Human PEX19: cDNA cloning by functional complementation, mutation analysis in a patient with Zellweger syndrome, and potential role in peroxisomal membrane assembly

Yuji Matsuono†, Naohiko Kinoshita‡, Shigeiko Tamura*, Nobuyuki Shimozawa*, Maho Hamasaki*, Kamran Ghaedi*, Ronald J. A. Wanders§, Yasuyuki Suzuki†, Naomi Kondo‡, and Yuki Fujiki*†

*Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-8581, Japan; ‡Department of Pediatrics, Gifu University School of Medicine, Gifu 500-8076, Japan; †Department of Pediatrics, Academic Medical Center, University of Amsterdam, P.O. Box 22700, 1100DE, The Netherlands; and ¶Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo 117-0013, Japan

Communicated by Christian de Duve, Christian de Duve Institute of Cellular Pathology, Brussels, Belgium, December 21, 1998 (received for review October 18, 1998)

ABSTRACT At least 11 complementation groups (CGs) have been identified for the peroxisome biogenesis disorders (PBDs) such as Zellweger syndrome, for which seven pathogenic genes have been elucidated. We have isolated a human PEX19 cDNA (HsPEX19) by functional complementation of peroxisome deficiency of a mutant Chinese hamster ovary cell line, ZP119, defective in import of both matrix and membrane proteins. This cDNA encodes a hydrophilic protein (Pex19p) comprising 299 amino acids, with a prenylation motif, CAAX box, at the C terminus. Farnesylated Pex19p is partly, if not all, anchored in the peroxisomal membrane, exposing its N-terminal part to the cytosol. A stable transformant of ZP119 with HsPEX19 was morphologically and biochemically restored for peroxisome biogenesis. HsPEX19 expression also restored peroxisomal protein import in fibroblasts from a patient (PBDJ-01) with Zellweger syndrome of CG-J. This patient (PBDJ-01) possessed a homozygous, inactivating mutation in the PEX19 gene, a 1-base insertion, A764, in a codon for Met255, resulting in a frameshift, inducing a 24-aa sequence entirely distinct from normal Pex19p. These results demonstrate that PEX19 is the causative gene for CG-J PBD and suggest that the C-terminal part, including the CAAX homology box, is required for the biological function of Pex19p. Moreover, Pex19p is apparently involved at the initial stage in peroxisomal membrane assembly, before the import of matrix protein.

Peroxisomal proteins, including membrane proteins, are encoded by nuclear genes, translated on free polyribosomes in the cytosol (1). Peroxisomes are thought to be formed by division of preexisting peroxisomes after import of newly synthesized proteins (1). It is noteworthy that recent evidence suggests involvement of the endoplasmic reticulum in peroxisomal membrane biogenesis in yeast (2). Peroxisomal functions are highlighted by the existence of a number of protein factors, called peroxins, that are essential for peroxisome assembly by genetic analysis of peroxisome-deficient mutants of yeast and mammalian cells (3, 10–12). To date, seven peroxin cDNAs have been cloned in mammals by genetic phenotype-complementation assay of Chinese hamster ovary (CHO) cell mutants and by the expressed sequence tag search of the human database by using the yeast PEX genes.

To investigate molecular mechanisms involved in peroxisome biogenesis and the genetic cause of PBD, we have so far isolated seven CGs of peroxisome-deficient CHO cell mutants (4–7, 13) (see Table 2). Very recently, we identified CG-J (8). Two CHO cell mutants, ZP119 and ZP165, were also found to belong to this group (8). In no CG-J mutant cell were peroxisomal ghosts found (8). We isolated PEX1, PEX2 (formerly PAF-1), PEX5, PEX6, and PEX12 by genetic phenotype-complementation assay of CHO cell mutants ZP107, Z65, ZP105/ZP139, ZP92, and ZP109, respectively (13–18), and demonstrated that these PEX genes are defective in the various patients with PBD (13, 14, 17–20). Thus, peroxisome biogenesis-defective CHO cell mutants are a useful mammalian somatic cell system for the investigation of peroxisome assembly at the molecular and cellular level, as well as for delineation of the genetic basis of PBD (3). However, until now no peroxins evidently required for the biogenesis of peroxisomal membranes have been elucidated in mammals.

In the study reported here, we isolated human PEX19 cDNA (HsPEX19) encoding a farnesylated protein that restored peroxisome biogenesis in a CHO cell mutant ZP119 devoid of peroxisomal membrane vesicles (8). HsPEX19 expression also complemented impaired peroxisome biogenesis in fibroblasts from a patient with CG-J PBD. In a patient with CG-J, we identified a mutation site that inactivated Pex19p. The biological function of Pex19p is also discussed.

MATERIALS AND METHODS

Cell Lines. Patient skin fibroblast cell lines and CHO cell mutants were cultured as described (4, 5). ZP119EG1, ZP119 stably expressing “enhanced” green fluorescent protein (EGFP) tagged with PTS1 (EGFP-PTS1), was isolated by transfecting pUcD2HygEGFP-PTS1, as described (14).

Abbreviations: CG, complementation group; CHO, Chinese hamster ovary; EGFP, “enhanced” green fluorescent protein; PBD, peroxisome biogenesis disorders; PEX19, cDNA encoding the peroxin Pex19p; PMP70, 70-kDa peroxisomal integral membrane protein; PTS1 and PTS2, peroxisome targeting signal types 1 and 2; RT, reverse transcription; ZS, Zellweger syndrome.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB018541).

†Y.M. and N.K. contributed equally to this work.

To whom reprint requests should be addressed at: Department of Biology, Kyushu University Faculty of Science, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. e-mail: yfujschb@mbbox.nc.kyushu-u.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.
Screening of a cDNA Library. A human liver cDNA library in pCMVSPORT vector divided into small pools, each containing about 4,000 clones (14), was transplanted to ZP119 cells with Lipofectamine (GIBCO/BRL), as described (14, 18). Among the cDNA pools examined, a positive one (F6–19) that restored peroxisomes in ZP119 was further divided into subpools and screened. A single positive clone, F6-19-30, was isolated. The nucleotide sequence of F6-19-30 was determined with the GENETYX-MAC program (Software Development, Tokyo). Western blot analysis was done with rabbit antibodies to rat acyl-CoA oxidase, 3-ketoacyl-CoA thiolase (thiolase), and Pex19p, as described (14, 18). The catalase latency assay with digitonin was done as described (5). PI2/UV and P90H/UV resistances were determined under conditions of 2 μM for 1.5 min and 6 μM for 2 min, respectively (4).

RESULTS

Cloning of a Human PEX19 cDNA. We used a transient expression assay (14, 18) to clone the PEX19 cDNA, making use of a human liver cDNA library. After transfection with a small pool of cDNA library, we searched for cells in which peroxisome biogenesis was restored, by staining ZP119 with anti-rat catalase antibody. In ZP119, catalase was localized in the cytosol (Fig. 1a), consistent with our earlier observation (8). Twenty-seven pools, which contained about 1.1 × 10^5 independent cDNA clones, were screened, and one pool (F6-19) yielded numerous catalase-positive particles, presumably peroxisomes, in several of ZP119 cells in a single dish (Fig. 1b, arrows). After a third round of screening, we isolated one positive clone, F6-19-30, that contained a 1.8-kb cDNA with an ORF encoding a 209-aa protein. A homology search suggested that this ORF was likely to be related to the yeast Saccharomyces cerevisiae (23) and identical to the amino acid sequence from residues at 91 to the C terminus of human HK33 (21), whereas the deduced sequence of isolated clone F6-19-30 was shorter by 90 aa compared with the sequence of HK33. F6-19-30 started the cDNA sequence nine nucleotides downstream of the initiation codon in the HK33, i.e., 261 nt upstream of a putative initiation codon in F6-19-30. Accordingly, we constructed a cDNA, termed HsPEX19, encoding the full-length, 299-aa PEX19 protein of 32,806 Da, Pex19p, by inserting a 9-mer oligonucleotide, ATGGCGCGCC (see Materials and Methods). HsPEX19 complemented peroxisomal import of catalase more efficiently than F6-19-30 in both ZP119 and ZP165 (data not shown). The human Pex19p was shorter in a rabbit reticulocyte cell-free protein-synthesizing system was performed with 1.2 mCi/ml [35S]methionine and [35S]cysteine (Amersham Pharmacia) or 80 μCi/ml [3H]farnesyl pyrophosphate (New England Nuclear). Immunoprecipitation of Pex19p was done as described (22).

Other Methods. The nucleotide sequence was determined by the dye-sequencing chain termination method with a dye terminator DNA sequencing kit (Applied Biosystems). Alignment was done with the GENETYX-MAC program (Software Development, Tokyo). Western blot analysis was done with rabbit antibodies to rat acyl-CoA oxidase, 3-ketoacyl-CoA thiolase (thiolase), and Pex19p, as described (14, 18). The catalase latency assay with digitonin was done as described (5). PI2/UV and P90H/UV resistances were determined under conditions of 2 μM for 1.5 min and 6 μM for 2 min, respectively (4).

In Vitro Translation. Anti-Pex19p antibody was raised in rabbits by immunizing them with synthetic peptide comprising the C-terminal, 19-aa sequence (see Fig. 2, underline) supplemented with cysteine at the N terminus that had been linked to key-hole limpet hemocyanin (15). In vitro transcription/translation was done essentially as described (22). Translation

FIG. 1. Restoration of peroxisomes in CG-J CHO mutant cells. (a) Peroxisome-deficient mutant ZP119 cells. (b) Peroxisome-restored ZP119, after lipofection with a combined pool (F6-19) of human cDNA library. Arrows indicate the complemented cells. Cytosolic appearance of catalase was apparent in the other cells. (c and d) 119P12 cells, stable HsPEX19-transformants of ZP119 cells. Cells were stained with antisera to catalase (a and b), PTS1 peptide (c), and PMP70 (d), respectively. (Magnification: ×630; bar = 20 μm.)
by 51 aa than *S. cerevisiae* Pex19p (23), with 20% amino acid identity, showing 92% identity to the same-length Chinese hamster (Cl) Pex19p (24) (Fig. 2). Hydropathy analysis suggested that Pex19p is a hydrophilic protein with no apparent membrane-spanning region (data not shown).

**Restoration of Peroxisome Biogenesis in ZP119 by PEX19.**

In 119P19, a stable *HsPEX19* transformant of ZP119, numerous peroxisomes were detected by immunofluorescent staining of PTS1 and PMP70 (Fig. 1c and d), which were seen in a diffuse staining in ZP119 (data not shown; ref. 8). Import of peroxisomal thiolase, a PTS2 protein, was also restored (data not shown). These results demonstrated that 119P19 cells had morphologically normal peroxisomes, as seen in the wild-type CHO-K1 cells.

In peroxisome-deficient cells, peroxisomal proteins are mislocalized to the cytosol, rapidly degraded, or not converted to mature forms, despite normal synthesis (4–8, 13, 14). According to the results of the digitonin titration assay, nearly 60% of catalase activity was latent at 100 μg/ml digitonin in the wild-type cells, consistent with earlier observations (5–8, 13, 14) (Fig. 3A). In ZP119 cells, full activity of catalase was detected at 100 μg/ml digitonin, which was also the case for a cytosolic enzyme, lactate dehydrogenase (Fig. 3A), suggesting that catalase is present in the cytosol, in agreement with the morphological observation (Fig. 1z; ref. 8). In 119P19 cells, catalase latency was comparable to that of the wild-type CHO-K1 cells (Fig. 3d). Moreover, 119P19 was resistant to P12/UV treatment and sensitive to P90H/UV, similar to CHO-K1 (Table 1). In contrast, mutant ZP119 was resistant to P90H/UV treatment and sensitive to P12/UV (8).

Acyl-CoA oxidase is synthesized as a 75-kDa polypeptide (A component) and is proteolytically converted into 53- and 22-kDa polypeptides (B and C, respectively) in peroxisomes (22). In immunoblots, all three polypeptide components, A, B, and C, were present in CHO-K1 as well as in 119P19 (Fig. 3B, lanes 1 and 3), but only the A component was seen in a smaller amount in the ZP119 (lane 2). Peroxisomal thiolase, a PTS2 protein, is synthesized as a larger 44-kDa precursor and undergoes maturation to the 41-kDa form in peroxisomes (14, 18). In wild-type CHO-K1 and 119P19 cells, only the 41-kDa mature thiolase was detected (Fig. 3B, lanes 4 and 6), whereas only the larger precursor was found in ZP119 (lane 5).

Taken together, these results indicate that *HsPEX19* can fully complement the ZP119 mutation. *HsPEX19* expression also complemented peroxisome assembly in ZP165 cells, the same CG as ZP119 (8), but did not restore peroxisome formation in six other CGs of peroxisome-deficient CHO cell mutants so far isolated (4–7), Z65, ZP92, ZP105, ZP109, ZP110, and ZP114. These data show that Pex19p is the peroxisome biogenesis factor for ZP119/1165 (Table 2).

**PEX19 Specifically Complements Fibroblasts from CG-J Patient with ZS.** ZP119 and ZP165 belong to the same CG as ZP119 and CG J of Gifu University, Japan, and groups II and III of the Kennedy-Krieger Institute, Baltimore. As expected, the fibroblasts derived from a CG-J patient (PBDJ-01) with ZS were morphologically restored for import of catalase (Fig. 4Ca and Cb), but no restoration of peroxisome formation was seen in fibroblasts from the other, nine CGs (Table 2). Restoration of peroxisome assembly was also assessed by staining the transfectants with antibodies to PTS1 and PMP70 (data not shown). These results strongly suggest that *HsPEX19* is the causal gene of CG-J PBD.

**CG-J Patient Analysis.** To determine dysfunction of PEX19 in the patient PBDJ-01, we isolated *PEX19* cDNA from PBDJ-01 fibroblasts by RT-PCR. A point mutation was detected by subsequent sequencing: a 1-base insertion, A764, in a codon for Met255, named PEX19A2546ins, resulting in a frameshift, inducing a 24-aa sequence distinct from that of normal Pex19p (Fig. 4A Left and Center and B). All of the 10 cDNA

![Fig. 2](Image 2)

**Fig. 2.** Amino acid sequence alignment of *PEX19* protein from two mammalian species and *S. cerevisiae* Pex19p. Deduced amino acid sequence of human (Hs) *PEX19* was compared with those of Pex19p from Chinese hamster (Cl) and *S. cerevisiae* (Sc), · a space. Identical amino acids between species, including two mammalian ones, are shaded, and the CAAX motif is boxed. The sequence used for chemical synthesis of Pex19p peptide is underlined. The solid arrowhead indicates the position of mutation in CG-J PBD patient (see Fig. 4). The database accession numbers for the human *PEX19* cDNA is AB018541.

![Fig. 3](Image 3)

**Fig. 3.** Complementation of biogenesis of peroxisomal enzymes. (A) Latency of catalase activity in CHO-K1, ZP119, and 119P19 cells. •, CHO-K1; ○, ZP119; ○, 119P19; ○, lactate dehydrogenase in ZP119. Relative free enzyme activity is expressed as a percentage of the total activity measured in the presence of 1% Triton X-100 (5). The results represent a mean of duplicate assays. (B) Biogenesis of peroxisomal proteins. Cell lysates (~1.8 × 10⁶ cells) were subjected to SDS/PAGE and transferred to polyvinylidene difluoride membrane. Cell types are indicated at the top. Immunoblotting was done with antibodies to acyl-CoA oxidase and thiolase. Arrowheads show the positions of acyl-CoA oxidase components, A, B, and C; open and solid arrowheads indicate a larger precursor (P) and mature protein (M) of thiolase, respectively. Dots indicate nonspecific bands (14).

**Table 1.** Properties of wild-type CHO-K1, ZP119, and *HsPEX19* transfected ZP119 (119P19) cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Peroxisome</th>
<th>Catalase latency, %</th>
<th>P12/UV, %</th>
<th>P90H/UV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>+</td>
<td>57</td>
<td>83</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>ZP119</td>
<td>−</td>
<td>0.6</td>
<td>&lt;0.01</td>
<td>98</td>
</tr>
<tr>
<td>119P19</td>
<td>+</td>
<td>39</td>
<td>73</td>
<td>12</td>
</tr>
</tbody>
</table>

Catalase latency represents peroxisomal catalase, calculated as described (5). For determination of P12/UV or P90H/UV resistance, 200 or 1 × 10⁵ cells were inoculated into 60-mm dishes and selected (4). The numbers of colonies were counted in duplicate experiments and expressed as percentages of that of unselected control.
Table 2. Complementation of CHO cell mutants and patient fibroblasts by HsPEX19

<table>
<thead>
<tr>
<th>CHO mutant</th>
<th>Peroxisome-positive clone</th>
<th>Patient fibroblasts from CG</th>
<th>Peroxisome-positive</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZP119</td>
<td>24/30</td>
<td>IPBDJ-01</td>
<td>+</td>
<td>PEX19</td>
</tr>
<tr>
<td>ZP165</td>
<td>21/30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZP107</td>
<td>–</td>
<td>E(I)</td>
<td>–</td>
<td>PEX1</td>
</tr>
<tr>
<td>ZP139</td>
<td>–</td>
<td>II</td>
<td>–</td>
<td>PEX5</td>
</tr>
<tr>
<td>ZP109</td>
<td>–</td>
<td>III</td>
<td>–</td>
<td>PEX12</td>
</tr>
<tr>
<td>ZP92</td>
<td>–</td>
<td>C(IV)</td>
<td>(VI)</td>
<td>PEX6</td>
</tr>
<tr>
<td>Z65</td>
<td>–</td>
<td>F(X)</td>
<td>–</td>
<td>PEX2</td>
</tr>
<tr>
<td>ZP110</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZP114</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Peroxisome-deficient CHO mutants of 8 CGs (4–8, 13–18) and fibroblasts from 10 CG PBD patients (3, 4, 8, 9) were transfected with pUCd2Hyg/HsPEX19 and examined for peroxisome assembly by immunostaining with antisera to rat and human catalase, respectively, at 3 days after transfection. Parentheses indicate fibroblasts of CG not used in this experiment. In CG-J ZP119 and ZP165, peroxisome-positive colonies were counted in 30 colonies; in other cells: +, complemented; –, not complemented; ND, not determined.

Clones isolated showed the same site mutation, evidently implying that the patient was a homozygote for the mutation. To confirm the homozgyosity of a PEX19A764ins allele, genomic PCR was done to amplify the sequence corresponding to nucleotide residues 691–908 in the PEX19 ORF. Only a single type of nucleotide sequence giving rise to the A insertion was identified in the PCR product (Fig. 4A Right), indicating that patient PBDJ-01 with ZS was a homozygote for the A764 insertion. This one-point mutation inactivated PEX19, as assessed by back-transfection of ZS fibroblasts to both PBDJ-01 fibroblasts (Fig. 4C) and ZP119 cells (data not shown). Collectively, these data demonstrate that the dysfunction of PEX19 is responsible for CG-J peroxisome deficiency.

**Pex19p Is Prenylated.** To investigate whether a conserved cysteine in the CAAX motif is essential for the function of Pex19p, Cys296 was mutated to Ser. Expression of C296S-mutated HsPEX19, termed HsPEX19C296S, could not rescue the impaired assembly of peroxisomes in PBDJ-01 fibroblasts (Fig. 4Cd) and ZP119 (data not shown). Flag-HsPEX19C296S was also inactive in complementing ZP119, where its expression was assessed (Fig. 4Ce and Cf). Therefore, prenylation of Pex19p is most likely essential for its biological activity.

Rabbit antibody raised against the C-terminal peptide (residues 276–294) of Pex19p reacted with two protein bands with apparent masses of 40 kDa and 39 kDa, respectively, in immunoblots of CHO-K1 cells (Fig. 5, lane 1). The mobility of these two bands shown by SDS/PAGE was indistinguishable from that of Pex19p likewise showing two bands, synthesized in vitro by using [35S]methionine and [35S]cysteine by coupled transcription/translation of HsPEX19 (lane 2). Both 35S-Pex19p proteins were specifically immunoprecipitated by anti-Pex19p antibody but not by preimmune serum (lanes 3 and 4), thereby indicating that two bands were Pex19p and that a cloned HsPEX19 encoded bona fide Pex19p. In vitro synthesized Pex19p using [3H]farnesyl pyrophosphate as a prenyl label showed a [3H]-protein band (Fig. 5, lane 5), exactly with the same mobility as 35S-Pex19p as well as an immunoblotted band with a higher mobility (lanes 1, 2, and 4), thus demonstrating that the lower band corresponds to the farnesylated Pex19p. Moreover, the transcription/translation product of HsPEX19C296S, 35S-labeled Pex19pC296S, migrated as a single band with the same mobility as the larger 35S-Pex19p and was immunoprecipitated by anti-Pex19p antibody (Fig. 5, lanes 7 and 8). In contrast, transcription/translation of HsPEX19C296S with [3H]farnesyl pyrophosphate resulted in no [3H]-labeled protein (Fig. 5, lane 6), indicating that Pex19pC296S was not prenylated. Together, the results demonstrate that Pex19p is partly farnesylated in vivo (see Fig. 5, lane 1).

**Intracellular Location of Pex19p.** Subcellular localization of Pex19p was determined by immunofluorescent microscopy of flag-Pex19p ectopically expressed in CHO-K1 cells. Flag-Pex19p was detected in both punctate structures and a diffuse staining pattern in the cytoplasm (Fig. 6a). The punctate pattern was superimposable on that obtained with anti-PTS1 antibody (Fig. 6b), thereby suggesting that flag-Pex19p was localized partly to peroxisomes. Punctate staining was hardly discernible, however, in flag-Pex19pC296S-expressing CHO-K1 as expressed in ZP119 (Fig. 4Cf), suggesting that prenylation is required for peroxisomal localization (data not shown). Membrane topology of Pex19p was determined by a differential permeabilization procedure. Upon cell permeabilization with 25 μg/ml digitonin (17, 18), flag-Pex19p was likewise observed in a punctate staining pattern, whereas there was hardly any staining of cells with anti-PTS1 antibody (Fig. 6c and d), thus strongly suggesting that the N-terminal part of peroxisomal Pex19p was exposed to the cytosol, presumably anchored by the farnesylated C terminus (see Figs. 2 and 5).
Localization of a part of Pex19p (Fig. 6c). It is noteworthy that ScPex19p (23) and CI/Pex19p, originally named PxF (24), were also shown to be localized to peroxisomes.

**Kinetics of Peroxisome Biogenesis.** We investigated the kinetics of peroxisome assembly with respect to membrane vesicle formation and matrix protein import. ZP119EG1, ZP19 stably expressing EGFP-PTS1, was transfected with HsPEx19 and monitored under a fluorescent microscope. HsPex19p was detectable by immunoblot analysis at 7 h after the transfection and increased with time (Fig. 7A). At 10 h, PMP70 became visible in punctate structures in a part of transfected cells, possibly representing assembled peroxisomal membranes, whereas EGFP-PTS1 showed a diffuse fluorescence pattern implying localization in the cytoplasm and membranes, whereas EGFP-PTS1 showed a diffuse fluorescence pattern implying localization in the cytoplasm and nonfarnesylated Pex19p, respectively (see text).


**FIG. 5.** Farnesylation of Pex19p. Size comparison of in vitro transcription/translation product of normal and mutated PEX19 cDNA and Pex19p of CHO cells. In vitro transcription/translation product of HsPEx19 and CHO-K1 cell-lysates were subjected to SDS-PAGE. Immunodetection was done for lane 1, as in Fig. 3B, by using anti-Pex19p antibody; radioactive bands were detected by a FujiX BAS1500 Bio-Imaging Analyzer at exposures for 16 h (lanes 2–4, 7, and 8) and 72 h (lanes 5 and 6). Lanes: 1, 40 µg of CHO-K1 cell-lysates; 2, in vitro transcription/translation product (1 µl) of HsPEx19 in the presence of [35S]methionine and [35S]cysteine as label; 3 and 4, immunoprecipitation of [35S]Pex19p (3.5 µl) was done with preimmune and anti-Pex19p immune sera, respectively; 5 and 6, in vitro transcription/translation product (15 µl) of HsPEx19 and HsPEx19C296S, respectively, using [3H]farnesyl pyrophosphate as label; 7 and 8, total (1 µl) and immunoprecipitate (3.5 µl) from in vitro transcription/translation product of HsPEx19C296S using [35S]methionine/cysteine. Solid and open arrowheads indicate farnesylated and nonfarnesylated Pex19p, respectively (see text).

**FIG. 6.** Intracellular localization and topology of Pex19p. N-terminally flag-tagged human Pex19p was expressed in CHO-K1 cells. Cells were treated with 0.1% Triton X-100 (a and b), or with 25 µg/ml of digitonin, under which the plasma membrane was permeabilized (17, 18) (c and d). Cells were stained with antibodies to flag (a and c) and PTS1 (b and d). Note that punctate structures, peroxisomes, are superimposable in a and b and that flag-Pex19p was detected after both types of treatments (a and c). A diffuse staining pattern was partly detected in a and c (see text). (Bar = 20 µm.)

**FIG. 7.** Kinetics of peroxisome biogenesis. (A) ZP119EG1 cells expressing EGFP-PTS1 were transfected with pUd2Hyg/HsPEx19, then monitored by fluorescence microscope. (a–c) PMP70 was visualized by using rabbit anti-PMP70 antibody and Texas Red-labeled goat anti-rabbit IgG antibody. (d–f) EGFP-PTS1. (g–i) Catalase in other cells detected by anti-catalase antibody, as for PMP70. (a, d, and g) 10 h after transfection, (b, e, and h) 16 h, (c, f, and i) 25 h. Note that PMP70, but not EGFP-PTS1 and catalase, is already in numerous vesicular structures at 10 h (see text). (Bar = 20 µm.) (B) Expression of human Pex19p in ZP119. Pex19p was detected by immunoblotting HsPEx19-transfected ZP119 lysates (1.3 × 10⁵ cells at 0 h), at indicated time, where cell-doubleing time was 22 h. Arrowhead, Pex19p.

nucleus (Fig. 7Aa and Ad) as seen in the untransfected cells (8). At 16 h, PMP70-positive vesicles increased in number, which were then partly colocalized with EGFP-PTS1 (Fig. 7Ab and Ae), although EGFP-PTS1 still showed a predominantly diffuse pattern. This was interpreted to mean that a part of the assembled peroxisomal membrane vesicles imported EGFP-PTS1. At 25 h, numerous PMP70-positive punctates were superimposable with those of EGFP-PTS1, demonstrating re-establishment of membrane assembly and matrix protein import of peroxisomes (Fig. 7Ac and Af). In contrast, catalase was noticeably imported only at 25 h after the HsPEx19 transfection (Fig. 7Ag–Ai), into PTS1-positive vesicles (data not shown), suggesting the import of catalase at a slower rate as compared with PTS1. Collectively, peroxisomal membrane vesicles containing PMP70 are likely to form before the import of matrix proteins. The import kinetics of matrix proteins appears to be variable.

**DISCUSSION**

Our earlier work showed that CHO cell mutants, ZP119 and ZP165, were defective in import of both matrix proteins and membrane polypeptides, a phenotype distinct from other CGs cells (8). Peroxisomal remnants were seen in the other 7 CGs of CHO cell mutants (4, 6, 7, 13) and 7 CGs of fibroblasts from PBD patients (25, 26). In the present work, we isolated a human Pex19p cDNA by functional complementation of peroxisome-deficient CG-J CHO cell mutants, although the initially isolated PEX19 cDNA clone was shorter by 9 nucleotides. The result implies that Pex19p translated at the second potential initiator methionine, comprising the primary sequence from residues 91–299 of the full-length Pex19p, is functional in complementing the impaired assembly of peroxisomes in CG-J cells. The entire N-terminal region of 90 amino acids appears not be required. Expression of the full-length HsPEx19 fully restored peroxisome biogenesis, including membrane assembly, in ZP119 and ZP165. Restoration of peroxisome-deficiency in fibroblasts from a CG-J patient indicated that the dysfunction of PEX19 is causal in this group. We delineated the
homozygous mutant PEX19 allele from a CG-J patient: one-base insertion, A\textsuperscript{764}, in a codon for Met\textsuperscript{255}, resulted in a frameshift, inducing a 24-amino acid sequence entirely different from the normal sequence. Accordingly, PEX19 is the eighth gene identified to date responsible for the peroxisome-deficient diseases (Table 2). Moreover, given the fact that a mutation C296S abolished the complementation of peroxisomes in ZP119 and CG-J patient fibroblasts, we conclude that prenylation is essential for the biological activity of Pex19p. Very recently, Goette et al. (23) reported similar findings, including farnesylation of \textit{S. cerevisiae} Pex19p being required for peroxisome biogenesis and growth in oleic acid.

Upon transfection of \textit{HsPEX19} into ZP119 devoid of peroxisomal “ghosts,” most striking was the formation of peroxisomal membranes, apparently followed by import of matrix proteins, including catalase and urate oxidase, if not all, into peroxisomal membrane vesicles. Interestingly, a temporally differential translocation of some matrix proteins, catalase at a different rate in \textit{S. cerevisiae} Pex19p binds Pex3p (23).

Further investigation, with ZP119 and ZP165 together with PEX19, should shed light on the molecular mechanisms involved in peroxisome biogenesis, and will allow dissection of the mechanism of membrane vesicle formation and import of matrix enzymes at the molecular level.

We thank T. Utsumi for helpful advice concerning the \textit{in vitro} prenylation assay and the members of the Fujiki laboratory for discussion. This work was supported in part by a Core Research for Evolutional Science and Technology (CREST) grant (to Y.F.) from the Science and Technology Corporation of Japan and Grants-in-Aid for Scientific Research (08557011 to Y.F.) from The Ministry of Education, Science, Sports and Culture.