Molecular Mechanisms of Import of Peroxisome-targeting Signal Type 2 (PTS2) Proteins by PTS2 Receptor Pex7p and PTS1 Receptor Pex5pL*

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In the present study, we investigated molecular mechanisms underlying the import of peroxisome-targeting signal type 2 (PTS2) proteins into peroxisomes. Purified Chinese hamster Pex7p that had been expressed in an SF9/baculovirus system was biologically active in several assays such as those for PTS2 binding and assessing the restoration of the impaired PTS2 protein import in Chinese hamster ovary (CHO) pex7 mutant ZPG207. Pex7p was eluted as a monomer in gel filtration chromatography. Moreover, the mutation of the highly conserved cysteine residue suggested to be involved in the dimer formation did not affect the complementing activity in ZPG207 cells. Together, Pex7p more likely functions as a monomer. Together with PTS1 protein, the Pex7p-PTS2 protein complex was bound to Pex5pL, the longer form of Pex5p, which was prerequisite for the translocation of Pex7p-PTS2 protein complexes. Pex5pL-(Pex7p-PTS2 protein complexes were detectable in wild-type CHO-K1 cells and were apparently more stable in Pex14 CHO cells deficient in the entry site of the matrix proteins, whereas only the Pex7p-PTS2 protein complex was discernible in a Pex5pL-defective pex5 CHO mutant. Pex7p-PTS2 proteins bound to Pex14p via Pex5pL. In contrast, PTS2 protein-bound Pex7p as well as Pex7p directly and equally interacted with Pex13p, implying that the PTS2 cargo may be released at Pex13p. Furthermore, we detected the Pex13p complexes likewise formed with Pex5pL-bound Pex7p-PTS2 proteins. Thus, the Pex7p-mediated PTS2 protein import shares most of the steps with the Pex5p-dependent PTS1 import machinery but is likely distinct at the cargo-releasing stage.

Peroxisome functions in various metabolic pathways including β-oxidation of very long chain fatty acids (1). With respect to biogenesis of peroxisomes, matrix proteins are synthesized on cytoplasmic free polysomes and are post-translationally transported to pre-existing peroxisomes (2). Two types of peroxisome-targeting signal (PTS)3 for the matrix proteins are identified: the C-terminal tripeptide-SKL and its conserved variants (PTS1) for most proteins (3, 4) and PTS2, the N-terminal cleavable nonapeptide, -{R/K}(L/V/I)X_4(H/Q)(L/A)-, present in several proteins such as 3-ketoacyl-CoA thiolase (thiolase) of fatty acid β-oxidation pathway (5, 6). More than 14 PEX genes are required for peroxisome biogenesis in mammals (7, 8). PEX5 and PEX7 are causal genes for peroxisome biogenesis disorders of complementation groups 2 and 11, encoding the PTS1 and PTS2 receptors, respectively (9–14). Pex5p and Pex7p, a Trp-Asp motif (WD) protein, recognize their cargoes in the cytoplasm and transport them to peroxisomes. We reported earlier that mutations in Pex7p such as L292ter, G217R, A218V, and W221ter abrogate the binding to Pex5pL (see below) and PTS2 (14).

Two isoforms of Pex5p, termed Pex5pS and Pex5pL, with 37-amino acid insertions, are identified in mammals (10). Pex5pL forms a complex with Pex7p-PTS2, being exclusively responsible for PTS2 protein import (15, 16). Thus, Pex5pL has distinct domains, including the N-terminal region for Pex7p-PTS2 binding and the C-terminal TPR motifs responsible for binding to PTS1 (16). However, whether Pex5pL simultaneously transports both PTS1 and PTS2 cargoes has not yet been elucidated. In Saccharomyces cerevisiae, Pex7p-PTS2 complexes are translocated to peroxisomes by Pex18p-Pex21p complexes (17), apparently the functional homologue of mammalian Pex5pL. Pex5pL docks with Pex14p to form complexes comprising Pex14p-Pex5pL-Pex7p-PTS2 (18). Contrary to this, Pex18p-Pex21p complexes do not directly bind to Pex14p when the docking complexes consisting of the Pex7p-PTS2 protein and Pex14p are formed (19). Hence, the mechanism of PTS2 import is partly distinct between the yeast and mammalian cells.

In this study, we attempted to address the molecular mechanisms underlying Pex7p-mediated PTS2 import. First, we made successful progress in expressing biologically active, recombinant mammalian Pex7p in an SF9/baculovirus system. Using the purified Pex7p, we show here that Pex7p-PTS2 protein complex can be loaded onto Pex5pL together with PTS1

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3 The abbreviations used are: PTS1 and PTS2, peroxisome-targeting signal types 1 and 2; CHO, Chinese hamster ovary; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; HA, influenza virus hemagglutinin; IgG, immunoglobulin G; thiolase, 3-ketoacyl-CoA thiolase; WD, Trp-Asp motif; WT, wild type.
Pex7p-mediated Transport of PTS2 Proteins

#### EXPERIMENTAL PROCEDURES

**Biochemicals**—Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene (Tokyo, Japan) and Takara (Kyoto, Japan). Fetal bovine serum and Hams F-12 medium were from Invitrogen. We used rabbit antibodies to thioloise (20), Pex7p (14), Pex5p (18), Pex13p (21), Pex14p (22), and guinea pig anti-Pex14p antibody (14). Rabbit antibody to green fluorescent protein (GFP), glutathione S-transferase (GST), and mouse anti-FLAG antibody were purchased from Sigma. Mouse antibody (16B12) to influenza virus hemagglutinin (HA) was from Covance.

**Construction for Protein Expression in Sf9 Cell/Baculovirus System**—To use a Bac-to-bac baculovirus expression system (Invitrogen), we made several constructs. Full-length Chinese hamster PEX7 was amplified with a pair of primers, PEX7 EcoRI-Fw (Table 1) and ClPEX7 SalI-Rv (14), and ligated into EcoRI-SalI site of pFastBac HTb vector. pFastBacHTb-FLAG-PEX7 was constructed as follows. BamHI, and introduced into the blunted RsrII and BamHI site between EGFP and PTS15 was ligated into the BamHI vector-derived sequence for 5 amino acids intervening PEX7 and pcDNA3.1zeo-HA2-EGFP-PTS1 and pcDNA3.1zeo-FLAG-EGFP-PTS1 were constructed by subcloning of BamHI-Apal fragment derived from pucd2SraMCSHyg-HA2-EGFP-PTS1 to the same site of pcDNA3.1zeo-HA2-ubiquitin* and of pcDNA3.1zeo-FLAG-ubiquitin. pcDNA3.1zeo-HA2-PTS2-EGFP and pcDNA3.1zeo-FLAG-PTS2-EGFP were constructed as follows. BamHI-Ncol fragment of the PCR product for PTS2 of pTZ18R-pTH using primers pTH-Fw+2-Fw and pTHM8-Rv was ligated together with the Ncol-NotI fragment of pEGFP into the BamHI-NotI site of pcDNA3.1zeo-HA2-ubiquitin and pcDNA3.1zeo-FLAG-ubiquitin. Flag-PEX7C199S was also constructed. The PCR products each with pairs of primers C1995-Fw and CIPEX7 SalI-Rv (14) and CIPEX7-Fw (14) and C1995-Rv using a template pucd2SraMCSHyg-FLAG-PEX7 were mixed and used as a template in PCR with CIPEX7-Fw and CIPEX7 SalI-Rv. The resulting product in pGEM T-Easy was digested with NotI and SalI. The NotI-SalI fragments was ligated into the NotI-SalI site of pFastBac HTb vector. pFastBacHTb-FLAG-EGFP-PTS1 encoding N-terminally FLAG-tagged and C-terminally PTS1- targeted enhanced GFP (EGFP) and pFastBacHTb-FLAG-PTS2-EGFP were constructed as follows. BamHI-Apal fragment of pucd2SraMCSHyg-HA2-EGFP-PTS1 encoding tandem double HA-tagged EGFP fused with -Ser-Lys-Leu and pEGFP-C1 vector-derived sequence for 5 amino acids intervening between EGFP and PTS1 was ligated into the BamHI-Apal site of pFastBac-FLAG vector. BamHI-Sall fragment of pcDNA3.1zeo-HA2-PTS2-EGFP (see the next section) was ligated into the BamHI-Sall site of pFastBac-FLAG vector.

**Construction of Expression Plasmids in E. coli and Mammalian Cells**—His6PTS2-EGFP was constructed as follows. PTS2 sequence coding for the 36-amino acid presequence plus N-terminal 8-amino-acid residues (23) of type-A precursor of rat 3-ketoacyl-CoA thiolase (24) was amplified by PCR with primers pTH-Fw and reverse pTHM8-Rv using as a template pTZ18R-pTH encoding the type-A thiolase precursor and was cloned into pGEM T-Easy. Its BamHI-Sall fragment was ligated into the BamHI-Sall site of pEGFP vector (Clontech). pEGFP-PTS2 was digested with BamHI and Spel, and then the resulting fragment was ligated into the BamHI-Nhel site of pQ30 vector (Qiagen), pcDNA3.1zeo-HA2-EGFP-PTS1 and pcDNA3.1zeo-FLAG-EGFP-PTS1 were constructed by subcloning of BamHI-Apal fragment derived from pucd2SraMCSHyg-HA2-EGFP-PTS1 to the same site of pcDNA3.1zeo-HA2-ubiquitin* and of pcDNA3.1zeo-FLAG-ubiquitin. pcDNA3.1zeo-HA2-PTS2-EGFP and pcDNA3.1zeo-FLAG-PTS2-EGFP were constructed as follows. BamHI-Ncol fragment of the PCR product for PTS2 of pTZ18R-pTH using primers pTH-Fw+2-Fw and pTHM8-Rv was ligated together with the Ncol-NotI fragment of pEGFP into the BamHI-NotI site of pcDNA3.1zeo-HA2-ubiquitin and pcDNA3.1zeo-FLAG-ubiquitin. Flag-PEX7C199S was also constructed. The PCR products each with pairs of primers C1995-Fw and CIPEX7 SalI-Rv (14) and CIPEX7-Fw (14) and C1995-Rv using a template pucd2SraMCSHyg-FLAG-PEX7 were mixed and used as a template in PCR with CIPEX7-Fw and CIPEX7 SalI-Rv. The resulting product in pGEM T-Easy was digested with NotI and SalI. The NotI-SalI fragments was ligated into the NotI-SalI site of pucd2SraMCSHyg-FLAG vector (14).

**Preparation of Recombinant Proteins and Protein Import Complexes**—For isolation of His6PTS2-EGFP, pQ30/PTS2-EGFP in XL1-Blue strain cultured in 2X YT medium at 37 °C was induced by 1 mM isopropyl-β-d-thiogalactoside. The cells were lysed by sonication in L buffer consisting of 20 mM Tris-HCl, pH 7.4, 0.3 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 20,000 × g for 15 min. After a 1-h rotation of soluble fraction with Talon resin harboring Co2⁺ (BD Biosciences), unbound proteins were thoroughly washed out with L buffer. Sf9 cells were infected with baculoviruses each harboring His6Pex7, FLAG-EGFP-PTS1, and FLAG-PTS2-EGFP. The cells were lysed at 48–72 h post-infection by sonication in L buffer without Triton X-100 but containing 25 µg/ml of antipain, pepstatin A, and leupeptin, and 50 units of aprotinin. From the soluble fraction, His6-Pex7p was purified with nickel-nitrilotriacetic acid resin (Qiagen) in the presence of 1% Triton X-100. FLAG-EGFP-PTS1 and FLAG-PTS2-EGFP were similarly purified using anti-FLAG immunoglobulin G (IgG) M2-agarose (Sigma) in the presence of 1% Triton X-100. Pex7p was isolated by digestion of His6-Pex7p with AcTEV protease (Invitrogen) in 20 mM Tris-HCl, pH 7.4, and 0.15 mM NaCl. Two FLAG-PTS

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**TABLE 1**

| Synthetic oligonucleotide primers used |
| Code* | Sequences (5’ to 3’ | Underlined |
|---|---|---|
| pTH-Fw | CCGGAGCTCGATGCCAGCTGCTAGCAG | BamHI site |
| pTH+2-Fw | TGGGACCTAGCTGCTGAGCTGCTAGCAG | BamHI site |
| pTHM8-Rv | TCTAAGGCGAGCTGCTGCTAGCAG | Ncol site |
| PEX7 EcoRI-Fw | GCGGAGCTCGATGCCAGCTGCTAGCAG | EcoRI site |
| C1995-Fw | GGCAGCTCGATGCCAGCTGCTAGCAG | Codon for Cys |
| C1995-Rv | GATCCATGGCCGAGGCCTGCGGGAAG | Codon for Cys |
| Hsp1ex13 H3-Fw | CGAATTCACCTAGCTGCTGCTAGCAG | EcoRI site |
| Hsp1ex13-Rv | TCTCAGCTGCTGCTGCTAGCAG | Codon for termination |

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

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4. K. Okamoto and Y. Fujiki, unpublished observations.
5. H. Otera and Y. Fujiki, unpublished observations.
model proteins, FLAG-EGFP-PTS1 and FLAG-PTS2-EGFP, were isolated by elution with FLAG peptide (Sigma).

Cargo-loaded Pex5pL was prepared by pull-down with Pex5pL of FLAG-EGFP-PTS1 bound to anti-FLAG-IgG beads in buffer B (20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). His$_6$-PTS2-EGFP-loaded Pex7p was isolated by incubation of Pex7p with His$_6$-PTS2-EGFP on Talon beads and following elution with buffer B containing 0.25 mM imidazole, 1 mM EDTA, and 1 mM dithiothreitol (DTT). His$_6$-Pex7p was isolated from His$_6$-Pex7p on nickel-nitritriacetic acid resin, as done for His$_6$-PTS2-EGFP. His$_6$-Pex7p-FLAG-PTS2-EGFP complexes were isolated by FLAG pull-down of FLAG-PTS2-EGFP on beads that had been incubated with His$_6$-Pex7p in Sf9 cell lysates. His$_6$-Pex7p-loaded GST-fused Pex13p (GST-Pex13p) and FLAG-PTS2-EGFP-His$_6$-Pex7p-bound GST-Pex13p were prepared by GST pull-down of GST-Pex13p after incubation with His$_6$-Pex7p and (FLAG-PTS2-EGFP)-His$_6$-Pex7p complexes, respectively. Other recombinant proteins used in this study, including GST-Pex5pS, GST-Pex5pL, GST-Pex13p, GST-Pex14p, Pex5pL, Pex5pLMut234 with mutations at the XXXX(2Y) motifs 2, 3, and 4, and Pex13p, were as described (16).

**Gel Filtration Chromatography**—Five μg of recombinant Pex7p was applied onto 1.6 × 60-cm Superdex S-200 (Amersham Biosciences) column equilibrated with 20 mM HEPES-KOH, pH 7.4, 150 mM NaCl, and 1 mM DTT. Elution was done at 1 ml/min and monitored at 280 nm by an AKTA system (Amersham Biosciences). Pex7p in the eluate fractions was detected by immunoblotting with anti-Pex7p antibody.

**Assessment of Disulfide Linkage by SDS-PAGE**—HEK293 cells transiently expressing FLAG-Pex7p were lysed in 50 mM Tris-HCl, pH 6.8, 4% SDS, and 20% glycerol in the presence or absence of 10 mM EDTA. Pex7p was as assessed by SDS-PAGE and immunoblotting with anti-Pex7p antibody.

**Cell Culture, cDNA Transfection, and Protein Introduction**—CHO cells, including wild-type CHO-K1, PEX7-defective (pex7) ZPG207 stably expressing GFP tagged with PTS2-containing 26-amino acid presequence of rat thiobase B (14, 25, 26), pex5 ZPG231 harboring a mutant Pex5p defective in binding to Pex7p (15), and pex14 ZP161 (22) were cultured as described (27). HEK293 cells were cultured as described (7). cDNA transfection into CHO and HEK293 cells were performed by lipofection method as described (7, 27). CHO pex14 mutant ZP161 stably expressing FLAG-EGFP-PTS1 was isolated in the presence of Zeocin after transfection to ZP161 of pcDNA3.1Zeo-FLAG-EGFP-PTS1. Purified soluble Pex7p was introduced into pex7 ZPG207 cells with Provenctin protein delivery reagents (Imgenex). Briefly, 1 μg of Pex7p was mixed with Provenctin and incubated for 5 min at room temperature. A protein-Provenctin mixture was transferred onto cells (~2 × 10$^5$) and incubated at 37 °C for 4 h in a 5% CO$_2$ incubator. The cells were further cultured for 8 h, as described (20).

**Assays for Multiple Component Complexes**—Various amounts of Pex7p-(His$_6$-PTS2-EGFP) complexes were incubated with Pex5pL-(FLAG-EGFP-PTS1) complex bound to anti-FLAG IgG beads, in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, and a protease-inhibitor mixture. FLAG-EGFP-PTS1 and His$_6$-PTS2-EGFP were detected by immunoblotting using antibodies to FLAG and His$_6$, respectively. Pex5pL and Pex5pLMut234 were likewise incubated with two different complexes each consisting of His$_6$-Pex7p-bound GST-Pex13p and FLAG-PTS2-EGFP-His$_6$-Pex7p-bound GST-Pex13p. His$_6$-Pex7p, FLAG-PTS2-EGFP, and GST-Pex13p were assessed by immunoblotting with antibodies to Pex7p, FLAG, and Pex13p, respectively.

Pex13p complexes in pex7 ZPG207 expressing Pex and PTS proteins including FLAG-Pex13p were investigated. At 12 h post-transfection, the cells were lysed in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and protease inhibitors). Immunoprecipitation of FLAG-Pex13p was done with anti-FLAG-IgG-agarose. Proteins in the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting. PTS2-GFP stably expressed in ZPG207 cells was detected with anti-GFP antibody. HEK293 cells transfected with HA$_2$-EGFP-PTS1 and/or HA$_2$-PTS2-EGFP were likewise verified at 12 h post-transfection.

**Protease Sensitivity Assay**—Cargo-loaded Pex7p was prepared as follows. Purified Pex7p (0.1 μg) was incubated with FLAG-PTS2-EGFP (0.8 μg) bound to anti-FLAG-IgG agarose. After thorough washing, the complexes were eluted with FLAG peptide and treated with 1, 10, and 50 μg/ml of Pronase on ice for 30 min (14). The reaction was terminated by boiling in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol. Pex7p and FLAG-PTS2-EGFP were detected using antibodies to Pex7p and GFP, respectively. Pex7p was also treated with Pronase in the presence of FLAG-EGFP-PTS1 equivalent to the amount to FLAG-PTS2-EGFP.

**Morphological Analysis**—Peroxisomes in CHO cells were visualized by indirect immunofluorescence light microscopy as described (27). Antigen-antibody complexes were detected using fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody (Alexa) and Texas Red-labeled goat anti-guinea pig IgG antibody (Alexa). GFP and EGFP fluorescence were directly observed by fluorescence light microscopy after fixation with 4% paraformaldehyde (26).

**Expression, Purification, and Antibody Preparation of Pex13p SH3 Domain**—EcoRI-Sall fragment of the PCR product using a template human (Hs) PEX13 (21) with primers, HsPex13SH3-Fw and HsPex13-Rv, was ligated into the EcoRI-Sall site of pGEX6P-1 (Amersham Biosciences). E. coli BL21 was transformed with pGEX6P-1-HsPex13pSH3 and grown according to the procedure recommended by the manufacture. A C-terminal part comprising amino acid residues 256 – 403 of HsPex13p, termed Pex13pSH3, was recovered from cell lysates by incubation at 4 °C for 2 h with glutathione-Sepharose (Amersham Biosciences). After thoroughly washing, Pex13pSH3 was isolated by cleaving the bound GST fusion protein with Prescission protease in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, and 1 mM EDTA. Rabbit anti-Pex13p antibody was raised by conventional subcutaneous injection of Pex13pSH3 (28).

**Immunoprecipitation**—Immunoprecipitation was performed in immunoprecipitation buffer with anti-FLAG IgG-conjugated agarose or antibodies to Pex14p and Pex13p bound to protein A-Sepharose (Amersham Biosciences).
Expression and purification of recombinant Pex7p. A, purification of Chinese hamster Pex7p. His<sub>6</sub>-Pex7p was expressed in Sf9 cells and purified as described under “Experimental Procedures.” His<sub>6</sub>-Pex7p was analyzed by SDS-PAGE and Coomassie Blue staining. Lane 1, His<sub>6</sub>-Pex7p purified from cell lysates (open arrowhead); lane 2, soluble Pex7p (solid arrowhead) obtained from His<sub>6</sub>-Pex7p (5 aliquots of the protein shown in lane 1) by digestion with ACTEV protease. B, purified Pex7p was introduced into pex7 ZPG207 cells stably expressing PTS2-GFP to assess its PTS2-transport activity. PTS2-GFP was monitored by fluorescence microscopy after cell fixation. ZPG207 cells. Panel a, mock treated; panel b, Pex7p-introduced. Scale bar, 10 μm.

Other Methods—Western blot analysis was performed as described (14). In detection by immunoblot of protein import complexes in HEK293 cells, monoclonal anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) and CDP-Star detection reagent (Amersham Biosciences) was used.

RESULTS

Expression and Purification of Pex7p Using Sf9 Cell/Baculovirus System—As a step to understanding at a molecular level the mechanisms of PTS2 import mediated by the PTS2 receptor, we attempted to express and purify recombinant Pex7p using an Sf9 cell/baculovirus expression system. Basically after two-step purification, soluble 40-kDa Pex7p was obtained (Fig. 1A). To assess the PTS2 receptor activity, the purified Pex7p was introduced into pex7 ZPG207 cells stably expressing PTS2-GFP (14, 26). PTS2 import was restored in ZPG207 cells upon introduction of Pex7p, but not in mock-treated ZPG207 (Fig. 1B). Next, a binding assay was performed with peroxisomal matrix proteins FLAG-EFGP-PTS1 (SGLRSSKL) and FLAG-PTS2-EFGP containing a 36-amino acid presequence plus N-terminal 8-amino-acid residues (23) of rat thiolase A (24), on agarose beads. Pex7p was detected only in the fraction bound to FLAG-PTS2-EFGP, but not to FLAG-EFGP-PTS1 (Fig. 2A). Pex7p binding to GST-Pex5p was also verified by GST pull-down assay. Pex7p bound to Pex5pL, not to Pex5pS, only in the presence of PTS2 cargo protein, FLAG-PTS2-EFGP (Fig. 2B), consistent with our earlier observation (14). Taken together, these results demonstrated that the purified recombinant Pex7p was biologically active. Furthermore, PTS2 cargo-dependent interaction between Pex7p and Pex5pL was assessed in vivo by co-immunoprecipitation assay upon their ectopic expression in CHO-K1 cells (Fig. 2C). HA<sub>2</sub>-Pex5pL was co-immunoprecipitated with FLAG-Pex7p and the endogenous thiolase precursor (Fig. 2C, lane 8) and more efficiently in the presence of excess amount of a PTS2 protein, HA<sub>2</sub>-PTS2-EFGP (lane 10), confirming the PTS2-dependent binding of Pex7p to Pex5pL.

Conformational Change of Pex7p Is Required for the Interaction with Pex5pL—How does Pex7p interact with Pex5pL in a PTS2-dependent manner? One possibility is that conformational change of Pex7p is induced when it binds to PTS2 protein. To verify such possibility, cargo-free Pex7p and PTS2 cargo-loaded Pex7p were treated with Pronase (14). Pex7p loaded with FLAG-PTS2-EFGP was detectable at up to 10 μg/ml of Pronase and completely digested with 50 μg/ml Pronase (Fig. 3, lanes 5–8), whereas cargo-free 40-kDa Pex7p was digested to a 35-kDa fragment in a manner dependent on Pronase concentration, in the presence of FLAG-EFGP-PTS1 (lanes 1–4), EFGP-PTS1 (data not shown), or EFGP (data not shown). Detection of the 35-kDa fragment with antibody against the C-terminal peptide of Pex7p (14) suggested that Pex7p was proteolytically cleaved at an N-terminal site. This difference in the sensitivity to the protease suggests that the conformational change occurs in Pex7p upon the cargo loading. It is also possible that FLAG-PTS2-EFGP binding to Pex7p protects the Pronase-sensitive site.

Conserved Cysteine Residue at 199 of Pex7p Is Not Critical for Function—In Pex7p from yeast to mammals including Chinese hamster (14, 29), only one cysteine residue located in the fourth WD region is conserved. Functional importance in vivo of this cysteine residue remained unclear, although it was shown to be required for dimerization of Pex7p in vitro (29). FLAG-Pex7p with mutation at the conserved cysteine at 199 (C199S) was expressed in pex7 ZPG207. Wild-type FLAG-PEX7 (WT) and FLAG-PEX7G212R (G212R) (14) were also transfected to ZPG207 as positive and negative controls, respectively. WT, C199S, and G212R were comparable in the expression level (Fig. 4A, panel d). C199S complemented the impaired PTS2 import in ZPG207 cells as efficiently as WT (Fig. 4A, panels c and a), whereas G212R failed to restore the PTS2 protein import (Fig. 4A, panel b). 44-kDa thiolase precursor was processed to the matured 41-kDa form in the ZPG207 cells each expressing C199S and WT, but not in those expressing G212R (Fig. 4A, panel d), implying the PTS2 import into peroxisomes. Next, recombinant Pex7p was analyzed by gel filtration chromatography (Fig. 4B). Pex7p was eluted as a single peak corresponding to ~40 kDa in size. Furthermore, cell lysates of HEK293 cells transfected with WT were processed in the presence and absence of a reducing reagent, DTT, for SDS-PAGE (Fig. 4C). Endogenous Pex7p and WT were each detected as a single band with ~40 and 41 kDa, respectively, in both conditions (Fig. 4C). A minor band with ~70 kDa was likely a nonspecific one, because no additional band appeared with WT expression. Accordingly, these results strongly suggest that the conserved cysteine is not essential for the physio-
logical activity of Pex7p. Pex7p most likely functions in a monomeric form.

Quaternary Complexes Formed in the Cytosol in Matrix Protein Import—The C-terminal half of Pex5p including TPR motifs binds PTS1 of its cargo, and the N-terminal half interacts with Pex14p, Pex13p, and Pex7p (16). Pex5pL also plays an essential role in PTS2 protein import (15). However, it remains unclear whether Pex5pL simultaneously loads PTS1 and Pex7p-PTS2 complexes. To address this issue, a binding assay was performed with PTS1-loaded Pex5pL and PTS2-saturated Pex7p (Fig. 5A). Soluble Pex7p-(His6)-PTS2-EGFP complexes were added to the assay mixture containing Pex5pL-bound FLAG-EGFP-PTS1 on FLAG-IgG beads (Fig. 5B). Pex7p-(His6)-PTS2-EGFP was detected in the fraction bound to (FLAG-EGFP-PTS1)-Pex5pL complexes, not to FLAG-EGFP-PTS1 (Fig. 5B, lane 1), and was elevated with the loaded amount (lanes 2–5). To assess this finding in vivo, CHO pex14 ZP161 cells stably expressing FLAG-EGFP-PTS1 were transfected with HA2-EGFP-PTS1 or HA2-PTS2-EGFP. Expression of HA2-EGFP-PTS1 and HA2-PTS2-EGFP did not alter the endogenous level of Pex5p and Pex7p to any greater extent (Fig. 5C, lanes 1–3). In the immunoprecipitates of FLAG-EGFP-PTS1, Pex5p was reduced when HA2-EGFP-PTS1 was expressed (Fig. 5C, lanes 4 and 5), apparently because of the competition in binding to Pex5p between two types of PTS1 cargoes. In contrast, HA2-PTS2-EGFP expression gave rise to an apparent increase in the amount of Pex5p, presumably Pex5pL, in the immunoprecipitates, where HA2-PTS2-EGFP and Pex7p were concomitantly co-immunoprecipitated (Fig. 5C, lane 6), hence suggesting that the complexes of (FLAG-EGFP-PTS1)-Pex5p-(HA2-PTS2-EGFP)-Pex7p were formed. It is also likely that (HA2-PTS2-EGFP-Pex7p)-bound Pex5pL was elevated. Co-immunoprecipitation of Pex7p in a PTS2 protein-dependent manner was consistent with our earlier finding (14). We therefore interpreted these results to mean that the quaternary complexes consisting of PTS1, Pex5pL, Pex7p, and PTS2 are formed in the cytosol. Thus, the Pex7p-PTS2 complexes are likely imported to peroxisomes with PTS1 via Pex5pL.

To assess any physiological importance of the interaction between Pex5pL and Pex7p in addition to the transport of Pex7p-PTS2 complexes, we characterized the Pex5pL-Pex7p-PTS2 complexes in the wild-type CHO-K1, pex5 ZPG231 de-
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FIGURE 4. Pex7p functions in a monomeric form. A, wild-type, FLAG-tagged Chinese hamster PEX7 (FLAG-PEX7, WT), FLAG-PEX7G212R (G212R), and FLAG-PEX7C199S (C199S) were respectively transfected to CHO pex7 ZPG207 cells (panels a–c). Restoration of PTS2-GFP import was monitored by fluorescence microscopy. Scale bar, 20 μm. Pex7p and thiolase were assessed by immunoblotting of cell lysates from respective transfectants with antibodies to Pex7p (panel d, upper panel) and thiolase (panel d, lower panel). p and m, precursor and mature forms of thiolase, respectively. B, Pex7p in gel filtration chromatography. Purified Pex7p (5 μg) was verified by gel filtration on Superdex S-200 column (upper panel). A280, absorbance at 280 nm. Molecular mass markers used were 200-kDa α-amylase, 66-kDa bovine serum albumin, and 29-kDa carbonic anhydrase. Pex7p in each fraction was also assessed by immunoblot (lower panel). C, endogenous Pex7p and FLAG-Pex7p expressed in HEK293 cells were verified by SDS-PAGE in the absence (lanes 1 and 2) or the presence of 10 mM DTT (lanes 3 and 4) and immunoblotting with anti-Pex7p antibody. Open and solid arrowheads indicate Pex7p and FLAG-Pex7p, respectively.

cient in the Pex5pL binding to Pex7p (15), and pex14 ZP161 cells. FLAG-PTS2-EGFP was transfected to these cell lines with three distinct phenotypes. The Pex5pL binding to Pex7p-FLAG-PTS2-EGFP complexes was indeed defective in ZPG231 cells, whereas the complex formation of Pex7p-FLAG-PTS2-EGFP was normal as in CHO-K1 and ZP161 cells (Fig. 5D). In pex14 ZP161 cells, Pex7p was more abundant than that in CHO-K1 and ZPG231 cells (Fig. 5D, lanes 1, 3, and 5). Pex5pL-Pex7p-FLAG-PTS2-EGFP complexes were formed and apparently more stable in ZP161 (Fig. 5D, lane 6), but not imported (18, 22), whereas Pex5p was less in the amount in such complexes in CHO-K1 cells (lane 2).

PTS2 Import Complexes at the First Docking Site—To dissect the steps as well as the interacting peroxins involved in the PTS2 import pathway, we carried out GST pull-down assays using recombinant proteins. All of the recombinant proteins, including FLAG-EGFP-PTS1, FLAG-PTS2-EGFP, Pex7p, Pex5pL, and Pex13p, were purified nearly to the homogeneity (Fig. 6A). GST-Pex13p and GST-Pex14p were similar to those used in our earlier studies (16, 30). Pex5pL and Pex5pL plus FLAG-EGFP-PTS1 were detected in fractions each bound specifically to GST-Pex14p (Fig. 6B, lanes 2–4), indicating the binding of Pex5pL and Pex5pL-PTS1 complexes to Pex14p, consistent with the report by Otera et al. (16). In contrast, neither Pex7p nor Pex7p plus FLAG-PTS2-EGFP were detectable in fractions bound to GST-Pex14p, suggesting no direct interaction of these proteins with Pex14p (Fig. 6B, lanes 5 and 6). Binding of Pex7p and FLAG-PTS2-EGFP to GST-Pex14p occurred only in the presence of Pex5pL (lane 7), possibly representing the initial docking step on peroxisome membranes in the PTS2 protein import. Binding of Pex7p, FLAG-PTS2-EGFP, Pex5pL, and Pex13p to GST-Pex14p was likewise detected (Fig. 6B, lane 8), suggesting that the hetero-pentameric complexes comprising Pex14p, Pex13p, Pex5pL, Pex7p, and PTS2 cargo were formed.

We verified the PTS protein import complexes formed in vivo by immunoprecipitating Pex14p from HEK293 cells transiently transfected with HA-EGFP-PTS1 and HA-PTS2-EGFP. Together with Pex5p, Pex13p, and Pex7p, both types of PTS cargos were co-immunoprecipitated (Fig. 6C, lane 3), whereas none of these proteins was detectable with preimmune serum (lane 2). These findings strongly suggest that the peroxin complexes...
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FIGURE 5. Loading of Pex7p-PTS2 complexes onto Pex5pL together with PTS1 protein. A, FLAG-EGFP-PTS1 loaded with Pex5pL (0.2 μg) and Pex7p (0.1 μg) -loaded His5-PTS2-EGFP were prepared as described under “Experimental Procedures” and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. The open arrowhead indicates Pex5pL; solid arrowheads show FLAG-EGFP-PTS1 (lane 1) and His5-PTS2-EGFP (lane 2). Open and solid circles indicate IgG heavy and light chains, respectively. Pex5pL, Pex7p, and both types of PTS cargoes were assessed by immunoblotting with antibodies to Pex5p, Pex7p, and GFP, respectively (lanes 3 and 4). B, Pex7p complexes with His5-PTS2-EGFP were added to the assay mixture containing Pex5pL-bound FLAG-EGFP-PTS1 complexes on beads at ratio: 1, 5, and 20 (Pex7p to Pex5p). After 1 h of incubation at 4 °C, the reaction mixtures were centrifuged, and the resulting pellet fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to Pex5p, Pex7p, His, and FLAG. Anti-FLAG IgG-agarose pellet from the reaction mixture containing only FLAG-EGFP-PTS1 was loaded as a mock-treated control in lane 1. The dot indicates a nonspecific band. C, HA2-EGFP-PTS1 (lane 2) and HA2-PTS2-EGFP (lane 3) were separately expressed in CHO pex14 ZP161 cells stably expressing FLAG-EGFP-PTS1. Immunoprecipitation (IP) was done from cell lysates (~5 × 106 each) with anti-FLAG IgG-agarose. Proteins were detected as in B. HA-tagged proteins was likewise assessed with anti-HA antibody. Lanes 1–3, 8% input; lanes 4–6, co-immunoprecipitated proteins. D, wild-type CHO-K1, pex5 ZPG231, and pex14 ZP161 cells (~5 × 106 each) were transfected with FLAG-PT2-EGFP (lanes 2, 4, and 6) or mock-transfected (lanes 1, 3, and 5). FLAG-PT2-EGFP was immunoprecipitated at 20 h post-transfection using anti-FLAG IgG-agarose. Co-immunoprecipitated endogenous Pex5p and Pex7p were detected with respective specific antibodies. FLAG-PTS2-EGFP was detected with anti-FLAG antibody. Lower panel, 8% input; upper panel, immunoprecipitates.

PTS1 to such a reaction mixture did not alter the result obtained in the assay without the PTS1 cargo (lane 9), thereby strongly suggesting that Pex5pL bound to Pex13p via the Pex7p-PTS2 complex in the PTS2 protein import pathway.

To confirm such findings, competition assays between Pex5pL and Pex7p or Pex5pL and Pex7p-PTS2 in binding to Pex13p were performed (Fig. 7B). Pex5pL was pulled down with GST-Pex13p loaded with His5-Pex7p and apparently more efficiently with that carrying His5-Pex7p-FLAG-PTS2-EGFP, in a concentration-dependent manner (Fig. 7B, lanes 1–4 and 9–12). Pex5pL did not compete out His5-Pex7p and His5-Pex7p-FLAG-PTS2-EGFP from His5-Pex7p- and (His5-Pex7p-FLAG-PTS2-EGFP)-loaded GST-Pex13p, respectively, hence suggesting that Pex5pL interacted with Pex13p via Pex7p-PTS2 complexes in addition to the direct binding to Pex13p. Pex5pLMut234, a mutant with mutations at pentapeptide motifs 2, 3, and 4 (16), did not bind to Pex7p-loaded GST-Pex13p (Fig. 7B, lanes 5–8), consistent with the earlier report (16), but formed a complex with (Pex7p-PTS2)-loaded Pex13p, although with less efficiency as compared with normal Pex5pL (lanes 13–16), thereby confirming the results using Pex5pL.

Moreover, essentially the same results were obtained by immunoprecipitation of FLAG-Pex13p from pex7 ZPG207 transfectants (Fig. 8A). HA2-Pex5pL, not HA2-Pex5pLMut234, was co-immunoprecipitated with FLAG-Pex13p, even in the presence of HA2-EGFP-PTS1 (Fig. 8A, lanes 7–10). HA2-Pex5pL appeared to be more efficiently co-immunoprecipitated with FLAG-Pex13p in the presence of Pex7p with PTS2-GFP (Fig. 8A, lane 11), whereas HA2-Pex5pLMut234 was detectable in the immunoprecipitates of FLAG-Pex13p only in the presence of Pex7p with PTS2-GFP (lane 12). We likewise assessed such complex formation in vivo. From HEK293 cells transiently transfected with HA2-EGFP-PTS1 or/and HA2-PTS2-EGFP, endogenous Pex13p was immunoprecipitated. Upon expression of HA2-EGFP-PTS1, Pex5p and Pex7p, but not HA2-EGFP-PTS1, were specifically co-immunoprecipitated with Pex13p (Fig. 8B, lanes 4 and 5), presumably with endogenous PTS2 proteins such as the thiolase precursor. When HA2-PTS2-EGFP was expressed, a higher level of Pex5p, but a similar level of Pex7 as in the

DISCUSSION

In the present study, we succeeded in expressing and isolating mammalian Pex7p using the S99/baculovirus system. Purified recombinant Pex7p was active in several assays including re-establishment of PTS2 protein import upon introduction of Pex7p into pex7 ZPG207 cells and in vitro binding to PTS2 proteins and Pex5pL. Therefore, this preparation is the first
recombinant Pex7p shown to possess biological activities. In this regard, it was possible that the cytosolic chaperonin TCP1 (t-complex protein 1)/TRiC/CCT (31) played a role in proper folding of Pex7p during its expression and purification, as reported for the yeast Pex7p as a model WD protein in S. cerevisiae (32).

We also attempted to address the molecular mechanisms underlying Pex7p-mediated PTS2 protein import, primarily using the highly purified Pex7p. PTS2 protein import is divided into several steps: Pex7p binding to PTS2 and formation of PTS2-Pex7p-Pex5pL complexes in the cytosol, their transloca-

tion to peroxisomes, and the release of PTS2 cargoes. Consistent with our earlier findings by GST pull-down assay using cell lysates (14), the isolated Pex7p readily bound to Pex5pL only in the presence of PTS2 protein in in vitro assays, presumably representing the initial step in the cytosol for the import of PTS2 proteins to peroxisomes. Moreover, the difference in the sensitivity to the protease digestion of Pex7p between the PTS2 cargo-free and cargo-loaded states suggested a conformational change of Pex7p upon its binding to the cargoes. Such a difference may also represent the specific interaction between Pex5pL and Pex7p in the cytosol at the early steps in the PTS2 protein import pathway. Apparently two domains of Pex7p are likely involved in conformational changes: one responsible for the protease sensitivity of the N-terminal 5-kDa domain and the other conferring a compact folding of the entire protein, i.e., the N-terminal region comprising 50 amino acids shows an "three-dimensional structure" conferring a compact folding of the entire protein, i.e., the N-terminal region comprising 50 amino acids shows an "three-dimensional structure"
A pex7 ZPG207

| Input | IP: α-Flag |
|-------|------------|
| PEX5-WT | + + + + + + + + + |
| PEX5-Mut234 | + + + + + + + + + |
| PEX7 | + + + + + + + + + |
| HA2-EGFP-PTS1 | + + + + + + + + + |
| FLAG-PEX13 | + + + + + + + + + |

HA2-Pex5pL

B HEK293

| Input | IP with α-Pex13p |
|-------|------------------|
| HA2-EGFP-PTS1 | + + + + + + + + + |
| HA2-PTS2-EGFP | + + + + + + + + + |
| Pex5p | + + + + + + + + + |
| Pex7p | + + + + + + + + + |
| HA2-PTS2-EGFP | + + + + + + + + + |
| HA2-EGFP-PTS1 | + + + + + + + + + |
| Pex13p | + + + + + + + + + |

FIGURE 8. Pex13p binds to Pex7p-PTS2 and Pex5pL-Pex7p-PTS2 complexes in vivo. A, interaction of Pex13p with Pex5pL, Pex7p, and PTS1 and PTS2 cargoes was assessed in vivo. FLAG-PEX13, HA2-PEX5L, HA2-PEX5L-Mut234 (see Fig. 7A), PEX7, and HA2-EGFP-PTS1 were transfected to CHO pex7 ZPG207 cells stably expressing PTS2-GFP, as indicated at the top. FLAG-Pex13p was immunoprecipitated (IP) with anti-FLAG IgG-agarose from respective cell lysates. HA2-Pex5pL, Pex7p, Pex13p, and both types of PTS proteins were detected using antibodies to Pex5p, Pex7p, and Pex13p, respectively. Dot, IgG heavy chain. Lanes 1–6, one-fifteenth of the input; lanes 7–12, immunoprecipitates. Untransfected pex7 ZPG207 cells were loaded in lanes 1 and 7. HA2-EGFP-PTS1 showed an apparently lower mobility than PTS2-GFP in SDS-PAGE (see text). B, endogenous Pex13p was immunoprecipitated with anti-Pex13p antibody from the lysates of HEK293 cells transiently transfected with HA2-EGFP-PTS1 and/or HA2-PTS2-EGFP. Immunoprecipitates were analyzed by immunoblotting with antibodies to Pex5p, Pex7p, HA, and Pex13p. Lanes 1–3, one-thirtieth of the input; lanes 4–7, immunoprecipitates with preimmune and anti-Pex13p sera.

FIGURE 9. Schematic model for Pex7p-mediated PTS2 protein import. Pex7p likely changes its conformation upon binding to PTS2 cargo in the cytosol (step 1). Pex7p-PTS2 protein complexes are then loaded onto the longer isoform of Pex13p receptor, Pex5pL (step 2). Pex7p-PTS2 protein complexes are also loaded onto PTS1 cargo-loaded Pex5pL. The PTS2 protein import complexes formed in the cytosol target Pex14p, the first docking site of Pex5p, associated with Pex13p on peroxisomal membranes (step 3, docking). Pex7p-PTS2 complexes are translocated to Pex13p independently or together with Pex5pL, where the PTS1 cargoes are unloaded from Pex5pL (step 4, translocation). PTS2 proteins are then unloaded into the matrix of peroxisomes, probably at the step involving Pex13p (shown in a dashed arrow; step 5). At the final step, both types of PTS receptors shuttle back to the cytosol for another round of the PTS protein import (step 6, recycling).
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free as well as PTS1-loaded Pex5pL; 2) these ternary and quaternary complexes dock onto Pex14p associated with Pex13p, as proposed for the transport of Pex7p-PTS2 protein complexes mediated by Pex18p-Pex21p complexes in S. cerevisiae (19); 3) PTS2 cargo is likely released at Pex13p; and 4) Pex7p and Pex5p shuttle back to the cytosol for another round of the PTS2 protein import. Together, the PTS2 proteins are more likely released from Pex7p at Pex13p, whereas the PTS1 proteins are unloaded from Pex5p before the step at Pex13p in mammalian cells (16). Successful expression and purification of recombinant peroxins such as Pex7p and PTS cargoes would define more detailed mechanisms underlying the protein import to peroxisomes.

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