Pex13p Is an SH3 Protein of the Peroxisome Membrane and a Docking Factor for the Predominantly Cytoplasmic PTS1 Receptor

Stephen J. Gould,*‡§ Jennifer E. Kalish,* James C. Morrell,* Jonas Bjorkman,‖ Aaron J. Urquhart,‖ and Denis I. Crane‖

The Departments of *Biological Chemistry and ‡Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, §The Kennedy Krieger Institute, Baltimore, Maryland 21205; and ‖The School of Biomolecular and Biomedical Science, Griffith University, Brisbane, QLD 4111, Australia

Abstract. Import of newly synthesized PTS1 proteins into the peroxisome requires the PTS1 receptor (Pex5p), a predominantly cytoplasmic protein that cycles between the cytoplasm and peroxisome. We have identified Pex13p, a novel integral peroxisomal membrane from both yeast and humans that binds the PTS1 receptor via a cytoplasmically oriented SH3 domain. Although only a small amount of Pex5p is bound to peroxisomes at steady state (<5%), loss of Pex13p further reduces the amount of peroxisome-associated Pex5p by ~40-fold. Furthermore, loss of Pex13p eliminates import of peroxisomal matrix proteins that contain either the type-1 or type-2 peroxisomal targeting signal but does not affect targeting and insertion of integral peroxisomal membrane proteins. We conclude that Pex13p functions as a docking factor for the predominantly cytoplasmic PTS1 receptor.

COMPARTMENTALIZATION of proteins within subcellular organelles is a hallmark of eukaryotic cells. Accordingly, eukaryotic cells have developed mechanisms for recognizing newly synthesized organellar proteins and directing them to their proper destination. In the case of protein import into peroxisomes, newly synthesized peroxisomal matrix proteins are distinguished from other cytoplasmic proteins by the presence of a peroxisomal targeting signal (PTS)[1] within their structure. The PTS1 consists of a COOH-terminal tripeptide of the sequence serine-lysine-leucine-$_{COOH}$, or a conservative variant, and is used by almost all proteins destined for the peroxisome lumen (Gould et al., 1989; Subramani, 1993). A second signal, PTS2, also directs proteins to the peroxisome lumen but differs from PTS1 because it is found at the NH_2 terminus and is used much less commonly (Subramani, 1993; Swinkels et al., 1991). Integral peroxisomal membrane proteins use neither PTS1 nor PTS2, but rather a distinct type of signal (McCammon et al., 1994; Dyer et al., 1996). Interestingly, proteins devoid of PTS1 and PTS2 can still be imported into the peroxisome lumen, provided that they oligomerize with a PTS1 or PTS2 protein before import (Glover et al., 1994; McNew and Goodman, 1994). The hypothesis that these oligomers may be translocated intact across the peroxisome membrane is supported by the observation that PTS1-coated gold particles can be imported into peroxisomes in vivo (Walton et al., 1995).

Import of PTS1 and PTS2 proteins requires a host of peroxisome assembly factors, or peroxins (Distel et al., 1996), that include specific PTS receptors (Dodt et al., 1995; McCollum et al., 1993; Marzioch et al., 1994), two ATPases (Erdmann et al., 1991; Spong and Subramani, 1993; Yahraus et al., 1996), a ubiquitin-conjugating enzyme (Crane et al., 1994; Wiebel and Kunau, 1992), three zinc-binding integral peroxisomal membrane proteins (IPMPs) (Kalish et al., 1995, 1996; Kunau et al., 1993; Tsukamoto et al., 1991; Gould, S.J., unpublished observations), and several other novel proteins (Kunau et al., 1993; Elgersma et al., 1993). Identification of these factors has depended upon the isolation of yeast mutants deficient in import of peroxisomal proteins (Subramani, 1993). Significant contributions to understanding peroxisome assembly and peroxisomal protein import have also been obtained from analysis of the peroxisome biogenesis disorders (PBD).

Please address all correspondence to either S.J. Gould, The Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205. Tel.: (410) 955-3085. Fax: (410) 955-0215. E-Mail: Stephen.Gould@qmail.bs.jhu.edu or D.I. Crane, The School of Biomolecular and Biomedical Science, Griffith University, Brisbane, QLD 4111, Australia. Tel.: 61 7 875 7253. Fax: 61 7 875 7656. E-Mail: D.Crane@sct.gu.edu.au

1. *Abbreviations used in this paper*: dbEST, database of expressed sequence tags; GFP, green fluorescent protein; IPMP, integral peroxisomal membrane protein; MBP, maltose-binding protein; ORF, open reading frame; SH3, src homology-3; PTS, peroxisomal targeting signal; TPR, tetratricopeptide repeat.

These genetically heterogeneous, lethal diseases are caused by a defect in import of at least one class of peroxisomal matrix proteins (Lazarow and Moser, 1995). Although ten distinct complementation groups (CGs) have been defined by cell fusion complementation analysis (Shimozawa et al., 1993), the genes defective in only three of these groups have been defined: *HsPEX2* (*PAF1*) in CG10 (Shimozawa et al., 1992), *HsPEX5* (*PXR1*) in CG2 (Dodt et al., 1995), and *HsPEX6* (*PXAAA1*) in CG4 (Yahraus et al., 1996).

While the role of most of these peroxisome assembly factors remains to be determined, there has been considerable research on the PTS1 receptor, encoded by *HsPEX5* (*PXR1*) in humans (Dodt et al., 1995). The product of *HsPEX5* (HsPex5p) is a tetratricopeptide repeat (TPR) protein that binds PTS1-containing peptides via its TPR domains. Although a missense mutation in *HsPEX5* was found to generate a specific defect in the import of PTS1-containing proteins, a more severe mutation in *HsPEX5* abolished import of PTS1 and PTS2 proteins, indicating that the human PTS1 receptor is required for import of both PTS1 and PTS2 proteins (Dodt et al., 1995; Slawecki et al., 1995; Braverman, N., G. Dodt, S. Gould, and D. Valle, manuscript submitted for publication). Interestingly, we have found that HsPex5p is a predominantly cytoplasmic protein that can cycle between the cytoplasm and peroxisome (Dodt et al., 1995; Dodt and Gould, 1996). At steady state, <3% of the protein is peroxisome-associated.

The PTS1 receptor was first identified in yeast as the product of the *P. pastoris PEX5* (*PAS8*) gene (McCollum et al., 1993). Like the human PTS1 receptor, PpPex5p is required for import of PTS1-containing proteins and binds PTS1 peptides via its TPR domains (Terlecky et al., 1995). However, PpPex5p is dispensable for import of PTS2-containing proteins and has been reported to be an integral peroxisomal membrane protein (McCollum et al., 1993; Terlecky et al., 1995). Thus, while the PTS1 receptor is required for import of PTS1 proteins in all species studied, the human and *P. pastoris* forms of this molecule appear to differ in (*1*) their role in import of PTS2 proteins, and (*2*) their subcellular distribution.

In this report, we describe a novel, conserved peroxisome assembly factor encoded by the *P. pastoris* and human *PEX13* genes. PpPex13p and HsPex13p are peroxisomal membrane proteins with a carboxy-terminal SH3 domain that extends into the cytoplasm. This domain is essential for activity of PpPex13p but is not involved in targeting PpPex13p or HsPex13p to the peroxisome membrane. We demonstrate that the SH3 domains of PpPex13p and HsPex13p bind the PTS1 receptors from *P. pastoris* and humans, respectively. We also find that the PTS1 receptor in *P. pastoris* is not a peroxisomal membrane protein, as previously reported, but is a predominantly cytoplasmic protein. Consistent with the hypothesis that Pex13p is a docking factor for the soluble PTS1 receptor, we find that loss of PpPex13p results in more than a 40-fold reduction in the amount of PpPex5p that associates with the peroxisome membrane. In addition, cells lacking Pex13p fail to import peroxisomal matrix proteins, but do not display any defect in the synthesis of peroxisomal membranes or targeting of integral peroxisomal membrane proteins.

Materials and Methods

Cloning and Sequencing the P. pastoris and Human PEX13 Genes

The *P. pastoris PEX13* gene was cloned by functional complementation of the methanol growth defects of a *pex13-1*, *his4Δ* strain (Gould et al., 1992). DNA was extracted from rescued strains, genomic DNA inserts were mapped, and subclones were inserted into *HIS4*-based replicating plasmids (Crane and Gould, 1994). *PEX13* activity of each subcloned DNA fragment was assessed by functional complementation assays. The smallest complementing clone was sequenced in its entirety on both DNA strands (GenBank accession No. U70067) using a modified version of the chain termination method (Sanger et al., 1977). BLAST (Altschul et al., 1990) searches of the existing genetic databases led to the identification of the *S. cerevisiae* YLR191W ORF (GenBank accession No. S51436) encoding ScPex13p, and the *C. elegans* F32A5.6 ORF (GenBank accession No. U20864) encoding CePex13p. The deduced amino acid sequence of CePex13p was then used to screen the database of expressed sequence tags (dbEST), yielding a single human EST (GenBank accession No. R10031). The cDNA corresponding to this EST was obtained from Lawrence Livermore National Laboratories (Livermore, CA) and used to probe a human fetal brain cDNA library. A full-length cDNA for *HsPEX13* was isolated and the sequence of its ORF (GenBank accession No. U71374) was determined on both strands.

Subcellular Fractionations, Protease Protection Experiments, and Immunoblots

All biochemical fractionations were performed as described in Crane et al. (1994) and Kalish et al. (1995) with the exception that NaF was added to a final concentration of 0.21 mg/ml in all solutions except growth media and solutions used for generating spheroplasts. Strains were grown in glucose medium to mid-log phase, transferred to oleate medium, and incubated for 12–16 h. Cells were harvested, converted to spheroplasts in isotonic buffer, resuspended in Dounce buffer (5 mM MES, pH 6.0, 1 M sorbitol, 1 mM KCl, 0.5 mM Na_2EDTA, 0.21 mg/ml NaF) and homogenized using a Dounce tissue grinder. Postnuclear supernatants were prepared by two successive 10-min spins at 1,500 *g*. Organelle pellet and supernatant fractions were generated by centrifugation of the postnuclear supernatants for 30 min at 25,000 *g*. The Nycodenz gradient was formed and used to separate organelles from a postnuclear supernatant as described (Erdmann and Blobel, 1995). Hypotonic lysis, 1 M NaCl extraction, and 100 mM Na_2CO_3, pH 11.5 extractions of purified peroxisomes were performed as described (Erdmann and Blobel, 1995), with the protein concentration <1 mg/ml. Membranes were collected by 30-min spins at 200,000 *g*. Protease protection experiments were performed on 25,000 *g* organelle pellets from the appropriate strain as described (Crane et al., 1994). Whole cell lysates of yeast protein were prepared by NaOH lysis of intact cells (Crane et al., 1994).

For immunoblotting, samples were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA), and processed as described (Crane et al., 1994). Polyclonal rabbit anti-Pex13p antibodies were raised against a bacterially synthesized form of the protein and affinity-purified. Polyclonal rabbit anti-thiolase and anti-PpPex10p (PpPas7p) antibodies have been described elsewhere (Kalish et al., 1995). Anti-PpPex5p (PpPas8p) antibodies were generated against a bacterially synthesized maltose-binding protein (MBP)-Pex5p fusion and affinity-purified from membranes as described (Crane et al., 1994). Development of immunoblots was by chemiluminescence.

Strains, Plasmids, and Yeast Two-Hybrid Assays

pDC1 was the original complementing clone of the *P. pastoris PEX13* gene. All DNA manipulations were performed using standard protocols (Sambrook et al., 1989). For deletion of the *PEX13* gene from the *P. pastoris* genome, the *P. pastoris LEU2* gene was cloned between the EcoNI sites flanking either side of the *P. pastoris PEX13* ORF in pDC1, creating pDC10. A linear fragment of DNA from pDC10 containing the *LEU2* gene flanked by 5′ and 3′ untranslated regions from the PpPEX13 locus was introduced into the *P. pastoris leu2Δ* mutant by electroporation (Crane et al., 1994). LEU+ colonies were selected and complete deletion of the *PpPEX13* gene was confirmed by Southern blot. The *pex13Δ*, *his4Δ* strain was generated by crossing the *pex13Δ* strain with a *his4Δ* strain, se-

lecting for diploids, and screening their meiotic products for strains with the appropriate phenotype (Gould et al., 1992).

For mutational analysis of *PpPEX13*, a 1.2-kb segment of DNA upstream of the *PEX13* ORF was cloned into the *P. pastoris HIS4*-based replicating vector pSG927, creating pDC100, the base plasmid for expression of wild-type and mutant *PEX13* genes. The wild-type and mutant *PEX13* genes used for functional studies in yeast were synthesized by PCR using pairs of oligonucleoties designed to amplify only the desired ORF. Each fragment was then cloned downstream of the *PEX13* promoter in pDC100 and the sequence of each was confirmed. These replicating plasmids were introduced into the *pex13Δ, his4Δ* strain by electroporation, HIS+ colonies were selected, and then assayed for complementing activity by testing for growth on methanol as sole carbon source.

Plasmids used for expression of HsPex13p and PpPex13p in human cells were based on the plasmid pcDNA3myc (Yahraus et al., 1996) which contains the strong cytomegalovirus promoter upstream of a polylinker, followed by the myc epitope and a stop codon. The entire ORF of *HsPEX13* cDNA was amplified by PCR, as was a truncated *HsPEX13* ORF lacking the COOH-terminal 130 codons (H235myc). Each was cloned into pcDNA3myc in frame with the myc epitope. Thus, pcDNA3-HsPex13pmyc and pcDNA3-HsPex13p/H235myc encoded HsPex13 proteins with the myc epitope tag at their COOH terminus. The full-length *PpPEX13* ORF, as well as a truncated *PpPEX13* ORF lacking the SH3 domain (F281myc) were also synthesized by PCR and cloned into pcDNA3myc, creating pcDNA3-PpPex13pmyc and pcDNA3-PpPex13p/F281myc, respectively.

The *S. cerevisiae* two-hybrid reporter strain, BY3168 (*MATa, ade2-101, leu2-3,112, trp1-901, his3Δ-200, lys2::pGAL1HIS3, pGAL1LacZ, SPAL10-URA3, gal4Δ, gal80Δ*), was obtained from J. Boeke (The Johns Hopkins University School of Medicine, Baltimore, MD). The two-hybrid vectors pBD and pAD are modified versions of the two-hybrid vectors pAS2 (*TRP1*) and pGAD424 (*LEU2*), respectively (Clontech, Palo Alto, CA). DNA fragments encoding the SH3 domain of *PpPEX13* and *HsPEX13* were amplified by PCR and cloned in frame at the 3′ end of the GAL4 DNA-binding domain ORF in pBD1. *PpPEX5*, *HsPEX5L*, and *HsPEX5S* were cloned in frame with the activating domain of GAL4 in the fusion vector pAD, creating pAD-PpPex5p, pAD-HsPex5pL, and pAD-HsPex5pS. The plasmids were introduced into the two-hybrid reporter strain BY3168. The resultant derivatives of BY3168 were spotted on minimal medium lacking tryptophan, leucine, uracil, and histidine, and supplemented with 100 mM 3-aminotriazole. Plates were incubated at 30°C for several days, after which the ability of the different strains to grow was scored.

Transfections, Immunofluorescence, and Fluorescence Microscopy

The pcDNA3myc-based plasmids were introduced into the human fibroblast cell line 8333T as described (Dodt et al., 1995). 2 d after transfection, the cells were fixed, permeabilized, and processed for immunofluorescence microscopy (Slawecki et al., 1995). The standard protocol involved permeabilization with 1% Triton X-100 for 5 min, a treatment that permeabilizes all cellular membranes. For selective permeabilization of just the plasma membrane, the Triton X-100 incubation was replaced by a 5-min incubation in 25 μg/ml digitonin. The anti-myc mouse monoclonal antibody 1-9E10 (Evan et al., 1985) and fluorescent secondary antibodies were obtained from commercial sources and the anti-SKL antibodies have been described (Gould et al., 1990*b*).

Blot Overlay Assay

The SH3 domain–encoding segments of *PpPEX13* and *HsPEX13* were amplified by PCR and cloned into the vector pMALc2 (New England Biolabs, Beverly, MA), creating pMALc2-PpPex13p/SH3 and pMALc2-HsPex13p/SH3. pMBP was created by engineering a stop codon at the terminus of the MBP sequences of pMALc2. Strains carrying pMBP, pMALc2-PpPex13p/SH3, and pMALc2-HsPex13p/SH3 were induced to express the MBP proteins as described by the manufacturer. Induced cells were lysed and crude soluble fractions were prepared from each strain (Sambrook et al., 1989). MBP and the MBP fusion proteins were purified by one-step affinity chromatography on amylose resin as described by the manufacturer (New England Biolabs). Protein samples were separated by SDS-PAGE and either stained for protein using Coomassie blue or transferred to Immobilon-P membranes. After transfer, the membranes were washed two times in TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl), and excess binding sites were blocked by incubation with 10% nonfat dry milk in TBS for 2 h. This procedure also removes SDS from proteins on the membrane and returns them to physiological pH and salt conditions, presumably allowing some protein molecules to attain their proper conformations. Biotinylated PpPex5p was synthesized by in vitro transcription and translation in a rabbit reticulocyte lysate supplemented with a biotinylated lysyl-tRNA (Promega, Madison, WI). Each preparation of biotinylated PpPex5p was tested to ensure that PpPex5p was the sole biotinylated protein in the lysate. After blocking, the membrane was incubated overnight in 10 ml 1% nonfat dry milk in TBST/0.1 (TBS containing 0.1% Tween 20) supplemented with the biotinylated PpPex5p translation product. The membrane was washed five times with TBST/0.5 (TBS containing 0.5% Tween 20) and placed in 10 ml TBST supplemented with streptavidin-alkaline phosphatase. After a 1-h incubation at 25°C, the membrane was washed three times with TBST/0.5, two times with TBS, then incubated with Western Blue Substrate (Promega) to visualize PpPex5p that had bound to the membrane.

Results

Identification of a Conserved SH3 Protein with a Role in Peroxisome Assembly

In *P. pastoris*, utilization of methanol and fatty acids requires distinct sets of peroxisomal enzymes. The *P. pastoris pex13-1* (*pas6-1*) strain exhibits specific defects in growth on these carbon sources and mislocalizes the peroxisomal enzyme catalase to the cytoplasm (Gould et al., 1992). Because these phenotypes suggest that the product of the *PpPEX13* gene may be involved in protein import into peroxisomes, we cloned this gene by functional complementation of *pex13-1* cells. Multiple complementing clones were identified, all of which contained an 1,140-bp-long open reading frame (ORF). Targeted deletion of this ORF from the *P. pastoris* genome generated a strain with the typical *P. pastoris pex* mutant phenotype: inability to grow on either fatty acids or methanol. Additional genetic analysis revealed that *pex13-1* was allelic to this deletion mutant (*pex13Δ*), demonstrating that we had cloned the *PpPEX13* gene and not an extragenic suppressor.

If *PEX13* was truly required for protein import into peroxisomes, the ubiquitous presence of peroxisomes in eukaryotic organisms predicts that orthologs of *PEX13* should exist in other species. By screening sequence databases for gene products similar to PpPex13p, we identified a closely related gene product from *S. cerevisiae*, ScPex13p (Fig. 1). Targeted disruption of the corresponding gene generated an *S. cerevisiae* strain with the *pex* phenotype, an inability to use fatty acids as sole carbon source and defective import of peroxisomal matrix proteins (data not shown). We also identified an ORF from the worm *Caenorhabditis elegans* with the potential to encode a protein (CePex13p) similar to *P. pastoris* Pex13p (Fig. 1), but screening the database of expressed sequence tags (dbEST) with the PpPex13p sequence failed to identify any *PEX13* orthologs from humans. However, screening dbEST with the CePex13p sequence did lead to the identification of a human cDNA with the potential to encode a similar protein. A full-length cDNA for this human *PEX13* gene was isolated and sequenced. The deduced amino acid sequence of its product, HsPex13p, exhibited a high degree of sequence similarity to CePex13p, as well as to the yeast proteins PpPex13p and ScPex13p (Fig. 1). While the amino acid sequence similarity between these proteins was significant, the similarity in their overall organization was even

Figure 1. Amino acid alignment of *P. pastoris*, *S. cerevisiae*, *C. elegans*, and human *PEX13* proteins. Conserved residues that are present in at least two of the four proteins are blocked. The putative transmembrane domain is denoted by a dotted overline and the SH3 domain is marked with the solid overline.

more striking. In particular, all four proteins contained: (*1*) a carboxy-terminal src homology-3 (SH3) domain; (*2*) a hydrophobic membrane-spanning domain located just amino-terminal to the SH3 domain; and (*3*) an amino-terminal glycine-rich region. Furthermore, the hydropathy profiles of each protein were quite similar (data not shown).

Yeast and Human Pex13p Reside in the Peroxisome Membrane with their SH3 Domains Exposed to the Cytoplasm

We generated affinity-purified antibodies to a carboxy-terminal fragment of PpPex13p that included the SH3 domain. Immunoblotting experiments and mutational analysis revealed that *PpPEX13* encodes two polypeptides, Pex13pL and Pex13pS, which are generated by translation initiation at Met1 and Met33 of the *PEX13* ORF, respectively (data not shown). To localize these proteins within the cell, a postnuclear supernatant was prepared from a yeast spheroplast homogenate. This supernatant was then subjected to centrifugation at 25,000 *g* for 30 min, generating an organellar pellet containing peroxisomes and mitochondria, and a supernatant comprised of cytosol and microsomes. These fractions will be referred to as the 25,000-*g* pellet and supernatant throughout the paper. Both Pex13 proteins were found exclusively in the organelle pellet (data not shown) and sucrose density centrifugation of the pellet revealed that Pex13pL and Pex13pS were peroxisomal proteins (Fig. 2 *A*). In addition, Pex13pL and Pex13pS remained predominantly membrane-associated after hypotonic lysis, salt extraction, and sodium carbonate extraction of purified peroxisomes (Fig. 2 *B*), indicating that both proteins were integral components of the peroxisome membrane. Residual release of integral peroxisomal membrane proteins during sodium carbonate extraction of purified peroxisomes has been noted by other researchers (Erdmann and Blobel, 1995). To determine the orientation of Pex13pL and Pex13pS in the peroxisome membrane, we performed a protease protection assay. Organelles were isolated from wild-type cells, incubated with varying amounts of trypsin in the presence or absence of detergent, and then assayed for size and abundance of Pex13p by immunoblot (Fig. 2 *C*). The anti-Pex13p antibodies were directed against the carboxy-terminal portion of the *PEX13* product. Therefore, if Pex13pL and Pex13pS were oriented with their COOH-terminal SH3 domain outside the peroxisome, the antigenic region of the protein

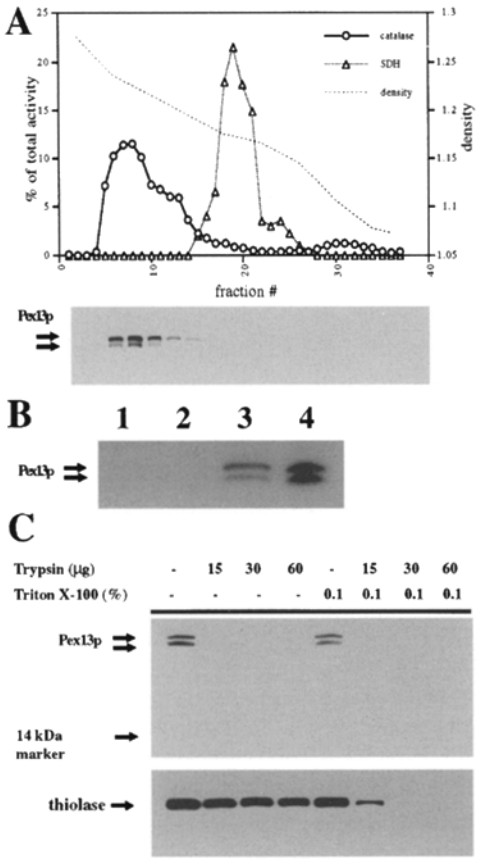

Figure 2. PpPex13p is an integral peroxisomal membrane protein with its COOH-terminal SH3 domain exposed to the cytoplasm. (*A*) A 25,000-*g* organelle pellet was separated by sucrose density centrifugation (Crane et al., 1994). Each fraction was assayed for catalase and succinate dehydrogenase (SDH) activity and even numbered fractions were assayed for density (by refractometry), as well as for PpPex13p by immunoblot using anti-PpPex13p antibodies. (*B*) Purified peroxisomes were extracted sequentially with 10 mM Tris-HCl, pH 8.5, 1 M NaCl and 100 mM Na_2CO_3, pH 11.5. Equivalent proportions of the supernatant from each extraction (lanes *1–3*, respectively) and the Na_2CO_3 extraction pellet (lane *4*) were separated by SDS-PAGE and assayed by immunoblot using anti-PpPex13p antibodies. (*C*) Protease protection analysis of PpPex13p. 200-μg protein equivalents of organelles (25,000 *g* pellet) were incubated with trypsin (~10,000 U per mg protein) in the absence or presence of 0.1% Triton X-100 for 25 min on ice. Reactions were stopped by addition of excess trypsin inhibitor. Equal proportions of each reaction were separated by SDS-PAGE and assayed by immunoblot using anti-PpPex13p antibodies.

would be digested by trypsin. However, if the Pex13p proteins were oriented with their SH3 domain within the peroxisome, an ~15-kD fragment of the protein would be detected. Both Pex13pL and Pex13pS were completely degraded by trypsin in the absence of detergent. In contrast, the matrix protein thiolase was degraded only when detergent was added.

To determine whether human HsPex13p exhibited the same subcellular distribution, we created a modified *HsPEX13* cDNA designed to append the myc epitope tag onto the carboxy terminus of HsPex13p. A plasmid designed to express HsPex13pmyc was transfected into human fibroblasts (8333T cells). At 2 d posttransfection the cells were fixed, incubated with Triton X-100 to permeabilize all cellular membranes, and processed for indirect immunofluorescence. HsPex13pmyc was detected exclusively in vesicular structures that had the typical appearance of peroxisomes (Fig. 3 *A*). The same cell population was also processed for indirect immunofluorescence after permeabilization with digitonin instead of Triton X-100. Under these conditions, only the plasma membrane is permeabilized and intra-peroxisomal antigens are inaccessible to exogenous antibodies (Swinkels et al., 1991). We detected vesicular HsPex13pmyc in digitonin-permeabilized cells at the same frequency as in cells permeabilized with Triton X-100 (Fig. 3 *B*), demonstrating that the COOH terminus of this protein extends into the cytoplasm. To determine whether the vesicular structures containing HsPex13pmyc were indeed peroxisomes, transfected cells were permeabilized with Triton X-100 and processed for double indirect immunofluorescence using both the anti-myc antibody and an anti-SKL antibody. The anti-SKL antibody recognizes multiple PTS1-containing proteins (Gould et al., 1990*b*). HsPex13pmyc (Fig. 3 *C*) and endogenous SKL-containing peroxisomal matrix proteins (Fig. 3 *D*) colocalized in these experiments, confirming that HsPex13pmyc was targeted to peroxisomes. We have also generated specific antibodies to the COOH terminus of

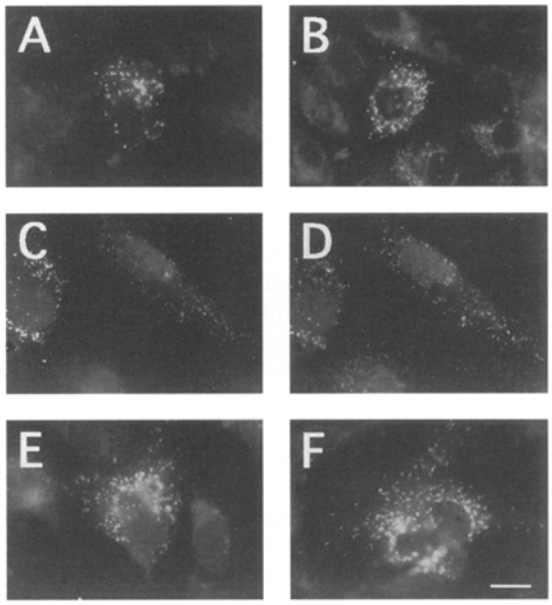

Figure 3. Peroxisomal targeting of HsPex13p and PpPex13p in human fibroblasts. The human fibroblast line 8333T was grown on glass coverslips and transfected with plasmids designed to express (*A–D*) HsPex13pmyc, (*E*) HsPex13p-H235myc, or (*F*) PpPex13p-F281myc. 2 d after transfection, the cells were fixed and permeabilized with either (*A*, *C–F*) 1% Triton X-100 or (*B*) 25 μg/ml digitonin for 5 min. The cells were then processed for (*A*, *B*, *E*, and *F*) single label indirect immunofluorescence using the anti-myc monoclonal antibody and a fluorescein-labeled goat anti–mouse secondary antibody, or (*C* and *D*) double label experiments using the anti-myc monoclonal antibody and a rabbit anti-SKL antibody, followed by fluorescein-labeled goat anti–mouse and rhodamine-labeled goat anti–rabbit secondary antibodies. Bar, 25 μm.

HsPex13p and confirmed that endogenous HsPex13p is exclusively peroxisomal and is oriented with its COOH-terminal SH3 domain exposed to the cytoplasm (data not shown).

SH3 domains are known to mediate protein–protein binding (Yu et al., 1994) and in some instances, these interactions are responsible for targeting an otherwise soluble protein to cellular membranes (Bar-Sagi et al., 1993). A mutant form of HsPex13p lacking its COOH-terminal 130 amino acids, but still containing a COOH-terminal myc tag (HsPex13p-H235myc), was efficiently targeted to peroxisomes (Fig. 3 *E*), demonstrating that the SH3 domain was not involved in targeting this protein to peroxisomes. Interestingly, a modified form of PpPex13p containing the c-*myc* epitope tag at its COOH terminus was also targeted to peroxisomes in human cells (data not shown), indicating that the mechanism of integral peroxisomal membrane protein targeting and insertion has been conserved between yeast and humans. The fact that a COOH-terminal truncation mutant lacking the SH3 domain was also targeted to peroxisomes (PpPex13p-F281myc; Fig. 3 *F*) further substantiates the hypothesis that the SH3 domain does not play a role in directing these proteins to peroxisomes. The peroxisomal distribution of these mutant proteins was confirmed by double indirect immunofluorescence experiments (data not shown).

The SH3 Domain Is Essential for PEX13 Function

The SH3 domain of yeast and human Pex13p is likely to be responsible for interaction with a specific partner protein. Furthermore, the importance of this interaction may be assessed by examining the effect of mutations which alter the SH3 domain of PpPex13p. Mutant forms of *PpPEX13* were created by site-directed mutagenesis, cloned downstream of the *PpPEX13* promoter in a *HIS4*-based *P. pastoris* replicating vector, and expressed in a *pex13*Δ, *his4*Δ strain of *P. pastoris*. *PEX13* activity was assessed by monitoring the growth of each strain on methanol (Table I). Deletion of the SH3 domain (Y286ter) abolished *PpPEX13* activity. Single substitution of conserved residues in the ALYDF motif of the SH3 domain (Y286A, D287A, and F288A) had no effect on complementing activity, consistent with the hypothesis that no single SH3 consensus residue is essential for establishing the basic architecture of the SH3 domain (Mussachio et al., 1994; Yu et al., 1994). In contrast, two substitution mutations in the RT loop of the SH3 domain, E291K and E296K, abolished activity. The RT loop of SH3 domains is immediately COOH-terminal to the ALYDF element and contributes to the specificity of ligand binding (Yu et al., 1994). There are two other acidic residues in this region of the protein, E293 and E294, but substitution at these positions did not affect PpPex13p activity (data not shown).

Table I. Complementing Activity of Wild-Type and Mutant Forms of PEX13

PpPEX13 gene	Complementing activity
WT	++
Y286ter	−
Y286A	++
D287A	++
F288A	++
E291K	−
E296K	−

Pex13p Binds Pex5p, the PTS1 Receptor

Two conclusions may be drawn from the observation that the SH3 domain is essential for PpPex13p activity: (*1*) peroxisome assembly requires the interaction between the SH3 domain of Pex13p and a partner protein; and (*2*) the putative partner protein must also be required for peroxisome assembly. Thus, the Pex13p ligand is likely to be the product of another *PEX* gene. We examined the capacity of different *P. pastoris PEX* proteins to interact with one another in the yeast two-hybrid system (Fields and Song, 1989).

We constructed derivatives of a two-hybrid reporter strain (BY3168) that express combinations of (*a*) fusion proteins between the GAL4 DNA-binding domain (BD) and the SH3 domains of PpPex13p and HsPex13p; and (*b*) fusion proteins containing the GAL4 transcriptional activation domain (AD) and the PTS1 receptors from *P. pastoris* (PpPex5p) and humans (HsPex5pS and HsPex5pL; there are two isoforms of the human PTS1 receptor [Dodt et al., 1995; Braverman, N., G. Dodt, S. Gould, and D. Valle, manuscript submitted for publication]). Cells expressing both BD-PpPex13p (SH3 domain only) and AD-PpPex5p fusion proteins grew on the two-hybrid indicator plates, as did cells which coexpressed the BD-HsPex13p fusion protein (SH3 domain only) with either AD-HsPex5pS or AD-HsPex5pL fusion proteins (Table II). Control strains coexpressing just the DNA-binding domain or transcriptional activation domain of Gal4p did not grow on these plates. A strain coexpressing the BD-PpPex13p (human) and AD-HsPex5p (yeast) fusion proteins also failed to proliferate on the indicator plates, indicating that the interaction between BD-PpPex13p and AD-PpPex5p was not due to a nonspecific affinity of the PTS1 receptor for SH3 domains.

These data suggested that the PTS1 receptor may bind the SH3 domain of Pex13p. To test this hypothesis, we examined the ability of biotinylated PpPex5p to bind the PpPex13p SH3 domain in a blot overlay assay. The SH3 domains of PpPex13p and HsPex13p were expressed in *E. coli* as maltose-binding protein (MBP) fusion proteins and purified by single step affinity chromatography on an amylose resin. Purified MBP (40 kD), MBP-PpPex13p/SH3 (55 kD), and MBP-HsPex13p/SH3 (53 kD) were separated by SDS-PAGE and either stained for protein (Fig. 4 *A*) or transferred to Immobilon-P membranes. After transfer, the membranes were twice washed with Tris-buffered sa-

Table II. Growth of BY3168 Derivatives Containing Various Combinations of GAL4 Two-Hybrid Expression Plasmids

	pBD	pBD-PpPex13p	pBD-HsPe13p
pAD	−	−	−
pAD-PpPex5p	−	++	−
pAD-HsPex5pS	−	n.d.	++
pAD-HsPex5pL	−	n.d.	++

++, denotes growth; −, denotes inability to grow.

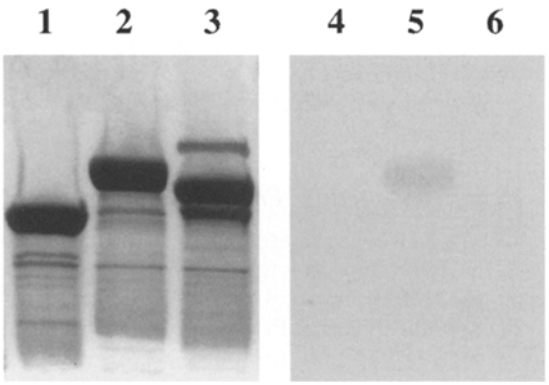

Figure 4. PpPex5p binds the SH3 domain of PpPex13p. (*A*) Coomasie-stained polyacrylamide gel of purified MBP (lane *1*), MBP-PpPex13p/SH3 (lane *2*), and MBP-HsPex13p/SH3 (lane *3*). (*B*) Purified MBP (lane *4*), MBP-PpPex13p/SH3 (lane *5*), and MBP-HsPex13p/SH3 (lane *6*) were separated by SDS-PAGE, transferred to Immobilon-P membranes, and probed with biotinylated PpPex5p.

line at physiological pH (TBS), which removed the denaturant (SDS). After a 2-h incubation in 10% nonfat dry milk/TBS, the membranes were probed with biotinylated PpPex5p (Fig. 4 *B*). PpPex5p was detected using a streptavidin-alkaline phosphatase conjugate and an alkaline phosphatase–specific colorimetric assay. In the experiment shown here, PpPex5p was detected only over MBP-PpPex13p/SH3 (Fig. 4 *B*, lane *2*). However, when equivalent membranes were extensively overdeveloped (resulting in high background) a small amount of PpPex5p was detected over MBP-HsPex13p/SH3 but not over MBP (data not shown).

The relatively weak signal detected in this experiment could be due to a variety of technical problems, including the lack of a specific renaturation step for the SH3 proteins after transfer to the membrane, low specific labeling of PpPex5p with biotin, or inappropriate folding of PpPex5p in the rabbit in vitro translation reaction. However, it is also possible that a low affinity of Pex5p for Pex13p may reflect the transient nature expected for interaction between the PTS1 receptor and its docking factor.

The P. pastoris PTS1 Receptor Is a Predominantly Cytoplasmic Protein, Not an Integral Peroxisomal Membrane Protein

While the above data suggest that the PTS1 receptor is a ligand for the SH3 domain of Pex13p, the conflicting reports on the subcellular distribution of the PTS1 receptor in humans as a predominantly cytoplasmic protein (Dodt et al., 1995), and in *P. pastoris*, as an exclusively peroxisomal membrane protein (McCollum et al., 1993; Terlecky et al., 1995), made it difficult to deduce the functional significance of this interaction. To investigate this discrepancy, we cloned the *P. pastoris PEX5* gene (by functional complementation of the *pex5-1* [*pas8-1*] mutant; Gould et al., 1992), sequenced the entire gene, generated affinity-purified anti-PpPex5p antibodies to a bacterially synthesized form of PpPex5p, and examined the distribution of the *P. pastoris* PTS1 receptor.

Wild-type *P. pastoris* cells grown in oleic acid medium were homogenized and separated by centrifugation at 25,000 *g* into an organelle pellet containing peroxisomes and mitochondria and a supernatant that contains cytosol and microsomes. Equal proportions of these fractions were separated by SDS-PAGE and immunoblotted with antibodies specific for PpPex5p, as well as for three peroxisomal marker proteins: PpPex12p (Pas10p), an integral peroxisomal membrane protein (Kalish et al., 1996); PpPex4p (Pas4p), an outer peripheral peroxisomal membrane protein (Crane et al., 1994); and thiolase, a soluble peroxisomal matrix protein. Quantitation of the immunoblot revealed that ~95% of PpPex5p exists in the cytoplasm at steady state (Fig. 5 *A*). In contrast, Pex12p was detected exclusively in the 25,000-*g* pellet, a distribution that is always observed for integral peroxisomal membrane proteins (Kalish et al., 1995). Only small amounts of

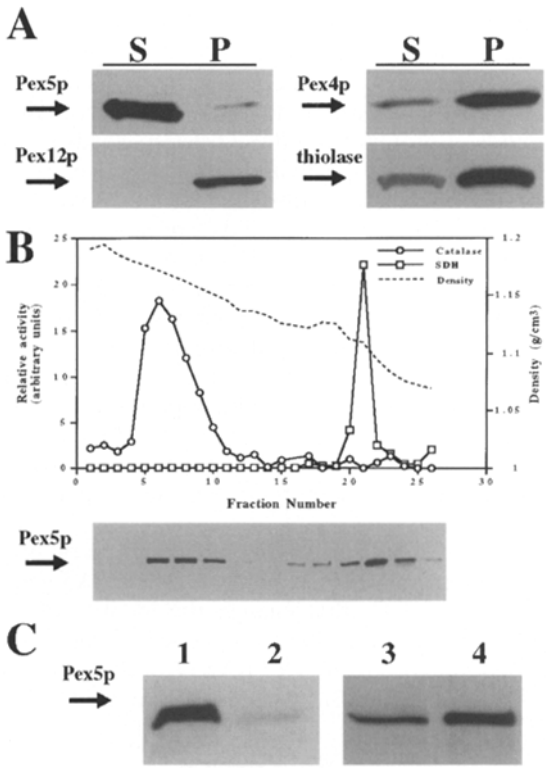

Figure 5. PpPex5p, a predominantly cytoplasmic protein, requires PpPex13p for association with the peroxisome membrane. (*A*) Equal proportions of 25,000 *g* supernatant and pellet fractions from wild-type cells were assayed for levels of PpPex5p, PpPex12p, PpPex4p, and thiolase by immunoblot. (*B*) A 25,000-*g* organelle pellet from WT cells was fractionated by Nycodenz gradient centrifugation. Each fraction was assayed for catalase, SDH, and density. Odd numbered fractions were assayed for PpPex5p. Of the PpPex5p in the 25,000-*g* pellet, only 1/2 was peroxisome-associated. (*C*) 25,000-*g* pellets from both wild-type and *pex13*Δ cells were resuspended in 10 mM Tris-HCl, pH 8.5, vigorously homogenized, incubated on ice for 30 min, and rehomogenized. Membranes were prepared from these lysates by centrifugation at 200,000 *g* for 30 min. Equal amounts of membrane proteins from wild-type (lane *1*) and *pex13*Δ cells (lane *2*) were resolved by SDS-PAGE and assayed by immunoblot using anti-PpPex5p antibodies. Equal amounts of total cellular protein from wild-type (lane *3*) and *pex13*Δ cells (lane *4*) were separated by SDS-PAGE and assayed for PpPex5p by immunoblot.

the peripheral membrane protein Pex4p and the matrix protein thiolase were released to the supernatant, demonstrating that the cytoplasmic distribution of Pex5p was not an artifact of harsh homogenization of the organelle fraction. Furthermore, of the Pex5p that did associate with the organelle pellet, only half was associated with peroxisomes (Fig. 5 *B*), indicating that peroxisome-associated Pex5p represents <3% of the total Pex5p in the cell. Thus, the PTS1 receptor has the same relative distribution in both *P. pastoris* and human cells: predominantly cytoplasmic with only small amounts (<3%) peroxisome-associated.

How can we reconcile our results with the reports by McCollum et al. (1993) and Terlecky et al. (1995), which concluded that PpPex5p was an exclusively peroxisomal protein and an integral component of the membrane? We have noted two significant differences between our experimental design and that reported in the earlier papers. First, while we assayed equal proportions of the 25,000-*g* supernatant and pellet fractions for levels of PpPex5p, the earlier reports assayed equal amounts of protein from these fractions. Because the supernatant fraction contains at least 50-fold more protein than the pellet, assaying equal amounts of protein from these fractions leads to an overestimation of the proportion of Pex5p that is peroxisome-associated. Second, we observed that the cytoplasmic form of the PTS1 receptor exhibits a pronounced protease sensitivity and that it could only be inhibited by addition of NaF to the lysis and fractionation buffers. To the best of our knowledge, this compound was not used in the previous studies on PpPex5p distribution. Proteolytic degradation has also been observed for the PTS1 receptor from humans (Dodt, G., and S. Gould, unpublished observations), *Saccharomyces cerevisiae* (van der Leij et al., 1993; Elgersma et al., 1996), and is apparent for the PTS1 receptor of *Hansenula polymorpha* (van der Klei et al., 1995).

During the course of these studies we identified two errors in the original published sequence of the *PEX5* gene (McCollum et al., 1993) that change the identity of 17 amino acids of the deduced Pex5p sequence. A corrected and expanded version of the *PpPEX5* (*PAS8*) sequence has been deposited in GenBank (accession No. U59222).

Levels of Peroxisome-associated PTS1 Receptor Are Severely Reduced in the pex13Δ Strain

The presence of most PTS1 receptor molecules in the cytoplasm at steady state suggests that the PTS1 receptor cycles between the cytoplasm and peroxisome and that it may require a docking factor for its association with the peroxisome membrane. Given that yeast and human Pex13p have the precise properties expected for a docking factor (both are membrane proteins that bind the PTS1 receptor via cytoplasmically oriented domains), we tested whether depletion of PpPex13p from cells reduced the amount of PpPex5p associated with the peroxisome membrane. 25,000-*g* pellet fractions were isolated from oleate-induced wild-type and *pex13Δ* cells and lysed by vigorous homogenization in hypotonic buffer (10 mM Tris HCl, pH 8.5). The membranes were recovered by centrifugation and equal amounts of each sample (by protein) were assayed for levels of Pex5p by immunoblot. Membrane-associated Pex5p was reduced by more than 40-fold in *pex13Δ* cells (Fig. 5 *C*, lanes *1* and *2*). This result does not reflect a general instability of Pex5p in the *pex13Δ* mutant since levels of Pex5p were somewhat elevated in *pex13Δ* cells (Fig. 5 *C*, lanes *3* and *4*).

Pex13p Is Required for Matrix Protein Import

The data presented above indicate that Pex13p may function to dock the PTS1 receptor to the peroxisome membrane during one step of matrix protein import. One prediction of this hypothesis is that Pex13p would be required for import of PTS1-containing proteins. To examine the effect of Pex13p depletion on peroxisomal protein import, postnuclear supernatants were prepared from oleate-induced wild-type and *pex13Δ* cells and resolved into supernatant and pellet fractions by centrifugation at 25,000-*g*. Equal proportions of these fractions were assayed for levels of catalase, a PTS1 protein (McCollum et al., 1993), thiolase, a PTS2 protein (McCollum et al., 1993), and PpPex10p (Pas7p), an integral protein of the peroxisome membrane (Kalish et al., 1995). Levels of catalase in the two fractions was determined by enzyme assay whereas abundance of thiolase and PpPex10p was determined by densitometry of immunoblots. While 55% of catalase and 68% of thiolase were found in the organelle pellet of wild-type cells, only 3% of catalase and 5% of thiolase were detected in the organelle pellet of *pex13Δ* cells (Fig. 6 *A*). Thus, Pex13p is required for import of PTS1 and PTS2 proteins. In contrast, Pex10p was exclusively associated with the organelle pellet in both wild-type and *pex13Δ* cells (Fig. 6 *A*). In an earlier report, we demonstrated that the IPMP Pex10p is located entirely within peroxisomes since it is resistant to exogenous protease in the absence of detergent but sensitive in its presence (Kalish et al., 1995). Organelles prepared from wild-type and *pex13Δ* cells were incubated with different amounts of trypsin in the absence or presence of detergent (Fig. 6 *B*). The protease sensitivity of Pex10p was identical in wild-type and *pex13Δ* cells, demonstrating that Pex10p had not only inserted into the peroxisome membrane, but had attained its proper orientation within the organelle.

The hypothesis that Pex13p is required for import of matrix proteins, was tested independently using peroxisomal forms of green fluorescent protein (GFP; Heim et al., 1995) that carry PTS1, PTS2, or IPMP targeting signals (PTS1-GFP, PTS2-GFP, and IPMP-GFP [Kalish et al., 1996]). These proteins were expressed in wild-type and *pex13Δ* cells and their subcellular distribution was determined by confocal fluorescence microscopy. We observed that both PTS1-GFP and PTS2-GFP were mislocalized to the cytoplasm in *pex13Δ* cells (Fig. 7, *A–D*), whereas the integral membrane protein, IPMP-GFP was targeted to vesicular structures in both wild-type and *pex13Δ* cells (Fig. 7, *E* and *F*).

Discussion

Transport of newly synthesized PTS1 proteins from the cytoplasm into the peroxisome lumen requires the PTS1 receptor. Studies in human cells (Dodt et al., 1995; Dodt and Gould, 1996) and now in the yeast *P. pastoris* have demon-

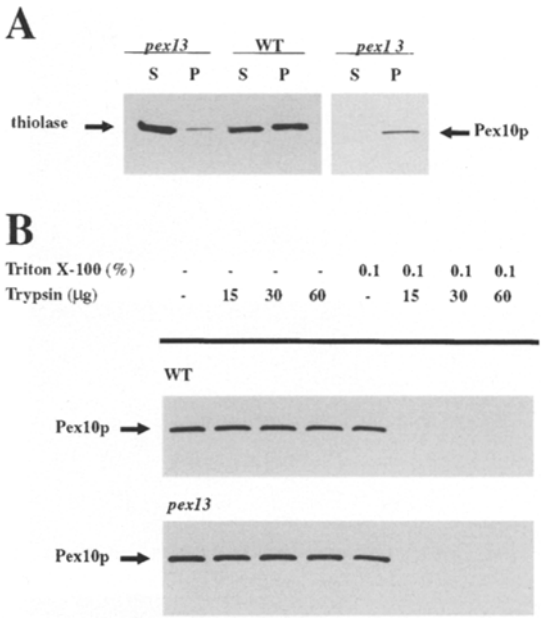

Figure 6. Biochemical analysis of the peroxisomal protein import defects of *pex13Δ* cells. (*A*) Equal proportions of 25,000 *g* supernatant (*S*) and pellet (*P*) fractions from *pex13Δ* cells and wild-type (*WT*) cells were assayed by immunoblot using affinity-purified anti-thiolase antibodies. Equal proportions of the supernatant and pellet fractions from *pex13Δ* cells were also assayed by immunoblot using affinity-purified anti-Pex10p antibodies (Kalish et al., 1995). (*B*) Protease protection analysis of Pex10p in wild-type and *pex13Δ* cells. 200-μg protein equivalents of organelles (25,000 *g* pellet) were incubated with trypsin (10,000 U/mg) for 25 min on ice in the absence or presence of 0.1% Triton X-100. Equal proportions of each reaction were assayed for levels of Pex10p by immunoblot.

strated that this receptor is a predominantly cytoplasmic protein and that only small amounts of it are peroxisome-associated at steady state. Furthermore, this distribution appears to reflect a dynamic, cycling receptor (Dodt, G., and S.J. Gould, 1996) rather than a static partitioning of distinct PTS1 receptor populations to these two compartments. Thus, the PTS1 receptor may function as a chaperone, binding newly synthesized PTS1 proteins in the cytoplasm and directing them to the peroxisome membrane. Inherent in this cycling receptor model is the requirement for a docking factor on the peroxisome membrane. Such a factor should (*1*) be an integral peroxisomal membrane protein, (*2*) be able to bind the PTS1 receptor via a cytoplasmically oriented domain, (*3*) require its receptor-binding domain for activity, (*4*) be required for association of the PTS1 receptor with the peroxisome membrane, and (*5*) be required for import of newly synthesized PTS1 proteins but not for targeting of integral peroxisomal membrane proteins. Pex13p satisfies all of these criteria.

Subcellular fractionation and protease protection studies revealed that PpPex13p is an integral peroxisomal membrane protein with its COOH-terminal SH3 domain exposed to the cytoplasm. Immunofluorescence microscopy experiments on human cells expressing HsPex13pmyc showed that this protein has an identical topology in the peroxisome membrane. Removal of the SH3 domain from human or yeast Pex13p failed to affect their subcellular distribution, demonstrating that the SH3 domain was not involved in targeting these proteins to the peroxisome membrane. Furthermore, the correct targeting of *P. pastoris* Pex13p to the peroxisome membrane of human cells suggests that the mechanism of IPMP targeting, like the machinery for matrix protein import (Gould et al., 1990*a*), has been conserved through evolution.

The second and third criteria for a PTS1 receptor docking factor are that it have the capacity to bind the PTS1 receptor via a cytoplasmic domain and require this domain for its activity. Both yeast and human Pex13p contain a cytoplasmically oriented SH3 domain that binds to their cognate PTS1 receptors. Furthermore, mutational analysis of PpPex13p revealed that the SH3 domain of PpPex13p is indeed essential for biological activity. There is a wealth of evidence demonstrating that SH3 domains are involved in protein binding (Musacchio et al., 1994), and that they do so by forming a binding pocket with affinity for "polyproline" helices. We have examined the sequences of the *P. pastoris* and human PTS1 receptors and were unable to find a segment that matched the putative consensus sequence of SH3 ligands (Yu et al., 1994). However, the structure recognized by SH3 domains is merely a left-handed helix (Yu et al., 1994) and more than 20% of known left-handed helices lack proline residues altogether (Adzhubei and Sternberg, 1993). Thus, the putative Pex13p binding site in Pex5p is not necessarily a proline-rich region. Identifying the region(s) of the PTS1 receptors that interact with Pex13p will be of particular interest in the near future.

The role of the PTS1 receptor in the cytoplasm is probably related to finding and binding newly synthesized PTS1 proteins and then directing these ligands from the cytoplasm to the peroxisome. However, once the receptor-ligand complex reaches the peroxisome surface, the role of the receptor may be much different, yet equally important. At the very least, the PTS1 receptor must dissociate from its ligands while transferring them to the translocation apparatus, and at the same time, ensure that its ligands are not released to the cytoplasm. Thus, the association of the PTS1 receptor with a docking factor in the peroxisome membrane is a crucial link between protein transport to the peroxisome and protein translocation into the peroxisome lumen. Loss of such a docking factor should reduce or eliminate the amount of PpPex5p associated with the peroxisome. We examined the levels of peroxisome-associated PpPex5p in wild-type and *pex13Δ* cells and found that loss of Pex13p reduced the levels of peroxisome-associated Pex5p by more than 40-fold. This decrease in peroxisome-associated Pex5p was not due to a general instability of Pex5p in the *pex13Δ* mutant since the abundance of Pex5p in these cells was slightly higher than in wild-type cells. Also, the lower levels of peroxisome-associated Pex5p in *pex13Δ* cells cannot be attributed to a general reduction in peroxisome membrane surface area in *pex* mutants: we have demonstrated elsewhere (Kalish et al., 1996) that levels of properly inserted integral peroxisomal membrane proteins are similar in *pex* mutants and wild-type cells. Thus, we may conclude that Pex13p is required for association of the PTS1 receptor with the peroxisome membrane.

The final criteria for a PTS1 receptor docking factor is that it be required for import of PTS1 proteins. This issue was addressed by two independent techniques: (*1*) classi-

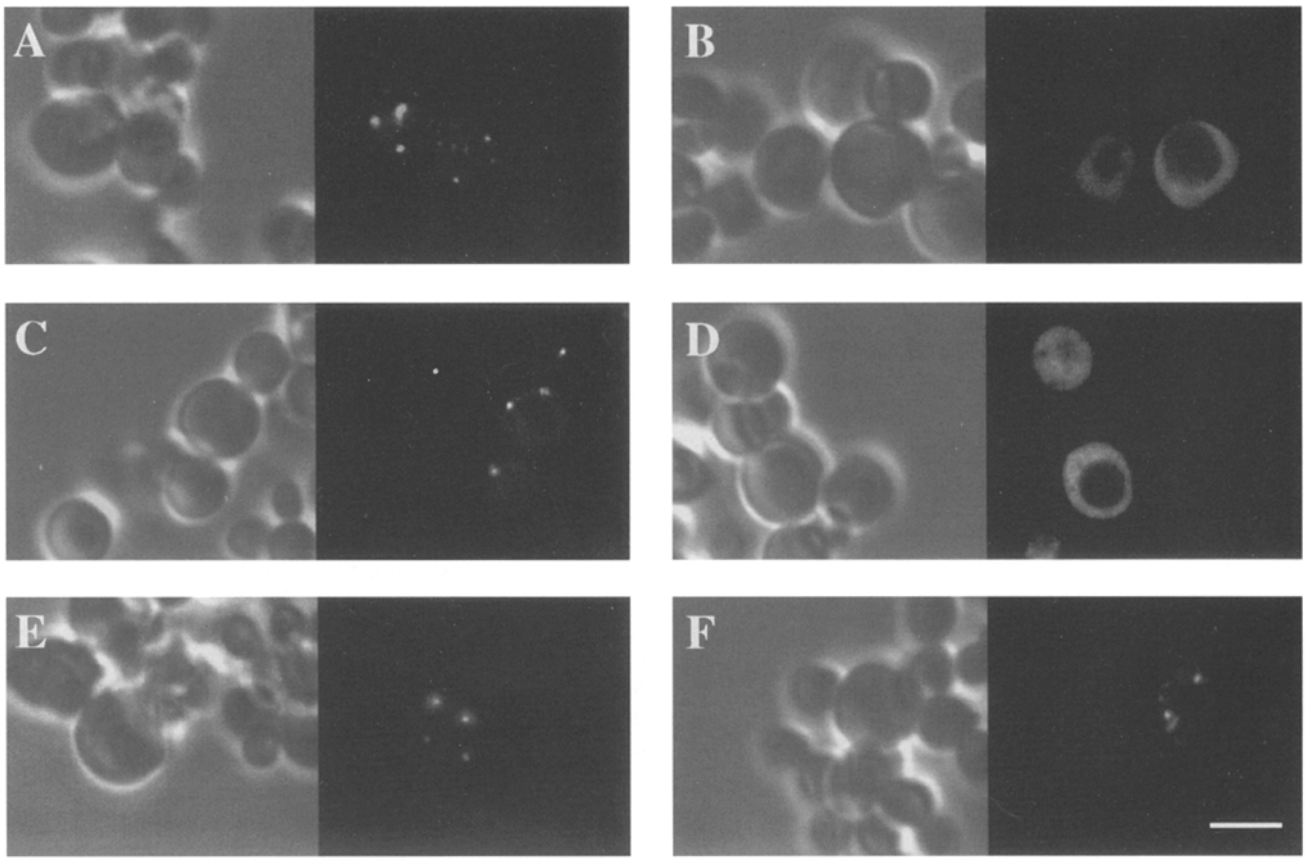

Figure 7. Pex13p is required for import of PTS1 and PTS2 proteins into peroxisomes. Wild-type (*A*, *C*, and *E*) and *pex13*Δ (*B*, *D*, and *F*) cells that express (*A* and *B*) PTS1-GFP, (*C* and *D*) PTS2-GFP, or (*E* and *F*) IPMP-GFP were grown in minimal medium containing glucose as sole carbon source and visualized by confocal phase contrast and fluorescence microscopy. (*Left panels*) Phase-contrast images; (*right panels*) fluorescence images of the same cell population. Variability of fluorescent protein expression was due to unregulated plasmid replication and inheritance. The small size and low abundance of peroxisomes in these cells reflects the fact that they were grown in glucose-containing medium which represses peroxisome proliferation.

cal subcellular fractionation and protease protection assays; and (*2*) fluorescence microscopy of *pex13*Δ cells expressing the fluorescent peroxisomal proteins PTS1-GFP, PTS2-GFP, and IPMP-GFP (Kalish et al., 1996). These studies revealed that Pex13p is required for import of both PTS1 and PTS2 proteins but is not involved in targeting or inserting IPMPs into the peroxisome membrane. By deduction, the correct targeting of PpPex10p and IPMP-GFP to peroxisome membranes in *pex13*Δ cells also means that synthesis of peroxisome membranes must occur in the absence of Pex13p. Thus, the role of Pex13p in peroxisome assembly appears restricted to the import of proteins into the peroxisome matrix.

Our data are consistent with a model in which newly synthesized PTS1-containing proteins are recognized in the cytoplasm by the PTS1 receptor, forming a receptor-ligand complex. Next, the putative complex must be transported to the peroxisome surface where it binds a docking factor in the peroxisome membrane, presumably Pex13p. After these steps, the simplest model would entail receptor-ligand dissociation with subsequent ligand translocation and return of the receptor to the cytoplasm. However, recent reports demonstrating translocation of the receptor into the peroxisome lumen in wild-type cells (van der Klei et al., 1995) and mutant cells (Dodt. G., and S.J. Gould, manuscript submitted for publication) raises the alternative possibility that docking could be followed by translocation of the intact receptor-ligand complex into the peroxisome, with subsequent receptor-ligand dissociation within the peroxisome and receptor export to the cytoplasm. Although such a model would require a translocation pore of prodigious proportions, peroxisomal import of oligomeric protein complexes (Glover et al., 1994; McNew and Goodman, 1994) and PTS1-coated gold particles (Walton et al., 1995) indicates that such a model is plausible. Distinguishing between these two possible modes of translocation will be one of the major challenges of the next few years. The identification of the docking factor for the PTS1 receptor should provide the necessary reagents for the eventual isolation and characterization of the protein translocation apparatus in the peroxisome membrane, the first step towards understanding translocation at the molecular level. Interestingly, loss of Pex13p also reduced the import of PTS2 proteins, providing support either for a role for the PTS1 receptor in PTS2 protein import, as observed in human cells (Dodt et al., 1995; Slawecki et al., 1995; Wiemer et al., 1995; Braverman, N., G. Dodt, S. Gould, and D. Valle, manuscript submitted for publication), or for Pex13p as a docking factor for the probable PTS2 receptor (Marzioch et al., 1994; Zhang and Lazarow, 1995, 1996).

We thank S. Mihalik for providing gradient samples and extractions of purified peroxisomes, W.-H. Lee and C.C. Chang for emergency aid, and M. Mendel for technical assistance. We also thank Y. Elgersma, B. Distel, and H. Tabak for communicating results before publication.

This work was supported by grants from the US National Institutes of Health (DK45787) and The Johns Hopkins University School of Medicine (IRG) to S.J. Gould and from the Australian National Health and Medical Research Council and Griffith University to D.I. Crane.

Received for publication 1 March 1996 and in revised form 11 July 1996.

References

Adzhubei, A.A., and M.J.E. Sternberg. 1993. Left-handed polyproline II helices commonly occur in globular proteins. *J. Mol. Biol.* 229:472–493.

Altshul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

Bar-Sagi, D., D. Rotin, A. Batzer, V. Mandiyan, and J. Schlessinger. 1993. SH3 domains direct cellular localization of signalling molecules. *Cell.* 74:83–91.

Crane, D.I., and S.J. Gould. 1994. The *Pichia pastoris HIS4* gene: nucleotide sequence, creation of a non-reverting *his4* deletion mutant, and development of *HIS4*-based replicating and integrating plasmids. *Curr. Genet.* 26:443–450.

Crane, D.I., J.E. Kalish, and S.J. Gould. 1994. The *Pichia pastoris PAS4* gene encodes a ubiquitin-conjugating enzyme required for peroxisome assembly. *J. Biol. Chem.* 269:21835–21844.

Distel, B., R. Erdmann, S.J. Gould, G. Blobel, D.I. Crane, J.M. Cregg, G. Dodt, Y. Fujiki, J.M. Goodman, W.W. Just, J.A.K.W. Kiel, et al. 1996. A unified nomenclature for peroxisome biogenesis factors. *J. Cell Biol.* 135:1–3.

Dodt, G., and S.J. Gould. 1996. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. *J. Cell. Biol.* In press.

Dodt, G., N. Braverman, C. Wong, A. Moser, H.W. Moser, P. Watkins, D. Valle, and S.J. Gould. 1995. Mutations in the PTS1 receptor gene, *PXR1*, define complementation group 2 of the peroxisome biogenesis disorders. *Nature Genet.* 9:115–124.

Dyer, J.M., J.A. McNew, and J.M. Goodman. 1996. The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. *J. Cell Biol.* 133:269–280.

Elgersma, Y., M. van den Berg, H.F. Tabak, and B. Distel. 1993. An efficient positive selection procedure for the isolation of peroxisomal import and peroxisome assembly mutants of *Saccharomyces cerevisiae. Genetics.* 135:731–740.

Elgersma, Y., L. Kwast, A. Klein, T. Voorn-Brouwer, M. van den Berg, B. Metzig, T. America, H.F. Tabak, and B. Distel. 1996. The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import of PTS1 containing proteins. *J. Cell. Biol.* 135:97–109.

Erdmann, R., and G. Blobel. 1995. Giant peroxisomes in oleic acid-induced *Saccharomyces cerevisiae* lacking the peroxisomal membrane protein Pmp27p. *J. Cell Biol.* 128:509–523.

Erdmann, R., F.F. Wiebel, A. Flessau, J. Rytka, A. Beyer, K.U. Frohlich, and W.H. Kunau. 1991. *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell.* 64:499–510.

Evan, G.I., G.K. Lewis, G. Ramsay, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610–3616.

Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature (Lond.).* 340:245–246.

Glover, J.R., D.W. Andrews, and R.A. Rachubinski. 1994. *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer. *Proc. Natl. Acad. Sci. USA.* 91:10541–10545.

Gould, S.J., G.A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1657–1664.

Gould, S.J., G.A. Keller, M. Schneider, S.H. Howell, L.J. Garrard, J. Goodman, B. Distel, H. Tabak, and S. Subramani. 1990*a*. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:86–90.

Gould, S.J., S. Krisans, G.A. Keller, and S. Subramani. 1990*b*. Antibodies directed against the peroxisomal targeting signal of firefly luciferase recognize multiple mammalian peroxisomal proteins. *J. Cell Biol.* 110:27–34.

Gould, S.J., D. McCollum, A.P. Spong, J.A. Heyman, and S. Subramani. 1992. Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. *Yeast.* 8:613–628.

Heim, R., A.B. Cubitt, and R.Y. Tsien. 1995. Improved green fluorescence. *Nature (Lond.).* 373:663–664.

Kalish, J.E., C. Theda, J.C. Morrell, J.M. Berg, and S.J. Gould. 1995. Formation of the peroxisome lumen is abolished by loss of *Pichia pastoris* Pas7p, a zinc-binding integral membrane protein of the peroxisome. *Mol. Cell. Biol.* 15: 6406–6419.

Kalish, J.E., G.A. Keller, J.C. Morrell, S.J. Mihalik, B. Smith, J. Cregg, and S.J. Gould. 1996. Characterization of a novel component of the peroxisomal protein import apparatus using fluorescent peroxisomal proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:3275–3285.

Kunau, W.H., A. Beyer, T. Franken, K. Gotte, M. Marzioch, J. Saidowsky, A. Skaletz-Rorowski, and F.F. Wiebel. 1993. Two complementary approaches to study peroxisome biogenesis in *Saccharomyces cerevisiae*: forward and reversed genetics. *Biochimie.* 75:209–224.

Lazarow, P.B., and H.W. Moser. 1995. Disorders of peroxisome biogenesis. *In* The Metabolic and Molecular Bases of Inherited Disease. C. Scriver, A. Beaudet, W. Sly, and D. Valle, editors. McGraw-Hill, New York. pp. 2287–2324.

Marzioch, M., R. Erdmann, M. Veenhuis, and W.H. Kunau. 1994. PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4908–4918.

McCammon, M.T., J.A. McNew, P.J. Willy, and J.M. Goodman. 1994. An internal region of the peroxisomal membrane protein PMP47 is essential for sorting to peroxisomes. *J. Cell Biol.* 124:915–925.

McCollum, D., E. Monosov, and S. Subramani. 1993. The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells. The PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal and is a member of the TPR protein family. *J. Cell Biol.* 121:761–774.

McNew, J.A., and J.M. Goodman. 1994. An oligomeric protein is imported into peroxisomes in vivo. *J. Cell Biol.* 127:1245–1257.

Musacchio, A., M. Wilmanns, and M. Saraste. 1994. Structure and function of the SH3 domain. *Prog. Biophys. Mol. Biol.* 61:283–297.

Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. Nicklin, and A. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Shimozawa, N., Y. Suzuki, T. Orii, A. Moser, H.W. Moser, and R.J.A. Wanders. 1993. Standardization of complementation grouping of peroxisome-deficient disorders and the second Zellweger patient with peroxisomal assembly factor-I (PAF-I) defect. *Am. J. Hum. Genet.* 52:843–844.

Shimozawa, N., T. Tsukamoto, Y. Suzuki, T. Orii, Y. Shirayoshi, T. Mori, and Y. Fujiki. 1992. A human gene responsible for Zellweger syndrome that affects peroxisome assembly. *Science (Wash. DC).* 255:1132–1134.

Slawecki, M., G. Dodt, S. Steinberg, A.B. Moser, H.W. Moser, and S.J. Gould. 1995. Identification of three distinct peroxisomal protein import defects in patients with peroxisomal biogenesis disorders. *J. Cell Sci.* 108:1817–1829.

Spong, A.P., and S. Subramani. 1993. Cloning and characterization of *PAS5*: a gene required for peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris. J. Cell Biol.* 123:535–548.

Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.* 9:445–478.

Swinkels, B.W., S.J. Gould, A.G. Bodnar, R.A. Rachubinski, and S. Subramani. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10: 3255–3262.

Terlecky, S.R., W.M. Nuttley, D. McCollum, E. Sock, and S. Subramani. 1995. The Pichia pastoris peroxisomal protein Pas8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:3627–3634.

Tsukamoto, T., S. Miura, and Y. Fujiki. 1991. Restoration by a 35K membrane protein of peroxisome assembly in a peroxisome-deficient mammalian cell mutant. *Nature (Lond.).* 350:77–81.

van der Leij, I., M.M. Franse, Y. Elgersma, B. Distel, and H.F. Tabak. 1993. PAS10 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA.* 90:11782–11786.

van der Klei, I.J., R.E. Hilbrands, G.J. Swaving, H.R. Waterham, E.G. Vrieling, V.I. Titorenko, W. Harder, and M. Veenhuis. 1995. The *Hansenula polymorpha PER3* gene is essential for the import of PTS1 proteins into the peroxisome matrix. *J. Biol. Chem.* 270:17229–17236.

Walton, P., P. Hill, and S. Subramani. 1995. Import of stably folded proteins into peroxisomes. *Mol. Biol. Cell.* 6:675–683.

Wiebel, F.F., and W.H. Kunau. 1992. The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. *Nature (Lond.).* 359: 73–76.

Wiemer, E.A.C., W.M. Nuttley, B.L. Bertolaet, X. Li, U. Franke, M.J. Wheelock, W.K. Anne, K.R. Johnson, and S. Subramani. 1995. Human peroxisomal targeting signal-1 receptor restores peroxisomal protein import in cells from patients with fatal peroxisomal disorders. *J. Cell Biol.* 130:51–65.

Yahraus, T., N. Braverman, G. Dodt, J.E. Kalish, J.C. Morrell, H.W. Moser, D. Valle, and S.J. Gould. 1996. The peroxisome biogenesis disorder group 4 gene, *PXAAA1*, encodes a cytoplasmic ATPase required for stability of the PTS1 receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2914–2923.

Yu, H., J.K. Chen, S. Feng, D.C. Dalgarno, A.W. Brauer, and S.L. Schreiber. 1994. Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell.* 76:933–945.

Zhang, J.W., and P.B. Lazarow. 1995. *PEB1* (*PAS7*) in *Saccharomyces cerevisiae* encodes a hydrophilic, intra-peroxisomal protein that is a member of the WD repeat family and is essential for the import of thiolase into peroxisomes. *J. Cell Biol.* 129:65–80.

Zhang, J.W., and P.B. Lazarow. 1996. Peb1p (Pas7p) is an intraperoxisomal receptor for the NH2-terminal, type 2, peroxisomal targeting sequence of thiolase—Peb1p itself is targeted to peroxisome by an NH_2-terminal peptide. *J. Cell Biol.* 132:325–334.