Intracellular Localization, Function, and Dysfunction of the Peroxisome-targeting Signal Type 2 Receptor, Pex7p, in Mammalian Cells*

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We previously isolated and characterized a Chinese hamster ovary (CHO) cell mutant, ZPG207, that is defective in import of proteins carrying a peroxisome-targeting signal type 2 (PTS2) nonapeptide. Herein we have cloned Chinese hamster (CI) PEX7 encoding the PTS2 receptor. CIPEX7p consists of 318 amino acids, shorter than human Pex7p by 5 residues, showing 91 and 30% identity with Pex7p from humans and the yeast Saccharomyces cerevisiae, respectively. Expression of CIPEX7 rescued the impaired PTS2 import in pex7 ZPG207. Mutation in ZPG207 PEX7 was determined by reverse transcription PCR; a G-to-A transition caused a 1-amino acid substitution, W221ter. We investigated the molecular dysfunction of Pex7p variants in mammals, including Pex7p-W221ter and Pex7p with one site mutation at G217R, A218V, or L292ter, which frequently occurs in the human fatal genetic peroxisomal disease rhizomelic chondrodysplasia punctata, showing a cell phenotype of PTS2 import defect. All types of the mutations affected Pex7p in binding to both PTS2 cargo protein and the longer isoform of PTS1 receptor Pex5pL, that is responsible for transport of the Pex7p-PTS2 complex. Subcellular fractionation and protease protection studies demonstrated bimodal distribution of Pex7p between the cytoplasm and peroxisomes in CHO and human cells. Moreover, expression of Pex5pL, but not of the shorter isoform Pex5pS, enhanced translocation of Pex7p-PTS2 proteins into peroxisomes, thereby implying that both PTS receptors shuttle between peroxisomes and the cytosol. Furthermore, a CIPEX7 mutant with a deletion of 7 amino acids from the N terminus retained peroxisome-restoring activity, whereas an 11-amino acid truncation abrogated the activity. CIPEX7p with a C-terminal 9-amino acid truncation, comprising residues 1–309, maintained the activity, whereas a 14-amino acid shorter form lacking several amino acids of the sixth WD motif lost the activity. Therefore, nearly the full length of Pex7p, including all WD motifs, is required for its function.

To elucidate the hierarchy of the highly organized biogenesis of intracellular organelles, the peroxisome, a single membrane-bounded essential organelle (1), has been used as a model compartment in mammalian and yeast systems (2, 3). Significant progress has recently been made in our understanding of the biogenesis of peroxisomes. For import, most of the peroxisomal matrix proteins are mediated by well characterized cis-acting peroxisomal targeting signals (PTSs): the C-terminal tripeptide SKL motif, PTS1 (4, 5), and the N-terminal cleavable presequence, PTS2, consisting of a nonapeptide with the conserved sequence (R/K)(L/V/I)(H/Q/L)A (6–8). Many peroxins required for peroxisome biogenesis have been identified in yeast and mammals (9–13).

By functional phenotype complementation assay using Chinese hamster ovary (CHO) cell mutants, we isolated nine cDNAs encoding peroxisome biogenesis factors, termed peroxins, PEX1 (14), PEX2 (formerly PAF-1) (15), PEX3 (16), PEX5 (17), PEX6 (18), PEX12 (19, 20), PEX13 (21), PEX14 (22), and PEX19 (23). We also showed that these PEXs, except for PEX14, are responsible for human fatal genetic disease, called peroxisome biogenesis disorders (PBD) such as Zellweger syndrome in which impaired peroxisome assembly is manifested (14, 17, 19, 20, 23–28). Thus, peroxisome assembly-defective CHO cell mutants have indeed proven useful for investigating molecular and cellular mechanisms involved in peroxisome biogenesis and for elucidating the primary defects of PBD (11, 28). Meanwhile, we have so far identified 15 different complementation groups (CGs) in mammals (11, 28, 29), including two recently isolated, novel CGs of CHO cell mutants, ZPG207 and ZPG208/ZPG209 (30).

ZPG207 is defective solely in PTS2 import and belongs to the same CG as fibroblasts from patients with rhizomelic chondrodysplasia punctata (RCDP). PEX7 encoding the PTS2 receptor is mutated in RCDP patients, where morphologically normal peroxisomes are present but defective in the import of PTS2 proteins (31–33). The most frequently occurring mutations in patients with RCDP have been identified at L292ter and less frequently at A218V and G217R (31, 32). The impaired PTS2 import has been assessed morphologically but not at the molecular level. Furthermore, intracellular localization of Pex7p

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The abbreviations used are: PTS, peroxisome targeting signal; AOX, acyl-CoA oxidase; BSA, bovine serum albumin; CG, complementation group; CHO, Chinese hamster ovary; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, influenza virus hemagglutinin; ORF, open reading frame; P90H, 91'-pyrene)nonanol; P12, 12'-pyrene)dodecanic acid; PBD, peroxisome biogenesis disorders; Pex5pS and Pex5pL, a shorter and a longer isoforms of Pex5p; PEX7, cDNA encoding the peroxin Pex7p; PMP70, 70-kDa peroxisomal integral membrane protein; PNS, postnuclear supernatant; RCDP, rhizomelic chondrodysplasia punctata; thiolase, peroxisomal 3-ketoacyl-CoA thiolase.

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is not well understood; epitope-tagged Pex7p expressed in Saccharomyces cerevisiae is mostly in the cytosol (34) or exclusively within peroxisomes (35). Overexpression of Myc-tagged mouse Pex7p in human fibroblasts morphologically showed mostly, if not all, cytoplasmic localization (31). Elgersma et al. (36) suggested that Pex7p is a mobile receptor as assessed by overexpression in the yeast Pichia pastoris. Therefore, it is apparent that Pex7p has not been defined unambiguously in terms of its subcellular location, presumably because of a lower level of expression in cells. Antibodies to mammalian Pex7p are not available, obviously due to the difficulty confronted in expressing a recombinant protein. Mammalian Pex7p may be less antigenic in immunizing animals such as rabbits, although rabbit antisera were successfully raised against P. pastoris Pex7p (36). The molecular properties of Pex7p such as the relationships between structure and function also have not been characterized. In mammals, two isoforms of the PTS1 receptor Pex5p of a tetratricopeptide repeat family were identified: a short form termed Pex5pS comprising 598 amino acids in CHO serves as a PTS1 receptor, and the longer Pex5pL with a 37-amino acid internal insertion functions in both the PTS1 and PTS2 import pathways (17, 37). Furthermore, we more recently demonstrated, using CHO pex5 cell mutants, that Pex5pL plays an exclusively pivotal role in PTS2 transport by interacting with Pex7p in mammals (38, 39).

In the present study, we have investigated the intracellular localization of Pex7p in CHO and human cells and found for the first time its bimodal distribution at the endogenous level, namely in the cytoplasm and peroxisomes. Furthermore, several lines of evidence clearly suggested that Pex7p traverses into peroxisomes in a Pex5pL-mediated manner. We have also defined the function and dysfunction of Pex7p at molecular and cellular levels in PTS2 protein transport by making use of a CHO pex7 mutant, ZPG207 (30). We report here that mutations of Pex7p identified in RCDP patients as well as ZPG207 cells affect the binding of Pex7p to both PTS2 protein and Pex5pL. Truncation analysis revealed that nearly the full length of Pex7p is required for the biological activity.

**Experimental Procedures**

Isolation of cDNA Clones Encoding Chinese Hamster PEX7—We isolated Chinese hamster (C3H) PEX7 cDNA clone by colony hybridization on a cDNA library from wild-type CHO-K1 cells (17) using human (h) HsPEX7 cDNA as a probe. The HsPEX7 cDNA was constructed by PCR amplification of the full-length of the HsPEX7 open reading frame (ORF) with a pair of primers, a forward HsPEX7Fw and a reverse HsPEX7I-Rv. The PCR product, termed ClPEX7SalI-Fw and HA-ClPEX7SalI-Rv. The second PCR was performed with ClPEX7FlagNotI and HA-ClPEX7SalI-Rv. The resulting fragment, termed pUCD2HygClPEX7W221ter as a template and primers HA-ClPEX7Fw and HA-ClPEX7SalI-Rv.
incubated with 35S-labeled 3-ketoacyl-CoA thiolase (thiolase) (44) (10 μl) at room temperature for 1 h in 200 μl of the binding assay buffer (50 mM Heps-KOH, pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40, 0.25% BSA, 25 μg/ml each of leupeptin and antipain, 50 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM dithiobisulfitol). Rabbit anti-HA antibody and protein A-Sepharose were added, and the reaction mixture was further incubated at 4°C overnight. Immunocomplexes bound to Sepharose beads were recovered by centrifugation, washed twice with the binding assay buffer minus BSA, and analyzed by SDS-PAGE. Radioactive protein bands were detected using a FujiX BAS1500 Bio-Autoimaging Analyzer (Fuji Photo Film, Tokyo, Japan). HA-Pex7p was assessed by immunoblot.

In vitro binding assays were also performed using HA-HsPex7p and Pts2 mutants. [35S]Thiolase precursor. Mutations at HI–17/E, R(−24)Q, and R(−24)H in PTS2 of the rat thiolase B precursor in pTz18R (8, 22) were done by PCR. cDNA for HI–17/E-thiolase type precursor was constructed using primers, pTH-H(−17)E–Fw and pTH-H(−17)E–Rv. Rabbit anti-human 35S-labeled IgG was used as forward primers pTH-R(−24)Q–Fw and pTH-R(−24)H–Fw, respectively. The EcoRI–SalI fragment of each product in pGEM T-Easy was replaced directly into the wild-type sequence at the equivalent site. Mature thio- lase cDNA was similarly constructed using primers TH-Fw and TH-Rv. The BamHI–SalI fragment of the products was likewise ligated into pTz18R vector. Glutathione S-transferase (GST) pull-down assays using GST-Pex5pS and GST-Pex5pPL were performed as described (38).

Protease Protection Assay—PNS fractions from normal and mutant types of cells (4 × 106 cells each) were treated with 250 and 500 μg/ml Pronase (Sigma) in the absence or presence of 0.2% Triton X-100 for 30 min on ice. The reaction was terminated with a protease inhibitor mixture containing 25 μg/ml each of leupeptin, antipain, aprotinin, and E64 (Peptide Institute, Osaka, Japan), 50 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM EGTA, and then the reaction mixture was separated into the organellar and cytosolic fractions. Peroxisomal proteins, including Pex7p, a cytoplasmically oriented membrane protein Pex14p (22), a matrix enzyme, acyl-CoA oxidase (AOx) (22), and 70-kDa peroxisomal integral membrane protein (PMP70) (41), in both fractions were assessed by SDS-PAGE and immunoblot.

Expression and Purification of HsPEX7—A HsPEX7 expression vector was constructed essentially as described (22). A blunt SalI–SalI fragment of HsPEX7 in pTz18R was ligated into the blunt SalI site of Escherichia coli expression vector pQE30 containing a His tag sequence (Qiagen, Hilden, Germany). E. coli BL21 was transformed with pQE30-HsPEX7 and grown according to the procedure recommended by the manufacturer. HsPEX7 was recovered from the cells using nickel-nitritrotriacetic acid-agarose (Qiagen) under the denatured condition recommended by the manufacturer. The protein-bound agarose beads were subjected to SDS-PAGE. The His6–Pex7p band was cut out from the Coomassie Blue-stained gel and was electrophoretically eluted using an Electro-Eluter (Bio-Rad). HsPEX7 was detected by immuno blotting using rabbit anti-Pex7p C-terminal peptide antibody and anti-His6 monoclonal antibody (Qiagen).

Preparation and Affinity Purification of Anti-Pex7p Antibodies—Antiserum to Pex7p was raised in rabbits by conventional subcutaneous injection of His6–HsPex7p (termed Pex7pF) and of the synthetic C-terminal peptide of residues 305–323 of human Pex7p (termed Pex7pC; see Fig. 1A, dashed underline). Respective antiserum were applied to a protein A-Sepharose (Amersham Biosciences, Inc.) column. After a thorough washing with chilled phosphate-buffered saline, bound IgG was eluted using 0.1 M acetic acid, pH 2.3, 0.15 M NaCl. The eluted IgG fraction was neutralized immediately with 0.25 M NaHPO4, and then was applied onto a His6–HsPex7p-conjugated NHS (N-hydroxysuccin-imide-activated) Hi-trap column (Amersham Biosciences, Inc.). After thorough washing in phosphate-buffered saline, bound IgG was likewise eluted and neutralized. Purified anti-Pex7p antibodies were stored at −20°C in 50% glycerol, 2 mg/ml BSA, 0.01% NaN3.

Other Antibodies—Rabbit antibodies to rat catalase (41), rat thiolase (41), rat AOx (41), rat PMP70 (41), and rat Pex14p (22) were described. Guinea pig anti-rat Pex7p raised to a C-terminal part comprising amino acid residues 241–376 of rat Pex14p (22), termed Pex14pC was also used. Rat anti-GFP antibody was purchased from Molecular Probes.

Other Methods—Northern blot analysis was done using total RNA from the liver of a rat treated for 7 days with clofibrate and of an untreated rat (14, 20) as described (38). Probes, the full-length of HsPEX7 cDNA, the EcoRI fragment (−1 kb) of rat thiolase cDNA (22), full-length CIEPE5XIL (17), the NcoI fragment (−600 bp) of rat AOx cDNA (5), and a 1.3-kb cDNA for human glyceraldehyde-3-phosphate dehydrogenase, were labeled with [α-32P]dCTP (Amersham Biosciences, Inc.). In vitro transcription and translation were carried out essentially as described (45). RNA translation in a rabbit reticulocyte cell-free protein-synthesizing system was performed using 1.2 μCi/ml [35S]methionine and [35S]cysteine (Amersham Biosciences, Inc.). Subcellular fractionation of cells was done as described (38). Western blot analysis was performed using electrophoretically transferred samples.

Table I

| Code*     | Sequence (5’ to 3’) | Underlined |
|-----------|---------------------|------------|
| HsPEX7Fw  | GCTTAAAGATGAGTGGCTGCCG |           |
| HsPEX7SalI-Rv  | GCCCGCGGGCGGAGATGAGAACG | Initiation codon |
| CIPEX7Fw  | GCGCGGGCGGAGATGAGAACG | Initiation codon |
| CIPEX7Rv  | GCGCGGGCGGAGATGAGAACG | Initiation codon |
| HA-HsPEX7Fw | GCGCGGGCGGAGATGAGAACG | Termination codon |
| HA-HsPEX7Fw | GCGCGGGCGGAGATGAGAACG | HA epitope |
| HA-CIPEX7Fw | GCGCGGGCGGAGATGAGAACG | HA epitope |
| HA-CIPEX7SalI-Rv | GCGCGGGCGGAGATGAGAACG | Termination codon |
| HA-HsPEX7SalI-Rv | GCGCGGGCGGAGATGAGAACG | Codon for Arg |

* Fw and Rv, forward and reverse primers, respectively.

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on polyvinylidene difluoride membrane (Bio-Rad) with primary antibodies and a second antibody, donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham Biosciences, Inc.). Antigen antibody complexes were visualized with the ECL Western blotting detection reagent (Amersham Biosciences, Inc.). Protein assay was conducted using a Bio-Rad protein assay kit (Bio-Rad).

**RESULTS**

**Isolation of Chinese Hamster PEX7**

Human PEX7 cDNA was isolated from a human liver cDNA library by amplifying the full-length HsPEX7 ORF. The nucleotide sequence and its deduced sequence of the cloned HsPEX7 was identical to the one reported (31–33). Next, we screened a cDNA library of CHO-K1 cells by colony hybridization using as a probe an 0.5-kb fragment of HsPEX7 ORF and obtained three positive colonies. The nucleotide sequence of plasmids from each colony was determined; one longer clone contained a 1,476-bp cDNA apparently encoding Pex7p consisting of 318 amino acid residues and six WD motifs (Fig. 1A). Chinese hamster Pex7p, named ClPex7p, showed 91 and 98% identity in the primary sequence with human and mouse Pex7p, respectively, but was shorter by 5 amino acids than human one, as mouse Pex7p (31).

**Expression Level of PEX7**

Peroxisomal proteins, including a PTS2 protein, 3-ketoacyl-CoA thiolase, are induced in rat by the administration of hypolipidemic agents such as clofibrate (46, 47). As a step toward characterizing the PEX2 receptor Pex7p, we investigated whether PEY7 mRNA is inducible. PEX7 mRNA, with an estimated size of ~3.7 kb, was apparently induced about 15-fold in clofibrate-treated rats as determined by Northern blot analysis, whereas it was detectable at a very low level in normal rats (Fig. 1B, upper panels). About 1.9 kb of thiolase mRNA was also elevated nearly 20-fold as compared with a lower level of expression in normal rats. Thus, it is likely that transcription of the PEX7 gene is concomitantly induced with PEX2 protein genes upon peroxisome proliferation. In contrast, PEX5 mRNA was not induced by clofibrate in rat liver, whereas mRNA for AOX, a PTS1 protein, was strikingly elevated ~30-fold (Fig. 1B, lower panels), consistent with our earlier observation (38).

**Specificity of Anti-Pex7p Antibodies**

Anti-Pex7p antibodies were raised in rabbits using the C-terminal 19-mer peptide Pex7pC comprising residues 305–323 of human Pex7p and His-tagged full-length HsPex7p, termed His-HsPex7pF. These two types of anti-Pex7p antibodies were verified by immunoblotting using wild-type as well as various site-mutated and truncated forms of Flag-Pex7p (see Figs. 3 and 5) that had been expressed in CHO-K1 cells. Both antibodies were specific and slightly more reactive to Flag-HsPex7p than Flag-CIPex7p (Fig. 1C, lanes 1, 2, and 6), apparently reflecting a difference between HsPex7p and CIpex7p in the sequence used for the antigen. HsPex7p, derived from RCDP or polypidemic agents such as clofibrate (46, 47). As a step toward characterizing the PTS2 receptor Pex7p, we investigated whether PEY7 mRNA is inducible. PEX7 mRNA, with an estimated size of ~3.7 kb, was apparently induced about 15-fold in clofibrate-treated rats as determined by Northern blot analysis, whereas it was detectable at a very low level in normal rats (Fig. 1B, upper panels). About 1.9 kb of thiolase mRNA was also elevated nearly 20-fold as compared with a lower level of expression in normal rats. Thus, it is likely that transcription of the PEX7 gene is concomitantly induced with PEX2 protein genes upon peroxisome proliferation. In contrast, PEX5 mRNA was not induced by clofibrate in rat liver, whereas mRNA for AOX, a PTS1 protein, was strikingly elevated ~30-fold (Fig. 1B, lower panels), consistent with our earlier observation (38).

**Specificity of Anti-Pex7p Antibodies**

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resultant PCR product had an expected size of ~1.0 kb (data not shown). Subsequent sequencing of all six of the independent cDNA clones indicated a mutation of nucleotide G to A at position 663 (the A of the initiation codon ATG being 1) in a codon for Trp<sup>221</sup>, resulting in a termination codon (Fig. 2B; see Fig. 1A, solid arrowhead), named ClPEX7W<sub>221</sub>ter. To assess the impaired function of Pex7p, ClPEX7W<sub>221</sub>ter was transfected back to ZPG207. PTS2-GFP remained in the cytosol in the transfected cells (Fig. 2A-f). Peroxisomal thiolase was likewise present in the cytosol in ClPEX7W<sub>221</sub>ter-transfected fibroblasts from a RCDP patient (data not shown), suggesting that ClPex7p-W<sub>221</sub>ter protein is not biologically active. Collectively, we conclude that the dysfunction of Pex7p caused by the W<sub>221</sub>ter mutation is responsible for the abnormal phenotype of ZPG207, e.g., the defect in PTS2 import (see Fig. 2A-a) and the resistance to P9OH/UV treatment (30).

**Impaired Interaction of Pex7p by Mutation**

We recently reported that the longer isofrom of the PTS1 receptor, Pex5pL, plays an indispensable role in the transport of PTS2 proteins by interacting with Pex7p (38, 39). Therefore, we investigated whether several types of mutations identified thus far in RCDP patients and the CHO cell mutant affect either or both of the two types of Pex7p interaction, one with PTS2 and the other with Pex5pL.

**Recognition of PTS2**—The wild-type and mutant forms of Flag-tagged Pex7p expressed in COS-7 cells were incubated with [³⁵S]rat thiolase precursor synthesized in vitro. Pex7p was then immunoprecipitated using anti-Flag antibody, where Pex7p and its variants were detected in similar amounts as verified by immunoblot (Fig. 3A, middle panel, lanes 2–7). [³⁵S]PTS2-thiolase was co-immunoprecipitated with the normal type of human Pex7p, whereas a near background level of [³⁵S]thiolase was detected with all three of the HaPex7p mutants with L292ter, A218V, or G217R, as well as when using mock-transfected cell lysate (Fig. 3A, top panel, lanes 1–5).

Wild-type CIPex7p interacted with [³⁵S]thiolase precursor, but ZPG207-derived Pex7p-W<sub>221</sub>ter did not (Fig. 3A, top panel, lanes 6 and 7). The binding of [³⁵S]thiolase to CIPex7p was comparable with that of HaPex7p (lanes 2 and 6). By quantitation of the co-immunoprecipitated [³⁵S]thiolase precursor, it was evident that PTS2 binding of all types of Pex7p mutants was at background level (Fig. 3A, bottom panel). Collectively, we concluded that the mutations so far identified in pex7 cell mutants, including RCDP fibroblasts, affect Pex7p in recognizing PTS2 proteins.

To verify whether Pex7p selectively interacts with the PTS2 sequence but not with the mature protein, several variants of the thiolase precursor with mutations (underlined) in the conserved motif of PTS2 (R/K)(L/V/I)(X₁)(H/Q)(K/L)/A), such as those with R→24Q, R→24H, and H→17E (the N-terminal residue of mature thiolase being +1) (8), were synthesized in vitro with [³⁵S]labeling and incubated with normal human Pex7p expressed in COS-7 cells. Only the wild-type PTS2 thiolase precursor was found in immunoprecipitates of Ha-Pex7p (Fig. 3B, lane 2), whereas all of the PEX7 mutants examined were not detectable with Pex7p (lanes 3–5). The mature form of thiolase lacking Pex7GFP was not co-immunoprecipitated with Pex7p (Fig. 3B, lane 1). Taken together, it is evident that Pex7p recognizes PTS2 but not PTS2 variants mutated in the conserved basic residues and the mature thiolase.

**Interaction with Pex5pL**—Next, we determined whether any mutation in Pex7p affects the interaction with Pex5pL. Cell lysates of COS-7 expressing HA-tagged Pex7p and its variants were incubated with two isoforms of Pex5p fused to GST, GST-Pex5pS and GST-Pex5pL. Bound protein fractions were ana-
The respective cell lysates were incubated with each of the Flag-tagged normal and mutant forms of Pex7p. Pex7p was immunoprecipitated using anti-Flag antibody and were analyzed by autoradiography. Top panel, [35S]thiolase at an exposure of 40 h; middle panel, recovered Flag-Pex7p was probed with anti-Flag antibody; bottom panel, [35S]thiolase was quantified and is represented as a percentage relative to the [35S]thiolase immunoprecipitated with human control Pex7p. B, binding of Pex7p to PTS2 variants. The binding assay was performed as described in A, using normal HA-HsPex7p and 3°S-labeled, 41-kDa matured thiolase as well as wild-type and mutant forms of the 44-kDa thiolase precursor. Pex7p was immunoprecipitated using anti-HA antibody. Lane 1, mature thiolase. Lane 2, normal thiolase precursor. Lanes 3–5, thiolase precursors with mutations (underlined) in the consensus nonapeptide PTS2 (R/K)(L/V/I). H is mutated to E at position –17 (by taking the N-terminal residue of mature thiolase as +1) (8); R to Q at –24; R to H at –24, respectively. Upper panel, in vitro synthesized [35S]thiolase (1 μl of each input). Lower panel, [35S]thiolase (5 μl) recovered by immunoprecipitation using anti-HA antibody. Note that Pex7p bound only to wild-type PTS2-thiolase.

For the interaction with Pex5pL, the turnover rate of Pex7p may be altered in these cell mutants as compared with that in CHO-K1. It is noteworthy that a similarly elevated level of Pex5p was also noted in these pex mutants (38). The Pex7p from these cell mutants was functional in binding to Pex5pL, as assessed by a GST pull-down assay using GST-Pex5pL (lanes 2, 4, 6, 8, 10, and 12), which was consistent with the notion that complementation of PTS2 import in pex mutants by expression of the selectively responsible PEX genes, such as PEX2 in pex2 Z85 (15). These results also suggested that the Pex7p-Pex5pL interaction does not require Pex2p, Pex12p, Pex13p, or Pex14p. The reason for apparently less binding of endogenous Pex7p to GST-Pex5pL, more notable in pex12 and pex13 (Fig. 4B, lanes 7–10) than in others such as CHO-K1, pex2, and pex14 (lanes 1, 2, 5, 6, 11, and 12), is presently unknown.

Functional Domain Mapping of Pex7p
Deletion of N-terminal Region—To search for the region of Pex7p required for biological activity, we constructed various deletion mutants of Chinese hamster Pex7p (ClPex7p) in which pex14 and pex2 genes, such as PEX7 (22), No protein band was discernible in pex7 ZPG207 (Fig. 4B, lane 3), where Pex7p could not be detected by anti-Pex7pC antibody, consistent with mutation analysis of PEX7 in ZPG207 (see Fig. 2). In pex mutants defective in the import of PTS1 and PTS2, Pex7p was found to be present stably at a significantly higher level, ranging from severalfold to more than 10-fold, as compared with that in CHO-K1 (Fig. 4B, lanes 1, 5, 7, 9, and 11). The turnover rate of Pex7p may be altered in these cell mutants as compared with that in CHO-K1. It is noteworthy that a similarly elevated level of Pex5p was also noted in these pex mutants (38). The Pex7p from these cell mutants was functional in binding to Pex5pL, as assessed by a GST pull-down assay using GST-Pex5pL (lanes 2, 4, 6, 8, 10, and 12), which was consistent with the notion that complementation of PTS2 import in pex mutants by expression of the selectively responsible PEX genes, such as PEX2 in pex2 Z85 (15). These results also suggested that the Pex7p-Pex5pL interaction does not require Pex2p, Pex12p, Pex13p, or Pex14p. The reason for apparently less binding of endogenous Pex7p to GST-Pex5pL, more notable in pex12 and pex13 (Fig. 4B, lanes 7–10) than in others such as CHO-K1, pex2, and pex14 (lanes 1, 2, 5, 6, 11, and 12), is presently unknown.

Functional Domain Mapping of Pex7p
Deletion of N-terminal Region—To search for the region of Pex7p required for biological activity, we constructed various deletion mutants of Chinese hamster Pex7p (ClPex7p) in which a Flag epitope was tagged at the N terminus. CHO pex7 ZPG207 cells and fibroblasts from a patient with RCDP were transfected with each of the PEX7 variants and assessed for complementing activity (Fig. 5A). Wild-type Flag-ClPex7p complemented the impaired import of PTS2-GFP in ZPG207 as well as that of the thiolase precursor in RCDP fibroblasts (see Fig. 2A). The deletion of 7 amino acids from the N terminus did not affect the PTS2 import-restoring activity in ZPG207, whereas an 11-amino acid truncated ClPex7p no longer
maintained the activity (Fig. 5A, a and b). Interestingly, ClPex7pΔ1–7 was longer only by 4 amino acid residues, -RVPG-, than nonfunctional ClPex7pΔ1–11. Essentially the same results were obtained when these Pex7p mutants were verified in RCDP fibroblasts (data not shown). However, none of the N-terminally further truncated Pex7p variants, Δ1–19, Δ1–25, and Δ1–51, restored PTS2 protein import in ZPG207 (Table II). These Pex7p variants were also verified with respect to processing of the thiolase precursor to the mature protein. The processed form of thiolase was detected in immunoblots of ZPG207 cells (Fig. 5, lanes 1 and 2) and not in other types of transfectants such as those of CIPEX7Δ1–11 and CIPEX7Δ1–19 (lanes 3 and 4). Collectively, the N-terminal region of Pex7p, containing at least amino acid residues 7–11, is required for Pex7p function.

A Pex7p variant with a point mutation at position 10 (P10A) showed a normal complementing activity (Table II). Several other types of mutations in the N-terminal region, including the mutation of three basic charges to noncharged Ala at positions 5, 8, and 12 (R5A/R8A/R12A) did not affect the Pex7p activity (Table II), suggesting that these basic residues are dispensable. In ClPex7p the sequence of amino acid residues at positions 26–34, RLACAAQKH, which is highly conserved between at least three species of mammals (see Fig. 1A), resembles the PTS2 motif, (R/K)(L/V)LXp(II/H)(Q/L)A/ (13). We investigated whether mutation of the conserved residues affects the biological activity of Pex7p. Pex7p with mutations at R26H or Q33E complemented the impaired PTS2 import of ZPG207 (Table II), suggesting that this sequence does not behave in the same manner as PTS2. Moreover, the in vitro transcription/translation product of CIPEX7 showed the same mobility in SDS-

| Pex7p variant | Complementing activity | Binding to Pex5pL | PTS2 |
|---------------|------------------------|-------------------|------|
| Wild-type     | +                      | +                 | +    |
| Δ1–7          | +                      | −                 | −    |
| Δ1–11         | −                      | −                 | −    |
| Δ1–19         | −                      | −                 | −    |
| Δ1–25         | −                      | ND                | ND   |
| Δ1–51         | −                      | ND                | ND   |
| Δ305–318      | −                      | −                 | −    |
| Δ310–318      | +                      | +                 | +    |
| Δ311–318      | +                      | ND                | ND   |
| G212R         | −                      | −                 | −    |
| P10A          | −                      | −                 | −    |
| R5A/R8A/R12A  | +                      | ND                | ND   |
| R26H          | −                      | ND                | ND   |
| Q33E          | +                      | ND                | ND   |
| W221ter        | −                      | −                 | −    |
| G217R         | −                      | −                 | −    |
| A219F         | −                      | −                 | −    |
| L292ter        | −                      | −                 | −    |

a Chinese hamster counterpart of human Pex7p-G217R.

b CIPEX7 mutant derived from ZPG207.

c Human Pex7p mutants identified in RCDP patients.

PAGE with ClPex7p expressed in CHO-K1 (data not shown), implying that the N-terminal sequence of Pex7p, including this PTS2-like sequence, is not cleaved. ClPex7p-G212R corresponding to the mutant Pex7p-G217R from RCDP patients did not complement ZPG207 cells (Table II), where the thiolase
precursor was not processed (Fig. 5B, lane 5).

Truncation of C-terminal Region—The WD motifs in Pex7p appear to be indispensable for the biological activity of Pex7p in peroxisome biogenesis, as depicted by several mutations that occurred in RCDP patients; the most frequent one, at L292Ter, resulted in the truncation of the sixth WD. To search for the minimal sequence required at the C-terminal region, we constructed C-terminal deletion mutants of Flag-ClPex7p. Mutant Flag-ClPex7pΔ310–318 lacking the C-terminal 9 amino acid residues (terminating at 1 amino acid upstream from the C terminus of the sixth WD motif) showed complementary activity in ZPG207, whereas deletion of the C-terminal 14 amino acids (Flag-ClPex7pΔ305–318) abrogated the activity (Fig. 5A, c and d). Flag-ClPex7pΔ311–318 was likewise functionally active (Table II). In an immunoblot of cell lysates of ZPG207 cells transfected with PEX7 variants, the mature thiolase was detectable in addition to the precursor in Flag-ClPEX7Δ310–318-transfected ZPG207 cells (Fig. 5B, lane 7) but not in Flag-ClPEX7Δ305–318-transfected cells (lane 6). The mature thiolase was also visible in an immunoblot of Flag-ClPEX7Δ311–318-transfected ZPG207 cells (data not shown). Therefore, only short stretches comprising less than 10 amino acids could be truncated from the N or C terminus to maintain the biological function of Pex7p.

Binding to Pex5pL and PTS2—Various ClPex7p mutants were also verified for binding to Pex5pL and PTS2, as done for Pex7p derived from RCDP patients and ZPG207 (see Figs. 3 and 4). Flag-ClPex7pΔ1–7 bound to Pex5pL less efficiently as compared with the wild-type ClPex7p, whereas normal ClPex7p did not interact with Pex5pS (Fig. 5C, lanes 1–3). Several other N-terminal truncation mutants of ClPex7p, Flag-ClPex7pΔ11 and Flag-ClPex7pΔ1–19, were not detected in fractions bound to GST-Pex5pL (lanes 4 and 5). Several C-terminal truncation variants such as Flag-ClPex7pΔ310–318 (lane 8) and Flag-ClPex7pΔ311–318 (data not shown), but not Pex7pΔ305–318 (lane 7), interacted with Pex5pL. Flag-ClPex7p–G212R, a CHO counterpart of HsPex7p–G217R, was eliminated in Pex5p binding activity (lane 6), consistent with the results obtained using HsPex7p–G217R (see Fig. 4A).

Furthermore, binding to PTS2 (∼35S-labeled thiolase precursor) was detectable in the ClPex7p variants Flag-ClPex7pΔ1–7 and Flag-ClPex7pΔ310–318 (Fig. 5D, lanes 3 and 7) but with less efficiency as compared with the wild-type Flag-ClPex7p (lane 2). Other variants of Pex7p, including Flag-ClPex7pΔ1–11, Flag-ClPex7pΔ305–318, and Flag-ClPex7p–G212R, which were defective of binding to Pex5pL (see Fig. 5C), barely showed binding to PTS2, as seen in a mock treatment (Fig. 5D, compare lanes 4–6 with lane 1).

Collectively, all of the functionally active Pex7p variants verified here were competent in binding to Pex5pL as well as PTS2.

Subcellular Localization of Pex7p

We investigated intracellular localization of Pex7p by several approaches.

Morphological Observation—CHO-K1 cells were stained using rabbit antibody raised against bacterially expressed human Pex7p. Pex7p was detectable by immunofluorescent microscopy in punctate structures (Fig. 6A–a) as numerous catalase-positive particles (Fig. 6A–c) (namely peroxisomes) in CHO-K1 cells that had been fixed and treated with 0.1% Triton X-100 in a superimposable manner with Pex14p staining pattern (Fig. 6A–b), suggesting that the endogenous Pex7p is localized in peroxisomes. Pex7p was also discernible in the cytoplasm in a diffuse staining pattern, suggesting cytoplasmic localization of Pex7p. CIPex7p was discernible mostly in the cytoplasm in a diffusion pattern when transiently over-expressed in CHO-K1 cells and detected using anti-Pex7pC antibody (Fig. 6A–d), similar to the finding on the overexpressed mouse Pex7p in human fibroblasts (31). Pex7p likewise was observed in 20T7P cells, a pex7 mutant ZPG207 (30) that had been stably transfected with HsPex7 (data not shown; see Fig. 8A). Moreover, Pex7p and catalase were not discernible in a punctate staining pattern in CHO-K1 cells that had been treated with 25 μg/ml digitonin (Fig. 6A, e and g) under conditions in which only plasma membranes were permeabilized, whereas Pex14p was discernible using antibody to the cytoplasmically oriented C-terminal part, Pex14pC (Fig. 6A–f). The results strongly suggested that Pex7p partly resides inside peroxisomes.

Subcellular Fractionation—When CHO-K1 cells were fractionated, Pex7p was found in both organelar and cytosolic fractions, apparently in greater amounts in the cytosol (Fig. 6B, upper panel, lanes 1–3), whereas Pex7p was not detected in fractions from a pex7 ZPG207 (lanes 4–6). Similar dual distribution of Pex7p was more distinctly observed on fractionation of ClPEX7-transfected CHO-K1 and 20T7P cells (Fig. 6B, lower panel, lanes 1–6). AOx, a peroxisomal matrix PTS1-type enzyme, was found exclusively in the organelar fraction, obviously in peroxisomes, from all types of cells examined here, confirming the adequate separation of organelles and cytosol (Fig. 6B, upper and lower panels). Similar results, cytoplasmic staining and bimodal distribution of Pex7p as assessed using anti-HA antibody (data not shown), were obtained using HA-ClPEX7-transfected COS-7 cells.

To confirm the findings described above with respect to the intracellular location of Pex7p, PNS fractions from CHO cells of wild type, pex7 ZPG231 solely deficient in Pex7 import (39), pex13 ZP128 (21), and pex14 ZP161 (22) were fractionated by isopycnic sucrose density gradient ultracentrifugation. In CHO-K1, a larger amount of Pex7p was nearly at the top of the gradient and a smaller amount of Pex7p was co-sedimented with catalase and a peroxisomal membrane protein, PMP70, which was indicative of localization in peroxisomes (Fig. 6C, top panel). This bimodal distribution was consistent with the recovery of Pex7p in the membrane and soluble fractions (see Fig. 6B). Pex5p showed a very similar distribution throughout the gradient (Fig. 6C, top panel) as reported elsewhere (38). A similar bimodal distribution was observed upon fractionation of PNS from 20T7P cells (data not shown). In contrast, in ZPG231 Pex7p was exclusively detected at lighter density fractions corresponding to the same density fractions as in the case of the wild-type cells, apparently representing cytosolic Pex7p (Fig. 6C, middle upper panel). Immunoblotting of a higher, 4-fold amount of fractions 11–19 plus 20–21 showed no Pex7p-positive signal, hence confirming the absence of the peroxisome-associated form of Pex7p. Pex5p showed a bimodal distribution similar to that noted in CHO-K1 cells, where the Pex5p detectable at a somewhat lower level in the higher density fractions co-sedimented with PMP70 (Fig. 6C, middle upper panel), thereby reflecting normal import of Pts1 proteins as reported elsewhere (39). In Pex13p-defective ZP128 (21), Pex7p was discernible nearly from the top to the middle of the gradient, where PMP70 representing peroxisomal remnants sedimented to the middle (Fig. 6C, middle lower panel). This was consistent with the recovery of a larger proportion of Pex7p in the membrane fraction of ZP128 (see Fig. 7A, lanes 2 and 3 in panel ZP128). Pex5p showed a distribution similar to Pex7p through the gradient in ZP128. Contrary to a mutant ZP128, in Pex14p-deficient ZP161 almost all of Pex7p remained near the top, showing a distribution very similar to Pex5p (38), whereas PMP70, a marker membrane protein for peroxisomal ghosts, was in the middle of the gradient (Fig. 6C, bottom panel).
Therefore, Pex7p is partly localized in the peroxisomal remnants in pex13 mutant, ZP128, whereas Pex7p is in soluble fraction in pex14 ZP161. Thus, it is most likely that Pex14p is required for the targeting of Pex7p to peroxisomes, apparently in a Pex5pL-dependent manner as described previously (38). Collectively, we conclude that Pex7p is localized in both peroxisomes and the cytoplasm in normal cells. Pex7p is also associated with peroxisomal remnants, most likely in a Pex5p-dependent manner.

Protease Protection Assay—To determine whether Pex7p sedimented in peroxisomal fractions resides inside peroxisomes, a so-called protease protection assay was performed.
mant of pex7 ZPG207 cells (Fig. 7A, lower left panel). These results were interpreted to mean that Pex7p detected in a protease-resistant form was localized inside peroxisomes, hence being protected from the protease digestion.

In contrast, in a pex5 ZPG231 defective only in PTS2 import (39), Pex7p was sensitive to Pronase as Pex14p and a membrane protein PMP70, whereas AOx was resistant to the treatment, indicating that Pex7p was localized exclusively in the cytosol (Fig. 7A, top right panel). This finding was consistent with the results obtained by isopinic ultracentrifugation, showing that all of Pex7p was sedimented near the top of the gradient (see Fig. 6C). In pex14 ZP161 Pex7p was similarly digested as PMP70, suggesting cytoplasmic localization of Pex7p (Fig. 7A, bottom right panel). In the case of pex13 ZP128, Pex7p recovered in the membrane fraction was partly protected from the digestion, whereas PMP70 and Pex14p were digested by the protease, demonstrating that Pex7p associated with peroxisomal remnants partly resided inside the membrane vesicles (Fig. 7A, middle right panel). Pex7p in membrane fractions from pex2 Z65 and pex12 ZP109 was likewise resistant to digestion (data not shown), suggesting that Pex7p was stuck inside the remnants as noted for Pex5p (38). Furthermore, in human fibroblasts from a normal control and pex13 fibroblasts manifesting an import defect of catalase, but not PTS1 (26) and PTS2 (data not shown), Pex7p was partly protected from the Pronase digestion, indicating that Pex7p is localized inside peroxisomes and the peroxisomal remnants, respectively (Fig. 7B).

Pex7p Translocates into Peroxisomes

Because Pex5pL was demonstrated to be responsible for Pex5p-mediated PTS2 import (38, 39), we transfected CIPEx5L-HA as well as CIPExSS-HA into 207P7 cells, to further investigate whether Pex7p translocates into peroxisomes with its cargo protein in a Pex5pL-dependent manner. Intracellular localization of Pex7p was determined by the differential permeabilization method (20, 43). Expression of Pex5p5-HA and Pex5pL-HA was apparently at the same level in the transfected 207P7 cells (48%) (Fig. 8A, a and e). PTS2-GFP fluorescence patterns were more distinct in cells expressing Pex5pL-HA (Fig. 8A, e and f, arrowheads), whereas Pex5p5-HA-expressing cells showed PTS2-GFP fluorescence intensity similar to that in untransfected cells (Fig. 8A, a and b). Pex7p was visualized in distinct and larger punctate staining as well as cytoplasmically diffused patterns in Pex5pL-HA expressing 207P7 cells as compared with that in PEX5S-HA-transfected cells (Fig. 8A, g and c). Brighter Pex7p-positive punctate structures were evidently superimposable with PTS2-GFP fluorescence patterns (g and h, arrowheads), strongly suggesting that Pex7p was more readily translocated to peroxisomes with its cargo PTS2-GFP by expressed Pex5pL-HA. Essentially the same results were obtained when the cells were stained using anti-Pex7pC antibody (data not shown). Enhancement of the intensity of PTS2-GFP-positive peroxisomes by expression of Pex5pL-HA was consistent with our earlier observation (38). Moreover, the punctate staining pattern of Pex7p and catalase was not discernible in PEX5L-HA-transfected 207P7 cells that had been treated with 25 μg/ml digitonin (Fig. 8B, a and b), under which conditions only plasma membranes were permeabilized; however, Pex14p was visualized using antibody to the cytoplasmically oriented C-terminal peptide (Fig. 8B-c). The results strongly suggested that Pex7p translocated to peroxisomes by Pex5pL resides inside peroxisomes.

Pex5pL-dependent translocation of Pex7p into peroxisomes was also assessed by the protease protection assay. Upon expression of PEX5L-HA, an increased level of Pex7p in organelle
fraction of 20T7P cells was evident (Fig. 8C, lower panel, lane 3) as compared with that in PEX5S-HA-transfected (Fig. 8C, upper panel, lane 3) and mock-transfected cells (Fig. 7A, lower left, lane 3). A protease-protected amount of Pex7p was likewise more distinct in the organelle fraction of PEX5L-HA-transfected 20T7P (Fig. 8C, lower panel, lanes 5 and 7) than in those of PEX5S-HA- or mock-transfected cells (Fig. 8C, upper panel; Fig. 7A, lower left), under which conditions a matrix control protein, AOx was also protected (data not shown). An imported PTS2 cargo, PTS2-GFP, was concomitantly enhanced on Pex5pL between PTS2 cargo-loaded and unloaded forms. Much larger amounts of both 35S-labeled Pex7p and [35S]thiolase precursor apparently bound to GST-Pex5pL than did Pex7p in the absence of [35S]thiolase precursor or in the presence of mature [35S]thiolase or only [35S]thiolase precursor (Fig. 8D, upper panel). Preferential binding to Pex5pL of Pex7p-PTS2 protein complexes, as compared with the unloaded Pex7p, was also evident in the GST pull-down assays using cell lysates from Flag-ClPex7-transfected CHO-K1 plus pex7 ZPG207 or CHO-K1 as a source of a PTS2 cargo PTS2-GFP or a control, respectively. Pex7p and PTS2-GFP were detected using antibodies to Pex7pC and GFP, respectively. Dot, a nonspecific band. Aliquot parts (one-tenth) of each of the respective total cell lysates used for the assay were loaded in lanes 1–3. Recovered GST-Pex5pL was assessed as above.
DISCUSSION

In 15 different genotypes of peroxisome biogenesis deficiency in mammals (11, 28–30, 51) including peroxisome biogenesis disorders in 12 CGs (13, 28, 29, 52), a common phenotype is the import defect of PTS1 and PTS2 matrix proteins, except for RCDP in which only PTS2 transport is impaired (43, 53). Mutation of PEX7 encoding the PTS2 receptor is the genetic cause of RCDP (31–33). Our novel CHO cell mutant, ZPG207, was deficient in PTS2 protein import and was classified into the same CG as RCDP patient-derived cells (30). Expression of human and Chinese hamster PEX7 morphologically and biochemically restored the impaired peroxisome biogenesis in ZPG207. Moreover, we found that a non-sense point mutation in a codon for W221 of PEX7 was responsible for the morphological as well as biochemical phenotype of a pex7 ZPG207 Chinese hamster Pex7p was 91% identical to human Pex7p at the amino acid sequence level and was also functional in restoring the impaired PTS2 import in RCDP patient fibroblasts, thereby implying that Pex7p is highly conserved in mammals.

All mutated forms of Pex7p, such as Pex7p with L292ter, Pex7p-A218V, and Pex7p-G217R, represented the frequently occurring mutations in RCDP patients, as well as the ZPG207-derived Pex7p-W221ter, were inactive in restoring the impaired PTS2 import when expressed in both ZPG207 cells and RCDP patients’ fibroblasts. We recently found a novel function of Pex5pL; Pex5pL is exclusively responsible for Pex7p-mediated PTS2 translocation to peroxisomes by mediating the binding of Pex7p-cargo complexes to the initial docking site, Pex14p (38, 39). In mammals, Pex7p plays a role in PTS2 import by directly binding to PTS2 as well as to Pex5pL, whereby translocation of the Pex7p-PTS2 cargo complexes to peroxisomes is mediated by Pex5pL. We investigated which role(s) of Pex7p, i.e., PTS2 recognition and/or binding to Pex5pL, are actually affected and are responsible for the pex7 phenotype noted in RCDP patient-derived fibroblasts and a pex7 CHO mutant. All of the Pex7p mutants examined, including Pex7p-L292ter, Pex7p-A218V, Pex7p-G217R, and Pex7p-W221ter, were shown to be primarily impaired in binding to PTS2. This finding may well explain the phenotypic PTS2 import defect. The mutation G217R was identified in the second allele in RCDP patients with the mutation L292ter in the first allele but was not determined with respect to its functional significance (31). In the present study, we have demonstrated that Pex7p-G212R, a CHO variant equivalent to human Pex7p-G217R, is dysfunctional. Although no distinct difference could be observed in the morphological phenotype between pex7 cells, both ZPG207 and RCDP fibroblasts, that had been transfected with Pex7p possessing G217R and other mutations, the homozygous G217R mutation may confer a clinical phenotype milder than classical L292ter-type RCDP, as noted in patients with a homozygous A218V mutation (31). Furthermore, the interaction of Pex7p with Pex5pL is also abrogated in all Pex7p mutants from RCDP patients and in the CHO pex7 mutant. Collectively, we conclude that the dysfunction of Pex7p in both PTS2 recognition and binding to Pex5pL is responsible for the cell phenotype of pex7 mutants, including fibroblasts from RCDP patients. Defect of either one of these two distinct functions of Pex7p, i.e., PTS2 and Pex5pL binding, may also show a similar cell phenotype, despite the fact that no such findings have been reported thus far. Of note, a pex7 ZPG231-carrying mutation of S214F in Pex5pL abolished the binding to Pex7p, giving rise to a phenotype defective solely in PTS2 import (39). Yeast PTS2 import is solely dependent on Pex7p (34–36), hence implying that Pex7p is most likely to be disturbed in PTS2 recognition in pex7 mutants. It is also noteworthy that the introduction of three independent mutations, corresponding to those of RCDP patients described above, into the yeast P. pastoris PEX7 affected the binding of P. pastoris Pex7p to PTS2 as assessed by the yeast two-hybrid assay (36), consistent with our observation in the in vitro co-immunoprecipitation assay using mammalian proteins. Impaired docking of Pex7p to the convergent receptor Pex14p may also be possible in yeast and remains to be defined.

Missense mutations, G217R and A218V, occurred at a conserved amino acid, Gly or Ala, in the fourth WD motif of Pex7p. Mutation of Gly217 to Arg results in a basic and bulky group, presumably affecting conformation of the WD motif. Mutation at G212R, equivalent to G217R in HsPex7p, eliminated the Pex7p function, hence confirming that Gly217 is not dispensable. Likewise, substitution of Val for a conserved residue Ala/Gly creates a bulky hydrophobic side chain and may also alter the WD motif structure. Such mutations indeed affected the interaction with its Partners, PTS2 protein and Pex5pL. Furthermore, termination at Trp221 completely abrogated the Pex7p function. Taking these findings together, it is evident that the fourth WD region is essential for the Pex7p. Another variant, Pex7p-L292ter, was expressed nearly at the same level as the wild-type in COS-7 cells but was inactive in peroxisome-restoring activity, demonstrating that impaired function of Pex7p-L292ter is not due to protein degradation. Hence this observation implies that the sixth WD motif locating at the C-terminal region is also required for the biological activity of Pex7p. Therefore, WD motifs 4 and/or 6 are likely to be involved in the interaction with Pex5pL and PTS2. Furthermore, deletion of 7 amino acid residues but not 11 residues from the N-terminus maintained the biological activity including PTS2 import in vivo and in vitro binding to PTS2 and Pex5pL. From the C-terminus, again only 9 residues but not 14 residues could be truncated without the loss of such biological activities. Therefore, based on the results obtained from these partial truncation analyses, we conclude that nearly the full length, including all WD motifs of Pex7p, is required for the biological role involving PTS2 protein import, namely PTS2 and Pex5pL binding, as well as Pex5pL-dependent translocation to peroxisomes.

The N-terminal part is more likely to be facing toward the surface of Pex7p molecule. In determining the accessibility of Pex7p to protease, the major cleaved fragment, smaller in size by ~5 kDa, was detected with anti-Pex7p antibody, implying that Pex7p was eliminated in the N-terminal part consisting of about 40 to 50 amino acid residues but was maintained intact the C-terminal portion. Of note, Pex7p was highly resistant to several proteases examined in this report. Pronase was found to be a more potent protease than trypsin, thermolysin, papain, or proteinase K. It is noteworthy that the propeller-like structure maintained by six WD motifs has been shown essential for the function of the Gβ-subunit (54), which is similar presumably to Pex7p in the WD motif family. Pex7p may possess a similar configuration. At any event, the precise regions of Pex7p responsible for interaction with Pex5pL and PTS2 binding remain to be defined.

The sequence RLACAAAQH of mammalian Pex7p at positions 26–34 in Chinese hamster and mouse (RLACATAQH at positions 31–39 in humans) resembled the PTS2 consensus motif, (R/K)(L/V/I)X(H/Q/L/A) (where X is any amino acid). But it is less likely to be a PTS2, because the mutations at R26H or Q33E did not affect the function of Pex7p. Such mutations eliminated the PTS2 function in the case of thiolase precursor (8). In contrast, S. cerevisiae Pex7p was shown to possess a PTS2-like activity in the N-terminal region (35). It is noteworthy that PTS2 of S. cerevisiae thiolase is not cleavable (35), whereas PTS2 in mammalian thiolase is processed after

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import into peroxisomes (41, 44). Such differences may explain the differences observed in PTS2-like activity between yeast and mammalian Pex7p. We have also shown in the present study that mutant PTS2 protein, the thiolase precursor with each of the mutations at the conserved residues for the PTS2 function, H(–17)E, R(–24)Q, and R(–24)H in RLQVVLGHHL, no longer binds Pex7p. Accordingly, this clearly explains our earlier observation that mislocalization of such mutant thiolase precursors to the cytosol (8) is because of the failure of Pex7p in binding to the mutated PTS2. In the consensus PTS2 sequence of S. cerevisiae thiolase, missense mutations R4G (34) and L5R (35) similarly caused significant decrease in interaction with Pex7p.

In the present study, we have found for the first time a bimodal distribution of Pex7p at the endogenous level, namely in the cytoplasm and peroxisomes in CHO and human cells, by making use of anti-Pex7p antibodies that we raised. Essentially the same bimodal distribution of Pex7p was observed in subcellular fractionation of CHO cells expressing Pex7p at a higher level, such as 20TP7 cells, thereby confirming the findings using normal control cells. Our conclusion is based on the findings in: 1) morphological observation on Pex7p inside peroxisomes, 2) subcellular fractionation studies, 3) protease protection assays, and 4) Pex5pL-mediated translocation of Pex7p and PTS2 protein. In our earlier report (38), we proposed a hypothetical model for the Pex5pS and Pex5pL complexes that shuttles between the cytoplasm and peroxisomes. Translocation of Pex7p to peroxisomes requires Pex5pL, which docks with Pex14p at the initial step (38). The addition of anti-Pex14p antibody to the import assay using a semi-intact CHO cell system apparently interfered with PTS2 import (55), consistent with our observation (38). Particulate-bound endogenous Pex7p was resistant to exogenously added protease and co-sedimented with peroxisomal marker proteins, whereas soluble Pex7p was protease-sensitive. These observations have provided evidence for a bimodal, cytosolic and peroxisomal distribution of Pex7p in cells. In isopycnic ultracentrifugation on a sucrose density gradient of PNS fractions, each from normal as well as pex mutant cells, Pex7p showed a sedimentation pattern very similar to that of Pex5p, possibly consisting of homodimeric and heterodimeric forms of Pex5pL and Pex5pS. Furthermore, overexpression of Pex5pS but not Pex5pS enhanced translocation of Pex7p and PTS2 protein into peroxisomes. Indeed, Pex5pL selectively bound to PTS2 cargo-loaded Pex7p in vitro. Given these findings, we propose that Pex7p is the mobile PTS2 receptor, like the PTS1 receptor Pex5p (38, 56), and translocates into peroxisomes in a Pex5pL-dependent manner. It is likely that Pex7p and Pex5pL then shuttle back to the cytosol either in a bound form or independently after unloading the cargoes. Such a mobile Pex7p has also been suggested by overexpressing Pex7p in P. pastoris (36). Moreover, Pex5p has very recently indeed been shown to be a shuttling receptor in human cells in a semi-intact cell system (57). Therefore, it is most likely that Pex7p is likewise recycled between peroxisomes and the cytoplasm.

It should also be noted that Pex7p is inducible at nearly 20-fold the normal level in rat, as determined by peroxisome proliferators such as clofibrate, as is also the case for thiolase (see Fig. 1B). However, expression of Pex5p, both Pex5pS and Pex5pL, is not elevated by clofibrate treatment, whereas PTS1 proteins such as AOx are induced at 30–50-fold (see Fig. 1B) (38). How can these apparently unparallelled expression levels of peroxins and cargo proteins be reconciled? The expression level of Pex5p is higher than Pex7p in mammalian cells,3 as noted in rat liver (see Fig. 1B), where PTS1 proteins are generally more abundant than PTS2 proteins. Pex5p forms not only a homodimeric but also a heteromeric form, wherein only the dimers including Pex5pL play a role in the PTS2 transport (38). Immunoprecipitation of Flag-tagged Pex7p expressed in CHO-K1 cells failed to co-immunoprecipitate the co-expressed, untagged Pex7p (data not shown), suggesting that Pex7p appears to be present in a monomeric form. Accordingly, the elevated level of PTS2 protein may require a stoichiometrically equivalent level of its receptor Pex7p, in which dimerization of Pex5p may also accommodate an import receptor adaptive from a normal level to a highly induced state of PTS1 and PTS2 proteins. The dimeric form of recombinant Pex5pS indeed shows a higher affinity to a PTS1 protein, AOx, as AOx concentration increases (58). To validate this hypothesis at the molecular level, further kinetics studies are required.

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