Abstract. Peroxisomal matrix protein import requires PEX12, an integral peroxisomal membrane protein with a zinc ring domain at its carboxy terminus. Mutations in human PEX12 result in Zellweger syndrome, a lethal neurological disorder, and implicate the zinc ring domain in PEX12 function. Using two-hybrid studies, blot overlay assays, and coimmunoprecipitation experiments, we observed that the zinc-binding domain of PEX12 binds both PEX5, the PTS1 receptor, and PEX10, another integral peroxisomal membrane protein required for peroxisomal matrix protein import. Furthermore, we identified a patient with a missense mutation in the PEX12 zinc-binding domain, S320F, and observed that this mutation reduces the binding of PEX12 to PEX5 and PEX10. Overexpression of either PEX5 or PEX10 can suppress this PEX12 mutation, providing genetic evidence that these interactions are biologically relevant. PEX5 is a predominantly cytoplasmic protein and previous PEX5-binding proteins have been implicated in docking PEX5 to the peroxisome surface. However, we find that loss of PEX12 or PEX10 does not reduce the association of PEX5 with peroxisomes, demonstrating that these peroxins are not required for receptor docking. These and other results lead us to propose that PEX12 and PEX10 play direct roles in peroxisomal matrix protein import downstream of the receptor docking event.

Key words: PTS1 receptor • PEX5 • PEX10 • Zellweger syndrome • peroxisome biogenesis disorder

Subcellular organelles are a defining feature of eukaryotes and make essential contributions to virtually all aspects of cellular metabolism. Although each organelle is physically and chemically unique, there are several common aspects to the biogenesis of all membrane-limited compartments. One is that all or most of their protein content must be imported. The protein uptake mechanisms used in the biogenesis of each organelle must account for the recognition of newly synthesized proteins that are destined for each organelle, the transport of these proteins to the surface of the appropriate compartment, and the vectorial translocation of these proteins across one or more lipid bilayers (Schatz and Dobberstein, 1996).

Several paradigms for protein import into organelles have emerged from studies of the ER (Matlack et al., 1998), the mitochondrion (Pfanner et al., 1997), and the nucleus (Matlack et al., 1998). However, the process of peroxisomal protein import does not conform to any of these paradigms (Subramani, 1993): proteins are posttranslationally imported into the peroxisome but cotranslationally inserted into the ER; peroxisomal membrane proteins (PMPs) do not use either PTS1 or PTS2.

Abbreviations used in this paper: MBP, maltose-binding protein; ORF, open reading frame; PBD, peroxisome biogenesis disorder; PMP, peroxisomal membrane protein.

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motifs for targeting and are efficiently imported into peroxisomes independently of PEX5 and PEX7 (Subramani, 1993; Dyer et al., 1996; Chang et al., 1999).

Current models of peroxisomal matrix protein import are heavily influenced by the fact that PEX5 is a predominant cytoplasmic protein in several species (Död et al., 1995; Wiemer et al., 1995; Elgersma et al., 1996; Gould et al., 1996) and appears to cycle between the cytoplasm and peroxisomes in mammalian cells (Död and Gould, 1996). The dynamic distribution of the PTS1 receptor implies that peroxisomal matrix protein import involves overlapping cytoplasmic and peroxisomal events in addition to the actual translocation process (Braverman et al., 1995; Rachubinski and Subramani, 1995). Before translocation, import of PTS1-containing proteins (ligands) is likely to involve recognition by PEX5 in the cytoplasm, followed by transport of the receptor-ligand complex to the peroxisomal surface and binding of the receptor-ligand complex to docking factors on the peroxisomal membrane. After ligand translocation, additional factors are thought to mediate the recycling of PEX5 back to the cytoplasm. PEX14, PEX13, and PEX17 have been implicated in receptor docking (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996; A Ibertini et al., 1997; Huhe et al., 1998; Gritzalsky et al., 1999), and PEX4 has been reported to act in the recycling of PEX5 from the peroxisome to the cytoplasm (van der Klei et al., 1998). Other PEX genes such as PEX1 (Portsteffen et al., 1997; Reuber et al., 1997; Chang et al., 1999), PEX2 (Shimozawa et al., 1992; Chang et al., 1999), PEX6 (Yahrus et al., 1996; Chang et al., 1999), PEX10 (Kalish et al., 1995; Warren et al., 1998; Chang et al., 1999), and PEX12 (Kalish et al., 1996; Chang et al., 1997; Chang and Gould, 1998; Chang et al., 1999) have also been implicated in peroxisomal matrix protein import. However, their roles in matrix protein import are only poorly understood and other reports have suggested that PEX1 and PEX6 may instead participate in the biogenesis of peroxisomal membranes (Edmond et al., 1991; Spong and Subramani, 1993; Heyman et al., 1994; Titorenko et al., 1997; Faber et al., 1998; Titorenko and Rachubinski, 1998a,b).

Although most PEX genes were originally identified in yeast, our understanding of peroxisome biogenesis has also been advanced by the analysis of this process in humans and how it is disrupted in the peroxisome biogenesis disorders (PBDs) (Lazarow and M. Oser, 1995). Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease represent a phenotypic continuum of diseases within the PBDs that we refer to as the Zellweger spectrum. Their clinical phenotypes include development delay, multiple neural, hepatic, and renal defects, and pronounced mental retardation. These clinical phenotypes are most severe in Zellweger syndrome patients, who rarely survive their first year, are milder in neonatal adrenoleukodystrophy, and even less pronounced in infantile Refsum disease. At the cellular level, virtually all Zellweger spectrum patients display defects in the import of both PTS1 and PTS2 proteins, though there are rare patients who are defective only in PTS1 protein import (Slawecki et al., 1995). There is significant genetic heterogeneity within the Zellweger spectrum and recent studies have identified the genes that are mutated in >95% of PBD patients and nine of the eleven known PBD complementation groups. These include PEX1 (Portstein et al., 1997; Reuber et al., 1997), PEX2 (Shimozawa et al., 1992), PEX5 (Död et al., 1995), PEX6 (Fukuda et al., 1996; Yahrus et al., 1996), PEX10 (Okumoto et al., 1998a; Warren et al., 1998), PEX12 (Chang et al., 1997; Okumoto et al., 1998b), PEX13 (Liu et al., 1999; Shimozawa et al., 1999), PEX16 (Honsho et al., 1998; South and Gould, 1999), and PEX19 (Masuzono et al., 1999). Variations in the clinical and cellular phenotypes within the Zellweger spectrum are related primarily to the severity of the affected alleles, with mild phenotypes arising from PEX gene mutations that only partially reduce PEX gene function (Reuber et al., 1997; Chang and Gould, 1998; Collins and Gould, 1999). In contrast to the Zellweger spectrum of diseases, rhizomelic chondrodysplasia punctata is caused by mutations in PEX7, which encodes the PTS2 receptor (Braverman et al., 1997; Motley et al., 1997; Purdie et al., 1997).

We initially identified PEX12 in the yeast Pichia pastoris as a gene that is required for normal peroxisome biogenesis (Kalish et al., 1996). More recent studies have described the human homologue of this gene and reported that mutations in PEX12 are responsible for complementation group 3 of the PBDs (Chang et al., 1997; Okumoto and Fujiki, 1997; Chang and Gould, 1998; Okumoto et al., 1998b). Both yeast and human PEX12 encode an integral peroxisomal membrane protein with a zinc ring motif at its COOH terminus (Kalish et al., 1996; Okumoto and Fujiki, 1997). These studies also established that PEX12 spans the peroxisomal membrane twice and extends its NH2 and COOH termini into the cytoplasm. In this report, we investigate the function of human PEX12 in peroxisome biogenesis. We find that PEX12 interacts with PEX5 and PEX10 via its COOH-terminal zinc-binding domain, that these interactions are biologically significant, and that PEX12 and PEX10 participate in an aspect of peroxisomal matrix protein import that occurs downstream of receptor docking.

Materials and Methods

Cell Lines, Transfections, and Indirect Immunofluorescence

Skin fibroblast cell lines from patients with peroxisome biogenesis disorders are referred to by their PBD numbers and were cultured in complete medium (DME supplemented with 10% FBS and penicillin/streptomycin). Transfections were performed by electroporation using the protocol outlined by Chang et al. (1997) and were processed 2 d later for immunofluorescence. Immunofluorescence experiments were performed essentially as described (Slawecki et al., 1995). In brief, cells were grown on cover glasses, fixed, permeabilized, washed, incubated with the primary antibodies, washed extensively, incubated with the secondary antibodies, washed extensively, and then mounted on glass slides. Standard permeabilization was for 5 min with 1% Triton X-100, which permeabilizes both plasma and peroxisome membranes. Differential permeabilization was for 2-4 min with 25 µg/ml digitonin, which permeabilizes the plasma membrane but does not permeabilize the peroxisome membrane. Hence, only cytoplasmically exposed antigens can be detected under these conditions. Differential permeabilization experiments were generally performed with additional controls to ensure that the incubation in digitonin had not permeabilized any intracellular membranes. Rabbit polyclonal antibodies against the PTS1 tripeptide ser-lys-leu-COOH have been described (Gould et al., 1990) and the anti-human PEX5 antibodies were generated
Table 1. Sequence of Primers Used in This Article

| Primer      | Sequence                                      |
|-------------|------------------------------------------------|
| Chang-10    | 5′-CCCGGATCCCTTTCAGGGGATGATTT-3′               |
| Chang-17    | 5′-AAAATGTTTTCACGGGGG-3′                      |
| Chang-20    | 5′-CCAGGATCCGTAGAAAGACGATGATCCG-3′            |
| Chang-21    | 5′-CAAGTACCAAAGTAGAAAAGCCGATCCCCAG-3′         |
| Chang-24    | 5′-CCACCAGCTCAGATTGTTGCC-3′                   |
| Chang-30    | 5′-ATGAGTGCCAAACCGACCAG-3′                    |
| 10.2HC5     | 5′-CCCGGATCCGAGGCGGAGGAGGAGGAGGAGGAGGAGG-3′   |
| 10.2H3      | 5′-CCCAAGCGCCTTACGAGGATGAGAGGAGGAGGAGGAGG-3′ |
| 12.2HC5     | 5′-CCCGTCGACGCGTCCTCGGAGCAGGCAGAG-3′         |
| 12.2H3      | 5′-CCCAAGCGCCTTACGAGGATGAGAGGAGGAGGAGGAGG-3′ |
| 14.2H3      | 5′-CCCGGTGCAGGCTGCTGCTTGCCAGACGGAGCAGAG-3′   |
| 14.2H3      | 5′-CCAAAAGCGTCTCAGAGATTGGC-3′                 |

against bacterially expressed forms of the protein. Mouse mAb bs against the c-myc epitope were obtained from the tissue culture supernatant of the hybridoma 1-9E10 (Evan et al., 1985). Rabbit polyclonal antibodies against c-myc, sheep anti-human catalase antibodies, and fluorescently labeled secondary antibodies were obtained from commercial sources.

Mutation Detection

We previously described a mutation detection strategy for human PEX12 (Chang et al., 1997; Chang and Goud, 1998). In brief, the entire 2.3-kb PEX12 gene was amplified from the total human genomic DNA using the primers Chang-21 and Chang-20 (Table I). The coding regions and intron/exon junctions were sequenced directly from the PCR product using the primers used for amplification as well as several additional gene specific primers (Table I, Chang-17, Chang-24, and Chang-30). Total genomic DNA was isolated from PBD054 cells using the PureGene kit (Gentra Systems, Inc.).

Plasmids

Most of the plasmids used for yeast two-hybrid studies were based on pcDNA2, a LEU2-based GAL4 DNA-binding domain fusion protein expression vector and pCR8. A TRP1-based GAL4 transcriptional activation domain (AD) fusion protein expression vector (Chevray and Nathans, 1992). These plasmids contain two PvuI sites in symmetric positions. The PvuI fragments of these plasmids were switched to create pljL59 (Vidal et al., 1996) and pPC86L2. The plasmid pljL59 is identical to pPC86L2 except that it contains the TRP1 gene in place of the LEU2 gene, and pPC86L2 is identical to pCR8 except that it contains the LEU2 gene in place of the TRP1 gene. The plasmid pGAD424 (COUNTECH Laboratories) was also used for expression of some GAL4-AD fusion proteins.

The plasmid pljL59-PEX12 was created by amplifying a subregion of PEX12 using the primers 12.2HC5 and 12.2H3 (Table I) and pcDNA 3-PEX10 (Warrren et al., 1998), and pcDNA 3-PEX12 (Chang et al., 1997). To create pcDNA 3-PEX12/S320F, the PEX12 ORF was amplified using the oligonucleotides Chang-21 and Chang-10 (Table I) and pcDNA 3-PEX12 as a template. These primers appended an ApaI site upstream of the ORF and replaced the stop codon with a BamH1 site. The resulting PCR fragment was cleaved with ApaI and BamH1 and cloned upstream of the triple c-myc tag in pcDNA 3-xmcy (Geisbrecht et al., 1998). To create the plasmid pcDNA3-PEX12/S320F, we first amplified the PEX12 ORF from PBD054 cDNA. Total RNA was extracted from PBD054 cells using the PureScript kit (Gentra Systems, Inc.) and PEX12 cDNA was synthesized as described (Chang and Goud, 1998). The PEX12 ORF was amplified from the first strand PCR products using Chang-21 and Chang-20 (Table I), cleaved with ApaI and BamH1, and cloned between the ApaI and BamH1 sites of pcDNA3, generating pcDNA3-PEX12/S320F. The sequence of the final plasmid was confirmed to ensure the presence of the S320F mutation and the absence of any undesired mutations. The plasmid pcDNA3-3xHA has a 114-bp DNA insert between the ApaI and XbaI sites of pcDNA3, which contains a BglII site (AGATCT) immediately upstream of short ORF encoding three repeats of the HA epitope tag (GRGFYDVPDYAGYDPVDPYAGYDPVDPYDALTSTOP), the HA epitopes are underlined).

To create pcDNA3-PEX10/S320A, we excised the PEX10 ORF (lacking its stop codon) from pcDNA3-PEX10mcy (Warren et al., 1998) using the restriction enzymes ApaI and BamH1, excised the XbaI tag from pcDNA3-3xHA by cleavage with ApaI and XbaI, and inserted these fragments in tandem between the ApaI and XbaI sites of pcDNA3. The regions of all plasmids that were generated by PCR were sequenced to confirm the absence of any unintended mutations. Any plasmids that did contain undesired mutations were discarded and additional clones were characterized until one with the desired sequence was obtained.

Two-Hybrid Analysis

The Saccharomyces cerevisiae two-hybrid reporter strain BY3168 was used for all experiments (Vidal et al., 1996). All strains were grown overnight on a nitrocellulose filter membrane (Schleicher & Schuell, Inc.) that was placed on a plate with minimal medium lacking tryptophan and uracil (5c-W-L). The cells were lysed by submerison in liquid nitrogen, and activity of the two-hybrid reporter gene β-galactosidase was assayed by placing the filter membrane onto a filter paper saturated with 0.1% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in 100 mM potassium phosphate buffer, pH 7.0. The filters were photographed after color development.

Protein extracts from BY3168 carrying either pljL59-PEX12C or pljL59-PEX12C/S320F were prepared according to established protocols.
(A dams et al., 1997). In brief, the yeast were grown in 4 ml Sc-W-L medium to an OD of 2. The cells were transferred to a solution containing 50 mM Tris, pH 7.5, and 10 mM sodium citrate, centrifuged (5,000 g) for 10 min. The pellets were resuspended in 30 μl of ESB (80 mM Tris, pH 6.8, 1.5% DTT, 2% SDS, 10% glycerol, and 0.1 mg/ml bromophenol blue), and immediately boiled for 3 min. The tubes were cooled on ice, and then mixed vigorously with 0.1 g of 425–600-micron glass beads (Sigma Chemical Co.) for 2 min to lyse the cells. The resulting lysate were pelleted and an additional 70 μl of ESB and boiled for 1 min. Equal amounts of each sample were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with antibodies that are present in yeast PEX12.

**Protein Purification, In Vitro Translation of Proteins and Overlay Assays**

A 1-liter culture of D H10B cells (Grant et al., 1990) carrying pBM-PEX12C was grown to an OD of 0.4, induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and grown overnight at 18°C. These cells were harvested and resuspended in column buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol) plus 0.5 mg/ml lysosome. The cells were frozen in liquid nitrogen, thawed, and lysed by sonication. The lysate was centrifuged for 20 min at 14,000 g, and the supernatant was loaded onto a column containing 10 ml of amylose resin. The column was washed with 12-bed volumes of column buffer and the protein was eluted with 10 mM maltose. The eluant was collected in 1.5-ml fractions and the purity was assessed using SDS-PAGE. MBP-PEX12C and MBP-LacZ fusion proteins were purified using the same protocol.

Filter binding experiments were performed as follows. 10 μg of purified MBP-PEX12C and 10 μg of MBP-LacZ were spotted separately onto Immobilon-P membranes (Millipore). Two identical membranes were prepared, one for overlay with HisPEX5S and one for HisPEX5L. The membranes were allowed to dry for 15 min at room temperature, and then washed in methanol for 30 s followed by Milli-Q H2O for 1 min. The membranes were washed twice for 5 min at room temperature with buffer C with shaking. The agarose beads were preincubated with 1% BSA, and then incubated with the membranes. The beads were centrifuged for 10 min, washed, resuspended in SDS-PAGE, and fractionated on a 12CA5 monoclonal anti-HA antibody (Boehringer Mannheim Corp.). Equivalent levels of PEX5 and PEX10 were detected using standard immunoblotting techniques.

**Cell Fractionation and Protease Protection Assay**

Skin fibroblast cells were grown to 90% confluency in 100-mm dishes, removed from the plate by trypsinization, washed, resuspended in lysis buffer, and lysed in a ball bearing homogenizer as previously described (Dodd and Gould, 1996). Postnuclear supernatants were prepared by successive 1,500 g spins, and then separated into organelle pellets and cytosolic supernatants by centrifugation at 15,000 g for 10 min. To determine the relative levels of PEX5 in the cytoplasm and pex6osome of human fibroblasts, organelle pellets and cytosolic supernatants were prepared as above, transferred to membranes, and probed with polyclonal anti-PEX5 antibodies. For protease protection experiments, organelle preparations were derived from PBD006 and PBD054 cells in the same manner and were each split into eight equal fractions of 8 μg of protein. Triton X-100 was added to four of the eight tubes to a final concentration of 1%. We placed the samples on ice and added 0, 15, 30, or 60 μg of a trypsin preparation (Calbiochem-Novabiochem) to the four samples lacking detergent and the four samples containing detergent. These mixtures were incubated on ice for 20 min. Reactions were terminated by adding a twofold excess of bovine trypsin inhibitor (Sigma Chemical Co.). Equal amounts of each sample were processed for immunoblotting with anti-PEX5 antibodies.

**Results**

The Zinc-binding Domain Is Required for PEX12 Function

We have identified an array of PEX12 mutations that cause Zellweger syndrome and the milder phenotypic variants of neonatal adrenoleukodystrophy and infantile Refsum disease (Chang et al., 1997; Chang and Gould, 1998). To better understand the regions of PEX12 that are important for its role in peroxisome biogenesis, we compared the deduced products of the PEX12 alleles in severely and mildly affected patients (Fig. 1). Severe defects in PEX12 activity were associated with mutations that truncated PEX12 upstream of the cytoplasmically exposed zinc ring domain. Furthermore, all moderately and mildly affected patients expressed at least one PEX12 allele capable of encoding a protein that contained the COOH-terminal zinc ring domain. This phenotype-genotype correlation suggested that the COOH-terminal zinc-binding domain is critical for PEX12 function. This hypothesis is also supported by the results from directed mutagenesis experiments on PEX12 in both yeast (Kalish et al., 1996) and mammalian cells (Okumoto et al., 1998b).

**PEX12 Binds PEX5, the PTS1 Receptor**

Previous studies have suggested that zinc ring domains may mediate protein–protein interactions (Borden, 1998), and the important role of this domain in PEX12 suggested that it may mediate interactions between PEX12 and other proteins that are involved in peroxisome biogenesis. We employed the yeast two-hybrid system to search for such proteins. A fusion between the GAL4 DNA-binding domain and the COOH-terminal 100 amino acids of PEX12 was used as bait to screen a library of fusions between the GAL4-activating domain and all known human peroxins. We detected a strong interaction between the
zinc-binding domain of PEX12 and PEX5, the PTS1 receptor (Fig. 2 A). Recent studies (Braverman et al., 1998; Otera et al., 1998) have established that two isoforms of PEX5, PEX5S and PEX5L, are synthesized in roughly equivalent levels in human cells, but we did not observe any difference in the interaction between PEX12 and PEX5S or PEX5L.

Next, we used a protein binding assay to independently assess the interaction between PEX12 and PEX5. We generated a recombinant fusion protein between maltose-binding protein (MBP) and the COOH-terminal 100 amino acids of PEX12, which includes its zinc-binding domain. The resulting protein, MBP-PEX12, was tested for its ability to bind PEX5 using a filter binding assay. Equal amounts of purified MBP-PEX12 and an MBP-LacZ fusion protein were spotted onto membranes and subsequently probed with 35S-labeled PEX5 that had been synthesized in vitro in a rabbit reticulocyte lysate. PEX5 was bound by MBP-PEX12 but not by MBP-LacZα, indicating that PEX12 was capable of binding PEX5 (Fig. 2 B).

To determine whether the physical interaction between PEX5 and the zinc-binding domain of PEX12 reflected an association between these proteins in vivo, we tested whether PEX12 and PEX5 formed a complex of sufficient stability to withstand coimmunoprecipitation from cell lysates. Human fibroblasts were transfected with either of two plasmids, pcDNA3-PEX12 or pcDNA3-PEX12/3xmyc. 2 d after transfection the cells were lysed, the lysates were subjected to immunoprecipitation with antimyc polyclonal antibodies, and the immunoprecipitates were separated by SDS-PAGE and probed with anti-PEX5 antibodies. Equal amounts of PEX5 were present in both crude lysates, but PEX5 was immunoprecipitated only from the lysate of cells expressing PEX12/3xmyc (Fig. 2 C).

We next mapped the PEX12-binding domain of PEX5 by expressing different regions of PEX5 in the yeast two-hybrid system and assaying their interaction with the PEX12 zinc-binding domain. These fragments of PEX5 were also assayed for their ability to interact with PEX14, a known docking factor for PEX5 (A liberti et al., 1997; Fransen et al., 1998; Schliebs et al., 1999) (Fig. 2 D). The PEX5-PEX12 binding site of PEX5 is contained within its COOH-terminal half, a region that contains seven tetratricopeptide repeats (Dodt et al., 1995; Terlecky et al., 1995). A fragment of PEX5 containing little more than the PEX5-PEX12 binding domain of PEX5 retained full binding to PEX12. However, it was unable to bind PEX14, as expected from the recent study by Schliebs et al. (1999) in which the PEX14 binding sites were localized to the NH₂-terminal half of PEX5. A diblithal truncation mutants failed to define a smaller PEX12-binding site within PEX5 (data not shown).

**The Zinc-binding Domain of PEX12 Binds PEX10**

In addition to the interaction between PEX12 and PEX5, the yeast two-hybrid screen also revealed an interaction between PEX12 and PEX10, an integral PMP that is required for peroxisomal matrix protein import (Fig. 3 A). Like PEX12, human PEX10 contains a cytoplasmically exposed zinc ring domain (Warren et al., 1998) and the interaction we detected between these two proteins was mediated through their COOH-terminal zinc ring domains (amino acids 240–326 of PEX10 and 260–359 of PEX5). Independent biochemical evidence for physical interaction between PEX12 and PEX10 was obtained using blot overlay experiments. Equal amounts of purified, recombinant MBP-PEX12 and MBP-LacZα were resolved by SDS-PAGE, immobilized on membranes, and probed with 35S-labeled PEX10 that had been synthesized in vitro in rabbit reticulocytes (Fig. 3 B). PEX10 was bound by the MBP-PEX12 fusion protein but not by MBP-LacZα, suggesting specific binding between PEX12 and PEX10. To assess whether these proteins were present in a complex in vivo, we transfected normal human fibroblasts with plasmids designed to express tagged forms of these proteins, PEX12/3xmyc and PEX10/HA, or PEX10/HA and an untagged version of PEX12. 2 d after transfection, lysates were prepared from the two sets of transfected cells, subjected to immunoprecipitation using an anti-myc
The polyclonal antibody, and then blotted with a monoclonal anti-HA antibody. Equivalent amounts of PEX10/HA were detected in both crude lysates, but PEX10/HA was only detected in the immunoprecipitate from cells expressing PEX12 and PEX5. Equal amounts of MBP-LacZ and MBP-PEX12C were spotted on membranes and probed with [35S]PEX5S (upper panel) or [35S]PEX5L (lower panel). (C) PEX5 coimmunoprecipitates with PEX12/3xmyc. Lysates were prepared from fibroblasts that had been transfected with either pcDNA3-PEX12 or pcDNA3-PEX12/3xmyc. After immunoprecipitation with anti-myc antibodies, the immunoprecipitates were analyzed by immunoblot with anti-PEX5 antibodies (upper panel). In addition, equal amounts of the crude lysate before immunoprecipitation were assayed for PEX5 levels by immunoblot (lower panel). (D) PEX5 coimmunoprecipitates with PEX10/3xmyc. Thus, PEX12 and PEX10 do appear to be present in a complex in vivo (Fig. 3 C). Control experiments revealed that these tagged forms of PEX12 and PEX10 have normal activity in vivo. This region of PEX12 failed to interact with any of the remaining 12 human peroxins in the yeast two-hybrid assay.

**Genetic Interactions between PEX12, PEX10, and PEX5**

The simplest explanation for the physical association of PEX12 with both PEX5 and PEX10 is that these interactions contribute to the biogenesis of peroxisomes. In such an instance, we might expect that high dosage, allele-specific extragenic suppression could be observed among the
corresponding three genes. Therefore, we tested whether overexpression of any one of these genes could suppress mutations in either of the other two genes. In brief, fibroblast cell lines with mild or severe mutations in PEX12, PEX10, or PEX5 were transfected separately with expression vectors designed to express these genes, as well as a vector control. 2 d later, each cell population was processed for indirect immunofluorescence using antibodies specific for a peroxisomal matrix protein marker, catalase. The relative rescue activity of each gene in each cell line was calculated by comparing the frequency of cells importing catalase in each set of transfected cells.

All three patients from complementation group 2 of the PBDs have mutations in PEX5 (Dodt et al., 1995; Slawek et al., 1995). Two of these patients (PBD018 and PBD093) are homozygous for a PEX5-N489K/N526K mutation (N489/N586 refers to the position of this asparagine in PEX5S and PEX5L, respectively). The other PEX5-deficient patient, PBD005, is homozygous for a PEX5 nonsense mutation, R390ter/R427ter, which inactivates PEX5. PBD018 and PBD005 cells were transfected with the plasmids pcDNA3, pcDNA3-PEX5, pcDNA3-PEX12, and pcDNA3-PEX10, incubated for 2 d, and then processed for immunofluorescence using antibodies specific for peroxisomal catalase. Although expression of PEX5 efficiently rescued catalase import in both cell lines, PEX12 and PEX10 were unable to restore catalase import in these lines (data not shown).

We have also characterized eight complementation group 3 PBD patients, all of whom are mutated in PEX12 (Chang et al., 1997; Chang and Gould, 1998). Fibroblast cell lines from each of these patients were transfected with the above vectors, and the import of peroxisomal matrix proteins was determined in each population of transfected cells. As expected, pcDNA3-PEX12 efficiently rescued the peroxisomal protein import defects in all of these cell lines. However, we failed to observe any evidence for extragenic suppression in the CG3 cells that were transfected with the PEX10 or PEX5 expression vectors (data not shown).

Our previous work has established that PEX10 is the gene defective in two patients from complementation group 7 (CG7), PBD052, and PBD100 (Warren et al., 1998). Although we have yet to identify the PEX10 mutations in four other CG7 patients, we used fibroblasts from all six available CG7 patients for our studies. Expression of PEX10 rescued peroxisomal matrix protein import in all six CG7 cell lines, whereas PEX12 and PEX5 failed to have any effect on the cytosolic catalase distribution in five of these cell lines. However, expression of PEX12 clearly led to the restoration of catalase import in PBD054 cells (Fig. 4, A–F). Expression of PEX5 also rescued peroxisomal matrix protein import in this cell line, though to a lesser extent (Table II).

To better understand the molecular basis of the apparent suppression of PEX10 allele(s) by overexpression of PEX12 or PEX5, we sequenced the PEX10 gene from PBD054 cells. Surprisingly, we failed to detect any alteration to the gene in this patient. This result, combined with the fact that PEX12 was more effective than PEX10 at rescuing peroxisomal protein import in PBD054 cells (Table II), led us to consider whether PEX12, rather than PEX10, might be mutated in PBD054. The PEX12 gene was amplified from PBD054 genomic DNA and all coding portions of the gene were sequenced directly from the PCR products. We detected a missense mutation, S320F, in the PEX12 gene from this patient and no evidence of the wild-type sequence, suggesting that this patient was homozygous for this mutation in PEX12 (Fig. 5A). Although S320 is a conserved residue of PEX12 from yeast to humans (always a serine or threonine), missense mutations may be silent. We engineered the S320F mutation into the PEX12 expression vector and used a functional complementation assay (Chang et al., 1997; Chang and Gould, 1998) to assess the effects of this mutation. PEX12-deficient cells were transfected with pcDNA3, pcDNA3-PEX10, or pcDNA3-PEX12/S320F, and 2 d after transfection the percentage of cells importing matrix proteins into

![Image](https://via.placeholder.com/150)

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**Table II. Suppression of Catalase Import Defect in PBD054 Cells by Overexpression of PEX5 and PEX10**

| PEX gene | Percent relative activity to restore catalase import in PBD054 cells* |
|----------|---------------------------------------------------------------|
| HsPEX12  | 100                                                          |
| HsPEX10  | 40 ± 7                                                       |
| HsPEX5S  | 19 ± 9                                                       |
| HsPEX5L  | 13 ± 3                                                       |

* n = 3.

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Figure 4. Peroxisomal protein import in PBD054 cells is restored by expression of PEX10 or PEX12. PBD054 cells were transfected with pcDNA3 (A and D), pcDNA3-PEX10 (B and E), or pcDNA3-PEX12 (C and F). 2 d after transfection, the cells were fixed, permeabilized with Triton X-100, and processed for double indirect immunofluorescence using anti-catalase antibodies (A–C) and anti-PMP70 antibodies (D–F). Bar, 25 μm.

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peroxisomes was determined by immunofluorescence using antibodies to the peroxisomal matrix protein catalase. In each of two trials the PEX12/S320F cDNA displayed 10–15% of the rescue activity of the wild-type PEX12 cDNA (data not shown). The fact that the S320F mutation reduced but did not eliminate PEX12 function was consistent with the relatively mild cellular and clinical phenotypes of this patient (PBD054 was diagnosed with neonatal adrenoleukodystrophy, and a previous study has demonstrated that PBD054 cells are able to import small amounts of some peroxisomal matrix proteins [Dodt and Gould, 1996]).

The ability of PEX10 and PEX5 to suppress the PEX12/S320F mutation was a clear example of allele-specific suppression rather than bypass suppression since neither PEX10 nor PEX5 were capable of rescuing peroxisomal protein import in any cells with severe mutations in PEX12. This finding, together with the fact that the PEX12-S320F mutation lies within the zinc-binding domain of PEX12, suggested that this mutation might reduce the interaction between the zinc ring domain of PEX12 and either PEX10 or PEX5. Using the two-hybrid assay and the blot overlay assay, we observed that the S320F mutation led to a marked reduction in the PEX12–PEX10 interaction (Fig. 5 B). Similarly, the PEX12/S320F mutation appeared to reduce the interaction between PEX12 and PEX5 in the yeast two-hybrid assay (Fig. 5 C). It is interesting to note that the placement of PBD054 cells into CG7 of the PBDs was based upon its noncomplementation with PBD052 cells. PBD052 cells are mutated in PEX10 and express one allele with a missense mutation (H290Q) in the PEX10 zinc-binding domain (Warren et al., 1998). These results indicate that the combination of the PEX12/S320F and PEX10/H290Q alleles may have a deleterious effect on peroxisomal matrix protein import even in the presence of normal alleles of each gene.

PEX12 Appears to Act Downstream of Receptor Docking

A variety of earlier studies have established that loss of PEX12 or PEX10 results in a severe defect in peroxisomal matrix protein import (Kalish et al., 1995; Liu et al., 1995; Slawecki et al., 1995; Kalish et al., 1996; Chang et al., 1997; Okumoto et al., 1998a,b; Warren et al., 1998). It is also known that PEX12 and PEX10 are not required for synthesis of peroxisome membranes or import of peroxisomal membrane proteins (Kalish et al., 1995, 1996; Chang et al., 1997, 1999; Chang and Gould, 1998; Warren et al., 1998). Furthermore, the morphological abnormalities that have been reported for peroxisomes in cells lacking PEX12 or PEX10 are indistinguishable from those of peroxisomes in PEX5-deficient cells and appear to be a secondary effect of the metabolic deficiencies that are caused by the matrix protein import defects in these cells (Baes et al., 1997; Chang et al., 1999). These results, together with our observation that PEX12 interacts with both PEX5 and PEX10, suggest that PEX12 and PEX10 participate in peroxisomal matrix protein import.

Current models suggest that there may be several steps of peroxisomal matrix protein import that are limited to the peroxisome membrane and could, therefore, involve...
PEX12 or PEX10. These include docking of receptor-ligand (matrix protein) complexes to the peroxisomal membrane, and receptor recycling (Braverman et al., 1995; Rachubinski and Subramani, 1995). To distinguish between these different possibilities, we first tested whether PEX12 or PEX10 were required for docking of the PTS1 receptor, PEX5, to peroxisomes. Postnuclear supernatants were prepared from wild-type (B and C), PEX12-deficient PBD 006 (D and E), and PEX10-deficient PBD 100 (F and G) cells using anti-PEX5 antibodies (B, D, and F) and anti-PMP70 antibodies (C, E, and G). Bar, 25 μm.
type, PBD006 and PBD054 fibroblasts, peroxisomes were separated from cytosol by differential centrifugation, and the levels of PEX5 in the cytosolic supernatant and organelle pellet fractions were determined by immunoblot. PBD054 cells contained more peroxisome-associated PEX5, as predicted (Fig. 8 C).

Discussion

In this paper we investigated the role of PEX12 in peroxisomal matrix protein import.

A Role for PEX12 in Peroxisomal Matrix Protein Import

It is generally accepted that PEX5 is the receptor for newly synthesized PTS1-containing proteins (McCollum et al., 1993; Dodt et al., 1995; Fransen et al., 1995; Terlecky et al., 1995; Elgersma et al., 1996) and is a predominantly cytoplasmic protein in mammalian cells (Dodt et al., 1995; Dodt and Gould, 1996) and several fungal species (van der Klei et al., 1995; Elgersma et al., 1996; Gould et al., 1996). Studies in human cells have suggested that PEX5 shuttles between the cytoplasm and peroxisome (Dodt and Gould, 1996), and several models predict that PEX5 moves through a variety of steps as it catalyzes peroxisomal matrix protein import. The proportion of PEX5 that resides in the cytoplasm at steady state probably reflects the needs of each cell to efficiently capture newly synthesized peroxisomal matrix proteins (ligands) from the cytoplasm. However, once these ligands are bound by PEX5 many additional events must occur. These may be grouped into the general processes of (1) the transport to and docking of PEX5-ligand complexes with the peroxisome membrane, (2) the translocation of ligands into the peroxisome lumen, and (3) the recycling of receptors back to the cytoplasm (Braverman et al., 1995; Rachubinski and Subramani, 1995). To distinguish which of these events may involve PEX12, we considered the phenotypes that are expected for a mutant in each process and compared them to the phenotypes of PEX12-deficient cells.

Several studies have implicated the integral peroxisomal membrane proteins PEX13 and PEX14 in docking of PTS...
receptors to the peroxisome membrane (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996; Albertini et al., 1997; Girzalsky et al., 1999). The defining features of these docking factors are as follows: (1) their ability to bind PEX5 and PEX7, either directly or indirectly, and (2) the fact that loss of PEX13 or PEX14 results in a significant reduction in the amount of peroxisome-associated PEX5. The fact that loss-of-function mutations in PEX12 do not result in any detectable reduction in the levels of peroxisome-associated PEX5 argues strongly against the hypothesis that PEX12 participates in receptor docking. In fact, our data indicate that the loss of PEX12 may actually increase the levels of peroxisome-associated PEX5. This observation, together with the fact that receptor docking is the first peroxisome-localized step of peroxisomal matrix protein import, demonstrates that PEX12 acts downstream of the docking event. Thus, PEX12 appears to be the first known PEX5-binding protein that is not required for docking the PTS1 receptor to the peroxisome.

Of the two remaining aspects of peroxisomal matrix protein import, protein translocation and receptor recycling, there is no report of a bona fide protein translocation factor, but there is one report that proposes a role for PEX4 in receptor recycling (van der Klei et al., 1998). This conclusion was based in part on the phenotypes of pex4 mutants, which display a very mild defect in peroxisomal matrix protein import and can be suppressed by overexpression of PEX5. However, we observed that cells lacking human PEX12 display a severe defect in peroxisomal matrix protein import that cannot be suppressed by overexpression of PEX5. Thus, PEX12 does not have the properties we might expect of a factor that is required for receptor recycling.

The remaining aspect of peroxisomal matrix protein import to consider is the protein translocation process. A crucial role for PEX12 in peroxisomal matrix protein translocation would fit well with the known properties of this protein. First, PEX12 has the appropriate physical characteristics for such a role: it is an integral peroxisomal membrane protein that spans the membrane twice and extends its NH₂ and COOH termini toward the cytoplasm where they may interact with other protein import factors (Kalish et al., 1996; Chang et al., 1997; Okumoto and Fujiki, 1997). Second, it utilizes its COOH-terminal zinc-binding domain to interact with PEX5, the PTS1 receptor. Third, cells with inactivating mutations in PEX12 are unable to import peroxisomal matrix proteins but do synthesize peroxisomes and import integral peroxisomal membrane proteins (Kalish et al., 1996; Chang et al., 1997, 1999; Chang and Gould, 1998). Fourth, PEX12 interacts with PEX10, another integral peroxisomal membrane protein that displays a specific defect in the import of peroxisomal matrix proteins (Kalish et al., 1995; Warren et al., 1998) and yet does not appear to participate in receptor docking or recycling. Fifth, a missense mutation in PEX12, S320F, appears to affect the specificity of the translocation apparatus, resulting in the import of PEX5 into the peroxisome lumen.

Although there is no established in vitro protein translocation assay that can be used to test this hypothesis directly, it is useful to consider possible roles for PEX12 in the translocation process. The main function of a matrix protein translocon would be to move matrix proteins from the cytoplasmic side of the peroxisome membrane to the lumenal side. Given that most of these proteins arrive at the peroxisomes in a complex with PEX5 and that studies of Yarrowia lipolytica PEX5 strongly suggest that PEX5 participates in the matrix protein translocation process (Szilard et al., 1995), we favor a model for matrix protein translocation that includes PEX5. Such a model may involve: (1) acceptance of PEX5-ligand complexes from PEX14, the primary PEX5 docking site; (2) retention of PEX5 at the translocation apparatus; (3) opening of the matrix protein translocation pathway; (4) PEX5-ligand dissociation and ligand translocation; (5) closure of the translocation pathway; and (6) release of the unoccupied receptor from the translocation apparatus. The ability of PEX12 to bind PEX5 suggests that PEX12 may contribute to retaining PEX5 at the translocation apparatus. Some additional support for this hypothesis comes from the fact that PEX5 appears to be imported into the peroxisome as a result of the PEX12/S320F mutation, which reduces the interaction between PEX12 and PEX5.

This model predicts that PEX5 enters the peroxisomal compartment during the normal course of peroxisomal matrix protein import. However, it also predicts that PEX5 should normally be retained at the translocation apparatus rather than being released to move freely through the peroxisome lumen. A low rate of PEX5 release from the translocation apparatus into the lumen could explain the detection of intraperoxisomal PEX5 in Hansenula polymorpha (van der Klei et al., 1995). Y. lipolytica PEX5, which is detected only on or in the peroxisome, could also function within such a model, provided that it participates in just the peroxisome-limited steps of matrix protein import (Szilard et al., 1995).

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