The Peroxin Pex14p

**cDNA CLONING BY FUNCTIONAL COMPLEMENTATION ON A CHINESE HAMSTER OVARY CELL MUTANT, CHARACTERIZATION, AND FUNCTIONAL ANALYSIS**

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Rat cDNA encoding a 376-amino acid peroxin was isolated by functional complementation of a peroxisome-deficient Chinese hamster ovary cell mutant, ZP110, of complementation group 14 (CG14). The primary sequence showed 28 and 24% amino acid identity with the yeast Pex14p from Hansenula polymorpha and Saccharomyces cerevisiae, respectively; therefore, we termed this cDNA rat PEX14 (RnPEX14). Human and Chinese hamster Pex14p showed 96 and 94% identity to rat Pex14p, except that both Pex14p comprised 377 amino acids. Pex14p was characterized as an integral membrane protein of peroxisomes, exposing its N- and C-terminal parts to the cytosol. Pex14p interacts with both Pex5p and Pex7p, the receptors for peroxisome targeting signal type 1 (PTS1) and PTS2, respectively, together with the receptors’ cargoes, PTS1 and PTS2 proteins. Mutation in PEX14 from ZP110, the same CG as ZP110, was determined by reverse transcription-PCR as follows. A 133-base pair deletion at nucleotide residues 37–169 in one allele created a termination codon at 40–42; in addition to this mutation, 103 base pairs were deleted at positions 385–487, resulting in the second termination immediately downstream the second deletion site in the other allele. Neither of these two mutant forms of Pex14p restored peroxisome biogenesis in ZP110 and ZP114, thereby demonstrating PEX14 to be responsible for peroxisome deficiency in CG14.

The peroxisome is a ubiquitous, spherical intracellular organelle bounded by a single membrane and with a diameter of 0.1–1 μm. Peroxisomes are present in eukaryotic cells, from yeast to humans. Peroxisomes function in a wide variety of metabolic pathways, including β-oxidation of very long chain fatty acids; biosynthesis of plasmalogens, structural ether lipids abundant in the central nervous system; interconversion of cholesterol to bile acids; and glyoxylate transamination (1). The functional significance of human peroxisomes is implicated by fatal genetic diseases such as Zellweger syndrome, linked to peroxisomal malfunction and failure of peroxisome biogenesis (2, 3). Genetic heterogeneity has been seen in subjects with these peroxisome-deficient disorders, comprising 12 different complementation groups (CGs) (4–7). Peroxisomal proteins, including membrane proteins, are encoded by nuclear genes, translated on free polyribosomes in the cytosol, and posttranslationally translocated to preexisting peroxisomes (8). Genetic analyses of peroxisome-deficient mutants of yeast and mammalian cells have led to identification of a number of protein factors essential for peroxisome biogenesis (3, 9). Cell fusion studies on peroxisome-deficient Chinese hamster ovary (CHO) mutant cell lines (4, 7, 10–14) and fibroblasts from patients with peroxisomal biogenesis disorders identified 13 CGs (4–7), including rhizomelic chondrodysplasia punctata manifesting the defect solely in peroxisome-targeting signal type 2 (PTS2) protein import. Furthermore, two novel CGs, CG14 and CG15, were identified by newly isolated CHO mutants, ZP110 and ZP114, respectively (15). Thereby, more than 15 genes are likely to be involved in mammalian peroxisome biogenesis. We delineated mammalian PEX1 (16), PEX2 (4, 17), PEX5 (14), PEX6 (18, 19), and PEX12 (20, 21), using peroxisome-deficient CHO cell mutants, demonstrating that peroxisome assembly-defective CHO cell mutants are a useful system to study peroxisome biogenesis and to elucidate primary defects of human peroxisomal biogenesis disorders. We herein isolated rat, human, and Chinese hamster PEX14, which restored peroxisome biogenesis in a CHO cell mutant ZP110, using a genetic complementation cloning strategy (17–22). Pex14p is a peroxisomal integral membrane protein, apparently involved in PTS1 and PTS2 protein import mediated by Pex5p and Pex7p, respectively.

**EXPERIMENTAL PROCEDURES**

Rat Liver cDNA Library and Search for Complementing cDNA—Rat liver cDNA library in a ZAP Express predigested vector (Stratagene, La Jolla, CA), containing unidirectionally inserted cDNA under the CMV promoter, was described (20, 21). The library was divided into small pools, each containing about 2,000 clones. Combined cDNA pools consisted of an equal amount of three small pool mixtures, containing approximately 6,000 independent clones. For transfection of a cDNA library, ZP110 cells were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum. Cells that had been plated on a coverslip 1 day before transfection at 1 × 10^5 cells/well of a six-well plate were washed twice with serum-free F-12 medium. One μg of plasmid DNA and 12 μg of Lipofectamine (Life Technologies, Inc.) were separately diluted with 100 μl of serum-free medium, Opti-MEM (Life Technologies, Inc.) and then mixed and left for 30 min at room temperature before transfection.

The abbreviations used are: CG, complementation group; AOX, acyl-CoA oxidase; CHO, Chinese hamster ovary; GST, glutathione S-transferase; Pex5p and Pex5pL, shorter and a longer isoforms of Pex5p; PEX14, cDNA encoding the peroxin Pex14p; FMP70, 70-kDa peroxisomal integral membrane protein; P90HUV, 9-(1′-pyrene)nonanol/ultra-violet; P12, 12-(1′-pyrene)dodecanic acid; PTS1 and PTS2, peroxisome targeting signal types 1 and 2; CMV, cytomegalovirus; PAG, polycrystalline gel electrophoresis; bp, base pairs.

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tured before pouring onto the cells. After incubation for 1 h at 37 °C, the DNA-liposome mixture was removed by aspiration, and fresh F-12 medium with fetal calf serum was added. The cells were cultured for 2 days and further incubated overnight in 2 ml of serum-free F-12 medium. Among cDNA pools examined, a positive one (A9) that restored peroxisomes in ZP110 was further selected into subpools and amplified. A cDNA fragment (SalI–NoRI fragment) of an isolated clone pBK-CMV-PAK-4, was blunt-ended and subcloned into the Smal site of pBlue-script II KS(−) (Stratagene). Nucleotide sequence of both strands was determined by the dideoxy chain termination method using Dye-termi-nator DNA sequence kit (Applied Biosystems, Foster City, CA). Align-ment was done using a CLUSTAL W program (EMBL, Heidelberg, Germany).

Screening of Human and Chinese Hamster cDNA Library—A 32p-labeled, 65-kilobase pair BamHI fragment of open reading frame from rat (Rattus norvegicus; Rn) PEX14, RnPEX14, was used as a probe to screen 2.4 × 106 colonies of human liver cDNA library (16) in pCMVSF-PORT I (Life Technologies). Three positive clones were isolated; a longer one was sequenced. About 1.5 × 106 independent colonies of a cDNA library from wild-type CHO-K1 cells (14) were also screened with a 32p-labeled RnPEX14 (0.85-kilobase pair SalI–EcoRI fragment). Two of 10 positive clones were subcloned into pBluescript II SK(−) (Stratagene) at SalI and NotI sites.

Transfection of PEX14—An expression plasmid pUCd2Hyg RnPEX14 was generated by inserting a SalI–Apal fragment containing nucleotides residues at −16 to 1,315, taking A of the initiation codon as 1) of the original RnPEX14 cDNA clone, pBK-CMV-PAK-4, to the pUCd28RSaMCMSHyg vector (20, 21). CHO cell mutant ZP110 was trans- fected with pUCd2HygRnPEX14, followed by selection in the presence of 200 μg/ml hygromycin B (Sigma). The hygromycin B-resistant colonies formed on the coverslips were examined for complementation of peroxisomes, 4 days after transfection. Peroxisome-restored colonies were counted. Seven other groups of CHO cell mutants, Z44, Z65, ZP92, ZP105, ZP109, ZP114, and ZP119, and fibroblasts derived from peroxisome-deficient patients were similarly transfected with pUCd2HygRnPEX14. A stable cell clone of the RnPEX14 transformant of ZP110 was isolated as follows. Three of five hygromycin B-resistant transformant clones were peroxisome-positive, on the basis of immunostaining; one of the three, named 110P14, was cloned by the limiting dilution method. Transfection with human and Chinese hamster PEX14 (HsPEX14 and ClPEX14, respectively) was likewise done, as with RnPEX14.

Morphological Analysis—Peroxisomes in CHO cells and human fibroblasts were visualized by indirect immunofluorescence light micros-copy (Olympus, Tokyo, Japan) and confocal laser scanning microscopy (Olympus, Tokyo, Japan) using a number 17 filter, as described by Tateishi et al. (15). Antibodies used were rabbit antibodies to rat liver catalase (11), human catalase (4), PTS1 peptide (14), 70-kDa peroxisomal integral membrane protein (PMP70) (11). Anti-Pex14 antibody was raised in rabbits by immunizing with synthetic peptide comprising the C-terminal, 19-amino acid sequence of rat Pex14p (24). Protein in each fraction was separated by SDS–PAGE, and electrophoretically transferred onto polyvinylidene difluoride membrane (Bio-Rad). Pex14p and marker proteins were probed with respective antibodies and then visualized with ECL Western blotting detection reagent (Amersham Pharmacia Biotech). For determination of intraperoxisomal localization, peroxisomal fraction was diluted with 20 mM Hepes–ROH, pH 7.6, and sonicated four cycles for 30 s each in the presence of 1 mM NaN3 (17). Membrane and soluable fractions were separated by centrifugation for 30 min at 100,000 × g. Sodium carbonate treatment (26) and Triton X-114 extraction (27) were performed as described.

Expression of Fusion Protein—Glutathione S-transferase (GST) fusions protein with rat Pex14p was constructed in Escherichia coli expression vector pGEX4T-1 (Amersham Pharmacia Biotech) as follows. The 3′-half of the PvuII fragment of pBS-RnPEx14 was inserted into the Smal site of pGEX4T-1. This vector expresses the C-terminal region of Pex14p, termed GST-C. The 5′-half of the RnPEx14 was amplified using PCR primers (forward, 5′-GCCGATCTGGCAGAAGTGGCTCCG-3′; reverse, 5′-TCAGGGCTGTGCCCGTCCT-3′). Flag-tagged RnPEx14 was in the pBluescript II SK(−) vector and then cloned into pBluescript II SK(−) (Stratagene) using SalI–XhoI restriction enzyme site. The recombinant plasmid was then digested with XbaI and NotI sites and ligated with the FLAG vector (pUCd2Hyg RnPEx14) to give the pUCd2Hyg RnPEx14-Flag plasmid. The 5′-end of the FLAG plasmid was then digested with NotI and SalI sites and ligated with the FLAG vector (pUCd2Hyg RnPEx14) to give the pUCd2Hyg RnPEx14-Flag plasmid.

Expression of Pex14p—An epitope, myc, tagging to the C terminus of Pex14p was done with a PCR-based technique, using a forward primer (5′-CTCGAGATCCCAAGAGGAGATGGCTCCCT-3′) and an antisense r1 (5′-GCCACACTGGCCATGATG-3′) to cover a full length of PEX14 Open reading frame. PCR products were cloned into pBluescript II SK(−) and sequenced as described (21). ZP161-derived PEX14 cDNA was cloned into pUCd28RSaMCMSHyg and transfected into CHO cells by lipofection.

Expression of Epitope-tagged Pex14p—An epitope, myc, tagging to the C terminus of Pex14p was done with a PCR-based technique, using a forward primer (5′-CTCGAGATCCCAAGAGGAGATGGCTCCCT-3′) and a reverse primer (5′-GCCACACTGGCCATGATG-3′) containing the myc epitope (underline) and a stop codon (RnPEx14). The pcDNA3 plasmid was cut with XbaI and NotI sites, and the RnPEx14 fragment was ligated with the myc epitope (underline) and a stop codon (RnPEx14). A cDNA fragment (SalI–NoRI fragment) of an isolated clone pBK-CMV-PAK-4, was blunt-ended and subcloned into the Smal site of pBlue-script II KS(−) (Stratagene). Nucleotide sequence of both strands was determined by the dideoxy chain termination method using Dye-termi-nator DNA sequence kit (Applied Biosystems, Foster City, CA). Alignment was done using a CLUSTAL W program (EMBL, Heidelberg, Germany).
Pex7p-expression vector was constructed as follows. Blunted SmaI–SalI fragment of human PEX7 in pTZ18R (28) was ligated into the blunted SalI site of E. coli expression vector pQE30, containing a histidine tag sequence (QIAGEN, Hilden, Germany).

**Purification of Fusion Protein**—Five hundred μl of each of 16-h culture of E. coli, separately expressing GST and GST fusion protein, were diluted with 10 volumes of YT medium at 2-fold concentration. After 1 h of culture at 37 °C, isopropyl-D-thiogalactoside was added to the final concentration of 1 mM. After 2 ha t 37 ° C, the cells were harvested, resuspended in 1 ml of chilled PBS containing 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and then sonicated. Lysates were mixed with Glutathione-Sepharose beads at 4 °C. After a thorough wash, the protein-bound Sepharose beads were suspended in 10 volumes of binding assay buffer (400–500 μI) described above. The reaction was done overnight at 4 °C. A binding reaction was also done, using GST-Pex7p (5 μg) each of two isoforms of Pex5p (3 μg) (i.e. Pex5pS or Pex5pL), 35S-labeled 3-ketoacyl-CoA thiolase (29) (5 μl) in the binding assay buffer (400–500 μl) described above. The reaction was done overnight at 4 °C. A binding reaction was also done, using GST-Pex14p (5 μg) each of the two isoforms of Pex5p (3 μg) (i.e. Pex5pS or Pex5pL), 35S-labeled rat acyl-CoA oxidase (29) (5 μl), GST-Pex14p (20 μg), and 35S-labeled rat acyl-CoA oxidase (29) (5 μl). Proteins were recovered using glutathione-Sepharose, washed four times with the binding assay buffer minus BSA, and analyzed by SDS-PAGE using 12% or 4–20% polyacrylamide gel. Radioactive protein bands were detected using a FujiX BAS1000 Bio-Autoimazing Analyzer (Fuji Photo Film, Tokyo, Japan) and a Fuji BAS1000 Bio-Autoimazing Analyzer (Fuji Photo Film, Tokyo, Japan).

**Other Methods**—Continuous cell labeling with [35S]methionine and [35S]cysteine was done by culturing cells for 24 h in a medium containing 20 μCi/ml [35S]methionine and [35S]cysteine (Amersham Pharmacia Biotech). In vitro transcription/translation (30) were performed as described. Immunoprecipitation of proteins using antibodies to rat AOx and 3-ketoacyl-CoA thiolase and a catalase latency assay using digitonin were described.

**Amino acid sequence alignment of PEX14 protein from three mammalian species and yeast Pex14p from S. cerevisiae and H. polymorpha**. Deduced amino acid sequence of rat (Rn) PEX14 was compared with those of Pex14p from human (HsPEX14), Chinese hamster (CiPEX14), S. cerevisiae (ScPEX14) and H. polymorpha (HpPEX14). Hyphens represent spaces. Identical amino acids between species, including two or more mammalian ones, are shaded. A putative membrane-spanning segment is underlined; the dashed underline indicates a predicted coiled-coil region, where conserved hydrophobic amino acids are marked by dots. The sequence used for chemical synthesis of Pex14p peptide is shown by a dashed underline. The solid arrowheads indicate the position of mutation in ZP161 (see Fig. 4). The GenBank™ data base accession numbers for rat, human, and Chinese hamster PEX14 genes are AB017544, AB017546, and AB017545, respectively.

Pex7p was isolated as follows. His-Pex7p was purified by affinity chromatography using a nickel-nitrolitriacetic acid-agarose column (QIAGEN), where elution was done with 0.5 M imidazole, 8 M urea, according to the procedure recommended by the manufacturer. His-Pex7p was used at a 10–20-fold dilution.

**In Vitro Binding Assay**—Binding reaction mixture contained protein components to be examined, including 20 μg of GST or GST-Pex14p, His-Pex7p (5 μg), and 35S-labeled 3-ketoacyl-CoA thiolase (29) (5 μl) in the binding assay buffer (400–500 μl) described above. The reaction was done overnight at 4 °C. A binding reaction was also done, using GST-Pex14p (5 μg) each of the two isoforms of Pex5p (3 μg) (i.e. Pex5pS or Pex5pL), 35S-labeled rat acyl-CoA oxidase (29) (5 μl), GST-Pex14p (20 μg), and 35S-labeled rat acyl-CoA oxidase (29) (5 μl). Proteins were recovered using glutathione-Sepharose, washed four times with the binding assay buffer minus BSA, and analyzed by SDS-PAGE using 12% or 4–20% polyacrylamide gel. Radioactive protein bands were detected using a FujiX BAS1000 Bio-Autoimazing Analyzer (Fuji Photo Film, Tokyo, Japan) and a FujiX BAS1000 Bio-Autoimazing Analyzer (Fuji Photo Film, Tokyo, Japan).

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nin were performed as described (11). For Northern blotting, RNA was isolated from CHO cells and the liver of a rat treated for 7 days with clofibrate and of an untreated rat. The blot was hybridized with the BamHI fragment (nucleotide residues at 232–605) of \( RnPEX14 \) labeled with \([\alpha-32P]dCTP \) (Amersham Pharmacia Biotech). Western blot analysis was done with primary antibodies, including anti-Pex12p antibody (20, 21), and a second antibody, donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech), using ECL Western blotting detection reagent (Amersham Pharmacia Biotech). 12-(19-pyrene)dodecanoic acid/long wavelength ultraviolet light (P12/UV) and 9-(19-pyrene)nonanol/UV (P9OH/UV) resistance was determined under conditions of 2 \( \mu \text{g}/1.5 \text{ min} \) and 6 \( \mu \text{g}/2 \text{ min} \) (4), respectively.

**RESULTS**

**Cloning of a Rat PEX14 cDNA**

We used a transient expression assay as a cDNA cloning strategy (16, 18, 20, 21) on a CG14 CHO cell mutant, ZP110, defective in peroxisome assembly (15) (Fig. 1a). A cDNA library divided into small pools was transfected to ZP110. Peroxisome-restoring positive cDNA clones were searched for by immunofluorescence staining of catalase, a peroxisomal matrix marker enzyme. One combined pool (A9) yielded several peroxisome-restored cells of ZP110, in a single dish (Fig. 1b, arrows). After a fourth round of screening, one positive clone, named pBK-CMV-zPAF-4, was isolated, which restored peroxisomal import of catalase in ZP110 (data not shown). The cDNA portion of pBK-CMV-zPAF-4 was sequenced on both strands, indicating that the cDNA was 1,921 bp in length with an open reading frame encoding a protein consisting of 376 amino acids (Fig. 2). The calculated molecular mass of its deduced amino acid sequence was 40,936 Da. The amino acid sequence showed 28 and 24% identity with those of Pex14p from *Hansenula polymorpha* (31) and *Saccharomyces cerevisiae* (32, 33), respectively (Fig. 2).
Dysfunction of Pex14p Impairs Peroxisome Assembly in Mammals

Table I

| Cell     | Peroxisome  | Catalase latency | P90H/UV | P12/UV |
|----------|-------------|------------------|---------|--------|
| CHO-K1   | +           | 70               | <0.01   | 87     |
| ZP110    | −           | 1.4              | 91      | <0.01  |
| 110P14   | +           | 63               | <0.01   | 90     |

Thus, we termed this cDNA rat PEX14, RnPEX14. RnPEX14 complemented peroxisomal import of catalase in ZP110 (Fig. 1c). Human (HsPEX14) and Chinese hamster (ClPEX14) cDNA were also cloned by colony hybridization from human and Chinese hamster cDNA libraries, using RnPEX14 as probe. Both HsPEX14 and ClPEX14 encoded 377-amino acid Pex14p, with 96 and 94% identity to rat Pex14p at a deduced amino acid sequence level, while rat Pex14p was shorter by one amino acid, at alignment position 329 (Fig. 2). Pex14p apparently contains at least one hydrophobic segment and a coiled-coil domain (Fig. 2, underline and broken overline).

**PEX14 Restored Peroxisome Biogenesis in ZP110**

Several phenotypic abnormalities due to peroxisome deficiency, such as impaired import of PTS1 and PTS2 proteins, were found in ZP110 (15). To determine whether RnPEX14 could correct these phenotypes, a stable RnPEX14 transformant of ZP110, named 110P14, was isolated. PTS1 proteins were noted in numerous vesicular structures, presumably peroxisomes, when stained with antibodies to PTS1 (Fig. 1d). Catalase and 3-ketoacyl-CoA thiolase (data not shown). Numerous PMP70-positive particles were detected in 110P14 cells (Fig. 1f), whereas peroxisomal remnants, larger PMP70-positive vesicles but fewer in number, were discernible in ZP110 (15) (Fig. 1e). These results strongly suggested that 110P14 cells had morphologically normal peroxisomes, as seen in the wild-type CHO-K1 cells. When other CHO cell mutants, ZP111 (15) and ZP161 (7), of the same CG as ZP110, were transfected with human PEX14 (HsPEX14), PTS1 proteins were likewise localized in peroxisomes, demonstrating HsPEX14 to be functional in CHO cells (see Table II).

In peroxisome-deficient cells, peroxisomal proteins are mislocalized to the cytosol, rapidly degraded, or not converted to mature forms, despite normal synthesis (4, 11, 13). In the digitonin titration assay, nearly 60% of the catalase activity was latent at the digitonin concentration of 100 μg/ml in the wild-type cells (Fig. 3A). In ZP110 cells, full activity of catalase was detected at 100 μg/ml digitonin, with the same latency profile as lactate dehydrogenase, a cytosolic enzyme; hence, catalase was present in the cytosol (Fig. 3A). This was consistent with our earlier observation (4, 11, 16, 21). In 110P14 cells, catalase showed almost the same latency as in the wild-type CHO-K1 cells, demonstrating restoration of peroxisome biogenesis.

AOx, the first enzyme of the peroxisomal fatty acid β-oxidation system, is synthesized as a 75-kDa polypeptide (A component) and is proteolytically converted into 53- and 22-kDa polypeptides (B and C components, respectively) in peroxisomes (11, 30, 34). All three polypeptide components were evident in CHO-K1 cells, upon continuous cell labeling with [35S]methionine and [35S]cysteine followed by immunoprecipitation (Fig. 3B, lane 1), but only the A component was seen in the ZP110, in a much smaller amount, probably due to rapid degradation (11) (Fig. 3B, lane 2). The three components of AOX were found in 110P14, as in CHO-K1 cells (Fig. 3B, lane 3), indicative of proper import and proteolytic conversion of AOX. Peroxisomal 3-ketoacyl-CoA thiolase, the third enzyme of the peroxisomal β-oxidation system, is synthesized as a larger precursor with an amino-terminal presequence that contains a coiled-coil domain (Fig. 3B, lane 4), thereby reflecting rapid processing of the precursor form. In ZP110 cells, only the larger precursor was found (lane 5), implying the absence of processing activity. 110P14 cells showed only the mature form of thiolase, demonstrating the complementation of PTS2 protein import and processing (Fig. 3B, lane 6). Proper processing of AOX and thiolase was also evident, as assessed by immunoblotting, in 161P14, i.e. ZP161 cells stably transfected with RnPEX14, confirming the restored biogenesis of peroxisomes (Fig. 3C). These results demonstrated that RnPEX14 restored peroxisome biogenesis in ZP110 and ZP161.

Treatment with P12/UV specifically kills peroxisome-deficient mutants, due to almost no synthesis of plasmalogen (4, 39), whereas P90H/UV selection kills wild-type cells, which incorporate this fatty alcohol analogue into plasmalogen molecules (4, 40). 110P14 cells showed the resistance to P12/UV treatment and P90H/UV-sensitive phenotype, as was the case for CHO-K1 cells (Table I). Conversely, ZP110 was sensitive to the P12/UV but resistant to the P90H/UV, consistent with our earlier observation (15). Taken together, RnPEX14 could fully complement the abnormality of CG14 ZP110.

At the 4th day after RnPEX14 transfection, CG14 CHO mutants, ZP110, ZP111, and ZP161, all were mostly complemented for peroxisome assembly, whereas none of seven other CGs of peroxisome-deficient CHO cell mutants, ZP92, Z24/ ZP107, Z65, ZP105, ZP109, ZP114, and ZP119, showed peroxisomes (Table II). Furthermore, RnPEX14 was introduced into

Table II

| CHO mutant | Peroxisome-positive clone | Patient fibroblasts | Peroxisome-positive | Gene |
|------------|---------------------------|---------------------|---------------------|------|
| ZP110      | 35/40                     |                     |                    | ZPE14 |
| ZP111      | 34/40                     |                     |                    |       |
| ZP161      | 32/40                     | A                   | (VIII)             |       |
|            |                           | B                   | (V)                | PEX10 |
| ZP92       | 0/40                      | (C)                 | IV                  |       |
| Z24/ZP107  | 0/40                      | (D)                 | IX                  |       |
| Z65        | 0/40                      |                     |                    |       |
| ZP105      | 0/40                      | (E)                 |                   |       |
| ZP109      | 0/40                      | (F)                 |                   |       |
| ZP114      | 0/40                      | (G)                 |                   |       |
| ZP119      | 0/40                      |                     |                    |       |
fibroblasts from patients with peroxisomal biogenesis disorders, such as Zellweger syndrome, of CGs A, B, D, and G of Gifu University (Gifu, Japan) (15) and CG-VI of the Kennedy-Krieger Institute (Baltimore, MD) (15) that were distinct from CHO mutants. As expected, none of the peroxisomal biogenesis disorder fibroblasts were morphologically restored for peroxisome assembly (Table II). These results, together with those described above, demonstrate that Pex14p is a peroxisome biogenesis factor only for CG14.

Expression of PEX14

Peroxisomes and peroxisomal proteins are induced in rat liver by administration of hypolipidemic agents, such as clofibrate (41, 42). RnPEX14 mRNA was not induced by clofibrate, as assessed by Northern blotting (Fig. 4A, lanes 1 and 2, upper panel), under which AOx mRNA was induced by about 50-fold (middle panel), findings consistent with previous observations (21, 42, 43). PEX14 mRNA was detected as a single band of ~2 kilobase pairs in CHO-K1 and ZP161 (Fig. 4A, lanes 3 and 4).

The amount and size of PEX14 mRNA were indistinguishable between the mutant and wild-type CHO cells, suggesting that alteration in the PEX14 gene in ZP161 was not at a transcriptional level. PEX14 mRNA in ZP161 cells was similar in size but at much lower levels, as compared with those in rat liver (Fig. 4A; note that poly(A)+ RNA was loaded on lanes 3–5). It is noteworthy that PEX14 mRNA in ZP110 was nearly undetectable, implying alteration at a transcriptional level or rather rapid degradation of PEX14 mRNA (Fig. 4A, lane 5). The undetectable level of PEX14 mRNA in ZP110 was confirmed by reverse transcription-PCR, using up to 2–5 μg of poly(A)+ RNA (data not shown).

Dysfunction of Pex14p Impairs Peroxisome Assembly in Mammals

PEX14 in Mutants—To investigate dysfunction of Pex14p in ZP161, we determined the nucleotide sequence of Pex14p cDNA isolated from ZP161 by reverse transcription-PCR. In 12 independent cDNA clones isolated, six clones showed a 133-bp deletion from nucleotide C at position 37 of a codon for Pro13 to

**Fig. 4. Complementation of ZP161 and mutation analysis.** A, Northern blot analysis of PEX14 mRNA. RNA was separated, transferred to Zeta-Probe GT membrane (Bio-Rad), and hybridized with 32P-labeled cDNA probes for rat PEX14 (upper panel), and rat acyl-CoA oxidase (AOx, middle panel), respectively. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, lower panel) cDNA was used as a control probe to check the amount of RNA loaded. Washing was done twice with 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA/0.5% SDS at 60 °C. Lanes 1 and 2, total RNA (20 μg) from the livers of a normal (NL) and a clofibrate-treated (CL) rat, respectively; lanes 3 and 4, poly(A)+ RNA (5 μg) from wild-type CHO-K1 and ZP161 cells, respectively; lane 5, poly(A)+ RNA (2 μg) from ZP110 cells. Exposure time was 72 h (lanes 1 and 2, upper panel), and 24 h (lower panel), and 28 h (middle panel). Radioactive bands in lanes 3–5 were detected using a FujiX BAS1000 Bio-Imaging Analyzer at an exposure for 18 h. B, mutation analysis of PEX14 in ZP161. Partial sequence and deduced amino acid sequence of PEX14 cDNA isolated from CHO-K1 and a mutant ZP161 are shown. ZP161Δ1 is a 133-bp deletion at nucleotide residues 37–169 (Fig. 2, solid arrowhead), creating a termination at 40–42 in ZP161 cells; ZP161Δ2 is a second 103-bp deletion at 385–487 resulting in a termination at 385–387 (see Fig. 2). C, transfection of mutated Chinese hamster PEX14. a, ZP161 cells were transfected with Chinese hamster PEX14, ClPEX14; b, ZP110 transformed with ZP161-derived ZP161Δ1, ClPEX14del133; c, ZP161 back-transfected with ZP161Δ2, ClPEX14del133/103. Fluorescence microscopic analysis was done using anti-PTS1 antibody. Magnification, × 630; bar, 20 μm.
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Complementation of Protein Transport by ClPEX14—When mutant ZP161 cells were transfected with ClPEX14 cDNA from the wild-type CHO-K1, PTS1 proteins were found in punctate staining, thereby indicating complementation of peroxisomal import (Fig. 4C, panel A), as was the case by HsPEX14 (data not shown). Transfection of a mock vector did not alter intracellular location of PTS1 proteins in ZP161 (data not shown).

To assess the impaired function of Pex14p in ZP161, two types of ZP161-derived PEX14 cDNAs, ClPEX14del133 with a 133-bp deletion and ClPEX14del133/103 with two-site, 133- and 103-bp deletions, were separately transfected back to the mutant cells. PTS1 proteins were present in the cytosol, in a diffuse manner, in both ZP110 transfected with ClPEX14del133 and ClPEX14del133/103-transfected ZP161 (Fig. 4C, b and c, respectively), hence demonstrating dysfunction of the mutated forms of Pex14p. Similar results were likewise obtained for ClPEX14del133-transfected ZP161 and ClPEX14del133/103-transfected ZP110 cells (data not shown). Import of catalase was not restored in these transfectants as assessed by cell staining with anti-catalase antibody (data not shown). Moreover, ZP111, another mutant of CG14 (15), showed cytosolic staining of PTS1 proteins in transfectants with ZP161-derived PEX14 (data not shown), confirming the impaired function of ClPEX14del133 and ClPEX14del133/103.

Collectively, we conclude that dysfunction of Pex14p caused by truncation is the primary defect in impaired peroxisome biogenesis in CG14 CHO mutant ZP161.

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Fig. 5. Subcellular localization of Pex14p. A, size comparison of in vitro transcription/translation product of rat PEX14 cDNA and rat liver Pex14p. [35S]Methionine-labeled, in vitro transcription/translation product of RnPEX14 and subcellular fractions of rat liver were subjected to SDS-PAGE and transferred to Biodyne B nylon membrane. Autoradiography for lanes 1-3 was exposed for 3 days; immunodetection was done for lanes 4-8 with rabbit anti-Pex14p peptide antibody. Lane 1, in vitro transcription/translation product (1 μl) of RnPEX14; lanes 2 and 3, immunoprecipitation of 35S-Pex14p (10 μl) was done with preimmune and anti-Pex14p immune sera, respectively; lane 4, liver homogenates (80 μg); lane 5, postnuclear supernatant fraction (PNS) from liver homogenates (80 μg); lanes 6 and 7, cytosolic and organelar fractions from PNS; lane 8, rat liver peroxisomes (20 μg), respectively. The arrowhead indicates Pex14p; the dot designates an apparently cleaved product of Pex14p, as reported for yeast Pex14p (32). A fuzzy band below Pex14p in lanes 4 and 5 is an unidentified protein. B, isogenic subcellular fractionation. Light mitochondrial fraction (12 mg) of rat liver was fractionated by isopycnic sucrose density gradient ultracentrifugation. The gradient was collected into 15 tubes. After marker enzyme and protein assays, an equal volume of each fraction (5 μl, 10 μg of protein at original fraction 13; 1 μl for detection of AOx) was analyzed by immunoblot. Upper panel, distribution of marker enzymes and protein: catalase for peroxisomes; glutamate dehydrogenase, mitochondria; esterase, microsomes; N-acetyl-β-D-glucosaminidase, lysosomes. Lower panel, immunoblots with antisera against Pex14p, Pex12p, and AOx. Arrowheads, AOx components A and B. Results are presented in the direction of lower to higher density of sucrose, from left to right.
Intracellular Localization of Pex14p

The C-terminal peptide of rat Pex14p (residues 357–376) was used to raise rabbit antibody. This antibody reacted with a protein of an apparent mass of ~56 kDa, larger than the predicted size, in immunoblot of rat liver homogenates (Fig. 5A, lane 4). The mobility of Pex14p in SDS-PAGE was indistinguishable from that of Pex14p synthesized in vitro by coupled transcription/translation of RnPEX14 that was specifically immunoprecipitated by anti-Pex14p antibody but not by preimmune serum (lanes 1–3), thereby indicating that a cloned RnPEX14 encodes bona fide Pex14p. This result implies the synthesis of Pex14p at its final size, consistent with a general rule for peroxisomal proteins (8) (see below). The reason for the reduced mobility of Pex14p in SDS-PAGE is presently unknown.

Intracellular localization of Pex14p was investigated by subcellular fractionation of rat liver. Pex14p was detected in a postnuclear supernatant fraction and then exclusively present in the organelar fraction, not in cytosol (Fig. 5A, lanes 5–7). Pex14p was apparently enriched in isolated peroxisomes (lane 8). Upon further fractionation of the light mitochondrial fraction by sucrose density gradient centrifugation, Pex14p was detected as a single band and co-sedimented with a peroxisomal marker enzyme, catalase, and AOX as well as peroxisomal integral membrane proteins, such as Pex12p, although less in amount (20, 21) and PMP70 (data not shown), thereby indicating that Pex14p is a peroxisomal protein (Fig. 5B). This was consistent with morphological observation (see below). The apparently slight difference in the distribution of Pex12p, as compared with that of Pex14p and AOX, is presently unknown. The distribution of Pex14p on the gradient was distinct from those of the marker enzymes, glutamate dehydrogenase for mitochondria, esterase for microsomes, and N-acetyl-β-glucosaminidase for lysosomes, confirming the peroxisomal location of Pex14p.

The subcellular localization of Pex14p was also determined by immunofluorescent microscopy using Pex14p tagged at its N terminus with an epitope Flag. In the wild-type CHO-K1 cells transfected with Flag-RnPEX14, Pex14p was detected in a punctate staining pattern with anti-Flag antibody (Fig. 6A, a). The pattern was superimposable on that obtained using anti-PTS1 antibody, thereby demonstrating that Flag-Pex14p was targeted to peroxisomes (Fig. 6A, b and c). Similar results were obtained when RnPEX14 was expressed in CHO-K1 cells and stained using anti-Pex14p peptide antibody (data not shown). The flag-Pex14p fully restored peroxisome assembly in ZP110, as efficiently as Pex14p and was colocalized with PTS1 protein, indicating that the N-terminal tagging did not interfere with Pex14p function (Fig. 6A, d and e). These results were interpreted to mean that Flag-Pex14p and Pex14p were translocated to peroxisomes. Flag-Pex14p was colocalized with PMP70 in peroxisomal ghosts in other complementation groups of CHO cell mutants, such as PEX2-defective Z65 (17) (Fig. 6A, f and g), as was the case for Pex12p (21). Thus, translocation of Pex14p does not appear to be impaired in these mutant cells. It is noteworthy that endogenous Pex14p was also detectable by anti-Pex14p antibody, in CHO-K1 and PEX6-deficient ZP92 (4, 18) (Fig. 6A, h and i). Collectively, the data demonstrate peroxisomal localization of Pex14p.

Hydropathy analysis of Pex14p suggested that Pex14p contains at least one hydrophobic segment (see Fig. 2, solid line). Pex14p was not extracted with 50 mM Hepes-KOH (pH 7.6), from freshly isolated rat liver peroxisomes (data not shown). The integrity of Pex14p was verified by sonication in 50 mM Hepes-KOH (pH 7.6), 1 M NaCl; treatment with 1% Triton X-114 (27); and extraction with 0.1 M sodium carbonate (pH 11.5) (26, 44) (Fig. 6B). Pex14p was not extractable with either 1 M NaCl or sodium carbonate, as was the case for peroxisomal integral membrane proteins, PMP70 (25) and Pex12p (20, 21), but in contrast to a matrix enzyme, AOX, thereby strongly suggesting that Pex14p is an integral membrane protein (Fig. 6B, lanes 2, 3, 6, and 7). Upon treatment with Triton X-114, Pex14p, but not PMP70 and Pex12p, was recovered more in an aqueous phase than in a detergent phase (lanes 4 and 5), probably due to the presence of a highly charged region at the N-terminal part of Pex14p. A similar observation for highly charged membrane glycoproteins was reported by Bodier (27).

Membrane Topology of Pex14p

Topology of Pex14p in peroxisomal membranes was investigated by the differential cell permeabilization procedure,
Proteins were detected in particulates similar in number, presolubilizes all cellular membranes, both Pex14p-transfected CHO-K1 cells were treated with Triton X-100, which pressing CHO-K1 cells were treated with Triton X-100, which

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whole cell proteins were analyzed by SDS-PAGE. Immunoblot was done with antibodies to Flag (tag antibody. When the wild-type CHO-K1 cells transfected with Flag-RnPEX14 or RnPEX14-myc were mock-treated (lane 1) or were treated with 25 μg/ml Proteinase K in the presence of 25 μg/ml digitonin (lane 2) or 1% Triton X-100 (lane 3) for 30 min on ice. The reaction was terminated by precipitation using trichloroacetic acid, and whole cell proteins were analyzed by SDS-PAGE. Immunoblot was done with antibodies to Flag (top), myc (middle), and AOx (bottom). Cells loaded were 1 x 10^5 (for the analysis of Flag and myc) and 2 x 10^6 (for AOx), respectively. The open arrowheads indicate AOx components. A band (upward solid arrowhead) with a slightly greater mobility than AOx-B shows a Proteinase K fragment of AOx (24, 30).

where detection of Pex14p was performed using anti-epitope tag antibody. When the wild-type CHO-K1 cells transfected with RnPEX14-myc encoding C-terminally myc-tagged Pex14p were permeabilized with 25 μg/ml of digitonin, under which plasma membranes are selectively permeabilized and intraperoxisomal proteins are inaccessible to exogenous antibodies (20, 21). Pex14p-myc was observed in a punctate staining pattern, whereas almost no staining of cells was noted with anti-PTS1 antibody (Fig. 7A, a and b). When the Pex14p-myc-expressing CHO-K1 cells were treated with Triton X-100, which solubilizes all cellular membranes, both Pex14p-myc and PTS1 proteins were detected in particulates similar in number, presumably peroxisomes (Fig. 7A, a and d). The data demonstrating exactly the same topology were also obtained with a set of antibodies to Pex14p C-terminal peptide and catalase (not shown). Similar results were likewise obtained in studies of RnPEX14-myc-transfected ZP110 cells (data not shown). CHO-K1 cells expressing Pex14p-myc were permeabilized as above and then incubated with Proteinase K. In an immunoblot of mock-treated cells, Pex14p-myc was detected using anti-myc antibody, where AOx was used as a matrix protein marker (Fig. 7B, lane 1). Upon cell permeabilization with digitonin, Pex14p-myc became sensitive to Proteinase K and was no more detectable, while AOx was not accessible to the protease (lane 2). Both Pex14p and AOx were no more discernible after protease treatment in the presence of Triton X-100 (lane 3). The data together strongly suggest that the C-terminal part of Pex14p-myc is exposed to the cytosol.

To determine the membrane topology of N-terminal part of Pex14p, N-terminally Flag-tagged Pex14p was expressed in CHO-K1 cells. Flag-Pex14p was detected in an immunoblot, using antibodies to Flag (Fig. 7B, lanes 1) and Pex14p C-terminal peptide (data not shown). After cell permeabilization using digitonin, Flag-Pex14p became accessible to Proteinase K digestion (Fig. 7B, lane 2), as was the case for Pex14p-myc, thereby indicating the Flag N-terminal part of Pex14p to be facing the cytosol. This conclusion was confirmed by using anti-Pex14p C-terminal peptide antibody (data not shown). It is noteworthy that Flag-RnPEX14-transfected CHO-K1 was not readily stained using anti-Flag antibody (data not shown). It is plausible that the Flag epitope was inaccessible to the antibody, presumably due to steric hindrance by other peroxisomal proteins.

Taken together, the results demonstrate that N- and C-terminal parts of Pex14p are exposed to the cytosol.

Expression and Purification of Fusion Proteins

To investigate whether Pex14p interacts with other peroxins such as a PTS2 receptor Pex7p and Pex5p, a PTS1 receptor, fusion protein of Pex14p with GST, GST-Pex5pS, GST-Pex5pL, and His-tagged human Pex7p were expressed in E. coli. Each protein was purified by affinity chromatography of E. coli lysates using their respective ligand columns. Pex5pS and Pex5pL were isolated from the GST fusion protein. The purity of all proteins was assessed by SDS-PAGE.

Pex14p Interacts with Pex5p and Pex7p

In an in vitro Pex14p-binding assay, ^35S-labeled 3-ketoacyl-CoA thiolase, a PTS2 protein (35, 38), was detected at 20% recovery of the input, as determined by quantitation of the autoradiogram, by incubation with GST-Pex14p plus His-Pex7p (Fig. 8A, lane 5), while ^35S-labeled thiolase was found at a nearly background level with His-Pex5p plus GST, GST-Pex14p, or GST (lanes 2–4), thereby demonstrating Pex7p-mediated interaction of Pex14p with PTS2 protein to be specific (see also Fig. 8A, lower panel). ^35S-labeled AOx, a PTS1 protein (24, 30), was found in an unbound fraction in the assay, thus indicating Pex7p to be specific for PTS2 (data not shown). Moreover, ^35S-labeled Pex7p was recovered by incubation with GST-Pex14p, indicating direct binding of Pex7p to Pex14p (data not shown).

We identified two isoforms of Pex5p, Pex5pS and Pex5pL, that restored peroxisome biogenesis in CG-II CHO cell mutants, ZP105 and ZP139 (14). Pex5pL is involved in peroxiso-

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FIG. 7. Topology of Pex14p. A, differential cell permeabilization study. a and b, CHO-K1 cells transfected with rat PEX14-myc were treated with 25 μg/ml digitonin, under which the plasma membrane was specifically permeabilized (20, 21); c and d, RnPEX14-myc-transfected CHO-K1 was treated with 1% Triton X-100. Cells were stained with mouse anti-myc antibody (a and c) and rabbit anti-PTS1 antibody (b and d). Note that Pex14p was detected after both types of treatments. Magnification, × 630; bar, 20 μm. B, sensitivity to protease treatment. CHO-K1 cells transfected with Flag-RnPEX14 or RnPEX14-myc were mock-treated (lane 1) or were treated with 25 μg/ml Proteinase K in the presence of 25 μg/ml digitonin (lane 2) or 1% Triton X-100 (lane 3) for 30 min on ice. The reaction was terminated by precipitation using trichloroacetic acid, and whole cell proteins were analyzed by SDS-PAGE. Immunoblot was done with antibodies to Flag (top), myc (middle), and AOx (bottom). Cells loaded were 1 x 10^5 (for the analysis of Flag and myc) and 2 x 10^6 (for AOx), respectively. The open arrowheads indicate AOx components. A band (upward solid arrowhead) with a slightly greater mobility than AOx-B shows a Proteinase K fragment of AOx (24, 30).
mal import of not only PTS1 proteins but also PTS2 proteins, while Pex5pS apparently transports only PTS1 proteins (14, 45). Both Pex5pS and Pex5pL bound to GST-Pex14p, as assessed by immunoblot, together with 35S-AOx in the Pex14p-binding assay (Fig. 8, lanes 3 and 4). GST-Pex14p showed almost no direct binding to 35S-AOx (lane 2), while both GST-Pex5pS and GST-Pex5pL bound to 35S-AOx at a similar level (data not shown), thereby implying that both Pex5 isoforms mediate the interaction of Pex14p with PTS1. Specific Pex5p-mediated PTS1 protein binding to Pex14p was envisaged by quantitation of 35S-AOx, where nearly 40% of the input AOx was recovered in the case of both Pex14p/Pex5pS and Pex14p/Pex5pL, evidently indicative of a complex comprising these three components (Fig. 8, lower panel). In contrast, no binding of 35S-labeled thioliase was observed in this assay, confirming Pex5p to specifically recognize PTS1, not PTS2 (data not shown). Similar results showing interaction of Pex14p with Pex5p5S-AOx were obtained using His-tagged protein such as His-Pex5p5, and 35S-AOx, A half aliquot of the input, 35S-AOx, was loaded in lane 1. Protein components used are indicated at the top. 35S-AOx was detected at an exposure for 14 h; Pex5pS and Pex5pL were detected by immunoblot using anti-Pex5p antibody. Lower part, 35S-AOx of each lane was quantitated and is represented as a percentage of the input.

To determine whether Pex14p interacts with itself, GST-Pex14p was incubated with 35S-labeled Pex14p. 35S-Pex14p was recovered with GST-Pex14p at a nearly 4-fold amount as compared with the amount with GST, suggesting that Pex14p proteins bind each other in a homomeric manner (Fig. 9, A and B). The forms of homomeric interaction, i.e. dimer or oligomer, are presently unknown.

**DISCUSSION**

In the present work, we isolated rat PEX14 cDNA encoding 41-kDa integral membrane protein of peroxisomes, by functional complementation of peroxisome-deficient CHO cell mutant, ZP110. CG14 mutants, ZP110, ZP111, and ZP161, were restored in peroxisome assembly, for both morphological and biochemical criteria. None of the fibroblasts from patients with peroxisome deficiency disorders of 11 CGs was complemented, indicating that the PEX14 gene is not the causal gene of any CG of human peroxisome-deficient disorders thus far classified. PEX14 is novel; the eighth gene required for peroxisome assembly in mammals, including humans; and responsible for the defects in peroxisome assembly of CG14. We have shown that a
cloning strategy using mammalian expression vector is highly efficient in delineating peroxisome biogenesis factors in mammals, i.e. PEX1 (16), PEX2 (17, 22), PEX5 (14), PEX6 (18, 19), and PEX12 (20, 21), including the present work. While several human orthologues of yeast genes have recently been cloned by expressed sequence tags. It is noteworthy that functional complementation is an independent, parallel approach that often complements the cloning of human cDNAs of interest using expressed sequence tags. It is noteworthy that Fransen et al. (55) very recently identified Pex14p, using a combination of a peroxisomal protein import system in semipermeabilized CHO cells and antiserum to peroxisomal membrane proteins. Human Pex14p was shown to bind Pex5p in ligand blots, in good agreement with our findings.

The peroxin Pex14p from three mammalian species (i.e. rat, hamster, and Chinese hamster, each comprising 376, 377, and 377 amino acids) all showed nearly 30% identity in the amino acid sequence to Pex14p of H. polymorpha (31) and S. cerevisiae PEX14 (32, 33), indicating a high degree of conservation of Pex14p in eukaryotic evolution. At present, however, we do not know if the yeast PEX14 gene can complement ZP110 and the mammalian PEX14 restores peroxisomal protein import in the yeast mutants, ZP110, ZP111, and ZP161.

In the present study, dysfunction of Pex14p in ZP161 cells was evidently demonstrated to be due to truncation in PEX14 gene transcripts. Furthermore, both alleles of PEX14 were found to be expressed in CHO cells, at least in ZP161, although the type of gene expression in CHO cells has been postulated to be hemizygotic. The level of PEX14 mRNA derived from each allele remains to be determined.

The present finding that Pex14p is located in the peroxisomal membranes as an integral PMP, with topology of the N- and C-terminal parts exposed to the cytosol, agrees well with the observation by Wendland and Subramani (56) demonstrating in patient fibroblasts that the molecular defects are mostly associated with the organelles, while cytoplasmic factors seem to be functionally normal. It is noteworthy that S. cerevisiae Pex14p has been shown to be peripheral (32) or integral (33) membrane protein of peroxisomes, H. polymorpha Pex14p being integral (31). While peroxisomal transport of membrane proteins, including PMP70, appears to be normal in the CG14 mutants, ZP110, ZP111, and ZP161, import of soluble proteins such as PTS1 and PTS2 proteins is defective. We showed that Pex14p interacts with a WD motif protein Pex7p (PTS2 receptor) and PTS2 protein, as was the case for S. cerevisiae Pex14p (32, 33). Pex14p likewise binds to a tetractipeptide repeat (TPR) protein Pex5p (PTS1 receptor), both isoforms Pex5pS and Pex5pL (14), carrying their cargo PTS1 protein. This is compatible with the finding in S. cerevisiae by in vivo studies using the yeast two-hybrid system (32, 33), although only one type of Pex5p is present in yeast.

Given the fact that Pex5p and Pex7p bind to PTS1 and PTS2 proteins, respectively, it is more likely that several types of the receptor-cargo complex, such as Pex5p-PTS1 protein and Pex7p-PTS2 protein, translocate to a peroxisomal import machinery, presumably comprising Pex14p as well as other peroxin(s) and mediating the transport of polypeptides into the peroxisomal matrix. Therefore, Pex14p possibly plays a pivotal role in the peroxisome assembly process as a factor, e.g. a convergent component of the import machinery of matrix proteins but not membrane polypeptides. The interacting site(s) of Pex14p with Pex5p and Pex7p, both carrying their cargoes, are presumably in the cytosolic phase of Pex14p. Precise domains of Pex14p, each responsible for the interaction with Pex5p and Pex7p as well as for the homeric binding, remain to be investigated. It would be also intriguing to study whether Pex14p interacts with other mammalian peroxisome biogenesis factors so far identified, including RING peroxins Pex2p (17, 22, 43), Pex10p (52, 53), and Pex12p (20, 21, 51); AAA family peroxins Pex1p (16, 46, 47) and Pex6p (18, 19, 57); a Src homology 3 peroxisomal membrane protein Pex13p (58); and Pex16p (54). Such studies may be done by in vitro binding assay developed in this report and using semipermeabilized CHO cells, including wild-type CHO-K1 as well as PEX14 mutants ZP110, ZP111, and ZP161.

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