Domain Mapping of Human PEX5 Reveals Functional and Structural Similarities to Saccharomyces cerevisiae Pex18p and Pex21p*

Pex5 functions as an import receptor for proteins with the type-1 peroxisomal targeting signal (PTS1). Although PEX5 is not involved in the import of PTS2-targeted proteins in yeast, it is essential for PTS2 protein import in mammalian cells. Human cells generate two isoforms of PEX5 through alternative splicing, PEX5S and PEX5L, and PEX5L contains an additional insert 37 amino acids long. Only one isoform, PEX5L, is involved in PTS2 protein import, and PEX5L physically interacts with PEX7, the import receptor for PTS2-containing proteins. In this report we map the regions of human PEX5L involved in PTS2 protein import, PEX7 interaction, and targeting to peroxisomes. These studies revealed that amino acids 1–230 of PEX5L are required for PTS2 protein import, amino acids 191–222 are sufficient for PEX7 interaction, and amino acids 1–214 are sufficient for targeting to peroxisomes. We also identified a 21-amino acid-long peptide motif of PEX5L, amino acids 209–229, that overlaps the regions sufficient for full PTS2 rescue activity and PEX7 interaction and is shared by Saccharomyces cerevisiae Pex18p and Pex21p, two yeast peroxins that act only in PTS2 protein import in yeast. A mutation in PEX5 that changes a conserved serine of this motif abrogates PTS2 protein import in mammalian cells and reduces the interaction of PEX5L and PEX7 in vitro. This peptide motif also lies within regions of Pex18p and Pex21p that interact with yeast PEX7. Based on these and other results, we propose that mammalian PEX5L may have acquired some of the functions that yeast Pex18p and/or Pex21p perform in PTS2 protein import. This hypothesis may explain the essential role of PEX5L in PTS2 protein import in mammalian cells and its lack of importance for PTS2 protein import in yeast.

Peroxisomes are ubiquitous organelles of eukaryotic cells that participate in a wide variety of metabolic functions (1, 2). Peroxisomes lack nucleic acids, and peroxisomal proteins are encoded by nuclear genes. Enzymes that are destined for the peroxisome lumen, or matrix, are synthesized in the cytoplasm and imported post-translationally (3). Two targeting signals direct proteins into the peroxisome lumen (4). The type-1 peroxisomal targeting signal, or PTS1, is found on the vast majority of matrix enzymes and consists of just three amino acids at the extreme C terminus of the enzyme (5). Although the canonical PTS1 is serine-lysine-leucine-COOH, many sequence variants of the PTS1 have been described in mammalian cells (6–12) and even more in yeast (13, 14), protozoa (15–17), and plants (18). The type-2 peroxisomal targeting signal, or PTS2, is found on only a small number of peroxisomal enzymes and is located at or near the N terminus of proteins (19, 20). The canonical PTS2 is arginine-leucine-X₅-histidine-leucine, though sequence variants of the PTS2 have also been described previously (21–23).

PEX5 serves as the import receptor for PTS1-containing proteins (24–31). Each PEX5 monomer contains a single, high affinity PTS1-binding site in its C-terminal half (32), a region that contains six tetratricopeptide repeats (TPRs). The crystal structure of human PEX5 bound to the PTS1 reveals the critical role of the TPR repeats in PTS1 binding and provides a molecular model that explains much of what we know about PTS1 function in mammalian cells (32). Although there is not yet a molecular model for the PEX7-PTS2 complex, PEX7 does display high affinity and specificity for the PTS2 (33–35), and a variety of studies supports the hypothesis that PEX7-PTS2 interaction is the first step in PTS2 protein import (34–39). PEX5 and PEX7 are predominantly cytoplasmic proteins that are thought to cycle between the cytoplasm and peroxisome as they direct newly synthesized matrix enzymes from the cytoplasm to peroxisomes (27, 30, 34–38, 40–42). Numerous other PEX gene products are also required for import of PTS1- and PTS2-containing proteins. These include docking factors for PEX5 and/or PEX7 (PEX13, PEX14) (43–53), putative translocation factors (PEX12, PEX10, PEX2, PEX8), and several peroxins with less defined roles in peroxisomal matrix protein import (PEX1, PEX4, PEX6, PEX15, PEX17, PEX22, PEX23) (for review, see Ref. 54).

The peroxisome biogenesis disorders (PBDs) are a group of lethal neurological diseases caused by defects in peroxisomal matrix enzyme import and peroxisome assembly (55). The PBDs can be caused by mutations in any of at least 12 different human PEX genes, including PEX5 and PEX7 (54, 56). Mutations in PEX5 cause a severe peroxisome biogenesis disorder, Zellweger syndrome (ZS), whereas mutations in PEX7 cause a mild peroxisome biogenesis disorder, the rhizomelic chondrodysplasia punctata (RCDP) phenotype. PBDs are characterized by an abnormality in the biogenesis of peroxisomes, which leads to accumulation of products from pathways that normally occur in peroxisomes, such as the oxidation of very long chain fatty acids, phenylketonuria, and defects in phytanic acid oxidation. PBDs are usually caused by mutations in genes encoding peroxisomal biogenesis disorder (PBD) proteins such as PEX5 and PEX7, which are required for the assembly of peroxisome biogenesis disorders, PBDs, peroxisome biogenesis disorders; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay.

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tions in PEX5 can cause Zellweger syndrome, neonatal adre- 
noleukodystrophy, or infantile Refsum disease (8, 27). These 
three diseases represent a phenotypic continuum and are char-
acterized by a loss or reduction of virtually all peroxisomal 
metabolic functions (56, 57). In contrast to the Zellweger spec-
trum of disease caused by PEX5 mutations, mutations in PEX7 
cause rhizomelic chondrodysplasia punctata (36, 37, 39, 58, 59).
Patients with rhizomelic chondrodysplasia punctata are char-
acterized by defects in PTS2 protein import, normal PTS1 
protein import, and a more restricted set of metabolic and 
developmental abnormalities that involve PTS2-targeted en-
zymes (55, 60).

Studies of human PEX genes and cells derived from PBD 
patients have contributed significantly to our understanding of 
peroxisome biogenesis (54, 56, 61). This is particularly true in 
regard to PEX5. Human cell studies were the first to show that 
PEX5 is a predominantly cytoplasmic, partly peroxisomal pro-
tein (27) that cycles between the cytoplasm and peroxisome (40, 
42), and the crystal structure of the human PEX5-PTS1 com-
plex has provided a molecular model of PTS1 recognition (32).
In addition, human cell studies were the first to show that 
mammalian PEX5 is required for PTS2 protein import (27, 29, 
62). The requirement for PEX5 in PTS2 protein import repre-
sents a significant departure from the PTS2 protein import 
pathway in yeast, in which PTS2 protein import occurs nor-
mally in the absence of PEX5 (24, 25). Studies in human cells, 
Chinese hamster ovary cells, and transgenic mice have also 
established that mammalian PEX5 is expressed in at least two 
forms, PEX5L and PEX5S, which are generated by alternative 
splicing of a single PEX5 gene (27, 63–65). PEX5L contains an 
insert 37 amino acids long that is positioned between amino 
acids 214 and 215 of PEX5S. PEX5L also differs from PEX5S in 
insert coding regions (see Figs. 1, 3, and 5) that cycles between the cytoplasm and peroxisome (40, 42), and the crystal structure of the human PEX5-PTS1 complex has provided a molecular model of PTS1 recognition (32). In addition, human cell studies were the first to show that mammalian PEX5 is required for PTS2 protein import (27, 29, 62). The requirement for PEX5 in PTS2 protein import represents a significant departure from the PTS2 protein import pathway in yeast, in which PTS2 protein import occurs normally in the absence of PEX5 (24, 25). Studies in human cells, Chinese hamster ovary cells, and transgenic mice have also established that mammalian PEX5 is expressed in at least two forms, PEX5L and PEX5S, which are generated by alternative splicing of a single PEX5 gene (27, 63–65). PEX5L contains an insert 37 amino acids long that is positioned between amino acids 214 and 215 of PEX5S. PEX5L also differs from PEX5S in that it can rescue PTS2 protein import in PEX5-deficient cells at a high frequency (64, 65) and appears to interact with PEX7, the PTS2 receptor (66).

These and other results have led to the hypothesis that 
PEX5L plays an essential role in PTS2 protein import and that 
this role involves binding to PEX7 and facilitating PEX7 trans-
port to peroxisomes (66). However, many issues remain to be 
addressed regarding the role of PEX5L in PTS2 protein import. 
In this report, we have identified sequences of PEX5L that are 
necessary for PTS2 protein import and targeting studies (see Figs. 1–3, and 7) are derivatives of pcdna3 (Invitrogen). These constructs were named based on the region of PEX5 encoded by a particular plasmid. The PEX5 coding region in each of these constructs was cloned into the HindIII and BamHI sites in pcdna3 in-frame with an 11-amino acid c-myc epitope tag (for the sequence of the tag, see below) located between the BamHI and XbaI sites. For example, PEX5L/1–335myc encodes amino acids 1–335 of the long isoform of PEX5 whereas PEX5S/1–298myc encodes amino acids 1–298 of the short isoform of PEX5 both in front of 
the myc tag. The sequence of nomenclature applies to PEX5L/1– 
304myc and PEX5S/1–263myc, PEX5L/1–264myc and PEX5S/1– 
227myc, PEX5L/48–639myc and PEX5S/48–620myc, PEX5L/1– 
241myc, PEX5L/1–230myc, PEX5L/1–222myc, PEX5L/1–214myc, 
PEx5l/1–157myc, and PEX5L/1–90myc.

showing the c-myc epitope encoding sequence upstream of the PEX7 coding region, and the multiple cloning site between the EcoRI and XbaI sites of pcdna3 downstream of the PEX7 coding region (see PEX7pcDNA3 (37)), was cloned into the HindIII and BamHI sites of pcdna3.1Zeo. The start codon of PEX7 was omitted. The sequence upstream of the second PEX7 codon reads as follows: (5'–AGC TTC ACC AAT C AGG TAG AGC CAT CCG AGA C GGC CAC GTG CAC TC CG AAT AAC GAG C TTA GAG TAG ATC CTG CG T–3'). The nucleo-
tides encoding the myc tag are underlined. PEX7 in pEGFP was 
cloned by creating an Acc65I and Apal fragment of pEB6.4 (PEX7 in 
pM, see below) into the corresponding sites of pEGFP-C1 
CLONTECH).

Yeast Two-Hybrid System—The full-length PEX5L and PEX5S 
coding regions were excised from pGD106 and pGD100 with NotI 
(blunted) and BgIII and cloned into the SmaI and BglII sites of the 
activating domain vector ppc86 (71). PEX5S/1–228 in ppc86 
pGD142 was generated from pJM165.1 that contained amino acids 
1–298 of PEX5 in pcdna3 by cleavage with NotI (blunted) and XbaI 
and subsequent cloning into the SmaI and SpeI site of ppc86. The 
plasmid pM165.1 was generated by PCR using primers DVI037 
and P299ter with pGD100 as template (Table I). The PCR fragment was 
cleaved with Sse8387I and XbaI and cloned into the corresponding 
sites of pGD100. PEX5L/1–251 in ppc86 (pEB4.2) was generated by 
PCR using the primer pair T7 and GD30 with pGD106 as template. 
The fragment was cleaved with EcoNI and NotI and cloned into the 
respective sites of pGD142. PEX5L/48–298 in ppc86 (pEB2.2) was 
made by PCR with primers GD51 and P299ter using pGD106 as 
template. The fragment was cleaved with Sall and cleaved 
directed into the Sall and SpeI site of ppc86. PEX5L/139–298 and PEX5L/ 
214–298 in ppc86 were cloned in the same way, but the sense 
primers GD32 and GD33 were used in the PCR reactions. The 
ScPEX7 DNA in pC97 was described by Rehling et al. (34).

Mammalian Two-Hybrid System—HsPEX7 in pM (pEB6.4) was 
generated from pEB6.6 by excision of the full-length DNA with EcoRI
and cloning it into the EcoRI site of pM. Plasmid pEB6.6 was generated by amplifying a fragment with primers GD47 and Sp6 using PEX7pcDNA3 (37) as template. This fragment was then subcloned into pGEM-T (Promega) following the manufacturer’s instructions. HsPEX7 in pVP16 was cloned in a similar way generating pEB6.5. To generate PEX5L and PEX5S in pcDNA5 (pEB7.5 and pEB8.5), corresponding fragments were cleaved with Ncol (blunted) and BglII from pGD106 and cloned into the EcoRI site (EcoRI) of pEG100, and BamHI site of pVP16. The fragment was isolated from the host vector pGD100, HsPEX5L/1–335 in pVP16 was generated by amplifying pJM164.7 with Ncol (blunted) and XbaI and ligating into the fragment into the EcoRI (blunted) and XbaI sites of pVP16. The short version HsPEX5S/1–298 in pVP16 (pEB10.5) was derived in a similar way from pJM165.1 that encoded amino acids 1–298 in pEDNA3. HsPEX5L/335–355 in pVP16 was generated by cleaving the Ncol (blunted) and EcoRI site of pGD106 and ligating it into the EcoRI (blunted) and XbaI sites of pVP16. Plasmid pEB4.1 encodes PEX5L/1–355 in pcDNA3 and was derived by PCR with the primer pair GD30 and T7 using pGD106 as template. An EcoRI/NcoI fragment was then ligated into the corresponding sites of pGD100 to generate pEB4.1. HsPEX5L/48–355 in pVP16 (pCK7) was generated by excising a Ncol (blunted)/XbaI fragment from pEB2.1 and cloning this fragment into the EcoRI (blunted) and XbaI sites of pVP16. To generate pEB2.1 (HsPEX5L/48–355pcDNA3) a fragment was amplified by PCR with primers GD31 and P299ter. To create pEB2.1 and ligated into the XhoI sites of pVP16. HsPEX5L/138–385 in pVP16 was generated by amplifying pJM165.1 (with 0.5% Triton X-100 on ice for 30 min. The lysates were cleared at 15,000 g for 15 min. Supernatants (600 µl) were incubated with 50 µl of anti-mouse coated mouse IgG Dynabeads (Dynal), saturated with anti-myc antibodies. Immunoprecipitates were collected using a magnet. The immunoprecipitates were washed five times with 1 ml of binding buffer with 0.3% Triton X-100, 0.05%/SDS, resuspended in 20 µl of SDS sample buffer, denatured for 5 min at 95 °C, and separated by SDS-PAGE. Supernatant and pellet fractions were loaded with a ratio of 12.5:1. The gel was soaked in 0.5 M sodium salicylate for 30 min, dried, and subjected to fluorography.

**TABLE I**

| Primer        | Sequence                                      |
|---------------|-----------------------------------------------|
| DV1037        | 5’-TCCTGACGGCGCCAGATGC-3’                     |
| F299ter       | 5’-CCTCTGAAGGTACATCTGGAATCGACCAAGG-3’         |
| GD30          | 5’-GGACGCGGCCGCTGTCTGCTGTATAAAA-3’           |
| GD31          | 5’-AGGTGCAGGACCATGGCCCTCAA-3’                 |
| GD32          | 5’-ATGGTGCCACCAGCTGGCCCTCAGAAAGA-3’          |
| GD33          | 5’-ATTGGTGCCACCAGCTGGCCCTCAGAAAGA-3’         |
| GD47          | 5’-GCCGATTTGACCCGAGCTTGCGGCAGTTAA-3’         |
| Ku664         | 5’-CTAGTTGGCTAGCCAGTCATGCTTCACCTCAGCAGCT-3’ |
| Ku582         | 5’-CTAGTTGGCTACTATACCACAGATGTCATCCACACC-3’  |
| GD144         | 5’-CAGGACGCGGCCGCTGTCTGCTGTATAAAA-3’         |
| GD145         | 5’-GGCTATGATGAAAGCTTGCTTTCATCACC-3’          |
| Ku541         | 5’-TCAGGATGTCGGATGACCATGAGTAACTCCAC-3’       |
| Ku342         | 5’-CATACTGACGGTCAGTCATTCATTGCTTACCCAC-3’     |

**Immunoprecipitations**

**Transcription/Translation**—PEX5S (pGD100), PEX5L (pGD106), and N-mycPEX7 (pEB13.10) were transcribed and translated in vitro for 1 h, using the TNT Coupled Reticulocyte Lysate system (Promega). PEX5 and PEX7 were labeled with [35S]Leucine (1075 Ci/mmol) (PerkinElmer Life Sciences). Equal amounts of the PEX5L, PEX5S, and N-mycPEX7 translation reactions (10 µl) were mixed and incubated together at an additional hour at 30 °C. The radioactive mixture was diluted to 175 µl with binding buffer (20 m× Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride, 21 µg/ml NaF) containing 0.3% Triton X-100 and incubated for 2 additional hours at 4 °C with 25 µl of anti-mouse IgG Dynabeads (Dynal), saturated with anti-myc antibodies. Immunoprecipitates were collected using a magnet. The precipitates were washed five times with 1 ml of binding buffer with 0.3% Triton X-100, 0.05%/SDS, resuspended in 20 µl of SDS sample buffer, denatured for 5 min at 95 °C, and separated by SDS-PAGE. Supernatant and pellet fractions were loaded with a ratio of 12.5:1. The gel was soaked in 0.5 M sodium salicylate for 30 min, dried, and subjected to fluorography.

**Mammalian Two-hybrid Methodology and ELISA**

To investigate a possible interaction between human peroxins we used the mammalian MATCHMAKER two-hybrid assay kit (CLONTECH). The corresponding cDNAs were cloned behind the GAL4 binding domain sequence of the pM vector or the VP16-activating domain sequence of the pVP16 vector, as described under “Materials.” Human wild-type skin fibroblasts (GM5756) or XPE-deficient fibroblasts (PBD061) were seeded onto 12-well plates to reach 50–80% confluency the next day. The cells were co-transfected with 0.24 µg of DNA of pM and pVP16 derivatives together with 0.5 µg of reporter plasmid pG5CAT, using 1.5 µl of LipofectAMINE. Two days after transfection, the cells were lysed in 0.2 ml of Mammalian Two-Hybrid Lysis Buffer (CLONTECH) for 20 min at 4 °C, and the supernatants were cleared at 15,000 × g for 15 min. The amount of proteins in cell lysates was estimated using the BCA protein assay reagent (Pierce) and albumin as standard. Lysates corresponding to 50 µg of protein were used to determine the amount of chloramphenicol acetyltransferase with the CAT ELISA kit. Similar transfected samples were controlled for expression of the fusion proteins and for CAT expression by immunofluorescence microscopy, using polyclonal antibodies against the GAL4 binding domain (Santa Cruz Biotechnology) and against chloramphenicol acetyltransferase (5 Prime → 3 Prime, Inc.).

**Yeast Two-hybrid System**

The cDNAs were fused to the activation domain of GAL4 in pPC86 or the GAL4 binding domain in pPC97 (71) as described above. Co-transfection was performed, and two-hybrid vectors into strain H767 (CLONTECH) or PCY27 (71) and PCY24-pez14 (73) were performed with lithium acetate following a protocol from CLONTECH (Yeast Protocols Handbook). The β-galactosidase filter assay and the test for HIS prototrophy were performed after selection on SD plates lacking leucine and tryptophan according to Rehling et al. (34).

**RESULTS**

**Identification of the Minimal PTS2 Import Domain of PEX5L**—PBD005 cells were derived from a severely affected Zellweger patient and are homozygous for a nonsense mutation in PEX5, R390ter (27). This mutation results in the destabilization of PEX5 mRNAs, the absence of detectable PEX5 pro-
protein, and eliminates import of both PTS1- and PTS2-containing proteins (27, 64). Additional studies have established that mammalian cells express two forms of PEX5 that differ by 37 amino acids and that these forms are generated by alternative splicing of a single exon of the PEX5 gene (27, 64, 65). These forms appear to differ in their biological activities, with PEXL efficiently rescuing both PTS1 and PTS2 protein import and PEXS5 involved only in PTS1 protein import. This difference can be detected using the following in vivo assay. Plasmids designed to express (a) PTS2-CAT, a plasmid designed to express bacterial chloramphenicol acetyltransferase (CAT) with a PTS2 at its N terminus (19) and (b) either PEX5S or PEX5L (64), were simultaneously introduced into PBD005 cells by electroporation. Two days after transfection, the cells were processed for indirect immunofluorescence microscopy (Fig. 1).

The relative ability of PEX5S and PEX5L to mediate PTS2 protein import was calculated by comparing the percentage of cells in which PTS1 protein import was detected to the percentage of cells in which PTS2 protein (PTS2-CAT) import was detected. For PEX5L, the values were equivalent whereas PEX5S was 100 times less efficient at rescuing PTS2 protein import than it was at rescuing PTS1 protein import.

To better understand the molecular basis of PEX5-mediated PTS2 protein import, we mapped the minimal PTS2-import domain of PEX5L using the immunofluorescence-based in vivo assay described above. The C-terminal half of PEX5S is involved in binding the PTS1, and we hypothesized that it might be dispensable for PTS2 protein import. We removed this domain in binding the PTS1, and we hypothesized that it might be dispensable for PTS2 protein import. We mapped the minimal PTS2-import domain of PEX5L using the immunofluorescence-based in vivo assay. The C-terminal half of PEX5S is involved in binding the PTS1, and we hypothesized that it might be dispensable for PTS2 protein import. We removed this domain in binding the PTS1, and we hypothesized that it might be dispensable for PTS2 protein import. We removed this domain in binding the PTS1, and we hypothesized that it might be dispensable for PTS2 protein import. We mapped the minimal PTS2-import domain of PEX5L using the immunofluorescence-based in vivo assay. The C-terminal half of PEX5S is involved in binding the PTS1, and we hypothesized that it might be dispensable for PTS2 protein import. We removed this domain in binding the PTS1, and we hypothesized that it might be dispensable for PTS2 protein import. We mapped the minimal PTS2-import domain of PEX5L using the immunofluorescence-based in vivo assay.
then subjected each sample to immunoprecipitation with anti-myc antibodies. We were unable to detect interaction between PEX5L and PEX7 in this system (Fig. 4b). We also tried to assess binding between PEX5L and PEX7 using purified recombinant proteins expressed in bacteria in a variety of blot overlay, bead binding, and other protein association assays. These experiments also failed to show an interaction between PEX5L and PEX7. These protein-protein interaction assays rely on a number of assumptions regarding the relative affinity of the binding reaction, the conformational constraints of the interaction, the conformational status of the bacterially expressed proteins, and the ability of the interaction to occur in the absence of other factors. Thus, these results should not be interpreted as evidence against direct PEX5L-PEX7 binding. They did force us, however, to search for another assay system in which we could examine the PEX5L-PEX7 interaction.

**FIG. 2.** The N-terminal but not the C-terminal region of PEX5L is required for PTS2 import. PBD005 cells were co-transfected with the constructs expressing the various PEX5 proteins shown in e and with the plasmid expressing PTS2-CAT. After 2 days the cells were processed for double-indirect immunofluorescence microscopy with anti-CAT antibodies (a, c) and anti-PMP70 antibodies (b, d). PEX5S/1–227myc (a, b) did not rescue PTS2 import (a) whereas the long isoform of that protein, PEX5L/1–264myc (c, d), does restore PTS2 import (c). White boxes represent the six TPR domains involved in PTS1 binding. The amino acids encoded by the alternative exon (AE) are indicated by the gray box.
The mammalian two-hybrid protein interaction assay makes fewer assumptions regarding the variables described above, and we therefore tested whether it could be used to map the region of PEX5L that interacts with PEX7. The mammalian two-hybrid system (74) operates on the same principle as the yeast two-hybrid system but offers certain advantages. These include the ability to more easily control for expression and localization of test proteins, adaptability to a wide range of cell lines, and the ability to assay reporter gene expression on a cell-by-cell basis as well as on an averaged basis for the entire cell population. The particular assay system we employed uses the cDNA for chloramphenicol acetyltransferase (CAT) as the reporter, which is under the control of a GAL4-responsive promoter. Test proteins are expressed as fusions to either the GAL4 DNA binding domain (BD) or the transcriptional activation domain of VP16 (AD). Cells are then co-transfected with all three plasmids, and the interaction is scored by the extent of CAT expression in lysates prepared from transfected cells, which can be determined by ELISA as well as by indirect immunofluorescence microscopy (IIF).

Isolated expression of BD-PEX7, AD-PEX5S, and AD-PEX5L fusion proteins had no effect on GAL4-mediated expression of CAT in this assay (Table II). Co-expression of BD-PEX7 and AD-PEX5S also failed to activate the CAT reporter gene, but simultaneous expression of BD-PEX7 and AD-PEX5L led to significant expression of CAT protein, as determined by ELISA of cell lysates (Table II) and by IIF microscopy (Fig. 3). The particular assay system we employed uses the cDNA for chloramphenicol acetyltransferase (CAT) as the reporter, which is under the control of a GAL4-responsive promoter. Test proteins are expressed as fusions to either the GAL4 DNA binding domain (BD) or the transcriptional activation domain of VP16 (AD). Cells are then co-transfected with all three plasmids, and the interaction is scored by the extent of CAT expression in lysates prepared from transfected cells, which can be determined by ELISA as well as by indirect immunofluorescence microscopy (IIF).
results suggest that amino acids 191–222 of PEX5L are sufficient for interaction with PEX7 and that the interaction is increased with a slightly longer fragment of PEX5L (PEX5L/191–251).

Negative results in this assay are more difficult to interpret. Loss of interaction may reflect an interesting phenomenon, such as elimination or disruption of a protein–protein interaction motif, but negative results can also be caused by other factors, including reduced expression, aberrant folding, and altered subcellular distribution. In these experiments, we were able to control for expression and subcellular distribution of the various AD-PEX5L fusions, which were equivalent, but controls for folding were obviously beyond the limits of our experimental approach. Nevertheless, the lack of interaction between PEX7 and fragments of PEX5 that do not contain the 37-amino acid insert of PEX5L is consistent with the hypothesis that all or part of this insert plays an important role in PEX7 interaction. In addition, the lack of interaction between PEX7 and PEX5L/214–335 raises the possibility that some or all of the 14 amino acids upstream of the 37-amino acid insert in PEX5L are also involved in the interaction with PEX7.

The hypothesis that the PEX7 interaction domain of PEX5L is important for PTS2 protein import predicts that expression of this domain alone should inhibit PTS2 protein import. To test this hypothesis we co-transfected normal human fibroblasts with the PTS2-CAT expression vector and with either pM or pM-PEX5L/191–251. Two days after transfection the cells were processed for IIF microscopy. The percentage of cells in each population that showed import of PTS2-CAT into peroxisomes was determined by assessing PTS-CAT distribution in hundreds of transfected cells. In cells co-transfected with pM, PTS2-CAT was peroxisomal in 85% of expressing cells whereas it was peroxisomal in only 22% of cells co-transfected with pM-PEX5L/191–251. Thus, overexpression of PEX5L/...

### Table II

PEX7 interacting region of PEX5L determined by two-hybrid analysis in human cells

| pM (binding domain) fused to | pVP16 (activation domain) fused to | CAT\(^a\) | Normal human fibroblasts | PEX6-deficient fibroblasts |
|-----------------------------|----------------------------------|---------|-------------------------|---------------------------|
| PEX7                        | PEX5S                            | 0.1     | 0                       | 0                         |
| PEX7                        | PEX5L                            | 0       | 0                       | 0                         |
| PEX7                        | PEX5S                            | 0       | 0                       | 0                         |
| PEX7                        | PEX5L                            | 2.9     | 7.5                     | 0                         |
| PEX7                        | PEX5S/1–298                      | 0.1     | 0                       | 0                         |
| PEX7                        | PEX5S/1–298                      | 0       | 0                       | 0                         |
| PEX7                        | PEX5L/1–335                      | 0.66    | 4.9                     | 0                         |
| PEX7                        | PEX5L/1–335                      | 0       | 0                       | 0                         |
| PEX7                        | PEX5L/1–251                      | 2.9     | 9.5                     | 0                         |
| PEX7                        | PEX5L/1–251                      | 0       | 0                       | 0                         |
| PEX7                        | PEX5L/48–335                     | 3.4     | NA\(^b\)                | NA                        |
| PEX7                        | PEX5L/48–335                     | 0       | NA                      | NA                        |
| PEX7                        | PEX5L/139–335                    | 2.0     | NA                      | NA                        |
| PEX7                        | PEX5L/139–335                    | 0       | NA                      | NA                        |
| PEX7                        | PEX5L/214–335                    | 0       | NA                      | NA                        |
| PEX7                        | PEX5L/214–335                    | 0       | NA                      | NA                        |
| PEX7                        | PEX5L/191–251                    | 8.3     | NA                      | NA                        |
| PEX7                        | PEX5L/191–251                    | 0       | NA                      | NA                        |
| PEX7                        | PEX5L/191–222                    | 3.4     | NA                      | NA                        |
| PEX7                        | PEX5L/191–222                    | 0       | NA                      | NA                        |
| PEX7                        | PEX5L/1–131                      | 0       | NA                      | NA                        |

\(^a\)The amount of chloramphenicol acetyl transferase (CAT) was determined by ELISA.

\(^b\)NA, not analyzed.

**Fig. 5.** HsPEX7 only interacts with the long form of HsPEX5 in a mammalian two-hybrid assay. Wild-type fibroblasts were co-transformed with plasmids encoding fusions of PEX5L (a) or PEX5S (b) with the VP16 activation domain (AD), fusions of PEX7 with the GAL4 binding domain (BD), and with a reporter plasmid encoding GAL4-dependent CAT. Activation of the expression of CAT was investigated by immunofluorescence microscopy using polyclonal antibodies against CAT (a, b). Only the co-expression of plasmids encoding AD-PEX5L and BD-PEX7 resulted in a cytosolic expression of CAT (a) whereas no CAT expression was found with AD-PEX5S and BD-PEX7 (b). The expression of the PEX7 binding domain plasmid in both co-transfections with AD-PEX5L (c) or AD-PEX5S (d) was controlled with anti-binding domain antibodies, and the equal expression rates of AD-PEX5S and AD-PEX5L were monitored with anti-PEX5 antibodies (data not shown).
The cytoplasmic localization of catalase (Fig. 6) was expressed in wild-type fibroblasts (a–d) or in PEX5-deficient PBD005 cells (e, f). Permeabilization was either done with Triton X-100 (a, b, e, f) or with digitonin (c, d), which did not permeabilize the peroxisome membrane. a, EGFP-PEX7 shows a cytoplasmic and peroxisomal distribution in wild-type cells visualized with antibodies against EGFP. b, the same cells stained for peroxisomal catalase. c and d, digitonin permeabilization reveals a peroxisomal and cytoplasmic localization of EGFP-PEX7 when the intrinsic fluorescence of EGFP, which is independent of permeabilization, is monitored (c). No peroxisomal staining is noticeable when antibodies against EGFP were used (d). This indicates that most of the peroxisomal EGFP staining accounts for intraperoxosomal EGFP-PEX7 molecules. e and f, EGFP-PEX7 has an exclusively cytoplasmic localization in PEX5-deficient PBD005 cells, when stained with antibodies against EGFP (e). The identical cell stained with antibodies against catalase, indicating the PT51 import defect of these cells that leads to a cytoplasmic localization of catalase (f).

191–251 in normal human fibroblasts inhibited PTS2 protein import by ~75%.

Could PEX5L Represent a Functional Homolog of Yeast Pex18p/Pex21p?—Like PEX5L in mammalian cells, S. cerevisiae Pex18p and Pex21p interact with S. cerevisiae Pex7 and are required for PTS2 protein import (77). We therefore tested whether mammalian PEX5 shared any other properties with yeast Pex18p and Pex21p. All three proteins interact with PEX7, play an important role in PTS2 protein import, and play an important role in directing PEX7 to peroxisomes (77), and we therefore investigated whether PEX5 was required for PEX7 targeting in human cells. Although yeast Pex7p has been described as a predominantly cytoplasmic, partly peroxisomal protein (34, 35, 38), certain tagged versions of Pex7p will actually accumulate within peroxisomes (33). A fusion protein between enhanced green fluorescence protein (EGFP) and human PEX7 (EGFP-PEX7) displays this property, as shown by its peroxisomal distribution in normal human fibroblasts (Fig. 6, a and b) that was not detectable when the cells were differentially permeabilized with digitonin indicating an intraperoxosomal localization (Fig. 6, c and d). We expressed the same EGFP-PEX7 fusion protein in PEX5-deficient PBD005 cells and found that it remained completely in the cytoplasm (Fig. 6, e and f), even though these cells contain numerous peroxisomes (27, 68, 78).

The hypothesis that PEX5L mediates the transport of PEX7 to peroxisomes predicts that the minimal PTS2 rescue domain of PEX5L contains at least two functional elements: one that is sufficient for interaction with PEX7 and another that is sufficient for targeting to peroxisomes. It is not a trivial issue to assay the targeting of PEX5 proteins to peroxisomes, because PEX5 is a predominantly cytoplasmic, partly peroxisomal protein (27). For example ~95% of the total PEX5 protein is located in the cytoplasm of normal human fibroblasts. However, certain PBD cells with mutations in other PEX genes trap PEX5 on or in peroxisomes (40, 79), and we took advantage of this phenotype to determine whether the minimal PTS2 import domain of PEX5L was sufficient for peroxisomal targeting. PBD054 cells are homozygous for a missense mutation (S320F) in PEX12 and accumulate PEX5 on or in the peroxisome (79). We transfected PBD054 cells with plasmids designed to express various myc-tagged PEX5 proteins. Two days after transfection the subcellular distribution of these proteins was assessed by immunofluorescence microscopy using anti-myc antibodies (Fig. 7). PEX5/1–214 was the smallest fragment that retained peroxisomal targeting, and this region is contained within the smallest fragment of PEX5L that is sufficient for PTS2 protein import (PEX5L/1–230myc). Smaller fragments of PEX5 (amino acids 1–157 and 1–90) failed to target to peroxisomes and were instead located only in the cytoplasm.

PEX5L shares three properties with the S. cerevisiae peroxins Pex18p and Pex21p. All three proteins interact with PEX7, play an important role in PTS2 protein import, and play an important role in directing PEX7 to peroxisomes. These common properties led us to examine PEX5L, ScPex18p, and ScPex21p for any shared sequence motifs, and we identified a 21-amino acid-long peptide motif in all three proteins (Fig. 8). This motif (amino acids 209–229) is located within the minimal PTS2 rescue domain of PEX5L (amino acids 1–230), and a PEX5L fragment that lacked the C-terminal 7 amino acids of this element (PEX5L/1–222) displayed only a borderline ability to rescue PTS2 protein import. Most of this motif also matches...
the smallest fragment of PEX5L that retains interaction with PEX7 in the mammalian two-hybrid assay (amino acids 191–222), indicating that it may play an important role in PEX5L/PEX7 interaction. The fact that this conserved motif is 8 amino acids longer than the smallest PEX7-interacting fragment and the fact that a somewhat longer PEX5 fragment PEX5L/191–251 shows even stronger interactions may indicate that indeed the region including amino acids 209–229 of PEX5L is most important for the interaction with PEX7. This hypothesis is supported by a recent study from the Fujiki laboratory, which showed that substitution of phenylalanine for a conserved serine of this motif (Ser-213 of human PEX5) eliminates PTS2 protein import and disrupts the PEX5L/PEX7 interaction (80). The hypothesis that this 21-amino acid motif may be important for interaction with PEX7 is also supported by the fact that this motif is located within regions of Pex51p...
of interact with yeast Pex7p (Fig. 9). In addition, the interaction did indeed interact with yeast Pex7p whereas PEX5S did not. Using the yeast two-hybrid assay, we observed that human PEX5L interacted with yeast Pex7p and/or Pex21p may interact with human PEX7. Using all three proteins. The motif 21 amino acids long common to ScPex18p and/or Pex21p predicts that PEX5L and Pex21p that are sufficient for interaction with yeast Pex7p (77).

The possibility that PEX5L may represent a functional homolog of S. cerevisiae Pex18p and/or Pex21p predicts that PEX5L may interact with S. cerevisiae Pex7p and that S. cerevisiae Pex18p and/or Pex21p may interact with human PEX7. Using the yeast two-hybrid assay, we observed that human PEX5L did indeed interact with yeast Pex7p whereas PEX5S did not interact with yeast Pex7p (Fig. 9). In addition, the interaction of S. cerevisiae Pex7p was mapped to the same region of PEX5L, as with human PEX7. Previous studies have reported that yeast Pex7p interacts with yeast Pex5p in the yeast two-hybrid assay, but this interaction is detected only in the presence of yeast Pex14p. Presumably, Pex14p serves as a bridge between Pex5p and Pex7p, which do not contact each other directly (50). In contrast, the interaction between human PEX5L and yeast Pex7p is not reduced in the absence of Pex14p and in fact may even be stronger in the pex14-deficient two-hybrid reporter strain. We also tested whether S.c. Pex21p interacted with human PEX7 using the mammalian two-hybrid system. We again found evidence of interaction (Table III) indicating that the interacting regions of both partners may be conserved.

The fact that the role of PEX5 in PTS2 protein import differs greatly between mammalian cells and yeast led us to consider the possibility that additional differences may exist in PTS2 protein import. We already supposed that PEX5L could transport PEX7 and its cargo to the peroxisomal membrane. The two candidates for docking this complex to the peroxisomal membrane are PEX13 and PEX14. Data available so far suggest differences between the human and yeast system. Previous studies have established that PEX5 and PEX14 has now been shown by several laboratories (14, 66) and in two-hybrid assays (50). The interaction between human PEX5 and PEX14 has now been shown by several laboratories (48, 52). Using the human two hybrid-system we could easily detect the PEX5/PEX14 interaction (52, 66). Performing co-immunoprecipitations from lysates Fujiki and co-workers found evidence for PEX13-PEX5 (66) and PEX14-PEX7 (53, 66) interactions; however, there is no evidence that these interactions are direct and the interactions could not be detected in other laboratories (48, 52). Using the human two hybrid-system we could easily detect the PEX5/PEX14 interaction (52, and this study) and the PEX5L-PEX7 interaction, but we find no indication for a PEX13-PEX5 interaction, even when we used the SH3 domain of PEX13 (Table IV). In addition, we did not detect an interaction between PEX13-PEX7 that was suffi-
cien tively high above background (Table IV). The lack of a PEX14-PEX7 interaction has been reported previously for the human system (52). Together our data support the idea that PEX14 is probably the main docking partner not only for the PTS1 receptor complex (48, 66) but also mediates PTS2 import via PEX5L and PEX7 in the human system. The lack of PEX13-PEX7 and PEX14-PEX7 interactions may explain why PEX7 was not localized to the peroxisomal membrane in PEX5 deficient cells (Fig. 7).

DISCUSSION

In their initial description of PEX5, McCollum et al. (25) reported that this peroxin was not required for PTS2 protein import in the yeast Pichia pastoris. The subsequent observation that PEX5 is essential for PTS2 protein import in human cells was therefore unexpected (27, 29). However, studies showing that PEX5 is also essential for PTS2 protein import in hamster cells (65) and mice (63) have firmly established the central role of PEX5 in PTS2 protein import in mammalian cells. The different roles of PEX5 in PTS2 protein import in fungi and mammals are likely to reveal core properties of the PTS2 protein import pathway, and this topic has attracted considerable attention within the field. Mammalian PEX5 RNAs are alternatively spliced to generate two isoforms of PEX5X, which differ by 37 amino acids. The longer isoform, PEX5L, is the form that participates in PTS2 protein import (64, 65). Recent studies from Fujiki and colleagues (48, 66) have furthered our understanding by establishing that PEX7 displays preferential binding to PEX5L as compared with PEX5S. In this report we used a combination of functional complementation assays, mammalian two-hybrid assays, and protein targeting assays to identify regions of PEX5L that are sufficient for PTS2 rescue, interaction with PEX7, and targeting to peroxisomes.

PEX5L is a protein 639-amino acids long. Previous studies have established that amino acids 299–639 are involved in PTS1 recognition (27, 31, 79) and interaction with PEX12 (79) and that amino acids 1–298 are involved in interaction with PEX14 (70, 81). Positive results in a variety of assays have allowed us to enrich the functional domain map of PEX5L (Fig. 10) by showing that amino acids 1–230 are sufficient for rescuing PTS2 protein import in PEX5-deficient human cells, amino acids 191–222 are sufficient for interaction with PEX7, and amino acids 1–214 are sufficient for peroxisomal targeting. These results support the view of PEX5 as a modular, multidomain protein that interacts with numerous proteins during the matrix protein import process. Other protein interaction sites that remain to be mapped in PEX5 include the binding sites for PEX10 (82), Pex8p (83), and perhaps other PEX5-binding proteins that remain to be described.

Prior studies showing that PEX5L, but not PEX5S, mediated PTS2 protein import and PEX7 interaction (64–66) suggested that the 37-amino acid insert of PEX5L played an important role in these processes. However, several lines of evidence suggest that PEX7 interaction requires additional sequences N-terminal to the insert and may require only the N-terminal portion of the insert. We observed that the C-terminal 21 amino acids of the 37-amino acid insert could be removed without significantly reducing the ability of PEX5L to rescue PTS2 protein import. We also identified a peptide motif at amino acids 209–229 that spans the N-terminal boundary of the insert, is shared by the PEX7 binding domains of yeast Pex18p and Pex21p proteins, and lies within the smallest PTS2 rescue and PEX7 interaction domains we identified in PEX5L. Matsumura et al. (80) identified a single amino acid substitution mutation in PEX5 that eliminated only PTS2 protein import and reduced the interaction of PEX5L with PEX7. This serine to phenylalanine missense mutation alters a serine of the conserved peptide motif that is located two amino acids N-terminal of the 37-amino acid insert, lending additional support to the hypothesis that the PEX7 interaction domain spans the N-terminal boundary of the insert in PEX5L. Finally, we observed that a fragment of PEX5L lacking sequences upstream of the 37-amino acid insert failed to interact with PEX7 in the mammalian two-hybrid assay. Although there is no control for the folding of this PEX5L fragment, the fragment was properly expressed and localized and the result is at least consistent with the hypothesis that PEX7 interaction requires amino acids upstream of the insert. Additionally, we emphasize that our data do not demonstrate direct interaction between PEX5L and PEX7. In fact, we would argue that the existing data in the literature also fail to demonstrate direct PEX5L-PEX7 binding (66). We also note that our negative results are largely uncontrolled, can be accounted for by experimental artifact, and should not be interpreted as evidence that the PEX5L-PEX7 interaction is indirect.

Like yeast Pex18p and Pex21p, PEX5L is required for PTS2 protein import, interacts with PEX7, and is necessary for PEX7 transport to peroxisomes. In addition to these functional similarities, the presence of a shared peptide motif in all three proteins shows that they also share some structural similarities. These common properties suggest that PEX5L may have acquired one or more of the roles that yeast Pex18p and Pex21p perform in PTS2 protein import. One possibility is that PEX5L may have replaced either Pex18p or Pex21p, but such a simple model ignores the fact that PEX5L is essential for PTS2 protein import, whereas PTS2 protein import in yeast is blocked only by the loss of both Pex18p and Pex21p. Therefore, a more likely model would have PEX5L replacing both Pex18p and Pex21p. Another attractive possibility is that the role of these PEX7-associated proteins has changed through evolution in such a way that the Pex18p- and Pex21p-like activities would both be essential for PTS2 protein import and PEX7 interaction. The identification of human orthologs of Pex18p and Pex21p would...
help distinguish between these various possibilities, and, although we have been unable to identify Pex18p or Pex21p genes in humans, this failure hardly represents strong evidence that Pex18p and Pex21p are absent.

A further comparison of all known oxP5 yeast strains revealed that the PTS2 pathway may be absent in T. brucei (86) but not in Caenorhabditis elegans (C34C6.6 (87); Drosophila melanogaster (AAF45676.1); or different yeast species. For all species that share the common PEX7 binding motif there is evidence for PEX7 as in plants and Arabidopsis thaliana (AABB8113), or at least evidence for proteins with a PTS2 targeting signal as in plants and T. brucei (88). Our results suggest that PTS2 import may be similar in mammals, plants, and protozoa but different in yeasts. For C. elegans, which appears to lack PEX7 (89), PEX5 (87) lacks the PEX7 interaction motif. In addition, orthologs of mammalian PTS2 proteins in C. elegans lack a PTS2 signal and instead are imported via a PTS1 domain, indicating that the PTS2 pathway may be absent in C. elegans (89).

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REFERENCES

1. Wanders, R. J. A., and van Bakel, H., C. J. M. (1998) Biochim. Biophys. Acta 1372, 53–60.
2. Lametschwandtner, G., Brocard, C., Fransen, M., van Veldhoven, P., Berger, A., and van Bakel, H., C. J. M. (1995) Cell 81, 497–506.
3. Gedda, L., Kulsion, P., and Ledbetter, D. A. (1993) J. Biol. Chem. 268, 13530–13537.
4. Gedda, L., Kulsion, P., and Ledbetter, D. A. (1993) J. Biol. Chem. 268, 13530–13537.
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80. Matsumura, T., Otera, H., and Fujiki, Y. (2000) J. Biol. Chem. 275, 21715–21721
81. Saidowsky, J., Doft, G., Kirchberg, K., Wegner, A., Nastainczyk, W., Kunau, W. H., and Schliebs, W. (2001) J. Biol. Chem. 276, 34524–34529
82. Warren, D. S., Wolfe, B. D., and Gould, S. J. (2000) Hum. Mutat. 15, 509–521
83. Behling, P., Skaletz-Rorowski, A., Girzalsky, W., Vorn-Brouwer, T., Franse, M. M., Distel, B., Veenhuis, M., Kunau, W. H., and Erdmann, R. (2000) J. Biol. Chem. 275, 3593–3602
84. Wimmer, C., Schmid, M., Veenhuis, M., and Gietl, C. (1998) Plant J. 16, 453–464
85. Jardim, A., Liu, W., Zheleznova, E., and Ullman, B. (2000) J. Biol. Chem. 275, 13637–13644
86. de Walque, S., Kiel, J. A., Veenhuis, M., Opperdoes, F. R., and Michels, P. A. (1999) Mol. Biochem. Parasitol. 104, 106–119
87. Gurvitz, A., Langer, S., Piskacek, M., Hamilton, B., Ruis, H., and Hartig, A. (2000) Yeast 17, 188–200
88. Blattner, J., Dorsam, H., and Clatyon, C. E. (1995) FEBS Lett. 360, 310–314
89. Motley, A. M., Hettema, E. H., Ketting, R., Plasterk, R., and Tabak, H. F. (2000) EMBO Rep. 1, 40–46
Domain Mapping of Human PEX5 Reveals Functional and Structural Similarities to Saccharomyces cerevisiae Pex18p and Pex21p

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