Molecular Anatomy of the Peroxin Pex12p

RING FINGER DOMAIN IS ESSENTIAL FOR Pex12p FUNCTION AND INTERACTS WITH THE PEROXISOME-TARGETING SIGNAL TYPE 1-RECEPTOR Pex5p AND A RING PEROXIN, Pex10p

The three peroxin genes, PEX12, PEX2, and PEX10, encode peroxisomal integral membrane proteins with RING finger at the C-terminal part and are responsible for human peroxisome biogenesis disorders. Mutation analysis in PEX12 of Chinese hamster ovary cell mutants revealed a homozygous nonsense mutation at residue Trp263Ter in ZP104 cells and a pair of heterozygous nonsense mutations, Trp170Ter and Trp114Ter, in ZP109. This result and domain mapping of Pex12p showed that RING finger is essential for peroxisome-restoring activity of Pex12p but not necessary for targeting to peroxisomes. The N-terminal region of Pex12p, including amino acid residues at positions 17–76, was required for localization to peroxisomes, while the sequence 17–76 was not sufficient for peroxisomal targeting. Peroxins interacting with RING finger of Pex2p, Pex10p, and Pex12p were investigated by yeast two-hybrid as well as in vitro binding assays. The RING finger of Pex12p bound to Pex10p and the PTS1-receptor Pex5p. Pex10p also interacted with Pex2p and Pex5p in vitro. Moreover, Pex10p was co-immunoprecipitated with Pex10p from CHO-K1 cells, where Pex5p was not associated with the Pex12p-Pex10p complex. This observation suggested that Pex5p does not bind to, or only transiently interacts with, Pex10p and Pex12p when Pex10p and Pex12p are in the oligomeric complex in peroxisome membranes. Hence, the RING finger of Pex10p is most likely to be involved in Pex5p-mediated matrix protein import into peroxisomes.

Most organellar proteins are synthesized on cytoplasmic polyribosomes and directed to their destined compartments (1). The protein transport is mediated by targeting sequences that are recognized either in the cytoplasm, as in a secretory pathway, or on the surface of organelles, such as mitochondria and chloroplasts. Peroxins matrix and membrane proteins are also synthesized on free polyribosomes and imported posttranslationally into peroxisomes (2). Two distinct topogenic signals, including peroxisome-targeting signal type 1 (PTS1) and PTS2 (3–5), are involved in the translocation of proteins across the peroxisome membrane (10, 11). In contrast, PTS for membrane proteins, termed mPTS, is not well understood, while mPTS for Candida boidinii PMP47 is better known (11).

In recent years, we and other laboratories have identified 16 different complementation groups (CGs) in mammals by CG analysis between Chinese hamster ovary (CHO) cell mutants of 13 CGs and fibroblasts from patients with peroxisome biogenesis disorders (PBDs) of 13 CGs (12, 13). Hence, at least 16 genes or their products are required for mammalian peroxisome biogenesis. Thirteen genes have been identified by functional complementation assay using CHO mutants and homology search on the expressed sequence tag data base. Several peroxins identified are involved in peroxisomal matrix protein import. Pex14p locates on peroxisome membranes and binds the tetracopeptide repeat peroxin Pex5p in both isoforms of the PTS1 receptor, Pex5pS and Pex5pL (14–16). Furthermore, Pex5pL plays the following pivotal role in peroxisomal PTS2 import. Pex5pL, but not Pex5pS, mediates the binding of PTS2 protein to Pex14p by translocating the WD motif peroxin Pex7p, which is a PTS2 receptor (17); disruption of Pex5pL-Pex7p interaction completely abolishes the PTS2 import in mammals (18). The C-terminal SH3 domain of Pex13p, another peroxisomal integral membrane peroxin, binds Pex14p in a ligand overlay assay (14) but not in a yeast two-hybrid assay (15). However, molecular mechanisms involved in the translocation of proteins across the peroxisome membrane are not well understood.

We isolated PEX2 and PEX12 by genetic functional complementation of CHO mutant cell lines, Z65 and ZP109, respectively (19–21), and human PEX10 by the expressed sequence tag data base search using yeast PEX10 (22). PEX2 (23), PEX10 (22, 24), and PEX12 (20, 21, 25) are shown to be responsible for PBD of CG-F (CG10 in the United States), CG-B (CG7), and CG3, respectively. These three peroxins, Pex2p, Pex10p, and Pex12p, are integral membrane proteins of peroxisomes and contain RING zinc finger at the C-terminal part. Impaired function of these three peroxins results in the failure of matrix protein import, implying that RING peroxins are...
involved in the process of protein transport across the membrane, possibly as members of import machinery constituents. Moreover, Pex2p, Pex10p, and Pex12p are mutually distinct in the primary sequence except for a commonly shared RING finger. Thus, the RING finger is probably important for the function of these peroxins.

As a step toward understanding the role of Pex12p in the translocation of proteins into peroxosomes, we investigated the functional region(s) of Pex12p using pex12 CHO cell mutants and searched for peroxins interacting with Pex12p. We found that RING finger was required for the biological activity of Pex12p, not for targeting of Pex12p to peroxosomes. Pex12p interacted with Pex5p and Pex10p through this RING finger. The N-terminal portion facing the cytoplasm was found to be responsible for the biological function and peroxisomal targeting of Pex12p. Chang et al. (26) very recently reported similar findings, using the fibroblasts of CG3 patients.

### EXPERIMENTAL PROCEDURES

**Cell Lines and Culture Condition**—CHO cells, including pex12 mutants ZP104 and ZP109 (21, 27), were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum under 5% CO2, 95% air (28).

**Fibroblasts from a patient (PBD3–02) with PBD of CG3 were cultured with 10% fetal calf serum**.

**Isolation of Total RNA**—Total RNA was isolated from each mutant by the acid guanidium/phenol/chloroform method (32). Using 5 μg of the total RNA as a template, first-strand cDNA was synthesized and amplified with Ex Taq DNA polymerase (Takara, Tokyo, Japan) and the primers, sense ClPEX12 RT.F (Table I) and antisense ClPEX12 RT.R. After digestion with BamHI–ApaI fragment of pUcD2Hyg/FLAG-PEX12, corresponding to amino acid residues 2–202 (21). One of four positive clones was cloned into pBluescript II SK(−) (pBS) (Stratagene) at the SalI–NotI site. The nucleotide sequence of pBS/CIPEX12 was determined on both strands, using a Dye-terminator cycle sequence kit and a 377 DNA sequencer (Perkin-Elmer). Alignment was done with a GENETYX-Mac (Software Development, Tokyo, Japan). Wild-type CIPEX12 cDNA was cloned into a mammalian expression vector pUcD2RSrMCSHyg (31) was prepared by PCR using pBS/CIPEX12 as a template and a set of primers: sense CIPEX12 RT.F (Table I) and antisense CIPEX12 RT.R. After digestion with BamHI, the amplified fragments were cloned into pUcD2RSrMCSHyg at the BamHI site, resulting in the product termed pUcD2Hyg/CIPEX12.

**Synthetic oligonucleotide primers used**

*F* (f) and *R* (r) represent forward and reverse primers, respectively.

| Code       | Sequence (5’ to 3’)                                                                 | Underlined |
|------------|--------------------------------------------------------------------------------------|------------|
| CIPEX12 RT.F | GCGCCGATCTCAGTTGCTGCAAGACGGGCTC                                                   | Initiation codon |
| CIPEX12 RT.R | GCGGGATCCTCGATGCCAGTATATG                                                        | Termination codon |
| del-N5.F | GCGGGATCCTCGATGCCAGTATATG                                                        |            |
| del-N6.F | GCGGGATCCTCGATGCCAGTATATG                                                        |            |
| del-N8.F | GCGGGATCCTCGATGCCAGTATATG                                                        |            |
| del-M2.R | GCGGGATCCTCGATGCCAGTATATG                                                        |            |
| del-M3.R | GCGGGATCCTCGATGCCAGTATATG                                                        |            |
| del-M5.R | GCGGGATCCTCGATGCCAGTATATG                                                        |            |
| 46SalI.f | GCTGCCTACCCGTTTG                                                                |            |
| 46SalI.r | GTGTCCTGAGCTGTCAG                                                               |            |
| ΔRMyr.f | CGTACCAGCGCCCTCACAAGTCTTCTCAAGAAATAAGCTTGGTTGCTTCAATTTGAGTTGAGG AGAGAAGAGAG     |            |
| ΔSalI.f | GCTGCCCTACCGGTGTCGAGGGG                                                          |            |
| ΔPS.f | CGAATTTCGTGACGGCAGTCTGAGTTGA                                                     |            |
| HsP14.SalF | GCGGATCCTCGATGCCAGGCTAGGCGGAGGC                                                  |            |
| HsP14.SalR | GCGGATCCTCGATGCCAGGCTAGGCGGAGGC                                                  |            |
| RnPEX2-SalF | AGCGGATCCTCGATGCCAGGCTAGGCGGAGGC                                                  |            |
| RnPEX2-NotI.R | TACCTAGGGGGCTAAAGAGCCATTCA                                                        |            |
| RnPEX2-Rsal.R | CGTACGCGCCGCTCAGCTGAGTTGA                                                     |            |
| GST-P10C.F | GCGGATCCTCGATGCCAGGCTAGGCGGAGGC                                                  |            |
| GST-P10C.R | GCGGATCCTCGATGCCAGGCTAGGCGGAGGC                                                  |            |
| His-P10C-HA2.F | GCCGATCAGCGCCGCGGAGGC                                                          |            |
| His-P10C-HA2.R | GCCGATCAGCGCCGCGGAGGC                                                          |            |

isoiothyocyanate-labeled sheep anti-rabbit IgG antibody (Cappel) or donkey anti-rabbit IgG antibody conjugated to rhodamine (Chemicon or Bio Source International) for staining with rabbit primary antibodies (21). In the case of staining with mouse monoclonal antibodies, antigen-antibody complexes were detected by fluorescein isothiocyanate- or Texas Red-labeled sheep anti-mouse IgG antibody (Amersham Pharmacia Biotech). Enhanced green fluorescent protein (EGFP) was directly observed by fluorescent microscopy with the use of the same filter for fluorescein isothiocyanate after fixation (13).

**Cloning and Transfection of Chinese Hamster PEX12 cDNA**—Chinese hamster (Cricetulus longicaudatus) PEX12 cDNA (CIPEX12 cDNA) was cloned by colony hybridization assay. About 2.5 × 105 independent colonies of cdna from CHO-K1 cells (30) were screened using as a probe 32P-labeled rat PEX12 cDNA for the N-terminal region (BamHI–SalI fragment from pUcD2Hyg/FLAG-RnPEX12, corresponding to amino acid residues 2–202) (21). One of four positive clones was subcloned into pBluescript II SK(−) (pBS) (Stratagene) at the SalI–NotI site. The nucleotide sequence of pBS/CIPEX12 was determined on both strands, using a Dye-terminator cycle sequence kit and a 377 DNA sequencer (Perkin-Elmer). Alignment was done with a GENETYX-Mac (Software Development, Tokyo, Japan). Wild-type CIPEX12 cDNA was cloned into a mammalian expression vector pUcD2RSrMCSHyg (31) was prepared by PCR using pBS/CIPEX12 as a template and a set of primers: sense CIPEX12 RT.F (Table I) and antisense CIPEX12 RT.R. After digestion with BamHI, the amplified fragments were cloned into pUcD2RSrMCSHyg at the BamHI site, resulting in the product termed pUcD2Hyg/CIPEX12.

**Cloning and Transfection of PEX12 in ZP104 and ZP109—**PEX12 cDNAs from ZP104 and ZP109 were isolated by the reverse transcription-PCR method. Total RNA was isolated from each mutant by the acid guanidium/phenol/chloroform method (32). Using 5 μg of the total RNA as a template, first-strand cDNA was synthesized and amplified with Ex Taq DNA polymerase (Takara, Tokyo, Japan) and the primers, sense CIPEX12 RT.F (Table I) and antisense CIPEX12 RT.R. After digestion with BamHI, the amplified fragments were cloned into pUcD2RSrMCSHyg at the BamHI site, resulting in the product termed pUcD2Hyg/CIPEX12.

**N-terminal Deletion Mutants of Rat Pex12p**—An N-terminal truncation mutant of rat Pex12p, termed ΔN1 (Δ1–78), comprising amino acid residues 77–359, was described (21). Pex12p mutants ΔN2 (Δ1–97) and ΔN3 (Δ1–202) were constructed by self-ligation of BamHI–SalI and BamHI–SalI fragments of pUcD2Hyg/FLAG-RnPEX12, respectively, after adjusting a frame by using an 8-mer BamHI linker. For mutant ΔN4 (Δ1–258), PstI–ApoI fragment of PEX12 from pUcD2Hyg/FLAG-RnPEX12 was blunt-ended only at the PstI end, and 8-mer BamHI linker was added. After digestion with BamHI, the BamHI–ApoI fragment was replaced at the sites of BamHI and ApoI in pUcD2Hyg/FLAG-
Domain Mapping and Interacting Partners of Pex12p

RnPEX12. For mutants ΔN5 (Δ1–25), ΔN6 (Δ1–48), ΔN7 (Δ1–16), and ΔN8 (Δ1–8), the BamHI–XhoI region of full-length FLAG-PEX12 in pUCD25RmMCHHyg was replaced by respective BamHI–XhoI fragments of PCR products amplified by forward primers delM5.F and delM6.R, and reverse primers, RnP12.R.

Several mutants, named the ΔM series, with shorter deletions in the N-terminal region of Pex12p, were constructed. For mutants ΔM1 lacking amino acid residues 17–25 (Δ17–25) and ΔM2 (Δ26–48), BamHI fragments, each encoding amino acid residues 2–16 and 2–25 of Pex12p, were prepared by PCR with each pair of primers, FLAG.F/delM1.R and FLAG.F/delM2.R, using pUCD25RmMCHHyg as a template. PCR products were amplified using forward and reverse primers and digested with BamHI and SalI, respectively. BamHI–SalI fragments were cloned into the BamHI site of ΔN5 and ΔN6, resulting in ΔM1 and ΔM2. Mutant ΔM3 (Δ67–75) was generated by replacing the BamHI–XhoI region of full-length FLAG–PEX12 in pUCD25RmMCHHyg with the BamHI–XhoI fragment of the PCR products amplified by forward primers FLAG.F and delM3.R.2. Mutant ΔM4 (Δ77–97) was constructed by self-ligation after blunting of the SpeI–SalI fragment of pUCD2Hyg/FLAG–RnPEX12. Mutant ΔM5 (Δ98–106) was generated by replacing the SacI–SalI part of pUCD2Hyg/FLAG–RnPEX12 with the SacI–SalI fragment of PCR products amplified by primers delM5.F and 46SalI.r.

C-terminal Deletion Mutants of Rat Pex12p—For C-terminal truncation mutants of Pex12p, pEGFP/RnPEX12 was constructed by cloning the coding region of the Apol fragment of the Pex12p cDNA from pUCD2Hyg/FLAG–RnPEX12 at the sites of BglII (blunted) and Apol in pEGFP-C1 (CLONTECH). pEGFP/RnPEX12 encodes EGFP-Pex12p, Pex12p fused to the C terminus of the EGFP. C-terminal truncation mutants of Pex12p, named ΔC1 (Δ255–359), ΔC2 (Δ203–359), and ΔC3 (Δ177–359), were generated by self-ligation after blunting the digest of pEGFP/RnPEX12 with PstI, SacI, and XhoI, respectively.

For Pex12p mutants with deletion around the RING finger motif at the C-terminal region, ΔR and ΔPL were constructed from pUCD2Hyg/ RnPEX12–nmyc. First, the PstI–Apol fragment of PCR products amplified by primers PstI.F and ΔR∥myc was replaced into the sites of PstI and Apol in pBS46myc, RnPEX12–myn in pBS (21). Resultant pBS/RnPEX12ΔR was then digested with XhoI and Apol, and the fragment was inserted into the sites of SacI and Apol in pUCD25RmMCHHyg vector. mutant ΔR lacking RING finger residues 301–359 (Δ301–359) was tagged with Myc epitope at the C terminus. For mutant ΔPL (Δ225–286), the Apol (blunted)–Apol fragment from pBS46myc was cloned into the PstI (blunted)–Apol site of pBS46myc vector that had been separately prepared. Resultant pBS/RnPEX12ΔPL was digested with XhoI and Apol and cloned into pUCD25RmMCHHyg vector, as done for ΔR. Each mutant ΔPSs were prepared: SacI–Apol fragment of PCR product amplified by primers 46SalI.F and ΔPSs, using pUCD2Hyg/FLAG–RnPEX12 as a template, and Apol–ΔPSs fragment from pBS46myc. The two inserts were simultaneously ligated into the sites of SacI and Apol in pUCD2Hyg/FLAG–RnPEX12. All constructs were confirmed by nucleotide sequencing. These plasmids were transfected into wild-type CHO-K1 and pex12Δ/Δ cells, as described above. At 3 days after transfection, cells were examined by immunofluorescence light microscopy, as described above.

Yeast Two-hybrid Assay—Yeast two-hybrid plasmids were made by inserting a desired gene fragment downstream of the GAL4 DNA binding domain (DB) in pDBLeu and the GAL4 activation domain (AD) in pPC86 (33). For cloning of PEX14 cDNA, full-length PEX14 fragment was amplified by PCR with primers Hps14.PstI and Hps14.SpeR, using PCSPORT/HsPEX14 (16) as a template. After digestion with SacI and SpeI, plasmids pDB/PX14 and pPC/PX14 were prepared by cloning the fragment into the SacI–SpeI site of pDBLeu and pPC86, respectively. For pDB/PX58, the SacI (blunted)–NcoI fragment of the full-length HsPX58 from pUCD2HsPX58–I (30, 34) was ligated into the sites of SpeI (blunted) and NcoI in pDBLeu. For pPC/PX58, the SacI–NcoI fragment of pUCD2HsPX1–I was cloned and blunted into the SalI site of pPC86. For pPC/PX58L, the SacI–NcoI fragment of full-length CYPE58L from pY220/CYPE58L (17) was ligated into the sites of SacI and NcoI of pPC86.

To clone the full-length of PEX12, BamHI (blunted)–DraI fragment of HsPX12 (blunted)–DraI fragment of pUCD2Hyg/FLAG–RnPEX12 was cloned into the SacI–BamHI sites of plasmid pDBLeu and pPC86, respectively. For pDB/PX12 and pPC/PX12, the BamHI–XhoI region of full-length HsPX12 was inserted into the SacI–BamHI sites of pDBLeu and pPC86, as described above. For plasmid pPC/PX12, the BamHI–XhoI fragment of pUCD2Hyg/FLAG–RnPEX12 (22) was inserted into the sites of SpeI (blunted) and BamHI in pPC86, named pPC/PX12. Plasmid pDB/PX12 was constructed by cloning the SacI–NcoI fragment from the pPC/PX12 into the SacI–NcoI site in pDBLeu. To clone the full-length PEX2, the open reading frame of rat PEX2 cDNA was amplified by PCR with the primers RnPEX2-SacI.F and RnPEX2-NcoI.R, using pTZ/ RnPEX2 (35) as a template. After digestion with SacI and NcoI, the fragment was ligated into the sites of SacI and NotI in pDBLeu and pPC86, termed pDB/PXE2F and pPC/ PXE2F, respectively. To clone PEX12 encoding the C-terminal region, the manipulating for removal of excess cell materials by velvet, termed red complex, was done to reduce the background levels. Activity of the HIS3 reporter gene was determined after an additional 48-h incubation at 30 °C by cell growth on SC-LWH plus 15 mM 3AT medium.

Construction for Expression of Fusion Proteins—Glutathione S-transferase (GST) fusion proteins with RING finger domain of three different pexins were prepared in the experiments: GST-Pex12p fusion vectors pGE6P-1 and pGE6P-2 (Amersham Pharmacia Biotech). RnPEX12C, the blunted PstI–Apol fragment of RnPEX12 (see above), was inserted into the SacI site of pGEX6P-1, creating pGEX/PSEX12C. To construct plasmid pGEX/RnPEX12C Mut1 encoding GST-Pex12pR lacking a point mutation at C304S in a RING finger (21), PCR was conducted, using pBS/RnPEX12 Mut1 (21) as a template and primers His-P10C.F and His-P10C.R was replaced at the XhoI site of pGEX6P-1. After Pst digestion, blunting, and SalI digestion, the PCR fragment was ligated into the sites of SacI and SalI in pGEX6P-1, resulting in pGEX/RnPEX12C Mut1. To generate pGEX/PSEX10C, the SacI–NotI fragment created from HsPSEX10 (see above) was ligated into the sites of SacI and NotI in pGEX6P-2. Plasmids pGEX/RnPEX12C coding for GST-Pex2pC (35), pGEX/HsPEX14 (16) coding for GST-Pex14p, and pPC/PSEX5S encoding the shorter form of Pex5p with a hexahistidine tag, His-Pex5pS (17), were as described above.

To construct a fusion protein of Pex12pC with maltose-binding protein (MBP), a PstI–Apol (blunted) fragment of RnPEX12 cDNA from pUCD2Hyg/FLAG–RnPEX12 was cloned into the sites of PstI and HindIII (blunt) in pMAL-c2X (Invitrogen), resulting in pMAL/PSEX12C. For construction of MBP/PSEX10C HA 2, an NheI–BamHI fragment of a PCR product that had been amplified using pUCD2Hyg/FLAG–HsPX10 as a template and primers His-P10C.F and His-P10C.R was replaced at the sites of NheI and BamHI in pBS/HsPX16 HA (38), hence producing pBS/PSEX10C HA 2. Plasmid pMAL/PSEX10C HA 2 was constructed by cloning the BamHI–SalI fragment from pBS/PSEX10C HA 2 into the sites of BamHI and SalI in pMAL-c2X.

Real-time Quantitative Polymerase Chain Reaction—E. coli transformed with GST and GST fusion protein expression vectors was cultured overnight. One hundred μl of each of the E. coli expressing GST and GST-Pex14p was diluted in 5 ml of YT medium. After culturing for 1 h at 37 °C, cells were further grown for 2 h at 37 °C in the presence of 0.1 mM isopropyl-β-D-
thiogalactoside (IPTG) to induce the expression of fusion proteins. In the case of GST-Pex12pC, GST-Pex12pCMut1, GST-Pex10pC, and GST-Pex2pC, 400 μl of each of the E. coli was diluted in 5 ml of YT medium supplement with 1 mM ZnSO4. After culturing for 2 h at 18 °C, cells were grown for another 6 h at 18 °C in the presence of 0.5 mM IPTG to recover the recombinant protein as soluble protein. Harvested cells were resuspended in 400 μl of ice-cold suspension buffer (20 mM Hepes-KOH, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100), sonicated, and centrifuged to remove cell debris. The resulting supernatant was diluted with 600 μl of the suspension buffer and mixed with 150 μl of a 50% slurry of glutathione-Sepharose beads (Amersham Pharmacia Biotech), washed three times with the suspension buffer, and then centrifuged. His-Pex5pS was purified from the resulting supernatant by affinity chromatography using nickel nitrotriacetic acid-agarose beads (QIAGEN), using as an elution solution His lysis buffer containing 0.5 mM imidazole, according to the manufacturer’s procedure.

To purify fusion proteins, MBP-Pex12pC, MBP-Pex10pC-HA2, and MBP-Pex2pC. E. coli cells expressing these respective proteins were induced with IPTG as described for GST-RING proteins, except that the culture medium was supplemented with 0.2 mg/ml of glucose. MBP fusion proteins were affinity-purified using amylose resin (New England Biolab), from cell lysates in suspension buffer containing 0.1% Triton X-100. Protein-bound resin was washed with the suspension buffer plus 0.1% Triton X-100. MBP was eluted with the suspension buffer containing 10 mM maltose according to the manufacturer’s procedure.

In Vitro Binding Assay—Two independent in vitro assays were performed. One used two RING finger domain proteins fused with GST and MBP. Two μg of GST or 1.5 μg each of GST-Pex12pC, GST-Pex12pCMut1, GST-Pex10pC, or GST-Pex2pC were incubated with glutathione-Sepharose beads. To the slurry, 1.5 μg of MBP-Pex12pC or MBP-Pex10pC-HA2, and His-Pex5pS (4.5 μg) was added to 1 ml of the binding buffer (40 mM Hepes-KOH, pH 7.4, 150 mM NaCl, 1% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM PMSF, 2 μg/ml each leupeptin and antipain, 50 units/ml of aprotinin, 1 mM NaF, and 0.2% bovine serum albumin). The other assay was done as follows. GST (2 μg); GST-Pex14p (1 μg); and GST-Pex12pC, GST-Pex12pCMut1, GST-Pex10pC, or GST-Pex2pC (1.5 μg each) were conjugated with glutathione-Sepharose beads separately added to His-Pex5pS (4.5 μg) in 1 ml of the binding buffer. In both assays, the binding reaction was done overnight at 4 °C. Sepharose beads were washed four times with the binding buffer minus bovine serum albumin. Proteins associated with GST fusion proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 10 or 12% polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride membrane (Bio-Rad) and analyzed by immunoblot.

Co-immunoprecipitation with FLAG-Pex10p—A stable transformant of wild-type CHO-K1 expressing FLAG-Pex10p, named K1-FLP10, was isolated by transfection of pUcD2Hyg/FLAG-HsPex10X (22) into CHO-K1 followed by selection in the presence of hygromycin B (22). CHO-K1 and K1-FLP10 cells in culture dish were directly lysed by adding lysis buffer (20 mM Heps-KOH, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 0.1 mM PMSF, 2 μg/ml of each leupeptin and antipain, 50 units/ml of aprotinin, and 1 mM NaF), scraped off, and collected into tubes. After centrifugation to remove insoluble cell debris, the resulting supernatants were mixed with 40 μl of a 50% slurry of anti-FLAG M2-agarose (Sigma) and washed three times with the lysis buffer. After conjugation overnight at 4 °C, the agarose was washed with the lysis buffer. Proteins bound to the agarose were analyzed by SDS-PAGE and immunoblot.

Other Methods—Western blot analysis was performed using primary antibodies and a secondary antibody, donkey anti-rabbit IgG antibody or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Antigen-antibody complexes were visualized with ECL Western blotting detection reagent (Amersham Pharmacia Biotech).

RESULTS

Cloning of Chinese Hamster PEX12 cDNA

To delineate the primary defect of ZP104 and ZP109 cells, we first isolated CIPEX12 cDNA by screening a CHO-K1 cDNA library using as a probe rat PEX12 cDNA encoding an N-terminal region, residues 2–202. We isolated four positive colonies containing a plasmid with the same insert. One of the plasmids was 2,797 base pairs long and apparently contained PEX12 cDNA with an open reading frame encoding 359-amino acid protein (Fig. 1A). As compared with rat and human Pex12p, Chinese hamster Pex12p was the same in length and showed 95 and 90% identity, at the deduced amino acid level. The C-terminal region, including a RING finger motif, of Chinese hamster, rat, and human Pex12p shows only one or two amino acid substitutions, indicating a higher degree of conservation of this region in eukaryote evolution. The RING finger
motif in Chinese hamster Pex12p contains one zinc finger with 5 conserved cysteine residues, as Pex12p from other species, but distinct from the typical RING finger motif of C3HC4 (39), as found in Pex2p (19) and Pex10p (22, 24).

In both CG3 ZP104 and ZP109 cells, peroxisomal matrix proteins, such as catalase, were mislocalized in the cytosol (Fig. 1B, a and c) (27). Upon expression of CIPEX12, numerous catalase-positive, punctate structures were observed in both cells, indicating restoration of peroxisomal protein import (Fig. 1B, b and d). Complementation of impaired protein import was also confirmed by cell staining using antisera to PTS1 and thiolase, a PTS2 protein (data not shown). CIPEX12 expression also restored peroxisome biogenesis in CG3 PBD patient fibroblasts (see Fig. 2C). Thus, CIPEX12 cDNA cloned was evidently shown to be biologically active, similar to rat and human PEX12 cDNA (20, 21).

Mutation of CIPEX12 in ZP104 and ZP109

To investigate dysfunction of PEX12 in ZP104 and ZP109, we isolated PEX12 cDNAs from mutants by reverse transcription-PCR and determined their nucleotide sequence. All of the five independent cDNA clones isolated from ZP104 possessed a point mutation at position 789, from nucleotide G to A, resulting in creation of a termination codon at Trp170, termed CIPEX12W170Ter (Fig. 2A, left panel). No other common mutations were detected in these clones, suggesting that mutation of PEX12 in ZP104 was homozygous. Next, we isolated 12 independent PEX12 cDNA clones from ZP109 and identified two types of mutations. One, found in 7 of 12 clones, was at position 510, nucleotide G of a codon for Trp170 mutated to A, creating a premature termination, named CIPEX12W114Ter (Fig. 2A, middle). Another one was, in the remaining five cDNA clones, a transition mutation from nucleotide G to A at position 342 of a codon for Trp114, likewise resulting in a termination codon, termed CIPEX12W114Ter (Fig. 2A, right). It may be that mRNAs for two types of truncated Pex12p are translated in ZP109 (Fig. 2B).

To verify whether CIPEX12 cDNAs from ZP104 and ZP109 are biologically active, these mutants were transfected into patient fibroblasts of CG3 (PBD3–02) (20). We earlier found that the patient PBD3–02 possessed a homozygous nonsense mutation at Lys231 (20) and that peroxisome matrix proteins were mislocalized to the cytosol (Fig. 2C, a). Upon expression of wild-type CIPEX12, numerous catalase-positive particles, peroxisomes, were observed in PBD3–02 fibroblasts (Fig. 2C, b), demonstrating that Chinese hamster Pex12p was functional in the patient’s fibroblasts. When ZP104-derived CIPEX12W263Ter was transfected to the PBD3–02 fibroblasts, catalase remained in the cytoplasm, thereby indicating dysfunction of CIPEX12W263Ter (Fig. 2C, c). When CIPEX12 cDNAs from ZP104 and ZP109 were transfected back to parent CHO mutants, nearly all of the cells were not complemented (data not shown). Interestingly, only a small number of cells showed catalase-positive particles, possibly indicative of peroxisomes, but the restored cells were much less in number than those transfected with wild-type CIPEX12 (data not shown). Such partial complementation might be reflecting overexpression of mutated Pex12p, as noted in pex13 mutants (40). Taken together, it is evident that dysfunction of Pex12p caused by truncation is the primary defect in ZP104 and ZP109.

To further analyze the phenotype of ZP104 and ZP109, the level of peroxisomal proteins was determined by immunoblot using antibodies to C-terminal peptides of Pex12p, Pex14p, Pex5p and catalase (Fig. 2D). As expected, Pex12p was not detected in both ZP104 and ZP109 (lanes 2 and 3), in contrast to CHO-K1 (lane 1). The amount of Pex14p, a peroxisomal membrane peroxin acting as a docking factor of the PTS1 receptor Pex5p, and catalase was not affected. It is noteworthy, however, that Pex5p was found at an ~2-fold higher level in ZP104 and ZP109 than in CHO-K1. The significance of the increased amount of Pex5p in PEX12-deficient mutants ZP104 and ZP109 is discussed below.

Domain Mapping of Pex12p

Deletion of N-terminal Region—Pex12p is an integral membrane protein with two transmembrane segments, exposing its N- and C-terminal parts to the cytoplasm (21) (Fig. 3A). To search for a region of Pex12p required for complementing activity and that responsible for localization to peroxisomes, we constructed various deletion mutants of rat Pex12p in which an epitope FLAG was tagged at the N terminus. CHO-K1 and ZP109 were transfected with each of PEX12 variants and dou-
ble stained using antibody to FLAG for detection of Pex12p and anti-PTS1 antibody for assessing complementing activity (Figs. 3, A and B). Wild-type FLAG-Pex12p complemented peroxisome deficiency of ZP109 and was co-localized with PTS1 proteins in CHO-K1 and ZP109 (Fig. 3, A and B, a and b), consistent with our earlier observation (21). None of the N-terminally truncated Pex12p variants, ∆N3, ∆N2, ∆N1, ∆N6, and ∆N5, each comprising residues 203–359, 98–359, 77–359, 49–359, and 26–359, respectively, showed PEX12-protein expressed and restored PTS1 protein import in ZP109 (∆N1, Fig. 3B, c and d; Fig. 3A). Mutant ∆N4, only the C-terminal part containing RING finger, was localized in the cytoplasm and showed no complementation activity (Fig. 3A). In contrast, mutants ∆N7 and ∆N8, comprising residues 17–359 and 9–359, respectively, were localized to peroxisomes and restored peroxisomal protein import as efficiently as the wild-type (∆N7, Fig. 3B, e and f; ∆N8, Fig. 3A). Interestingly, ∆N7 was longer only by 9 amino acid residues than nonfunctional ∆N5.

A Pex12p variant with an internal deletion of 9 amino acid residues at positions 17–25, named ∆M1 (∆17–25), was not detectable and was biologically inactive when expressed in CHO-K1 and ZP109 (Fig. 3B, g and h). Mutants ∆M2 (∆26–48) and ∆M3 (∆67–75), containing a short deletion at the C-terminal side of ∆M1, were also biologically inactive and undetectable (Fig. 3A). In ZP109, the mutant ∆M4 (∆77–97) was expressed at a lower level and localized in large PMP70-positive particles (data not shown), presumably peroxisomal remnants, where PTS1 protein import was not restored (Fig. 3A). When expressed in CHO-K1, ∆M4 was detected in particles (Fig. 3B, i and j), in a superimposable manner with PMP70 (data not shown), thereby indicating that ∆M4 was imported in peroxisomes (Fig. 3A). To our surprise, PTS1 proteins in the CHO-K1 expressing ∆M4 were localized in the cytoplasm, not in peroxisomes, suggesting that ∆M4 inhibited the PTS1 protein import, presumably acting as a dominant-negative factor (Fig. 3B, i and j, arrowheads). ∆M5 (∆98–106) was biologically active, similar to wild-type FLAG-rat Pex12p (a and b), mutant ∆N1 (c and d), ∆N7 (e and f), or ∆M1 (g and h). Mutant ∆M4 was transferred into CHO-K1 (i and j). Cells were stained with antibodies to FLAG (left panels) and PTS1 (right panels). Note that no staining for import of FLAG and PTS1 was detected in ZP109 that had been transfected with ∆N1 and ∆M1. CHO-K1 cells expressing ∆M4 (arrowheads) showed cytoplasmic staining of PTS1 proteins. Bar, 20 μm.

Deletion of C-terminal Region—RING finger motif in Pex12p was important for biological function in peroxisome biogenesis, as depicted by mutagenesis of RING finger (21). Various deletion mutants of rat Pex12p were C-terminally tagged with Myc, N-terminally tagged with FLAG, or N-terminally fused with EGFP (Fig. 4A). When wild-type Pex12p fused with EGFP (EGFP-Pex12p) was expressed in CHO-K1 and ZP109, fluorescence of EGFP was observed in numerous punctate structures and co-localized with PTS1 proteins in both types of cells, indicating that EGFP-Pex12p was functionally active and prop-

Fig. 3. N-terminal deletion mutants of Pex12p. A, intracellular localization and complementing activity of N-terminal deletion mutants. Wild-type and deletion mutants of rat Pex12p were transiently expressed in CHO-K1 and ZP109. Cells were stained using antibodies to FLAG and PTS1 peptides. Complementing activity was verified by restoration of PTS1 protein import in ZP109. +, complemented; −, not complemented. Intracellular localization was as follows. Ps, peroxisomes; Cyto, cytosol; −, not detectable. Wild-type and mutant PEX12 proteins used were wild-type FLAG-Pex12p (comprising amino acid residues 2–359), mutant ∆N1 residues (77–359, termed ∆1–76), ∆N2 (98–359, ∆1–97), ∆N3 (203–359, ∆1–202), ∆N4 (259–359, ∆1–258), ∆N5 (26–359, ∆1–25), ∆N6 (49–359, ∆1–48), ∆N7 (17–359, ∆1–16), ∆N8 (9–359, ∆1–8), ∆M1 (∆17–25), ∆M2 (∆26–48), ∆M3 (∆67–75), ∆M4 (∆77–97), and ∆M5 (∆98–106). TM, transmembrane segment. B, morphology of ZP109 expressing N-terminal deletion mutants of Pex12p. ZP109 cells were transfected with plasmid for wild-type FLAG-rat Pex12p (a and b), mutant ∆N1 (c and d), ∆N7 (e and f), or ∆M1 (g and h). Mutant ∆M4 was transfected into CHO-K1 (i and j). Cells were stained with antibodies to FLAG (left panels) and PTS1 (right panels). Note that no staining for import of FLAG and PTS1 was detected in ZP109 that had been transfected with ∆N1 and ∆M1. CHO-K1 cells expressing ∆M4 (arrowheads) showed cytoplasmic staining of PTS1 proteins. Bar, 20 μm.
Domain Mapping and Interacting Partners of Pex12p

Fig. 4. C-terminal deletion mutants of Pex12p. A, subcellular localization and peroxisome-restoring activity of C-terminal deletion mutants were verified as in Fig. 3A. Cells transfected with Myc-tagged constructs, Pex12p-Myc, SPL, and ΔR, were double stained using antibodies to Myc and PTS1. Cells expressing ΔPS were stained as in Fig. 3B. Cells expressing EGFP-Pex12p fusion proteins were stained with anti-PTS1 antisera and rhodamine-conjugated secondary antibody and were then assessed by GFP and rhodamine fluorescence. Complementing activity and cellular location were determined as in Fig. 3A. Wild-type Pex12p and its variants used were wild-type Pex12p-Myc (comprising amino acid residues 1–359), mutant ΔPL (lacking amino acid residues 259–286, 259–286, ΔR (301–359), ΔPS (Δ279–298), ΔC1 (Δ255–359), ΔC2 (Δ203–359), and ΔC3 (Δ77–359). B, expression of Pex12p lacking the RING finger domain. ΔR. Plasmid ΔR was transfected into CHO-K1 (a and b) and ZP109 (c–e). Cells were stained with antibodies to Myc (a and c), PMP70 (b and d), and PTS1 (e) respectively. Note that ΔR was co-localized with PMP70 in ZP109, but no restoration of PTS1 import was observed. Bar, 20 μm.

dually translocated to peroxisomes (Fig. 4A). Mutant ΔC1 (comprising residues 2–257, Δ258–359) showed no complementing activity (Fig. 4A) and was targeted to a few large compartments, distinct from peroxisomes (data not shown). Like ΔC1, mutant ΔC2 lacking the C-terminal 158 amino acid residues was not detectable, presumably due to rapid degradation (Fig. 4A). ΔC3, consisting of only N-terminal 76 amino acid residues fused with EGFP, was inactive and localized in the cytoplasm (Fig. 4A). Interestingly, ΔC3 corresponds to the sequence required for Pex12p targeting to peroxisomes (see above), implying that the N-terminal 76 amino acid residues are necessary but not sufficient for localization of Pex12p in peroxisomes.

The C-terminal 100 amino acid residues deleted in ΔC1 contain two interesting portions: the RING finger domain, starting from a conserved residue Cys3104, and a proline-rich segment of residues LPTPPPVPVLHLDYNSDPLLP at positions 279–298. ΔPL, a mutant Pex12p lacking 28 amino acid residues, immediately downstream of the second transmembrane segment to the middle of proline-rich segment, was undetectable and inactive (Fig. 4A). ΔR, a Pex12p variant, with a deletion of 59 residues from the RING to the C terminus, could not restore PTS1 protein import in ZP109 (Fig. 4B, e), but it was targeted to peroxisomes in CHO-K1 (Fig. 4B, a and b). ΔR was also transported to peroxisomal remnants in ZP109, as assessed by co-localization with PMP70 (Fig. 4B, c and d), implying that ΔR is properly localized as a peroxisomal membrane protein. ΔPS, a mutant lacking 7 amino acid residues (LPTP-PPP) in the proline-rich segment showed a phenotype slightly different from mutant ΔR. In CHO-K1, ΔPS was localized in peroxisomes like ΔR in CHO-K1, although it was not detectable in any compartments and was inactive in ZP109 (Fig. 4A). Collectively, both RING finger and the proline-rich segment of Pex12p are required for biological function but not for translocation to peroxisomes.

RING Finger of Pex12p Interacts with Pex5p and Pex10p

As a step toward understanding the functional role(s) of RING peroxins, interaction with other peroxins was investigated using a yeast two-hybrid system. Mammalian PEX cDNAs, RnPEX12, HsPEX10, and RnPEX2, coding for the full-length protein and those for the C-terminal region including the RING finger were fused to the DB in pDBLeu vector or the AD in pPC86 vector. Other mammalian PEX genes involved in matrix protein import, including PEX5 for the PTS1 receptor (the shorter form of HsPEX5 and the longer one of CIPEX5) and HsPEX14 for the Pex5p-docking factor, were also fused to downstream AD of GALA in pPC86 vector. Other mammalian PEX genes involved in matrix protein import, including PEX5 for the PTS1 receptor (the shorter form of HsPEX5 and the longer one of CIPEX5) and HsPEX14 for the Pex5p-docking factor, were also fused to downstream AD of GALA. Interaction of these PEX genes was verified by assaying β-galactosidase activity in Saccharomyces cerevisiae. Several pairwise combinations of the peroxins showed specific interaction. Yeast cells expressing both the C-terminal region of Pex12p with DB (DB-Pex12pC) and that of Pex10p with AD (AD-Pex10pC) showed a blue color in β-galactosidase assays (Fig. 5A). The DB-Pex12pC also gave positive β-galactosidase activity in combination with AD-Pex10F (full-length Pex10p fused to AD) as well as by a reverse combination (DB-Pex10pF and AD-Pex12pC) (Fig. 5A). How-
ever, no interaction was apparent between full-length Pex12pF and Pex10pF (Table II). Moreover, the Pex12pC interacted with both shorter and longer forms of Pex5p (Fig. 5B). Interestingly, full-length Pex12pF failed to bind to both types of Pex5p (Fig. 5B), Pex10p and Pex2p (Table II). We could not determine whether only the RING finger of Pex10p or Pex2p interacts with Pex5p, because yeast cells expressing the DB each fused to the C-terminal part of Pex10p and Pex2p, and Pex5p (both shorter and longer forms) gave a positive β-galactosidase activity, presumably by self-activation (Table II). Pex2p, one of the RING peroxins, had no binding partner in this assay (Table II). Taken together, these results imply that the RING-containing C-terminal region of Pex12p evidently interacts with Pex5p and Pex10p in yeast two-hybrid assays.

### Interaction of RING Finger Peroxins

To confirm the interaction observed in the yeast two-hybrid assay, an _in vitro_ binding assay was performed using bacterially expressed recombinant proteins. For binding of RING peroxins, MBP fused to the C-terminal part of Pex10p with tandem HA epitope (MBP-Pex10pC-HA2) and that to Pex12pC (MBP-Pex12pC) were incubated with GST or GST fused to the C-terminal part of Pex10p and Pex2p, and Pex5p (both shorter and longer forms) gave a positive β-galactosidase activity, presumably by self-activation (Table II). MBP-Pex12pC was barely detectable with GST-Pex12pCMut1 bearing a mutation of C304S in the RING finger (21) (lane 3), thereby suggesting that conformation of RING finger in Pex12p, presumably containing a zinc ion, is required for binding to the C-terminal part of Pex10p. When a similar _in vitro_ assay was repeated using MBP-Pex12pC and GST or GST fusion proteins, the interaction of Pex12pC with Pex10pF was also found, as well as with Pex12pC itself (Fig. 6A, lower left panel, lanes 2 and 4). The homomeric interaction of Pex12pC was abolished in the experiment using GST-Pex12pCMut1 (lane 3). No MBP-Pex12pC was pulled down with GST-Pex2pC and GST alone (lanes 5 and 1, respectively). These results are consistent with the result of the yeast two-hybrid assay, suggesting that the C-terminal part of Pex12p directly binds to that of Pex10p, apparently dependent on the conformation of the RING finger.

### Interaction between Pex5p and RING Finger Peroxins

Interaction between Pex5p and the C-terminal part of RING finger peroxins was likewise assessed, as described above. A shorter form of Pex5p with an N-terminal hexahistidine tag, His-Pex5pS, was pulled down with GST-Pex12pC and GST-
Pex10pC (Fig. 6A, right panel, lanes 2 and 4). The amount of His-Pex5pS recovered with GST-Pex12pC and GST-Pex10pC was nearly one-tenth of that with GST-Pex14p (Fig. 6A, right panel, lane 6), a docking site of Pex5p on peroxisome membranes (16, 17). It is possible that the difference in the amount of Pex5p bound to Pex14p versus Pex10p and Pex12p reflects the intermolecular affinity. The binding between Pex5pS and Pex12pC was abrogated by mutation of a conserved cysteine in RING finger of Pex12pC (lane 3). His-Pex5pS showed no binding to GST-Pex2pC and GST alone (Fig. 6B, lanes 5 and 1). These results provide evidence that Pex5pS binds to both Pex12p and Pex10p at the RING finger-containing C-terminal part.

Pex12p Interacts with Pex10p in Vivo

We verified in vivo the in vitro findings with respect to interaction of RING peroxins. Immunoprecipitation was done from K1-FLP10, CHO-K1 cells stably expressing FLAG-tagged Pex10p. In the K1-FLP10 cells, FLAG-Pex10p was targeted to normal peroxisomes as assessed by co-localization with PTS1, thus indicating that stable expression of FLAG-Pex10p does not morphologically affect peroxisome biogenesis (data not shown). FLAG-Pex10p was detected in whole cell lysate from K1-FLP10, not in that from CHO-K1, as assessed by immunoblot using anti-FLAG antibody (Fig. 6B, top panel, lanes 1 and 2). No difference between CHO-K1 and K1-FLP10 was observed in expression of Pex12p, Pex5p, and Pex14p (three lower panels, lanes 1 and 2). The FLAG-Pex10p in K1-FLP10 was immunoprecipitated using anti-FLAG antibody conjugated to agarose beads (Fig. 6B, lanes 3 and 4). Pex12p was found only in the immunoprecipitates from K1-FLP10, not from CHO-K1 (upper middle, lanes 3 and 4), thereby indicating that Pex12p bound to Pex10p in vivo. Interestingly, neither Pex5p nor Pex14p was recovered with FLAG-Pex10p (two lower panels, lane 4). Collectively, Pex12p forms a complex with Pex10p, but such a complex binds little to Pex5p in wild-type cells.

DISCUSSION

We isolated Chinese hamster PEX12 cDNA by colony hybridization, using rat PEX12 cDNA. Expression of CIPEX12 fully restored peroxisome biogenesis in pex12 CHN mutants, ZP104 and ZP109, and patients' fibroblasts, as efficiently as rat and human PEX12. Such interspecies complementation could be implicated by the highly conserved sequence homology between PEX12 proteins from these three species. Mutations of Pex12p found in CHO cell mutants as well as CG3 patients (20, 21, 25) all resulted in premature termination, where the RING finger domain was commonly eliminated. The importance of RING domain for Pex12p function was also inferred from the findings using various C-terminally truncated mutants of rat Pex12p, in good agreement with the genotype-phenotype relationship in CHO mutants and fibroblasts from PBD patients. ZP104-type Pex12p, lacking C-terminal residues at 264–359, is similar to an inactive Pex12p variant ΔC1 (Δ258–359). A nonsense mutation of PEX12 from ZP109 (Trp170Ter) and that from PDB3-03 fibroblasts of CG3 (Arg180Ter), both located upstream of the second membrane-spanning segment, are comparable with a mutant ΔC2 comprising residues 2–202. Base-transition from G to A found in the PEX12 gene of both ZP104 and ZP109 cells is consistent with the mode of action of N-methyl-N’-nitro-N-nitrosoguanidine, an alkylating agent used as a mutagen (27).

Site mutation studies of RING finger demonstrated that out of the five conserved cysteine residues, four (Cys304, Cys307, Cys325, and Cys326) appear to be indispensable (21), hence possibly forming a zinc finger. Neither RING mutagenesis nor deletion of a RING finger, such as a variant ΔR, affected targeting of Pex12p to peroxisomes. Instead, RING finger was required for the complementing activity. This requirement is inferred from ZS patient PDB3-01, possessing Pex12p comprising 291 amino acid residues (21), shorter by only 9 amino acids than ΔR, which might be localized in peroxisomes. Interestingly, a Pex12p variant, ΔN4 consisting of only the C-terminal part with RING, is diffused in the cytosol and is inactive, implying that this portion needs to be localized on peroxisome membranes for the function. The proline-rich cluster region, residues 280–298, was essential for the function of Pex12p but was not required for translocation to peroxisomes.

The amino acid sequence in N-terminal residues at 1–95 is highly conserved, where 87 amino acids are identical between three mammals, thus suggesting the importance of this region for Pex12p. The sequence from residue 17 to 76 was indeed required for localization of Pex12p to peroxisomes as well as its stability in cells. It is possible that this part of Pex12p, exposed to the cytosol (21), may interact with factor(s) involved in the import of Pex12p. However, this region was not sufficient for targeting to peroxisomes; as noted in the cytoplasmic localization of mutant ΔC3, the N-terminal 76 amino acid residues fused with EGFP. Furthermore, a fusion protein of EGFP with the N-terminal 154 amino acid residues of Pex12p, corresponding to the entire N-terminal portion facing the cytoplasm, remained in the cytoplasm (data not shown). Recently, the N-terminal region consisting of residues positions 1–40 of a peroxisomal membrane peroxin Pex3p was shown to be necessary and sufficient for peroxisomal targeting in mammals (41–43) and in yeast, Pichia pastoris (44) and Hansenula polymorpha (45). The highly conserved residues at 9–15, LKRHKKK of human Pex3p was proposed as a targeting sequence. Neither this sequence nor the basic loop identified in C. boidinii PMP47 (11) was found in the N-terminal region and even in the entire sequence of Pex12p, thereby suggesting that mechanisms of Pex12p targeting differ from those of Pex3p and PMP47. Moreover, all of the Pex12p mutants defective in complementing activity, except for those with deletion of RING finger, are more likely to be degraded before translocation to peroxisomes. The N-terminal region and two transmembrane segments appear to be responsible for the stability and precise targeting of Pex12p.

We identified several pairs of interactions between mammalian peroxins by in vivo and in vitro binding assays. The C-terminal region of Pex12p binds to full-length Pex10p as well as to its C-terminal region, as assessed by a yeast two-hybrid assay. The interaction was specific and apparently direct, as demonstrated by an in vitro binding assay using respective recombinant proteins. In the immunoprecipitation of FLAG-Pex10p from CHO-K1 expressing FLAG-Pex10p, Pex12p was co-immunoprecipitated, suggesting that Pex12p and Pex10p form a stable oligomeric complex in wild-type cells. Both isoforms of Pex5p, Pex5pS and Pex5pL (30), interacted with the C-terminal region of Pex12p in a yeast two-hybrid assay. Likewise, the C-terminal region of both Pex10p and Pex12p independently bound to Pex5pS in vitro. Moreover, Pex12p interacts with GST-Pex5pS and GST-Pex5pL in vitro (17). However, Pex12pF failed to bind both isoforms of Pex5p in a yeast two-hybrid assay, where all combinations of the assays using Pex12pF in the DB construct resulted negative, implying that Pex12pF may not be used as a bait. To our surprise, Pex5p was not co-immunoprecipitated with FLAG-Pex10p that associated with Pex12p. We interpret this finding to mean that Pex5p does not bind to, or only transiently interacts with, Pex10p and Pex12p when Pex10p and Pex12p are in the complex in peroxisome membranes. This is consistent with the observation that less than 5% of Pex5p is present in the organelle fraction in wild-type CHO-K1 (17) as well as in normal fibroblasts (46).
Pex5p is proposed as the mobile receptor that binds its cargo PTS1 proteins in the cytoplasm; it targets them, transports them across the peroxisomal membrane, and shuttles back to the cytoplasm (17, 46).

In the assay systems used for peroxin interaction, we observed several cases giving mutually inconsistent results between the in vivo and in vitro assays. Homomeric interaction of Pex12pC was detectable in the binding assay using the recombinant protein, while the yeast two-hybrid assay resulted negative; full-length Pex10p and Pex12p were co-immunoprecipitated, although this interaction was not observed in the yeast two-hybrid assay. Since the two-hybrid assay using yeast may reflect physiological relevance in general, we cannot conclude that Pex12p proteins interact with each other in a homomeric manner. Regarding the interaction of Pex12p and Pex10p, there are two different criteria dealing with co-immunoprecipitation from peroxisome membranes and interaction of two membrane proteins in the nucleus. “Co-immunoprecipitation” possibly means their in vivo association, including other factor-mediated binding. In the two-hybrid assay, however, two integral membrane proteins, Pex12p and Pex10p, may not form their proper configuration in nuclei that reflects a bona fide topogenic state. Hence, both peroxins are most likely to be present as a complex in peroxisome membranes. Our interpretation of these results is in good agreement with the most recent report by Chang et al. (26).

Several more membrane peroxins, including Pex13p (40, 47), are possibly involved in the Pex5p-mediated protein import process (17). When one essential factor such as a membrane peroxin involved in Pex5p translocation across the peroxisome membrane is defective, Pex5p might be stuck at an adjacent membrane protein with which it interacts. This may explain the observation that Pex5p increased in amount in ZP104 and ZP109, as compared with the level in wild-type CHO-K1 (Fig. 2D). Moreover, nearly half of the Pex5p was recovered in the organelle fraction from ZP109, after centrifugation at 100,000 × g, while almost all Pex5p was present in the cytosol fraction in wild-type CHO-K1 (data not shown). A similar observation was noted in other CHO mutants, such as PEX2-defective Z65 and PEX13-impaired Z128 (17). A moderately increased level of Pex5p was also found in fibroblasts of pex12C G3 as well as pex2 CG10 and pex10 CG7 (46). In contrast, in pex14 CHO mutant ZP161, no Pex5p was accumulated in the organelle fraction, thus suggesting that Pex5p with its cargo proteins is targeted on the peroxisome membrane by initially docking to Pex14p (17). Therefore, Pex5p might temporarily bind to the import machinery complex containing Pex10p and Pex12p, after anchoring to the Pex14p, and can then translocate toward the luminal side of peroxisomes, through the luminal or cytosolic face of Pex12p-Pex10p (Fig. 7). These observations imply that the RING peroxins are involved in the Pex5p-mediated protein import. The RING finger peroxins, Pex10p and Pex12p, possibly including Pex2p, are more likely to be involved in Pex5p-mediated protein import. While this manuscript was being prepared, Chang et al. (26) very recently reported similar findings, such as interaction of Pex12p with Pex5p and Pex10p, using CG3 patients’ fibroblasts. It is also possible that Pex10p and Pex12p form a complex via their RING finger in an another factor-mediated manner. Other interactions observed in vitro, such as homomeric binding of Pex12pC and Pex10pC as well as the interaction between Pex10pC and Pex2pC, remain to be addressed in vivo. The finding of proteins directly interacting with membrane-associated Pex5p, with the use of CHO mutants and patients’ fibroblasts, will clarify the molecular mechanisms of protein import across the peroxisome membrane.

The absence of binding of Pex5p to Pex2pC, despite a cellular phenotype similar to pex10 and pex12 mutants showing accumulation of Pex5p in peroxisomial remnants (17), may imply that Pex2p plays a role in peroxisome biogenesis, protein translocation, and more likely in recycling of mobile signal receptors, Pex5p and Pex7p, back to the cytosol. It is noteworthy that overexpression of Pex2p truncated in the C-terminal, RING-containing part restores peroxisomal import of matrix proteins in pex2 mutants, including fibroblasts derived from a patient with Zellweger syndrome (48). Contrary to this, impaired import is not complemented by overexpression of Pex10p and Pex12p mutants where C-terminal parts are deleted (21, 22, 24, 25). Therefore, it is possible that Pex2p may play a role distinct from those of Pex10p and Pex12p in peroxisome biogenesis.

The RING finger domain and the immediate flanking sequences represent the highly conserved region in Pex12p. The RING finger domain has now been identified in over 80 proteins (39). Recent studies have implicated this domain as an important element in the function of various proteins. For example, the cancer-predisposing mutation C61G in the breast cancer 1 (BRCA1) RING domain disrupts homodimer formation (49). Our findings provide clear evidence for a critical role played by the RING finger domain in the biological function of Pex12p as a peroxisome assembly factor via interaction with Pex10p. It is also possible that the RING finger domains of Pex2p, Pex10p, and Pex12p may mediate interactions with other proteins besides Pex5p. It is also noteworthy that the RING finger domains may mediate binding of ubiquitin-conjugating enzymes (50). The RING finger of these peroxins might recruit ubiquitin-conjugating enzyme components of the ubiquitination machinery, independently or in a concerted manner, whereby peroxisome biogenesis or turnover may be regulated. Moreover, a typical C4HC4 RING finger found in Pex2p and Pex10p, as opposed to one zinc ion-bound “half” RING in Pex12p, may distinguish the biological function(s) of respective peroxins. Further studies designed to assess the biochemical function(s) of the RING finger domain, Pex2p, Pex10p, and Pex12p using cell mutants will address the mechanism and roles of the peroxins involved in peroxisome biogenesis.
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