150 kDa Pex1p, was detectable (Figure 5, band marked with a dot). We do not yet know whether or not
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Figure 5 Expression level of Pex1p in normal fibroblasts and in fibroblasts derived from PBD patients

Cells were cultured for 3 days at 37 °C (left panel) or 30 °C (right panel). Cell lysates of fibroblasts (approx. 10^5) from a normal control (N.C.) and PBD patients (PBDE-04, PBDE-05, PBDE-06, PBDE-24, PBDE-13 and PBDE-14) were analysed by SDS/PAGE and immunoblotting with anti-Pex1p antibody. The solid arrowhead indicates the position of normal Pex1p; two mutated forms of Pex1p in PBDE-04 are designated by open arrowheads. Dots show unidentified bands. The positions of molecular mass markers are indicated at the left of each panel.

Figure 6 Subcellular localization of Pex1p

Fibroblasts from a normal control (N.C.) and from IRD patient PBDE-06 (E-06) were cultured for 3 days at 37 or 30 °C. (A) Cytosolic (S) and organelar (P) fractions from PNS of fibroblasts (8 × 10^5 cells each) were analysed by SDS/PAGE and immunoblotting. Immunoblotting was performed with anti-Pex1p antibody. The arrowhead indicates Pex1p. The positions of molecular mass markers are indicated at the left. (B) Isopycnic subcellular fractionation. PNS fractions from a normal control and from IRD patient PBDE-06 (2 × 10^7 cells each) were fractionated by isopycnic sucrose-density-gradient ultracentrifugation. The gradient was collected into 20 fractions (fractions 1–3, 1 ml each; others, 0.5 ml each). An equal volume (20 μl) of each fraction was analysed by SDS/PAGE followed by immunoblotting with antisera against Pex1p (top panel), Pex6p (middle panel) and PMP70 (bottom panel). Solid arrowheads show Pex1p, Pex6p and PMP70 respectively in each panel. A protein band (dot) with a faster migration than Pex6p was unidentified. Results are presented in the direction of lower to higher density of sucrose from left to right. Only fractions 1–14 were shown because protein bands were below the limit of detectability in fractions 15–20.

This protein is related to Pex1p. In PBDE-06 fibroblasts that had been cultured at 30 °C, Pex1p was clearly visible as in normal cells (Figure 5, right panel, lanes 1 and 4), suggesting that Pex1p-G843D was not degraded and was quite stable at 30 °C. Pex1p-G843D was similarly detectable in fibroblasts from IRD PBDE-24 but apparently not in IRD PBDE-05-derived fibroblasts cultured at 30 °C (Figure 5, right panel, lanes 5 and 3 respectively). The lower temperature seems to stabilize the mutant Pex1p-G843D. In PBDE-13 and PBDE-14 fibroblasts, protein bands were not clear at 30 °C (Figure 5, right panel, lanes 6 and 7).

Intracellular localization of Pex1p

Subcellular fractionation

The intracellular localization of Pex1p was determined by subcellular fractionation of fibroblasts. In normal fibroblasts cultured at 37 °C, Pex1p was detected by immunoblot, mostly in the organelle pellets and slightly in the cytosolic fraction, when the PNS was fractionated (Figure 6A, lanes 1 and 2). In contrast, Pex1p was barely detectable in fractions from PBDE-06 fibroblasts, suggesting a much lower level, or degradation, of Pex1p in cell culture at 37 °C (Figure 6A, lanes 3 and 4), which is consistent with the results for total cell lysate (see Figure 5). Subcellular fractionation of PBDE-06 fibroblasts that had been cultured for 3 days at 30 °C showed Pex1p mostly in the organellar fraction, strongly suggesting that Pex1p-G843D is a ts property and is functional at 30 °C, apparently in a membrane-associated form (Figure 6A, lanes 7 and 8). The membrane-associated form of Pex1p also increased in amount in normal cells cultured at 30 °C (Figure 6A, lanes 5 and 6).

Isopycnic centrifugation

To confirm the findings described above with regard to the intracellular location of Pex1p, PNS fractions from a normal control and from IRD patient PBDE-06 were fractionated by isopycnic sucrose-density-gradient ultracentrifugation. In normal cells cultured at 37 °C, Pex1p was mostly co-sedimented with PMP70, suggesting that Pex1p was associated with peroxisomes (Figure 6B, top and bottom panels). Pex1p from control cells cultured at 30 °C was sedimented similarly to that from cells cultured at 37 °C. Most of the Pex6p of normal cells was detectable in fractions with a lower gradient density, whereas only part of the Pex6p was sedimented into the middle of
observed at a higher level at 30 °C than at 37 °C (Figure 7A, lanes 1 and 2), suggesting that Pex1p was stable at the lower temperature. Pex1p-L664P and Pex1p-L664P were similarly increased in amount at 30 °C (Figure 7A, lanes 3–6), implying that the stability of these Pex1p variants was not affected. In contrast, Pex1p-G843D was detectable at a very low level at 37 °C, whereas the protein level at 30 °C was comparable with that of normal Pex1p as well as two other Pex1p variants (Figure 7A, lanes 7 and 8). Pex1p-G843D seemed to be rapidly degraded by shifting the temperature from 30 to 37 °C, suggesting a protein degradation system of some abnormal proteins in CHO cells in a temperature-dependent manner. We interpreted these thermostability profiles to mean that temperature-sensitive complementation resulted from the protein stability of Pex1p-G843D. It is also possible that the turnover rate of Pex1p varies depending on the types of mutation.

To determine whether the mutations at L664P, 634del690 and G843D in Pex1p interfered with the interaction of Pex1p with Pex6p [17,18], we expressed N-terminally FLAG-tagged mutant Pex1p together with Pex6p at 30 °C in ZP107 cells. Flag-Pex1p was immunoprecipitated from cell lysates at 4 °C by the use of anti-FLAG antibody conjugated with agarose beads, then analysed by SDS/PAGE. Pex1p and Pex6p were assessed by immunoblotting. Pex6p was detectable with normal Pex1p (Figure 7B, lane 1), which is consistent with our earlier observation that externally added Pex6p or Pex1p was co-immunoprecipitated with Pex1p or Pex6p expressed in CHO-K1 cells [17]. Pex6p was similarly co-immunoprecipitated with FLAG-Pex1p-G843D, although with a lower efficiency, approx. 50% of the level of normal control (Figure 7B, lane 4). In contrast, Pex6p was barely discernible in immunoprecipitates of Pex1p-L664P or Pex1p-L664P-634del690 lacking the Walker motif B-1 region, in which only 5% or 8% of the control Pex6p was detected (Figure 7B, lanes 2 and 3). Taking these results together, we consider it most likely that heterozygous mutations L664P and 634del690 carried by ZS patient PBDE-04 severely impaired the interaction between Pex1p and Pex6p and that the G843D mutation of IRD patient PBDE-06 maintained approx. 50% of the Pex6p-binding activity.

Next we investigated whether any difference could be observed in the binding of Pex1p with Pex6p at different temperatures, such as 30 and 37 °C. Pex1p and Pex6p were expressed at 30 °C in ZP107. FLAG-Pex1p, presumably in the form of heteromeric complexes with Pex6p, was first immunoprecipitated at 4 °C by the use of anti-FLAG antibody. Immunocomplexes with agarose beads were washed extensively and then incubated for a further 2 h at 30 or 37 °C to verify the stability of the Pex1p-Pex6p complexes. The amount of Pex1p-G843D and Pex6p was not distinctly altered by shifting the temperature to 30 or 37 °C, with an apparent slight increase at 37 °C similar to that observed with normal Pex1p (Figure 7C). This indicates that mutation G843D does not greatly affect the binding of Pex1p to Pex6p but instead drastically decreases its stability in a temperature-dependent manner.

**DISCUSSION**

In the present study we investigated phenotype-genotype relationships in PBDs of CG-E (CG1). The most frequently occurring mutation in the PEX1 gene is a point mutation in a codon resulting in G843D [16,18]. We previously showed that this mutation is responsible for a milder form, IRD, of CG-E PBDs [16]. A ts phenotype such as temperature-dependent import of catalase was evident in cells derived from IRD patients. Pex1p was found to be associated mostly with peroxisomes in normal cells, whereas Pex1p-G843D was at a very low level owing to
degradation and was not bound to PMP70-positive particles at normal temperature. On shifting the cell culture to 30 °C, Pex1p-G843D became co-sedimentable with PMP70 and a concomitant increase in catalase import was noted, where Pex6p also sedimented in the same density fractions in the gradient. It is therefore most likely that the G843D mutation affects the stability of Pex1p, giving rise to catalase-deficient and PTS2-deficient peroxisomes. This implies that there is a temperature-dependent protein degradation system, possibly on peroxisomal membranes.

Pex1p binds to Pex6p in vitro as well as in vitro ([17,18,28], and this study). Mutation at G843D decreased by approx. 50 % the binding activity to Pex6p. Impaired peroxisome biogenesis in PEX1G843D-IRD fibroblasts and in the PEX1G843D-transfected CHO pex1 mutant could not be explained by a decrease of 50 % in the Pex6p-binding activity of Pex1p-G843D. The protein level of Pex1p-G843D was also significantly decreased. We therefore conclude that the phenotype of CG-E IRD fibroblasts carrying at least a homozygous PEX1G843D mutation arose from a very low level of Pex1p, not simply a severe decrease in the activity in binding to Pex6p. These results agree only partly with those of Geisbrecht et al. [15], who showed that the amount of Pex6p bound to Pex1p-G843D was 30 % of that bound to normal Pex1p and that this mutation attenuated the interaction between Pex1p and Pex6p.

To our surprise, we found that the import of PTS1 seemed to be normal in IRD cells cultured at normal temperature (summarized in Table 1). In contrast, catalase and PTS2 were localized in the cytoplasm, apparently causing anomalies in IRD patients. On shifting the cell culture from 37 °C to the permissive temperature, 30 °C, the impaired import of catalase and PTS2 was normalized with a concomitant increase in the Pex1p level, although the potency of binding to Pex6p remained at the same level, nearly half that of normal Pex1p. It is noteworthy that PTS1 and PTS2, but not catalase, were moderately restored in import in fibroblasts from ZS patient PBDE-04, whereas all types of matrix proteins remained in the cytoplasm at 30 °C in fibroblasts from a typical ZS patient, PBDE-14. Pex1p-L664P was evidently shown to be responsible for such a ts phenotype with regard to import of PTS1 and PTS2, as assessed by expressing PEX1-L664P in a CHO pex1 line. ZP107. Pex1p-L664P could not bind to Pex6p, which might explain the import defect of catalase in ZP107 cells. Pex1p-Pex6p binding was nearly abolished in PBDE-04 cells, similarly reflecting the fact that catalase import was not restored at 30 °C. Accordingly, it is less likely that the interaction of Pex1p and Pex6p is required for import of PTS1 and PTS2. Such an interaction might be involved in the import of catalase, although catalase is apparently transported in a Pex5p-dependent pathway [22]. On the basis of the observation that no temperature-dependent re-establishment of catalase import was evident in cells from PBD patients with the severest form, ZS, we proposed previously that such a ts phenotype reflects a milder form of IRD of CG-E [16] as well as other CGs [15]. The function of Pex1p in peroxisomal protein import remains to be defined.

With regard to the molecular forms of the Pex1p–Pex6p complex, we do not know exactly how such heteromeric complexes are constituted. Faber et al. [28] suggested that such heteromeric complexes are assembled in an ATP-dependent manner. It is also noteworthy that Pex1p and Pex6p have recently been shown to be involved in vesicle fusion events at early steps of peroxisomal biogenesis in the yeast Yarrowia lipolytica [29]. However, a mechanistic insight into biological activities such as the relationship of ATPase activity to membrane fusion remains to be investigated. Defining the molecular mechanisms involved in mammalian peroxisome biogenesis might now be possible by the use of ts cell mutants as described in this report, including CHO mutants and fibroblasts from IRD patients, as well as the temperature-sensitive peroxins such as Pex1p-G843D. We are making progress in this direction in investigating the AAA peroxins Pex1p and Pex6p.

Furthermore, we identified in the present study several types of aberrant splicing in one allele of NALD patient PBDE-13 and NALD patient PBDE-14. However, we do not yet know how these aberrant splicing events were generated. Mutations in consensus sequences of introns are known to result in aberrant splicing. These mutations cause exon skipping, activation of a cryptic site, creation of a pseudogene within an intron, and intron retention to redefine exon–intron structures [30]. Exon skipping caused by nonsense mutations in several diseases and restoration of an open reading frame by exon skipping have been reported in some cases [31]. It would be intriguing to determine the genomic sequence and address clinical severity in relation to biochemical phenotype such as the dominant-negative effect of the truncated protein derived from aberrant mRNA.

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