Identification of PEX3 as the gene mutated in a Zellweger syndrome patient lacking peroxisomal remnant structures

Nobuyuki Shimozawa+, Yasuyuki Suzuki, Zhongyi Zhang, Atsushi Imamura, Kamran Ghaedi1, Yukio Fujiki1 and Naomi Kondo

Department of Pediatrics, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8076, Japan and Department of Biology, Kyushu University Graduate School of Science, Fukuoka 812-8581, Japan and 1Department of Biology, Kyushu University Graduate School of Science, Fukuoka 812-8581, Japan

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Peroxisome biogenesis disorders, of which 13 complementation groups have been identified, are subdivided with regard to two major dysfunctions: peroxisomal matrix protein import and peroxisomal membrane synthesis. Detectable remnant membrane structures are evident only in the former. Molecular defects have been defined in 10 PEX genes, including eight related to protein import and two to membrane synthesis. We now have evidence that the human complete cDNA encoding Pex3p, a peroxisomal membrane protein (PMP) factor for the proper localization of PMPs, rescues the import of both PMP and the matrix protein in fibroblasts from a Zellweger syndrome patient of complementation group G. This patient was homozygous for a 1 base insertion in the codon for V182, which resulted in a change of codon (182–183) and introduced a termination codon (184), which inactivated PMP and matrix protein import by Pex3p. A PEX3-defective CHO mutant clone, ZPG208, was of the same complementation group as group G.

INTRODUCTION

Peroxisome biogenesis disorders (PBDs) are genetically heterogeneous and can be classified into >13 complementation groups [A–H, J, 2, 3, 6 and rhizomelic chondrodysplasia punctata (RCDP)] (1). We have also subdivided them into two major categories. Groups D, G and J fibroblasts lack not only peroxisomal matrix protein import but also peroxisomal membrane structures, which may be caused by defects in peroxisomal membrane protein (PMP) biogenesis, including their import. The other category includes 10 complementation groups caused by defects in PMP import but not in PMP biogenesis. Hence, these fibroblasts were found to have peroxisomal remnant membrane structures (peroxisomal ghosts) (1).

RESULTS

Studies in fibroblasts from a group G patient

Eight PEX genes involved in peroxisomal matrix protein import, PEX1, -2, -5, -6, -7, -10, -12 and -13, have been identified as pathogenic genes for PBD groups E, F, 2, C, RCDP, B, 3 and H, respectively (2). Furthermore, the PEX16 and 19 genes, which are involved in an early stage of peroxisomal membrane assembly, are the genes involved in PBD groups D (3,4) and J (5). However, the gene responsible for group G has not been identified.

A human homolog of the yeast PEX3 gene was cloned (6,7) and localized to chromosome 6q23–24 (8). In Saccharomyces cerevisiae, a PEX3-defective mutant completely lacked any detectable peroxisomal membrane structures and Pex3 protein was required for proper localization of PMPs, in collaboration with Pex19 protein (9); however, a PBD group caused by the PEX3 gene has not been identified. We report here that the human PEX3 gene restores both peroxisomal membrane assembly and matrix protein import in fibroblasts from a Zellweger syndrome (ZS) patient of complementation group G and we identify an inactivating mutation of the PEX3 gene in this patient. Additionally, complementation analysis between group G and a PEX3-defective CHO mutant, ZPG208 (10), was performed.

+To whom correspondence should be addressed. Tel: +81 58 265 1241; Fax: +81 58 265 9011; Email: nshim@cc.gifu-u.ac.jp
Expression of human PEX3: restoration of peroxisomal membrane assembly and matrix protein import in fibroblasts from a group G ZS patient

We transfected an expression vector containing the human PEX3 cDNA (pCMVPEX3) (11) into fibroblasts from a patient of group G (G-01), in which no peroxisomal membrane structures were detected. A yeast PEX3-defective mutant also completely lacked these structures (9). Numerous punctate structures were seen in G-01 fibroblasts, determined using immunofluorescent staining by anti-human PMP70 and catalase antibodies (Fig. 1g and h), whereas particle-bound immunofluorescence with PMP70 and catalase before transfection was nil (Fig. 1c and d). Thus, PEX3 restored the peroxisomal membrane structures and matrix protein import in G-01 fibroblasts. In fibroblasts from the remaining complementation groups peroxisomal assembly was not rescued (data not shown). We interpret our findings to mean that human PEX3 is the pathogenic gene of group G.

Mutation analysis of human PEX3 in G-01 fibroblasts

To determine the dysfunction of PEX3 in patient G-01, the coding region of the complete cDNA for human PEX3 was amplified by RT–PCR. Direct sequencing of cDNA from G-01 indicated a 1 base insertion at position 544 (544insT) in codon 182Val, resulting in a change of codon (182–183) and introduction of a termination in codon 184 (Fig. 2). To determine the zygosity of the 544insT mutation allele, genomic DNA fragments corresponding to exons 6 and 7 of the PEX3 cDNA (8) were amplified by PCR. Only a single nucleotide sequence giving rise to the 544insT mutation was identified in the PCR products (data not shown). These results suggest that patient G-01 was homozygous for the 544insT mutation. When pCMVPEX3/544insT was transfected back into G-01 fibroblasts, no particles stained with anti-PMP70 and anti-catalase antibodies were evident (Fig. 1i and j), which means that the 544insT mutation probably has a dramatic effect on the function of Pex3p.
Complementation analysis between G-01 fibroblasts and a PEX3-defective CHO mutant

We examined the restoration of peroxisome assembly in fused cells between G-01 fibroblasts and a PEX3-defective CHO mutant, ZPG208 (10,11). Although no catalase-positive particles were detected in the fused cells (Fig. 1k), particles were numerous in fusion cells between group D fibroblasts and ZPG208 (Fig. 1l), which means that ZPG208 belongs to PBD complementation group G (Table 1).

DISCUSSION

In yeast mutants defective in peroxisome assembly (pex), pex3 and pex19 completely lack detectable peroxisomal membrane structures (13,14), which suggests that Pex3p and Pex19p might be implicated in the early stages of peroxisome assembly. Recently, Hettema et al. (9) showed that Pex3p and Pex19p are required for proper localization and stability of PMPs in S.cerevisiae.

In humans, PEX19 cDNA was cloned and was shown to be the causative gene in a ZS patient from PBD complementation group J who possessed a homozygous inactivating mutation (1 base insertion) in PEX19 and no peroxisomal membrane structure in fibroblasts (1,5). A human homolog for yeast PEX3 has been cloned (6,7); however, no data on a PBD patient defective in PEX3 have been published (7,8). Whether or not Pex3 is functional in humans remains unknown.

In PBD patients, we classified 13 complementation groups including RCDP and found that the fibroblasts from all patients belonging to groups D, G and J lacked detectable peroxisomal membrane structures and that only the severe phenotype of ZS was manifested (1). The causative gene for group G remained to be determined, but it was suspected that the gene would be involved in the formation of membrane structures and import of PMPs. Recently, it was shown that groups D and J were caused by inactivating mutations in PEX16 and PEX19, respectively (3–5). We provide evidence in this paper that a human cDNA encoding Pex3p rescues the import of both PMP and matrix protein in fibroblasts from a group G ZS patient (G-01) with a homozygous inactivating mutation in PEX3. Therefore, the human homolog of yeast PEX3 is indeed essential for PMP assembly and matrix protein import in humans.

The mutation in G-01 is a 1 base insertion in the codon for Val182, introducing a termination codon 184 (Fig. 2). Human PEX3 is localized to peroxisomes (6), and as a result of expression of the PEX3 truncations fused to green fluorescent protein (GFP) in human fibroblasts, it was suggested that the

![Figure 2. Mutation analysis of PEX3 from a complementation group G patient. Partial nucleotide sequence and deduced amino acid sequence of PEX3 cDNA isolated from ZS patient G-01 and a normal control are shown. A 1 base insertion of nucleotide T at position 544 in codon 182Val resulted in a change of codon (182–183) and introduced a termination in codon 184.](image)

Table 1. Complementation groups of PBD patients and CHO cell mutants

<table>
<thead>
<tr>
<th>Gifu</th>
<th>KKI*</th>
<th>Phenotype</th>
<th>Ghosts</th>
<th>CHO</th>
<th>Gene</th>
<th>Mapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>ZS, NALD, IRD +</td>
<td>ZP124</td>
<td></td>
<td>PEX10</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>7 (5)</td>
<td>ZS, NALD +</td>
<td>ZP92</td>
<td>PEX6</td>
<td>6p21.1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>ZS, NALD +</td>
<td>Z24, ZP101</td>
<td>PEX1</td>
<td>7q21–22</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>ZS –</td>
<td>ZP19</td>
<td>PEX16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>ZS, NALD, IRD +</td>
<td>Z65</td>
<td>PEX2</td>
<td>8q21.1</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>ZS, IRD +</td>
<td>ZP28</td>
<td>PEX13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>13</td>
<td>ZS, NALD +</td>
<td>ZP119</td>
<td>PEX19</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>2</td>
<td>ZS, NALD +</td>
<td>ZP102</td>
<td>PEX5</td>
<td>12p13.3</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>3</td>
<td>ZS, NALD, IRD +</td>
<td>ZP104, ZP109</td>
<td>PEX12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>6</td>
<td>ZS, NALD +</td>
<td>ZP207</td>
<td>PEX7</td>
<td>6q22–24</td>
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</table>

Gifu, Department of Pediatrics, Gifu University School of Medicine; KKI, Kennedy Krieger Institute, Johns Hopkins University School of Medicine; Ghosts, empty peroxisomal membrane structures (PMP70 in vesicles); NALD, neonatal adrenoleukodystrophy; IRD, infantile Refsum disease.

*Numbering of KKI according to Moser (2) and Liu et al. (12).
N-terminal 33 amino acids of PEX3 are necessary and sufficient to direct a reporter protein to peroxisomes and that the N-terminal 16 amino acids of PEX3 were not able to target GFP to peroxisomes but did localize to mitochondria (7). Furthermore, as mitochondrial localization was only observed in the case of C-terminal truncated PEX3 fusion proteins containing N-terminal amino acids, the C-terminal part of PEX3 might prevent exposure of an otherwise cryptic mitochondrial targeting signal (7). PMP import and peroxisomal assembly, as related to characterization and function of these proteins, are being addressed in ongoing studies.

MATERIALS AND METHODS

Patient

The patient was a male infant (G-01) born to consanguineous parents and showed typical phenotypic expression of ZS, as described for patient 1 elsewhere (15).

Complementation and morphological analysis

Complementation studies on human fibroblasts from various PBD patients and between the CHO mutant and PBD fibroblasts were done as described (16,17). Peroxisomes in fibroblasts were visualized by indirect immunofluorescence light microscopy as described (17), using rabbit antibodies to human and rat catalase, AOX, PT and human PMP70 (18).

Transfection of human PEX3 cDNA

Plasmid pCMVPEX3 (11), in which human PEX3 (GenBank accession no. AB033537) was inserted into the pCMVSPORT vector, was transfected into fibroblasts from PBD patients using a Gene Pulser II electroporator (Bio-Rad, Hercules, CA) at 300 V and 400 μF (19) and the transfectants incubated for 10 days, followed by immunocytochemical observation.

Mutation analysis of human PEX3 in group G

Human PEX3 cDNA was synthesized from fibroblast RNA by reverse transcription (RT)–PCR. The coding region of the cDNA (1–1122, starting from the first nucleotide of the initiator methionine codon) was amplified by PCR with primer sets 5′-CCACACCCTAGGGCCTAAA-3′ (–21 to –2) and 5′-CAGGCCCTTCAACAAAGCTAG-3′ (355–336) to obtain a 376 bp product (–21 to 355), 5′-GAGGCTTCAACCCTAG-3′ (285–304) and 5′-GGTCGGTCACAC-3′ (655–636) to obtain a 371 bp product (285–655), 5′-CGACAGGGTTTAGAAAGTG-3′ (561–580) and 5′-GGTCGACACTGAGAAAGCTA-3′ (958–939) to obtain a 389 bp product (561–958) and 5′-CCAGGGTTTAGAGCTC3′ (855–874) and 5′-GTAATGAACTC-3′ (1160–1141) to obtain a 306 bp product (855–1160) for 40 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The amplified DNA fragments were directly sequenced, using an automated DNA sequencer. Genomic DNA fragments containing 544insT in PEX3 were obtained from cultured fibroblasts of G-01 by PCR for 40 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min with primer set 5′-CAGATGTCCAACAGCAGTAT-3′ and 5′-CTTCTAAACCTCTTCGCAC-3′ (8). The amplified DNA fragment was directly sequenced.

To assess the effect of mutations in the G-01 patient, pCMVPEX3/544insT was constructed using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) from normal complete human PEX3 cDNA and transfected into G-01 fibroblasts by electroporation. The immunofluorescence study was done 10 days later, using anti-human PMP70 and anti-catalase antibodies.

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