The Mammalian Peroxin Pex5p\(^L\), the Longer Isoform of the Mobile Peroxisome Targeting Signal (PTS) Type 1 Transporter, Translocates the Pex7p-PTS2 Protein Complex into Peroxisomes via Its Initial Docking Site, Pex14p*  

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In mammals, two isoforms of the peroxisome targeting signal (PTS) type 1 receptor Pex5p, i.e. Pex5p\(^S\) and Pex5p\(^L\) with an internal 37-amino acid insertion, have previously been identified. Expression of either type of Pex5p complements the impaired PTS1 import in Chinese hamster ovary pex5 mutants, but only Pex5p\(^L\) can rescue the PTS2 import defect noted in a subgroup of pex5 mutants such as ZP105. In this work, we found that Pex5p\(^L\) directly interacts with the PTS2 receptor Pex7p, carrying its cargo PTS2 protein in the cytosol. Pex5p\(^L\), but not Pex5p\(^S\), mediated the binding of PTS2 protein to Pex14p by translocating Pex7p, demonstrating that Pex5p\(^L\) plays a pivotal role in peroxisomal PTS2 import. Pex5p was localized mostly in the cytosol in wild-type CHO-K1 and Pex14p-deficient mutant cells, whereas it accumulated in the peroxisomal remnants in cell mutants defective in Pex13p or the RING family peroxins such as Pex2p and Pex12p. Furthermore, overexpression of Pex14p, but not Pex10p, Pex12p, or Pex13p, caused accumulation of Pex5p in peroxisomal membranes, with concomitant interference with PTS1 and PTS2 import. Therefore, Pex5p carrying the cargoes most likely docks with the initial site (Pex14p) in a putative import machinery, subsequently translocating to other components such as Pex13p, Pex2p, Pex10p, and Pex12p.

Peroxisomes are ubiquitous intracellular organelles that are found in organisms ranging from yeasts to human beings. The organelles are spherical, with a diameter of 0.1-1 \(\mu\)m, and they are bounded by a single membrane. Peroxisomes function in a wide variety of metabolic pathways, including the catabolism of very long chain fatty acids by \(\beta\)-oxidation and the biosynthesis of plasmalogon-type glycerolipids (1). The functional significance of human peroxisomes is highlighted by fatal human genetic diseases called peroxisome biogenesis disorders (PBDs)\(^1\) such as Zellweger syndrome (2). Peroxisomes are formed by division of preexisting peroxisomes after post-translational import of newly synthesized proteins (3). In yeast, the endoplasmic reticulum has recently been suggested to be involved in peroxisomal membrane biogenesis (4). More than half of peroxisomal matrix proteins are mediated by well-characterized cis-acting peroxisome targeting signals: C-terminal SKL motif PTS1 and N-terminal cleavable presequence PTS2 (2). Genetic analyses of peroxisome-deficient mutants of yeast and mammalian cells have led to the identification of a number of protein factors, peroxins, essential for peroxisome biogenesis (2, 5-7). PEX5 and PEX7 encoding the receptors for PTS1 and PTS2, respectively, have been identified both in yeast and mammals, including humans (for review, see Ref. 8). Deficiency of Pex5p causes PBDs of complementation group II (CG2) manifesting protein import defects (9-11).

To search for a clue to the mechanisms involved in protein import to peroxisomes, we earlier isolated two phenotypically distinct groups of PEX5-defective CG2 CHO cell mutants (11). One group of the pex5 mutants, such as ZP105, showed an import defect of both PTS1 and PTS2 proteins, whereas another group of pex5 mutants represented by ZP19 showed impaired transport of PTS1 proteins, but not PTS2 proteins. Fibroblasts with such distinct phenotypes from CG2 patients were also identified (9, 12). In yeast, PEX5 expression complements a mutant phenotype that is defective solely in PTS1 import (13-16). In mammals, including Chinese hamster and humans, two isoforms of Pex5p, termed Pex5p\(^S\) and Pex5p\(^L\), have been identified (9, 11, 12). Chinese hamster Pex5p\(^S\) and Pex5p\(^L\) consist of 595 and 632 amino acids, respectively (11) (see Fig. 1E). A G-to-A transition resulted in one amino acid substitution, G298E of Pex5p\(^S\) (G335E of Pex5p\(^L\)) in ZP105 and G485E of Pex5p\(^S\) (G522E of Pex5p\(^L\)) in ZP139 (11). Both mutations were in the tetratricopeptide repeat (TPR) domains, i.e. TPR1 and TPR6, respectively. In ZP105, expression of Chinese hamster (Cl) PEX5S complements import of PTS1 (but not PTS2) as in yeast, whereas ClPEX5L restores import of both PTS1 and PTS2, implying that Pex5pL is apparently involved in PTS2 import (11). Likewise, only Pex5pL can restore PTS2 import in fibroblasts from a CG2 patient with Zellweger syndrome (12). However, the molecular mechanisms involved in such mammalian specific import pathway(s) mediated by Pex5p are not understood.

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‡‡ The abbreviations used are: PBDs, peroxisome biogenesis disorders; PTS1 and PTS2, peroxisome targeting signal types 1 and 2, respectively; CG, complementation group; CHO, Chinese hamster ovary; TPR, tetratricopeptide repeat; GFP, green fluorescent protein; HA, hemagglutinin; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; FNS, post-nuclear supernatant.

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Pex5pL Targets PTS1/PTS2 Proteins to the Import Site, Pex14p

Pex5pL, a SH3 domain-containing protein, interacts with both Pex5p and Pex7p in a mammalian system (19). Pex5pL contains an SH3 domain, which is identified as a Pex5p-binding protein on peroxisomal membranes in yeast (17–19). Pex5pL interacts with both Pex5p and Pex7p in a mammalian system (20) as well as in yeast (21, 22). Contrary to matrix proteins, import of peroxisomal membrane proteins is normal in PEX13-defective CHO cell mutants (23) and PBD patient fibroblasts (24, 25) and CHO pex14 mutants (20, 26), as in most of the other mutants so far isolated (2, 27). Therefore, Pex5pL and Pex13p are likely to play an important role in the import of matrix proteins, possibly as components of "import machinery."

We report herein several lines of evidence that in addition to Pts1 import, Pex5pL mediates PTS2 protein transport into peroxisomes by interaction with Pex7p and a Pex5p-docking protein, Pex14p. Intracellular location, molecular forms, and a temperature-sensitive phenotypic property of Pex5pL are described. We also propose a putative peroxisomal import apparatus in relation to a mobile shuttle signal receptor, Pex5p, and its initial docking site, Pex14p.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection of PEX cDNAs, and Morphological Analyses—CHO cells (CHO-K1 cells and CHO pex5 mutants ZP105 and ZP193 (11) (see Table I) were cultured at 30 °C to examine a temperature-sensitive phenotype (20). In addition, we used a stable cell transformant termed 207P7,2 which is the PEX7-defective CHO cell mutant ZPG207 expressing PTS2-GFP (30) that had been stably transfected with human PEX7. cDNA transfection was performed with expression plasmids in the pUC2Hyg vector of CHO mutant ZP105-derived PEX5, CIPESX1/S298E, and CIPESX1/S335E (11). We also used influenza virus hemagglutinin (HA)-tagged CIPESX variants (see below), rat FLAG-PEX5 (12), FLAG-PEX13 (23), and rat PEX14-myc (20), using LipofectAMINE (Life Technologies, Inc.) as described (32). Peroxisomes in CHO cells were visualized under a CAR Zeiss Axioskop FL microscope as described. We also propose a putative peroxisomal import apparatus in relation to a mobile shuttle signal receptor, Pex5p, and its initial docking site, Pex14p.

Preparation of Epitope-tagged Pxsps—HA tagging was performed essentially as described (20). The reaction mixture (400–500 μl) contained protein components to be examined, including GST-Pex5p (3 μg), Pex5pS or Pex5pL (3 μg), 3S-labeled Pex7p (10 μl), and 3S-labeled-5'-ketocyl-CoA thiolase (referred to hereafter as thiolase; 10 μl) (34), in binding assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 25 μg/ml each of leupeptin and antipain, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM dithiothreitol). After incubation for 2 h at 4 °C, proteins were recovered using glutathione-Sepharose (Amersham Pharmacia Biotech). Rat liver cytosol was isolated by centrifugation of the homogenates at 200,000 g (Bio-Rad Japan). Rat liver cytosol was isolated by centrifugation of the homogenates at 200,000 g (Bio-Rad Japan). Pex5pS antibody (see below) in cells that had been permeabilized for 5 min with either 25 μg/ml digitonin or 1% Triton X-100, Pex5p-HA (33), FLAG-Pex5p (31), FLAG-Pex14p (31), and Pex14p-Myc (20) were detected as described. Antigen-antibody complexes were detected using fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody (Cappel), except for Pex7p in 207P7, for which we used Texas Red-labeled goat anti-rabbit IgG antibody (Leinco Technologies).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—RT-PCR was done with Superscript RT (Life Technologies, Inc.) and 5 μg of total RNA each from rat liver as well as CHO-K1 cells and pex5 mutants ZP105 and ZP193 as described (11). Each set of primers used contained a sense and an antisense primer, respectively, as follows: 5′-GGTCTCTAGGGTGCCGC-3′; nucleotide sequence at positions 36–52 in CIPESX cDNA, where the initiation codon starts at position 86); and 5′-GGCTTACGGTGCCGC-3′; nucleotide sequence at positions 1253 to 1273 for CHO previously described (43). Primer sequences are as follows: 5′-GGTGGTCACCATG-3′ (positions 1253 to 1237) for CHO-K1; 5′-GGTGGTCACCATG-3′ (positions 1268 to 1252). Amplified DNA products were analyzed by agarose gel electrophoresis.

Expression of Cationic Protein—Cationic proteins were constructed in Escherichia coli expression vector pGEX6P-1 (Amersham Pharmacia Biotech, Tokyo, Japan) as follows. The Ncol-BglII site was created in pGEX6P-1 by inserting 5′-CATGGAAGCTT-3′ and its complementary oligonucleotide into the SmaI site. Ncol-BglII fragments of pBS-CIPESX and pBS-CIPESX5L (11) were separately ligated into the Ncol-BglII site of pGEX6P-1. Ncol-BglII fragments, each from ZP105-derived pBS-CIPESX5L and ZP193-derived pBS-CIPESX5L, were separately cloned into pUC2Hyg, and recombined were visualized under a Carl Zeiss Axioskop FL microscope as described (30). Peroxisomes in CHO cells (23), and rat FLAG-ClPEX13, and Pex14p-Myc (20) were detected by SDS-PAGE by replacing the BamHI-NolI fragment of each pUC2D-CIPESX and pUC2DHyg-CIPESXL with the respective BamHI-NolI fragment of the PCR-amplified products. Plasmid pUC2DHyg-CIPESX5-HA and pUC2DHyg-CIPESX5L-HA were constructed by inserting the BamHI-NolI fragments of pUC2D-CIPESX5-HA and pUC2D-CIPESX5L-HA into pUC2DRal-Phyg-Hygg-CIPESX5L, respectively. All plasmid constructs were assessed by nucleotide sequence analysis and used for transfection.

In Vitro Binding Assay—The binding reaction was performed essentially as described (20). The reaction mixture (400–500 μl) contained protein components to be examined, including GST-Pex5p (3 μg), Pex5pS or Pex5pL (3 μg), 3S-labeled Pex7p (10 μl), and 3S-labeled-5′-ketocyl-CoA thiolase (referred to hereafter as thiolase; 10 μl) (34), in binding assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 25 μg/ml each of leupeptin and antipain, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM dithiothreitol). After incubation for 2 h at 4 °C, proteins were recovered using glutathione-Sepharose (Amersham Pharmacia Biotech), washed four times with binding assay buffer minus glycerol, and then analyzed by SDS-PAGE. Radiolabeled protein bands were detected using a Fujix BAS1500 Bio-Autoimaging Analyzer (Fuji Photo Film, Tokyo), and Pex5pL was detected by immunoblotting. Such a GST pull-down assay was likewise done with GST fused to Pex5p or its variants derived from ZP105 and ZP193 and 3S-labeled Pex7p and [35S]thiolase. In vitro interaction assay was also performed using CHO cell lysates. CHO-K1, mutant, and CHO pex5 cells were lysed in 10 μl of binding assay buffer (2 ml) for 1 h and centrifuged at 10,000 × g for 40 min at 4 °C. The supernatant fraction (typically a 400-μl aliquot) was incubated with GST or GST fusion protein (~5 μg)-bound glutathione-Sepharose (30 μl) in 700 μl of buffer by rotating for 2 h at 4 °C. The Sephadex beads were washed three times with binding assay buffer minus glycerol. Bound proteins were detected by SDS-PAGE and immunoblotting. CHO-K1 cells expressing Pex5pL-HA or Pex5p-L-HA were lysed in 2 ml, and one-fifth aliquot each was incubated with cell-free synthesized [3S]-labeled Pex5pS and Pex5pL (3 μl each). Pex5pHA was immunoprecipitated with rabbit anti-HA antibody and formalin-fixed Staphylococcus aureus cells (Pansorb; Calbiochem).

Co-immunoprecipitation Assay—to verify the findings in vitro, we conducted immunoprecipitation with anti-HA antibody using a lysate of 207P7 cells transfected with PEX5S-HA or PEX5L-HA. Co-immunoprecipitated proteins were assessed by SDS-PAGE of the immunoprecipitates, followed by immunoblotting as described below.

Gel Filtration Chromatography—To estimate the molecular mass of a Pex5p oligomer, if any, of purified recombinant Chinese hamster Pex5pS, gel filtration was performed on a Bio-Gel A-0.5m column (1 × 90 cm; Bio-Rad Japan, Tokyo) in 50 mM Hepes-KOH, pH 7.4, and 0.1% NaCl. Molecular mass standards used were thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) (Bio-Rad Japan). Rat liver cytosol was isolated by centrifugation of the homogenates at 200,000 g for 60 min in a Beckman Optima L-70K ultracentrifuge. The cytosol was dialyzed against 50 mM Hepes-KOH, pH 7.4, and 0.15 M NaCl then analyzed by gel filtration. Each fraction of the eluates were assayed for Pex5p S and Pex5p L-HA.

Subcellular Fractionation—CHO-K1 and peroxisome biogenesis-defective CHO cell mutants (2 × 106 each) were homogenized in 1.5 ml of homogenization buffer (0.25% sucrose, 5 mM Hepes-KOH, pH 7.4, and 25 μg/ml each of leupeptin and antipain) by 30 strokes of an Elvehjem-Potter homogenizer. A post-nuclear supernatant (PNS) fraction, prepared by centrifugation of homogenates at 750 g for 10 min, was
centrifuged at 100,000 × g for 40 min to separate organelles (heavy and light mitochondrial and microsomal fractions) and the cytosol. The PNS from 1 × 10^7 cells was treated with 10 μg/ml protease K on ice for 30 min and was then separated into the organelar and cytosolic fractions. To biochemically study peroxisomal remnants, the PNS fraction from CHO mutant cells was centrifuged into a linear sucrose density gradient from 0.6 to 1.8 M in a Beckman SW 41 rotor at 35,000 rpm for 16 h at 4 °C. The gradient was collected into 23 fractions. Each fraction was analyzed by SDS-PAGE and immunoblotting.

Other Methods—For Northern blotting, RNA was isolated from CHO cells and from the livers of a rat treated for 7 days with clofibrate and an untreated rat as described (31). The RNA blots were hybridized with full-length ClPEX5L labeled with [α-32P]dCTP (Amersham Pharmacia Biotech). In vitro transcription/translation was done as described (35). Antiserum to Pex5p was raised in rabbits by conventional subcutaneous injection of bacterially expressed and purified Chinese hamster Pex5p. Western blot analysis was done using electrophoretically transferred samples on polyvinylidene difluoride membrane (Bio-Rad) with primary antibodies, including those raised against Pex5p, rat acyl-CoA oxidase (28), rat catalase (28), human Pex7p, Pex12p (31), and a second antibody, donkey anti-rabbit IgG antibody conjugated to horse-radish peroxidase (Amersham Pharmacia Biotech). Antigen-antibody complexes were visualized with ECL Western blotting detection reagent (Amersham Pharmacia Biotech).

RESULTS

Expression of PEX5

We investigated the expression level of PEX5 mRNA and Pex5p in rat liver and CHO cells.

Northern Blot Analysis—Peroxisomal proteins are induced in rat liver by administration of hypolipidemic agents such as clofibrate (36, 37). A rat PEX5 mRNA band with an estimated size of ~3.3 kilobases was detected on a Northern blot of RNAs from normal and clofibrate-treated rats and was apparently not induced by clofibrate, where PEX5S and PEX5L RNAs were indistinguishable in size (Fig. 1A, upper panel, lanes 1 and 2). The ~3.5-kb mRNA for acyl-CoA oxidase, a PTS1 protein, was elevated ~30-fold (middle panel), a finding consistent with previous observations (20, 31, 37, 38). Thus, it is likely that Pex5p functions as a shuttling PTS1 receptor (see below) or in a catalytic manner, even under the conditions in which peroxisomal matrix proteins are massively induced. Alternatively, it is also plausible that the turnover rate of Pex5p is drastically altered.

RT-PCR Analysis—The expression level of PEX5 mRNA in rat liver was also verified by RT-PCR. RT-PCR of rat RNA with a set of primers (sense primer 269S and antisense primer 834R) designed specifically for ClPEX5L yielded a single DNA band with an expected size from normal and clofibrate-treated rats obtained using ClPEX5L cDNA as a control template (Fig. 1B, upper panel). By PCR with another set of primers (269S and 1268R), giving the products from both ClPEX5S and ClPEX5L, two types of PEX5 DNA, i.e. PEX5S and PEX5L, were obtained from rat RNA, therefore indicating that both forms of PEX5 are expressed in rat (Fig. 1B, lower panel). Two DNA products were likewise produced with primers 494S (nucleotide sequence at 215/216, Open arrowhead) and 1253R (nucleotide sequence at positions 494–510) and 1142R (data not shown), confirming the expression of PEX5S and PEX5L RNAs in rat.

PCR of RNA from CHO-K1 cells with a set of primers (36S and 1253R) yielded DNA of two different but expected sizes, indicating that both of the PEX5 isoforms are expressed in CHO-K1 cells (Fig. 1C). The apparently larger product was more abundant than the smaller, plausibly implying that PEX5L mRNA is expressed at a higher level than PEX5S mRNA. The PCR products from CG2 pex5 mutant ZP19-de- derived RNA and primers 131S and 1127R were likewise in two bands, one for PEX5L in a greater amount than for PEX5S.

Fig. 1. Expression levels of Pex5p in rat and CHO cells. A, Northern blot analysis of liver RNA from normal and clofibrate-treated rats. RNA was separated, transferred to Zeta-Probe GT membrane (Bio-Rad), and hybridized with [32P]-labeled cDNA probes for ClPEX5L (upper panel) and rat acyl-CoA oxidase (AOx; middle panel). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; lower panel) cDNA was used as a control probe to check the amount of RNA loaded. Washing was done twice with 0.3 M NaCl, 30 mM sodium citrate, and 0.5% SDS at 60 °C. Lanes 1 and 2, total RNAs (15 μg) from the livers of a normal (NL) and a clofibrate-treated (CL) rat, respectively. Exposure times were as follows: upper panel, 24 h; middle panel, 28 h; and lower panel, 24 h, kb, kilobases. B, expression level of PEX5 mRNA. RT-PCR was done using, as templates, ClPEX5S (lane 1) and ClPEX5L (lane 2) cDNAs and total RNAs from the livers of normal (lane 3) and clofibrate-treated (lane 4) rats. PCR products were obtained with a set of primers: a forward primer (269S) and a reverse primer (834R) specific for ClPEX5L (upper panel) or primers 269S and 1268R for both ClPEX5S and ClPEX5L (lower panel). C, RT-PCR of RNA derived from wild-type and CG2 pex5 mutant CHO cells. Cell types are designated at the top. PCR was done with total RNA from each type of cell and a set of primers: sense primer 36S and reverse primer 1253R for CHO-K1; primers 66S and 1142R for ZP105; and primers 131S and 1127R for ZP139. Open and closed arrowheads indicate the PCR products corresponding to PEX5S and PEX5L, respectively, in each cell type. D, expression levels of Pex5p in wild-type and pex5 mutant CHO cells determined by immunoblotting using anti-Pex5p antibody. E, schematic representation of Pex5pL and Pex5pS and mutation sites (G335E in Pex5pL, A315/316, insertion site of a 37-amino acid (a.a.) sequence).

whereas PCR of ZP105 RNA using primers 66S and 1142R resulted in two products, more of the shorter than the longer. These results are in good agreement with findings by Northern blot analysis (11). Collectively, the two isoforms of Pex5p (Fig. 1E) are indeed expressed in at least three species of mammals, whereas only a single isoform is expressed in yeast.

Western Blot Analysis—The panel of Pex5p in CHO-K1 cells and pex5 (ZP105 and ZP139) was determined by immunoblotting. Pex5p was detected in CHO-K1 cells and in a lesser amount in ZP139, whereas Pex5p was under the detectable level in ZP105 cells (Fig. 1D), possibly due to rapid degradation (see below). Two bands, distinct in a prolonged SDS-PAGE and showing migration with apparent masses of 78 and 80 kDa, larger than

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Temperature-sensitive peroxisome assembly in pex5 mutant ZP105. A, immunofluorescence staining of wild-type CHO-K1 and pex5 mutant ZP105 cells. Cells were cultured for 3 days at 37 °C (panels a, b, and e) or 30 °C (panels c and f) and then stained with antibodies to PTS1 (panels a–c) and rat 3-ketoacyl-CoA thiolase (panels d–f). Magnification × 630; bar = 20 μm. B, biogenesis of peroxisomal proteins. CHO-K1 and pex5 mutant (ZP105 and ZP139) cells (5 × 10^5 each) were cultured for 3 days at 37 °C (lanes 1, 2, and 4) or 30 °C (lanes 3 and 5). Cell lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis was performed using rabbit antibodies to Pex5p (upper panel), rat acyl-CoA oxidase (middle panel), and thiolase (lower panel). Cell types are indicated at the top. Arrowheads A–C designate acyl-CoA oxidase components. αTH and mTH stand for larger precursor and mature protein of 3-ketoacyl-CoA thiolase, respectively. C, back-translation to ZP105 cells. The longer mutant isoform PEX5L335E and the shorter mutant isoform PEX5S298E, both derived from ZP105, were separately transfected into ZP105 cells. Cells (3 × 10^5 each) were verified for the expression of Pex5p by immunoblotting (upper panel) as well as for PTS2 import by immunostaining with anti-thiolase antibody (lower panel). Note that peroxisomal localization of Pts2 protein was evident in PEX5G335E-transfected cells (panel b). Magnification × 630; bar = 20 μm.

The calculated values from the deduced amino acid sequences (11), were noted in Pex5p from CHO-K1 cells, presumably representing two isoforms of Pex5p. The lower mobility of Pex5p in CHO-K1 cells, presumably due to rapid degradation. Peroxisomal 3-ketoacyl-CoA thiolase, a PTS2 protein, is synthesized as a 44-kDa larger precursor and processed to its mature protein in peroxisomes in mammals (28, 31, 40, 41). In CHO-K1 cells, only the mature thiolase was detected at 37 °C (Fig. 2f, lower panel, lane 1), therefore demonstrating rapid processing of the precursor form. In ZP105, only the larger precursor was found at 37 °C as in ZP139 at both temperatures (lanes 2, 4, and 5), implying the defect in import and processing activity. When ZP105 was cultured for 3 days at 30 °C, the thiolase precursor was processed to its final size, although less efficiently compared with CHO-K1 cells (lane 3), reflecting the complementation of the impaired thiolase biogenesis. Taken together, these results demonstrate that cell culturing at permissive temperature can complement the abnormality in biogenesis of peroxisomal proteins in ZP105 cells, in good agreement with the morphological findings described above. Furthermore, similar but partial complementation of PTS2 import was noted at 37 °C in ZP105 cells overexpressing ZP105-derived PEX5L335E, but not in those with its shorter form, PEX5S298E, where nearly the same amount of both forms of mutant Pex5p was detectable with mutually distinct mobility in SDS-PAGE (Fig. 2C). This was interpreted to indicate that the Pex5pL G335E mutant is biologically active in PTS2 transport.

Binding of Pex5p to Peroxisomal Proteins

GST fusion proteins of both forms of wild-type Pex5p (GST-Pex5pS and GST-Pex5pL) as well as those of two types of mutant Pex5pL, one with the mutation G335E in TFR1 (GST-G335E) and the other with G522E in TFR6 (GST-G522E), from pex5 ZP105 and ZP139 (11), respectively, were expressed in E. coli. Fusion protein was purified by affinity chromatography from E. coli lysates using a glutathione-Sepharose column. Purity of the fusion proteins was verified by SDS-PAGE (data not shown).

To investigate whether Pex5p interacts with other proteins, including several peroxins, each type of GST-Pex5p fusion protein was incubated with CHO-K1 cell lysates. Bound protein fractions were analyzed by SDS-PAGE and immunoblotting using specific antibodies. Acyl-CoA oxidase, a PTS1 protein, apparently bound to both forms of wild-type Pex5pS and Pex5pL and less efficiently to GST-G335E, but neither to GST-G522E nor to GST (Fig. 3A). Acyl-CoA oxidase forms oligomers in contrast, no PTS1- and thiolase-positive peroxisomes were found in ZP139, even at 30 °C (data not shown). Together, these data strongly suggest that the cellular phenotype of PEX5-defective ZP105 is ts.

Pex5p was hardly detectable in ZP105 when cultured for 3 days at 37 °C, whereas Pex5p was distinct in CHO-K1 cells (Fig. 2B, upper panel, lanes 1 and 2). After shifting to 30 °C, Pex5p was clearly discernible at 3 days of culture, as much as in ZP139 cultured at both temperatures (Fig. 2B, lanes 3–5). In normal mammalian cells, proteolytic conversion of the 75-kDa acyl-CoA oxidase A component to 53-kDa B and 22-kDa C polypeptides occurs in peroxisomes (35, 40), whereas in CHO cell mutants and PBD patient fibroblasts, the conversion of acyl-CoA oxidase fails and is instead rapidly degraded (31, 40, 41). The acyl-CoA oxidase A component was almost undetectable in ZP105 at 37 °C (Fig. 2B, middle panel, lane 2), presumably due to rapid degradation. The acyl-CoA oxidase A and B components were evidently increased in ZP105 cells after cell culturing for 3 days at 30 °C, indicating that the conversion of acyl-CoA oxidase proceeded in ZP105, but apparently at a slower rate compared with that in CHO-K1 cells (Fig. 2B, lanes 1 and 3). In contrast, neither the acyl-CoA oxidase A component nor its conversion was evident in ZP139, even at 30 °C (lanes 4 and 5), despite the normal synthesis of acyl-CoA oxidase A (11), therefore indicating a rather rapid degradation. Peroxisomal 3-ketoacyl-CoA thiolase, a PTS2 protein, is synthesized as a 44-kDa larger precursor and processed to its mature protein in peroxisomes in mammals (28, 31, 40, 41). In CHO-K1 cells, only the mature thiolase was detected at 37 °C (Fig. 2f, lower panel, lane 1), therefore demonstrating rapid processing of the precursor form. In ZP105, only the larger precursor was found at 37 °C as in ZP139 at both temperatures (lanes 2, 4, and 5), implying the defect in import and processing activity. When ZP105 was cultured for 3 days at 30 °C, the thiolase precursor was processed to its final size, although less efficiently compared with CHO-K1 cells (lane 3), reflecting the complementation of the impaired thiolase biogenesis. Taken together, these results demonstrate that cell culturing at permissive temperature can complement the abnormality in biogenesis of peroxisomal proteins in ZP105 cells, in good agreement with the morphological findings described above. Furthermore, similar but partial complementation of PTS2 import was noted at 37 °C in ZP105 cells overexpressing ZP105-derived PEX5L335E, but not in those with its shorter form, PEX5S298E, where nearly the same amount of both forms of mutant Pex5p was detectable with mutually distinct mobility in SDS-PAGE (Fig. 2C). This was interpreted to indicate that the Pex5pL G335E mutant is biologically active in PTS2 transport.
proteins were separately incubated with CHO-K1 cell lysates (5 × 10^7 cells). GST-G335E and GST-G522E, representing GST-Pex5pL with site mutations at G335E and G522E from ZP105 and ZP139, respectively, were also used. After thorough washing, proteins bound to glutathione-Sepharose were analyzed by SDS-PAGE. Acyl-CoA oxidase (AOx), Pex13p, Pex14p, and catalase were probed with antibodies to the respective proteins, consistent with a phenotype of ZP139 mutant cells (11). To study whether Pex5pS and Pex5pL interact with themselves and/or each other, C-terminally HA-tagged Pex5pL and Pex5pS that had been expressed in Pex5p-deficient ZP105 cells were incubated with cell-free synthesized [35S]-labeled Pex5pS and Pex5pL, and were then immunoprecipitated with anti-HA antibody. [35S]-Labeled Pex5pS and Pex5pL were recovered in the immunoprecipitates of Pex5pL-HA (Fig. 4A, lanes 4 and 6), suggesting that both forms of Pex5p interact with Pex5pL. Nearly the same level of [35S]-labeled Pex5pS and Pex5pL were co-immunoprecipitated with a Pex5pL-HA, suggesting the similar affinity in the homomeric and heteromeric interaction. [35S]-Labeled Pex5pL and Pex5pS were likewise co-immunoprecipitated with Pex5pS-HA (lanes 5 and 7), confirming the findings using Pex5pL-HA. In contrast, neither Pex5pS-HA nor [35S]-labeled Pex5pS was discernible in the immunoprecipitate fraction from mock-transfected ZP105 (lane 3), indicating that the reactions noted above were specific. Similar results were likewise obtained from lysates of CHO-K1 cells expressing Pex5pL-HA or Pex5pS-HA (data not shown). Taken together, it is most likely that Pex5pS and Pex5pL form homomeric as well as heteromeric oligomers.

Chinese hamster Pex5pS purified from GST fusion protein expressed in E. coli was estimated in size by the gel filtration method (Fig. 4B). Pex5pS was eluted in a nearly single protein peak with an estimated molecular mass of ~120 kDa as assessed by absorbance at 280 nm as well as by immunoblotting, which was similar to the size of a dimeric form of the 66-kDa monomer (11). Moreover, the elution profile of Pex5p in gel filtration of rat liver cytosol, as verified by immunoblotting, was nearly the same as that of purified recombinant Chinese hamster Pex5pS, strongly suggesting that Pex5p, presumably including Pex5pL, forms a dimer (Fig. 4B, lower panel).
Pex5pL Targets PTS1/PTS2 Proteins to the Import Site, Pex14p

Pex5pL Interacts with Pex7p

From the studies of the phenotype of pex5 ZP105, we earlier suggested that Pex5pL is likely to be involved in PTS2 transport (11). We examined here whether Pex5p interacts with the PTS2 receptor Pex7p by the same approach described for Fig. 3. The fractions of CHO-K1 cell lysates bound to various GST-Pex5 fusion proteins were probed with rabbit anti-Pex7p antibody. Pex5w was in fractions bound to GST-Pex5pL, GST-G335E, and GST-G522E, but not to GST and GST-Pex5pS (Fig. 5A, lanes 5–9). The binding assay was performed with 35S-labeled Pex7p synthesized in vitro. 35S-Labeled Pex7p was detected after incubation only with GST-Pex5pL, strongly suggesting a direct interaction of Pex5pL with Pex7p, whereas 35S-labeled Pex7p was found nearly at the background level with GST and GST-Pex5pS (lanes 1–4).

Pex7p was expressed at a rather low level in normal CHO-K1 and mutant cells (data not shown). To assess the Pex5p-Pex7p interaction in vivo, we used 207P7 cells (Table I), stable human PEX7 transfectants of pex7 mutant ZPG207 (30) expressing a higher level of Pex7p. Immunoprecipitation of HA-tagged Pex5pS and Pex5pL, each ectopically expressed in 207P7 cells, was done. Pex7p was detected only with Pex5pL-HA, but neither with Pex5pS-HA nor in mock-transfected cells (Fig. 5B), strongly suggesting that Pex5pL binds to Pex7p in vivo. The apparent failure of the co-immunoprecipitation of Pex7p using Pex5pS-HA can be explained. Pex7p expected, if any, in the immunoprecipitate with endogenous Pex5pL that had been bound to Pex5pS-HA is likely to be under the detectable level compared with that co-immunoprecipitated with overexpressed Pex5pL-HA. Collectively, only Pex5pL from the wild-type cells

Table I

| per mutant | CHO mutant | Complementing gene | Ref. |
|------------|------------|--------------------|-----|
| pex5       | ZP105, ZP139 | PEX5               | 11  |
| pex2       | Z65        | PEX2               | 45  |
| pex7       | ZPG207     | PEX7               | 30  |
| pex12      | ZP109      | PEX12              | 31  |
| pex13      | ZP128      | PEX13              | 23  |
| pex14      | ZP161      | PEX14              | 20  |

as well as from pex5 mutants ZP105 and ZP139 functions in direct binding to Pex7p, and the mutation G335E or G522E does not affect such an interaction.
Pex5pL Targets PTS1/PTS2 Proteins to the Import Site, Pex14p

Pex5pL Mediates Binding of Pex7p and PTS2 Proteins to Pex14p

To elucidate the underlying mechanisms by which Pex5pL functions in PTS2 protein transport, an in vitro binding assay was performed with GST-Pex5pS, GST-Pex5pL, 35S-labeled Pex7p (open arrowhead), and 3-ketoacyl-[35S]CoA thiolase (closed arrowhead). One-fifth aliquot each of input 35S-labeled Pex7p and [35S]thiolase were used for the assay was loaded in lanes 1 and 2, respectively. Components added to the assay mixture are indicated at the top. After thorough washing, proteins bound to glutathione-Sepharose were analyzed by SDS-PAGE. 3S-labeled Pex7p and [35S]thiolase were detected as described in the legend to Fig. 5 at an exposure of 14 h. A, an in vitro binding assay was done as described in the legend to Fig. 3 by incubating GST and GST-Pex5p fusion protein with lysates of 207P7 cells expressing a higher level of Pex7p and PTS2-GFP. Pex7p and PTS2-GFP were detected by immunoblotting using antibodies to Pex7p and GFP, respectively. C, shown are the results from co-immunoprecipitation of Pex5pL with Pex7p and PTS2 protein. CIPEX5S-HA and CIPEX5L-HA were separately transfected into PEX14-deficient ZP161, and CIPEX5L-HA was transfected into PEX15-defective ZP128. At 5 days, Pex5p was immunoprecipitated with anti-HA antibody, and the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting. Immunoblotting was done using antibodies to HA (upper panel), Pex7p (middle panel), and thiolase (lower panel). First lane, immunoprecipitates using cell lysates from mock-transfected ZP161; second and third lanes, immunoprecipitates from CIPEX5S-HA- and CIPEX5L-HA-transfected ZP161, respectively; fourth lane, immunoprecipitates from CIPEX5L-HA-transfected ZP128. Closed, open, and shaded arrowheads indicate Pex5p, Pex7p, and Pex14p, a precursor of thiolase, respectively. The dot designates an apparently nonspecific band recovered in the immunoprecipitates. Note that Pex7p as well as PTS2 thiolase were found only with Pex5pL. D, left panel, an in vitro binding assay was done using GST-Pex14p, purified recombinant Pex5pL and Pex5pL, 35S-labeled Pex7p (open arrowhead), and 3S-thiolase (closed arrowhead). One-fifth aliquot each of input 35S-labeled Pex7p and 3S-thiolase was loaded in lanes 1 and 2, respectively. Components added, including GST in place of GST-Pex14p, are indicated at the top. 3S-Labeled Pex7p and 3S-thiolase were detected as described for A at an exposure of 14 h; Pex5pS and Pex7p were detected by immunoblotting. Right panel, [35S]thiolase (TH) and Pex7p in each of lanes 4–6 were quantitated and represented as their ratio.

Pex5pL Translocates Pex7p into Peroxisomes

Pex7p was morphologically detectable only in 207P7 cells, whereas it was under a detectable level in all of the other cell types investigated so far, including wild-type CHO-K1 cells. To examine whether intracellular localization of Pex7p is altered by a higher level of Pex5p expression, we transfected PEX5L-HA as well as PEX5S-HA into 207P7 cells. Pex7p ap-

4 S. Mukai and Y. Fujiki, unpublished data.
The intracellular localization of Pex5p was determined by immunofluorescence microscopy of CHO cells. In CHO-K1 cells, Pex5p was seen mostly in the cytosol in a diffused staining pattern with anti-Pex5 antibody (Fig. 8A, panel a). A similar localization of Pex5p was observed in a pex14 mutant, ZP161 (20) (panel b). In contrast, pex5 ZP105 cells were not stained with anti-Pex5 antibody (data not shown), consistent with an undetectable level of Pex5p expression (Fig. 1D). Strikingly, Pex5p was found mostly in particulates in PEX2-defective Z65 cells (38, 45) that had been transfected with FLAG-PEX12 (31) (Fig. 8A, panel c). The Pex5p-positive particulate structures were superimposable with FLAG-positive particles, strongly suggesting that Pex5p is located in peroxisomal remnants or "ghosts" (40) (panel d). Pex5p and FLAG-Pex12p were stainable in pex2 Z65 cells treated with 25 \mu g/ml digitonin, under which conditions only plasma membranes are permeabilized. Treatment of the cells with 1% Triton X-100 gave essentially the same staining pattern (data not shown). This result was interpreted to mean that Pex5p is localized on or in the membrane of the peroxisomal remnants where antibody is accessible (panel c and d). Similar punctate staining of Pex5p was discerned in PEX12-defective ZP109 (31) and PEX13-impaired ZP128 when treated with not only Triton X-100 (panels e and g), but also digitonin (panels f and h). However, Pex5p was not seen in a punctate staining pattern in ZP139 expressing both Pex5pS-G485E and Pex5pL-G522E and PEX7-defective ZPG207 (data not shown).

Subcellular Fractionation—The intracellular localization of Pex5p was determined also by subcellular fractionation of CHO cells. Pex5p was found by immunoblotting mostly in the cytosolic fraction and a little in organelle pellets when the PNS fraction of CHO-K1 cells was fractionated (Fig. 5B, lanes 1 and 2). In contrast, Pex5p was hardly detectable in fractions from ZP105, suggesting a degradation of Pex5p in cell culture at 37 °C (lanes 3 and 4), consistent with the results using total cell lysate (Figs. 1D and 2B). PM70 was recovered exclusively in the membrane fractions of CHO-K1 and CHO cell mutants such as pex5 ZP105 and ZP139 and pex2 Z65, whereas catalase was largely in organelle pellets of CHO-K1, obviously in peroxisomes, and in the cytosolic fraction of the cell mutants, all consistent with our earlier morphological and biochemical findings (11, 28). Accordingly, these results confirmed the adequate separation of cytosolic and membrane fractions. In the same CG2 pex5 mutant (ZP139), Pex5p was at a lower level regarding the total amount compared with that in CHO-K1 cells, mostly in the cytosolic fraction and less in the membrane fraction (Fig. 5B, lanes 5 and 6). In contrast, a larger amount of Pex5p was found in pex2 Z65, pex13 ZP128, and pex12 ZP109, almost equally in both cytosolic and particulate fractions (lanes 7–10, 15, and 16). The localization of Pex5p in membrane fractions, including that in Z65, ZP109, and ZP128, was in good agreement with the findings using total cell lysate (Figs. 1D and 2B). Pex5p was mostly in the cytosol, as in CHO-K1 cells (Fig. 5B, lanes 11–14).

To confirm the findings described above with respect to the intracellular location of Pex5p, PNS fractions from CHO-K1, Z65, ZP128, and ZP161 cells were fractionated by isopycnic sucrose density gradient ultracentrifugation. In CHO-K1 cells, Pex5p was nearly at the top of the gradient, and a small amount of Pex5p cosedimented with Pex14p, indicative of localization in peroxisomes (Fig. 8C). In Pex2p-defective Z65 (38), Pex5p was discernible nearly from the top to the middle of the gradient, where Pex14p representing peroxisomal remnants (20) sedimented to the middle. This was consistent with the recovery of more than half of Pex5p in the membrane fraction of Z65 (Fig. 8B, lanes 7 and 8). Similar sedimentation of Pex5p in the gradient was seen in the case of Pex13p-defective ZP128. Contrary to these two mutants, in Pex14p-deficient ZP161, most of Pex5p remained nearly at the top, whereas PMP70, a marker membrane protein for peroxisomal ghosts, was in the middle of the gradient. Collectively, Pex5p is localized in the peroxisomal remnants in pex2 and pex13 mutants (Z65 and ZP128, respectively), whereas Pex5p is mostly in soluble fractions in pex14 ZP161, as in CHO-K1 cells. Thus, it is conceivable that Pex14p is required for targeting of Pex5p to peroxisomes.

Moreover, Pex5p found in the organellar fraction of Z65 was

**Fig. 7. Pex5pL translocates Pex7p into peroxisomes.** HA-tagged Pex5p was expressed in 207P7 cells as described in the legend to Fig. 5B. A, transfection was performed with the vector only (panels a and d), ClPEX5S-HA (panels b and e), and ClPEX3L-HA (panels c and f). Pex7p was visualized with anti-Pex7p antibody (panels a–c), and PTS2-GFP was visualized by fluorescence microscopy (panels d–f). Cells designated in the insets in panels c and f are also shown at larger magnification in panels g and h, respectively. Bars = 20 μm. Note that Pex5p7 was seen partially in a punctate staining pattern, in superimposable manner with PTS2-GFP, in Pex5pL-overexpressing cells. B, levels of Pex5p-HA and Pex7p in each cell type were assessed by immunoblotting using antibodies to HA and Pex7p, respectively. Two protein bands immunoreactive to anti-Pex7p antibody were discernible; the one with a higher mobility is presumably the product translated from the second Met located 10 amino acids downstream of the initiator.

**Intracellular Localization of Pex5p**

**Morphological Analysis**—The intracellular localization of Pex5p was determined by immunofluorescence microscopy of CHO cells. In CHO-K1 cells, Pex5p was seen mostly in the cytosol in a diffused staining pattern with anti-Pex5 antibody (Fig. 8A, panel a). A similar localization of Pex5p was observed in a pex14 mutant, ZP161 (20) (panel b). In contrast, pex5 ZP105 cells were not stained with anti-Pex5 antibody (data not shown), consistent with an undetectable level of Pex5p expression (Fig. 1D). Strikingly, Pex5p was found mostly in particulates in PEX2-defective Z65 cells (38, 45) that had been transfected with FLAG-PEX12 (31) (Fig. 8A, panel c). The
moderately resistant to treatment with exogenously added protease K, and part of membrane-associated Pex5p in Pex13p-deficient ZP128 was also resistant, under which conditions cytosolic Pex5p in both mutants was completely digested (Fig. 8D). However, Pex5p (in a rather smaller amount) in the membrane fractions of CHO-K1 and ZP161 cells was fully sensitive to protease K treatment. These results were interpreted to mean that the protease-resistant form of Pex5p is in the membrane of and/or inside the peroxisomal remnants in Z65 and ZP128, presumably representing the Pex5p stuck in the putative import machinery. The higher protease resistance, apparently indicative of being more deeply buried, of Pex5p in Pex2p-defective cells also implies that Pex2p functions as a component of the import machinery, downstream of Pex13p. It is possible that Pex5p partly localizes on the surface of peroxisomes in CHO-K1 cells and on the peroxisomal ghosts in ZP161 lacking Pex14p. Conceivably, Pex5p that was found accessible to the anti-Pex5p antibody after permeabilization of cell membranes with digitonin (Fig. 8A) may represent protease-sensitive Pex5p.

Accumulation of Pex5p in Pex14p Affects PTS1 and PTS2 Import

To search for a potential docking site of the apparently mobile receptor Pex5p, we took an approach to address whether

accumulation of Pex5p in Pex14p affects the import of PTS1 and PTS2 proteins.
overexpression of putative anchoring factors such as Pex14p causes a distinct change in the morphological phenotype of Pex5p. PEX14-myc encoding C-terminally Myc-tagged rat Pex14p and active in restoring peroxisomes in PEX14-impaired CHO mutants (20) was expressed under a strong promoter, SRα, in CHO-K1 cells. In Pex14p-Myc-expressing cells, the level of Pex14p-Myc was ~10-fold higher than that of endogenous Pex14p as assessed by immunoblotting (data not shown). Pex5p apparently accumulated in peroxisomal membranes, as seen in pex2 Z65, pex12 ZP109, and pex13 ZP128 (see above), with which Pex14p-Myc was co-localized as assessed by immunostaining after digitonin treatment of cells (Fig. 9A, panels a and b). Strikingly, in Pex14p-Myc-positive cells, both Pts1 proteins and Pts2 thiolase were not in punctate structures, but mostly rather diffused in the cytosol (panels c-f), therefore demonstrating that import of Pts1 and Pts2, but not peroxisomal membrane proteins, was affected by overexpression of Pex14p and concomitant accumulation of Pex5p. A larger excess of Pex14p may also lead to an unbalanced assembly of a putative import machinery. In contrast, we found in CHO-K1 cells that nearly a 10-fold overexpression of FLAG-tagged Chinese hamster Pex13p relative to endogenous Pex13p caused no apparent accumulation of Pex5p, resulting in normal import of Pts1 proteins and Pts2 (Fig. 9B). Likewise, the intracellular location of Pex5p and import of Pts1 and Pts2 proteins were not affected in CHO-K1 cells overexpressing FLAG-tagged Pex16p or Pex12p (data not shown). Together, it is very likely that Pex5p binds to Pex14p at the first step in the import process of matrix polypeptides, including Pts1 and Pts2 proteins, into peroxisomes.

**DISCUSSION**

In addition to a defect in Pts1 transport, the typical phenotype of mammalian and yeast pex5 mutants, the impaired Pts2 import in pex5 mutants of mammals (including fibroblasts from a CG Zellweger syndrome patient), and its restoration by Pex5pL are the novel findings (11, 12). As a step toward addressing this issue, we recently demonstrated that Pex14p binds both isoforms of Pex5p (Pex5pS and Pex5pL) and, with their cargo, Pts1 protein as well as Pex7p with Pts2 protein (20). In the present work, we showed that both Pex5pS and Pex5pL, in a homomeric and/or heteromeric dimer, bind to Pts1 proteins. More strikingly, studies in vivo as well as in vitro demonstrated that Pex5pL interacts with Pts2 protein in a Pex7p-mediated manner, with a concomitant increase in Pts2 protein bound per Pex7p, implying the dual function of Pex5p in mammals, i.e. transport of both Pts1 and Pts2 proteins. Pex5pL directly binds to Pex7p, whereby a Pex5pL homodimer or a heterodimeric form with Pex5pS transports the Pts2 proteins. In contrast, in yeast, an indirect interaction of Pex5p with Pex7p, apparently requiring Pex14p, was found (46).

The intracellular location of Pex7p is still under debate, particularly in Saccharomyces cerevisiae, arguing that Pex7p is mostly in the cytosol (47) or is intraperoxisomal (48). In human cells, overexpression of human Pex7p resulted in its localization exclusively in the cytosol (49). In the present work, we also observed such an intracellular location of Pex7p in 207P7 cells, stable human PEX7 transfectants of a Pts2-GFP-transformed CHO pex7 mutant, ZPG207 (Fig. 7A). To elucidate the physiological consequence of the interaction between Pex5pL and Pex7p, we investigated whether Pex5pL affects the intracellular localization of Pex7p. The higher level of Pex5pL expression gave rise to peroxisomal accumulation of Pex7p and enhanced Pts2-GFP import in 207P7 cells, evidently demonstrating that Pex5pL functions in the transport of the Pex7p/Pts2 complex. It is noteworthy that structurally and functionally homologous peroxins, S. cerevisiae Pex18p and Pex21p, were recently isolated as factors required for targeting of Pex7p and Pts2 to peroxisomes (50). It is tempting to speculate that Pex5pL functions like these peroxins in the transport of Pex7p and Pts2. It is also possible that the orthologues of yeast Pex18p and Pex21p may exist in mammals.

The ZP105-type mutation G335E in TPR1 reduces the activity of Pex5pL in recognizing Pts1, whereas ZP139-derived...
Pex5pL with the mutation G522E in TRP6 is incapable of binding PTS1. However, both Pex5pL mutants are indistinguishable from wild-type Pex5pL in binding to Pex7p. These findings can explain the ZP139-type phenotype showing the defect in import of PTS1, but not PTS2 (11). A similar phenotype was noted in fibroblasts from a CG2 patient with neonatal adrenoleukodystrophy with the missense mutation N489K in Pex5pS (N526K in Pex5pL) (9, 12). The undetectable level of Pex5p in ZP105 owing to its degradation apparently reflects the phenotype, an impaired import of both PTS1 and PTS2. It is noteworthy that PEX5 mRNA with an intragenic termination codon (R390Ter) was under the detectable level in a CG2 Zellweger syndrome patient manifesting PTS1 and PTS2 import defects (12). Pex5pL-G335E is active in vivo as well as interacting with the Pex7p-PTS2 complex, as witnessed in complementation of PTS2 import in ZP105 in a ts manner and by its overexpression. Given the fact that import of both PTS1 and PTS2 is affected in ZP105 cells presumably possessing functional Pex7p and Pex14p, one of the two potential translocation pathways, namely Pex14p-Pex5pL-Pex7p-PTS2 protein, is likely to be primarily utilized for PTS2 import in vivo, rather than the import route of Pex14p-Pex7p-PTS2 protein. It is of interest to note that Pex5p is also extensively reduced in the import route of Pex14p-deficient ZPG207 (20) as in CHO-K1 cells, whereas ZP161 shows a typical mutant phenotype, i.e. the impaired import of PTS1 and PTS2, possibly owing to the loss of the Pex5p-anchoring factor. The reverse, overexpression of Pex14p, results in a striking increase in Pex5p accumulated in peroxisomes and the concomitantly affected import of PTS1 and PTS2. No such phenotype was envisaged in CHO cells overexpressing Pex13p, Pex12p, or Pex10p. Therefore, Pex14p appears to function at the earliest and prerequisite step as a docking site of Pex5p carrying its cargoes, PTS1 and PTS2 matrix proteins, but not membrane polypeptides, thereby playing a pivotal role in the peroxisome assembly processes. A similar schematic view of Pex14p has recently been suggested (20, 21, 55). Furthermore, given the fact that Pex5p not only accumulates in peroxisomal remnants in PEX2-, PEX10-, and PEX12-defective mutant cells, but also interacts with Pex12p, it is conceivable that the RING peroxins Pex2p, Pex10p, and Pex12p function as a part of the putative translocation machinery, downstream of Pex14p and Pex13p. Similar findings using PEX12-defective PBD patient fibroblasts have been recently reported (56).

Based on the data we reported here, we propose a schematic model of a peroxisomal import machinery tightly linked to the function of Pex5p (Fig. 10). The receptor and cargo complexes, including Pex5p dimer-PTS1 proteins, Pex5p-Pex7p-PTS2 proteins, and Pex5p-Pex7p-cargo proteins, each with PTS1 or PTS2, are formed in the cytosol, traverse to peroxisomes, bind to Pex14p in the potential translocation machinery, and translocate to Pex13p subsequently to the RING peroxins. The interaction of Pex5pS and Pex5pL with Pex14p does not require Pex13p, although we do not know whether the interaction noted between Pex5p and Pex13p as well as Pex14p and Pex13p is direct or is mediated by other peroxisomal proteins. After emerging at the matrix side of the machinery, PTS1 and PTS2 proteins will be released from the receptors. PTS1 and PTS2 can be simultaneously and/or independently imported, as noted in pex5 ZP139 showing a defect in only PTS1 import, but not PTS2 import (11), as well as in pex7 ZPG207 solely deficient in PTS2 import (30). Import of PTS1 and PTS2 is likely to share the common, if not exclusive, translocase, as inferred from the common phenotype, the impaired import of both PTS1 and PTS2, in CHO cell mutants pex2, pex12, pex13, and pex14. It is also plausible that Pex7p translocates into peroxisomes, as shown in yeast (57). At the last step of protein import, Pex5p and Pex7p in a bound form or independently cycle back to the cytosol.

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5 S. Tamura and Y. Fujiki, unpublished data.
6 T. Harano and Y. Fujiki, manuscript in preparation.
The Mammalian Peroxin Pex5pL, the Longer Isoform of the Mobile Peroxisome Targeting Signal (PTS) Type 1 Transporter, Translocates the Pex7p-PTS2 Protein Complex into Peroxisomes via Its Initial Docking Site, Pex14p

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