Identification of Pex13p, a Peroxisomal Membrane Receptor for the PTS1 Recognition Factor

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Abstract. We have identified an *S. cerevisiae* integral peroxisomal membrane protein of M_r of 42,705 (Pex13p) that is a component of the peroxisomal protein import apparatus. Pex13p's most striking feature is an src homology 3 (SH3) domain that interacts directly with yeast Pex5p (former Pas10p), the recognition factor for the COOH-terminal tripeptide signal sequence (PTS1), but not with Pex7p (former Pas7p), the recognition factor for the NH_2-terminal nonapeptide signal (PTS2) of peroxisomal matrix proteins. Hence, Pex13p serves as peroxisomal membrane receptor for at least one of the two peroxisomal signal recognition factors. Cells deficient in Pex13p are unable to import peroxisomal matrix proteins containing PTS1 and, surprisingly, also those containing PTS2. Pex13p deficient cells retain membranes containing the peroxisomal membrane protein Pex11p (former Pmp27p), consistent with the existence of independent pathways for the integration of peroxisomal membrane proteins and for the translocation of peroxisomal matrix proteins.

LIKE several other cellular membranes, the peroxisomal membrane is endowed with the ability to translocate specific proteins from the cytosol to the peroxisomal lumen. Some of the initial events in the sorting of peroxisomal proteins have been elucidated. Peroxisomal matrix proteins are synthesized on free polyribosomes and are imported posttranslationally (for review see Lazarow and Fujiki, 1985). Import of peroxisomal matrix proteins requires both ATP and cytosolic factors (Imanaka et al., 1987; Wendland and Subramani, 1993), and recent evidence suggests that peroxisomal proteins can be imported in a folded state (McNew and Goodman, 1994; Glover et al., 1994; Walton et al., 1995; Häusler et al., 1996). At least two different types of conserved signal sequences function to target proteins for translocation across the peroxisomal membrane. One used by the majority of peroxisomal matrix proteins is a COOH-terminal tripeptide, for peroxisomal targeting signal one (PTS1); Gould et al., 1987; Subramani, 1992. A second one is an NH_2-terminal nonapeptide, termed PTS2 (Swinkels et al., 1991; Osumi et al., 1991; Subramani, 1992). Soluble proteins that serve as cognate signal recognition factors for PTS1 and PTS2 and termed PTS1R (Pex5p, former ScPas10 and PTS2R (Pex7p, former ScPas7), respectively, have been identified by genetic approaches (for review see Rachubinski and Subramani, 1995). By analogy to the signal recognition- and targeting systems of other cellular membranes, binding of PTS1 and PTS2 to PTS1R and PTS2R is presumably followed by targeting of PTS1R and PTS2R to the peroxisomal membrane. The cognate peroxisomal membrane proteins that may serve as receptors for PTS1R and PTS2R have hitherto not been identified.

In addition to the identification of the PTS1 and PTS2 recognition factors, the genetic approaches led to the discovery of 16 additional *PEX* genes, formerly known as *PAS*, *PER*, *PEB*, or *PAY* genes (see accompanying letter in this issue), whose gene products, designated peroxins, were shown to be essential for peroxisome assembly (for review see Erdmann and Kunau, 1992; Lazarow, 1993). It remains to be determined whether some of these peroxins are structural components of the peroxisomal protein import machinery as well.

We have previously purified a peroxisomal membrane fraction from *S. cerevisiae* that contains at least two dozen distinct integral membrane proteins (Erdmann and Blobel, 1995). In this paper we report on an integral membrane protein of 43 kD of this fraction. Microsequencing data showed that this protein is identical to data bank ORF L9470.1 which encodes a protein of 386 amino acid residues with a calculated M_r of 42,705 D. We termed this protein Pex13p (for peroxin 13). Pex13p's most striking structural feature is an SH3 domain. In vivo and in vitro analyses revealed that Pex13p's SH3 domain binds to PTS1R, but not to PTS2R. Pex13p is essential for peroxisomal import of both PTS1- and, surprisingly, also for PTS2-containing proteins. Pex13p deficient cells retained

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1. *Abbreviations used in this paper*: GST, glutathione-*S*-transferase; MBP, maltose-binding protein; PTS, tripeptide signal sequence; SH3, src homology 3.

membranes that contain the integral peroxisomal membrane Pex11p (former Pmp27p; Erdmann and Blobel, 1995; Marshall et al., 1995), consistent with the existence of distinct pathways for the integration of peroxisomal membrane proteins and the translocation of peroxisomal matrix proteins.

Materials and Methods

Strains, Growth Conditions, and General Methods

The yeast strains used in this study were *S. cerevisiae* wild-type UTL-7A (*MAT*a, *ura*3-52, *trp*1/*his*3-11,15, *leu*2-3,112), *Δpex13* (*MAT*a, *ura*3-52, *trp*1/*his*3-11,15, *leu*2-3,112, *pex13::URA3*), *Δpex13* [*PEX11*] and UTL-7A [*PEX11*] expressing Pex11p (Erdmann and Blobel, 1995), *Δpex13* [*PEX13*] and UTL-7A [*PEX13*] expressing Pex13p, *Δpex13* [*PEX13*myc] expressing myc-epitope tagged Pex13p, and *Δpex13* [*PEX13*HA] expressing HA-tagged Pex13p. Yeast complete (YPD) and minimal (SD) media have been described previously (Erdmann et al., 1991). Oleic acid medium (YNO) contained 0.1% oleic acid, 0.02% Tween 40, 0.1% yeast extract, and 0.67% yeast nitrogen base. For oleic acid induction, cells were precultured in SD containing 0.3% dextrose to midlog phase, shifted to YNO medium, and incubated for 9–12 h. When necessary, auxotrophic requirements were added as described (Ausubel et al., 1992). Whole yeast cell extracts were prepared from 30 mg of cells according to Yaffe and Schatz (1984).

Standard recombinant DNA techniques, including enzymatic modification of DNA, Southern blotting, and double-stranded sequencing of plasmid DNA, were performed essentially as described (Ausubel et al., 1992). Yeast transformations were performed according to Bruschi et al. (1987).

Isolation and Extraction of Peroxisomes

Peroxisomes were isolated from oleic acid induced SKQ2N and from complemented *Δpex13* cells by differential centrifugation and successive isopycnic sucrose and Accudenz gradient centrifugation as described previously (Erdmann and Blobel, 1995). The suborganellar localization of proteins was determined by extraction of 25,000 *g* organellar pellets with low salt (10 mM Tris/HCl, pH 8.0; 1 mM PMSF), high salt (10 mM Tris/HCl, pH 8.0; 500 mM KCl, 1 mM PMSF), or pH 11-buffer (0.1 M Na_2CO_3, 1 mM PMSF) according to Erdmann and Blobel (1995).

Organelle Flotation

A cell free extract was prepared from oleic acid induced cells according to Erdmann and Blobel (1995) and solid sucrose was added to 56% (wt/wt). 26 ml of a linear 20–54% (wt/wt) sucrose gradient according to Erdmann and Blobel (1995) was overlaid on 4 ml of the cell free extract. Gradients were centrifuged for 3 h in an SV288 rotor at 20,000 rpm (Sorvall Instruments, Wilmington, DE). 22 fractions were collected from the bottom and processed for enzyme measurements and Western blotting according to Erdmann and Blobel (1995).

Purification and Amino Acid Sequencing of Pex13p

High salt extracted peroxisomal membranes were prepared from oleic acid induced SKQ2N cells. Further separation of the peroxisomal membrane proteins was achieved by reverse-phase HPLC according to Erdmann and Blobel (1995).

For sequencing of Pex13p, the SDS samples of HPLC fractions containing this protein (fractions 63-66, see Fig. 1) were pooled and separated on a 12% polyacrylamide gel. Polypeptides were electrophoretically transferred onto a polyvinyldiene difluoride membrane and visualized with 0.1% amidoblack in 10% acetic acid. Pex13p was excised and subjected to internal sequence analysis on a gas phase sequenator (Applied Biosystems, Foster City, CA).

Isolation of PEX13

A *PEX13*-containing DNA fragment was amplified from yeast genomic DNA (100 μg; Promega Corp., Madison, WI) by PCR using oligonucleotide sense primer 5′GACTCGAGGTGTCGTCTAAGCAAATACCCCGC3′ and antisense primer 5′CGATTTTGAATTCGGTGATGACGA3′. The amplification product of the expected of size (1,909 bp) was isolated and subcloned (XhoI/BamHI) into pSK(+)ΔXbaI, a derivative of bluescript SK (Stratagene, La Jolla, CA) in which the XbaI and SpeI sites of the vector had been destroyed by XbaI/SpeI digestion and religation. The authenticity of the *PEX13* insert in the resulting pSK43-1 was confirmed by sequencing.

Plasmid pSK43-1 served as template in Klenow DNA polymerase reactions to generate *PEX13*-specific ^{32}P-labeled probes. The primers used were 5′CCGTATAGTATGAACTCT3′, 5′TCGCAGAATCTGAAGGAA3′, 5′CGACTCGAGCCTTCTTGTGGCTTTCTC3′, and 5′AAGAAGTACTTCGTCTCG3′. The resulting probes were pooled and used to screen a subgenomic library from *S. cerevisiae*. For the construction of the subgenomic library, *S. cerevisiae* genomic DNA was digested with PstI/EcoRI. Fragments of 1.7–2.1 kb were isolated and subcloned into bluescript SK(+)ΔXbaI, yielding about 15,000 independent clones. The library screening was performed in aqueous solution according to Ausubel et al. (1992). Plasmid DNA from positive clones was isolated and the presence of *PEX13* was confirmed by sequence analysis. The isolated plasmid, designated pSK-*PEX13*, did contain the *PEX13* open reading frame as well as 356 bp of the 5′- and 381 bp of the 3′-noncoding region of *PEX13*.

For complementation studies the 1.9-kb XhoI/PstI fragment of pSK-*PEX13* was subcloned in the yeast *CEN*-plasmid pRS315 (Sikorski and Hieter, 1989) resulting in pRS5-*PEX13*.

Epitope Tagging of Pex13p

The *CUP1*mycUb cassette from plasmid SK/mycUb (Marzioch et al., 1994) was subcloned into the SacI/XhoI sites of the *CEN*-plasmid pRS415 (Stratagene), resulting in pRS5myc. A BamHI site was introduced in front of the *PEX13* orf by PCR using primers 5′GTGAATTCGGATCCATAT-GTCATCCACAGCAGTA3′ and 5′CGACTCGAGCCTTCTTGTGGCTTTCTC3′ and resulting in plasmid pSP43D. The BamHI/XhoI fragment of pSP43D, containing the *PEX13* orf plus 381 bp of the 3′ noncoding region, was inserted in frame into pRS5myc (BglII/XhoI). The resulting plasmid, designated pRS5-*PEX13*myc, encoded the myc-epitope tagged Pex13p whose expression was under the control of the *CUP*1 promoter from a *CEN*-plasmid.

Construction of a PEX13 Null Mutant

Deletion of the *PEX13* gene was achieved by integrative transformation using the procedure of Rothstein (1991). A PCR product consisting of the *URA3* gene flanked by 65 bp of the 5′ and 3′ noncoding regions of *PEX13* was generated using the *URA3* gene as template and hybrid *URA3-PEX13* primers. Forward primer comprised nucleotides −65 to −1 of *PEX13* fused to nucleotides 1–18 of the yeast *URA3* gene. The second primer was complementary to the first 65 nucleotides of the *PEX13* 3′ noncoding region fused to the last 18 nucleotides of the *URA3* gene. The hybrid PCR product was transformed into the *S. cerevisiae* haploid strain UTL-7A. URA$^+$ transformants were isolated and tested for the correct insertion by PCR.

Immunofluorescence-, Electron-, and Immunoelectron Microscopy

Immunofluorescence microscopy was performed essentially according to Rout and Kilmartin (1990) with modifications as previously described (Erdmann, 1994). Rabbit antisera against yeast thiolase (Erdmann, 1994) and yeast Pcs60p (Blobel and Erdmann, 1996) were used in dilutions 1:3,000; monoclonal 9E10 antibody against the c-*myc* epitope (Evan et al., 1985) was used in a dilution of 1:50. 6 μg/ml solutions of Cy3-conjugated donkey anti-mouse IgG (cross absorbed against rabbit IgG) and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used for detection.

For electron microscopy washed cells were fixed with 1.5% $KMnO_4$ for 20 min at room temperature. After dehydration in a graded ethanol series, the samples were embedded in Epon812, ultrathin sections were cut with a diamond knife and examined. Immunogold labeling of yeast cells was performed as described (Erdmann and Blobel, 1995).

Immunoblots

Western blot analysis was performed according to standard protocols (Harlow and Lane, 1988) using anti-rabbit or anti-mouse IgG-coupled HRP as second antibody (Amersham Corp., Arlington Heights, IL). Pro-

tein-antibody complexes were visualized by treatment with HRP chemoluminescence developing reagents (ECL system; Amersham). Polyclonal rabbit antibodies against Fox3p (Erdmann and Kunau, 1994), Fox1p (Will, 1994), and Pcs60p (Blobel and Erdmann, 1996) were used at dilutions of 1:10,000. HA-tagged Pex11p (Erdmann and Blobel, 1995) was detected with monoclonal 12CA5 antiserum (BAbCO, Richmond, CA; dilution of 1:1,000). c-*myc*–epitope tagged Pex13p was detected with monoclonal 9E10 antiserum (Evan et al., 1985) in a dilution of 1:1,000.

Two Hybrid Methodology

Amino acids 286 to 386 of Pex13p were fused to either the activation domain or the DNA-binding domain of yeast Gal4p in vectors pPC86 or pPC97 (Chevray and Nathans, 1992), the PCR using primers 5'TCCAGAATTCGGATCCTACAGACCTCTGGAACCATA3' and 5'CAGTCTAGACTGCAGCTAGTGTGTACGCGTTTCATC3' and pSK-*PEX13* as a template. The resulting amplification construct was subcloned EcoRI/XbaI into bluescript SK(+) revealing pSK43hyb. The fragment was subsequently subcloned EcoRI/NotI in pPC86, resulting in pPC86-SH3, and BamHI/SacI in pPC97 (BglII/SacI), resulting in pPC97-SH3. The complete orf encoding the yeast PTS1R Pex5p (van der Leij et al., 1993) was amplified by PCR using primers 5'TAGAATTCGGATCCATATGGACGTAGGAAGTTGC3' and 5'CAGCTCGAGACTAGTTCAAAACGAAAATTCTCC3' and yeast genomic DNA (100 μg, Promega Corp.) as template. The *PEX5* PCR product was digested with EcoRI/XhoI, subcloned into bluescript SK(+), and the resulting plasmid was designated pSK-*PEX5T*. Pex5p was fused to the GAL4 activation domain by subcloning the EcoRI–SpeI fragment of pSK-*PEX5T* in pPC86, resulting in pPC86-*PEX5T*. Further *PEX* genes fused to either the activation or DNA-binding domain of *GAL4* in pPC86 or pPC97 were provided by Kunau and coworkers.

Strain HF7c (Clontech Laboratories, Palo Alto, CA) was transformed according to the matchmaker protocol supplied by the manufacturers. Double transformants were selected on SD medium lacking tryptophane and leucine. Colonies were transferred to nitrocellulose filters and lysed by immersion in liquid nitrogen for 20 s. The filters were incubated for up to 4 h at 30°C on Whatman 3-mm paper, saturated with 1 mg/ml X-Gal in 100 mM KPi, pH 7.0. Blue staining of colonies indicated β-galactosidase activity. To assay the expression of *HIS3*, double transformants of strain HF7c were transferred to SD plates lacking tryptophane, leucine, and histidine, but containing 10 mM 3-aminotriazole. His prototrophy of transformants indicated His3p expression.

In Vitro Binding Assay

Amino acids 286–386 of Pex13p comprising the Pex13p SH3 domain were fused to the COOH terminus of the *E. coli* maltose-binding protein (MBP). The SH3 domain encoding region of *PEX13* was amplified from plasmid pSK-*PEX13* by PCR with primers 5'TCCGAATTCGGATCCCTACAGACCTCTGGAACCATA3' and 5'CAGTCTAGACTGCAGCTAGTGTGTACGCGTTTCATC3'. The PCR product was subcloned EcoRI/PstI in pMAL-c2 (NEB, Beverly, MA), to produce pMAL-SH3. The MBP-SH3 fusion protein was expressed from pMAL-SH3 in *E. coli* strain TG1.

The EcoRI–PstI fragment of pSK-*PEX5T*, containing the *PEX5* orf (see above) was subcloned into pGEX4T-2N (Pharmacia, Uppsala, Sweden), resulting in pGEX-*PEX5*. Transformation of *E. coli* strain M15 (pREP4) with pGEX-*PEX5* resulted in the IPTG inducible expression of the glutathione-*S*-transferase (GST)-Pex5p fusion protein.

Expression of MBP and GST fusion proteins in *E. coli* was induced with 0.2 mM IPTG at an OD_{600} of 0.6. 200-ml cultures were induced for 3 h at 30°C. 500-μl aliquots were taken for the preparation of whole cell extracts, the remaining cells were sedimented and resuspended in 10 ml PBS containing 1 mM PMSF, 1.25 mg/ml pepstatin, 1.25 mg/ml leupeptin, 1.25 mg/ml antipain, 1.25 mg/ml chymostatin, and 10 mg/ml lysozyme. After 1 h incubation on ice, the cells were lysed by sonication. Triton X-100 was added to 1% (vol/vol) and the lysate was clarified by centrifugation at 25,000 *g* for 20 min. For each binding assay, 2-ml lysate containing GST-fusion proteins were added to 200 μl glutathione-Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ), equilibrated with wash buffer (PBS plus 1% Triton X-100 [vol/vol]), and incubated for 1 h on a rotary shaker at room temperature. The matrices were washed three times with wash buffer and incubated as above with 2 ml of lysate containing MBP fusion proteins. The matrices were washed twice with wash buffer, transferred to mini columns and washed again with 100 column volumes. Bound proteins were eluted with 100 ml of 10 mM glutathione in 100 mM Tris, pH 7.4, and analyzed by SDS-PAGE and Western blotting.

Analytical Procedures

Catalase (EC 1.11.1.6), thiolase (EC 2.3.1.16), and fumarase (EC4.2.1.2.) were assayed using standard protocols (Moreno de la Garza et al., 1985). Protein determination was performed as described (Bradford, 1976).

Results

Identification of Pex13p

Peroxisomal membranes were isolated from oleate-induced *S. cerevisiae* and successively extracted by low salt and high salt (see Materials and Methods). The membrane proteins were solubilized by SDS and separated by HPLC and by subsequent SDS-PAGE of the HPLC fractions (Fig. 1). Partial sequencing of a very minor protein of the preparation (barely visible and indicated by an arrowhead in Fig. 1) gave a peptide sequence that matched an ORF, L9470.1, in the yeast genome data bank. ORF L9470.1, in the following referred to as *PEX13* coded for a protein of 386 amino acids and a calculated M_r of 42,705 D (Fig. 2). Pex13p consists of three distinct domains: an NH_2-terminal hydrophilic domain (residues 1-150) rich in Gly, Asn, Gln, Ser, and Tyr; a central rather hydrophobic domain (residues 151-286) containing at least one region (residues 264-280) predicted to be a membrane-spanning α-helix; and a COOH-terminal region (residues 287-386) that contains a Src homology 3 (SH3) domain. An alignment of Pex13p's SH3 domain with SH3 domains of other protein is shown in Fig. 2 *A*. Search of the database also revealed striking similarities of Pex13p with an ORF of *Caenorhabditis elegans* and two human expressed sequence tags (Fig. 2 *B*).

Δpex13 Cells Are Defective for Growth on Oleic Acid

The genomic copy of *PEX13* in wild-type UTL-7A cells was replaced with *URA 3*, yielding the null mutant strain *Δpex13* (see Materials and Methods). *Δpex13* cells were viable on YPD, SD, and ethanol media, but were unable to use oleic acid as a single carbon source, indicating that Pex13p is essential for growth on oleic acid medium (Fig. 3 *A*). The impaired growth phenotype on oleate medium was restored when mutant cells were transformed with plasmids expressing either wild-type Pex13p or an NH_2-terminally, myc epitope-tagged Pex13p, indicating that the tagging had no obvious effect on the function of the protein (Fig. 3). However, expression of Pex13p containing an influenza hemagglutinin tag at the COOH terminus of Pex13p did not result in functional complementation of the *Δpex13* phenotype (data not shown). This observation underscores the importance of the COOH-terminal SH3-containing domain for Pex13p function.

Δpex13 Cells Fail to Import PTS1 and PTS2 Containing Peroxisomal Proteins

Electronmicroscopic analyses indicated that Pex13p is essential for peroxisome assembly. The characteristic electron dense peroxisomes present in wild-type cells (Fig. 3 *B*) were absent in *Δpex13* cells (Fig. 3 *C*), but were restored in

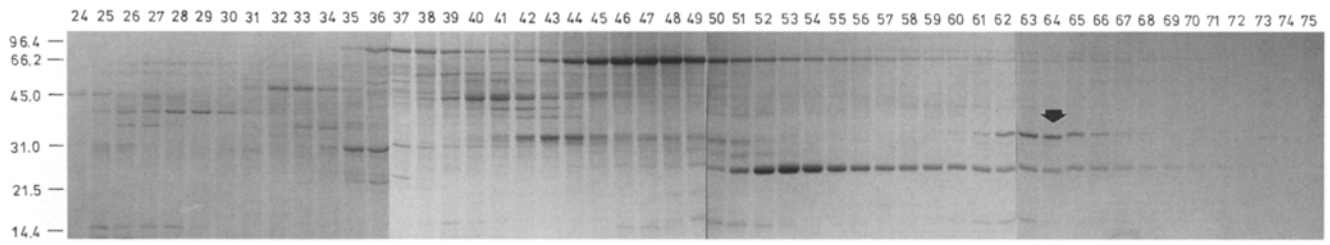

Figure 1. Preparative chromatographic separation of peroxisomal membrane proteins. High salt-extracted peroxisomal membranes (1 mg protein) were solubilized in SDS, and separated by reverse phase HPLC. Polypeptides of selected fractions were separated by SDS-PAGE and visualized by Coomassie blue staining. The very faint band of Pex13p is indicated by the arrowhead. The amount per lane corresponded to 5% of the total fraction. Molecular weight standards are indicated on the left.

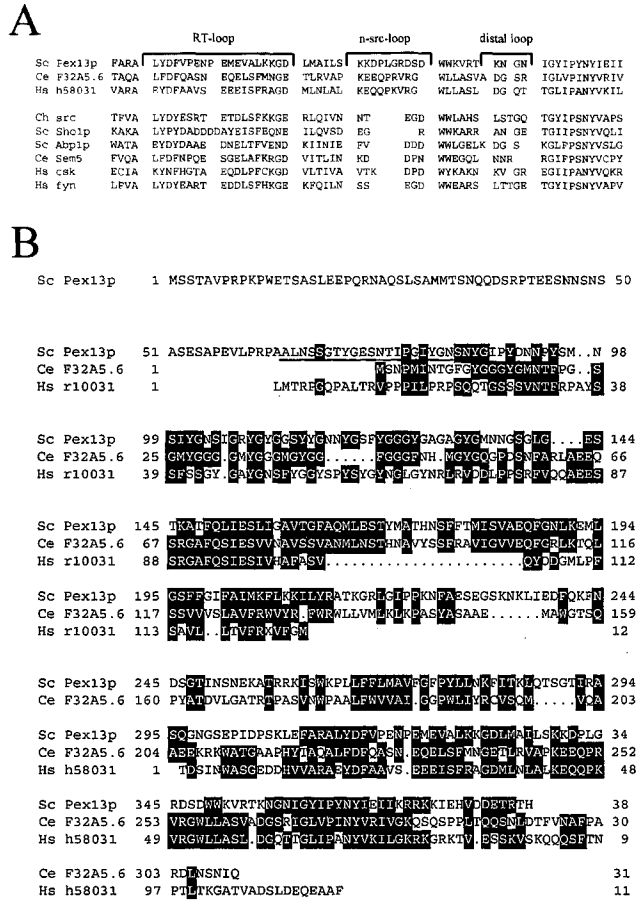

Figure 2. (*A*) Comparison of Pex13p SH3 domain with a subset of the known SH3 domain sequences. F32A5.6 and h58031 are putative *C. elegans* and human homologues of Pex13p. Other sequences are from chicken c-src (Takeya and Hanafusa, 1983), *S. cerevisiae* Sho1p (Maeda et al., 1995), *S. cerevisiae* Apb1p (Drubin et al., 1990), *C. elegans* Sem5p (Clark et al., 1992), human CSK (Partanen et al., 1991), and human fyn (Semba et al., 1986). (*B*) Comparison of the deduced amino acid sequences for *S. cerevisiae* Pex13p (accession number S51436), *C. elegans* orf F32A5.6 (accession number U20864), and human expressed sequence tags r10031 and h58031. The underlined amino acid sequence of Pex13p was obtained by peptide sequencing of purified Pex13p. Two or more identical or similar amino acids are shown in bold. Similarity rules: G=A=S; A=V; V=I=L=M; I=L=M=F=Y=W; K=R=H; D=E=Q=N; S=T=Q=N.

Δpex13 cells transformed with plasmids expressing Pex13p (Fig. 3 *D*).

Cell fractionation and enzymatic analyses indicated that *Δpex13* cells fail to import peroxisomal proteins. When wild-type cell homogenates were centrifuged to sediment organelles, the majority of the peroxisomal marker enzymes catalase and thiolase as well as of the mitochondrial marker enzyme fumarase cosedimented with the organelle fraction (Table I). In contrast, in *Δpex13* cells the majority of the two peroxisomal marker enzymes did not cosediment with the organelles, whereas the mitochondrial marker enzyme fumarase still did (Table I). In *Δpex13* cells that were transformed with plasmids expressing Pex13p, cosedimentation of the two peroxisomal marker enzymes with the organelles indicated restoration of peroxisomal protein import (Table I).

A failure of *Δpex13* cells to import peroxisomal proteins, both of the PTS1 and the PTS2 variety, was shown by immunofluorescence microscopy analyses as shown in Fig. 4. Wild-type cells exhibited the peroxisome-characteristic punctate staining for both thiolase (PTS2 peroxisomal protein) and Pcs60p (a PTS1 peroxisomal matrix protein associated with the inner aspects of the peroxisomal membrane, Blobel and Erdmann [1996]). In contrast, both of these peroxisomal matrix proteins gave a diffuse cytosolic immunofluorescence in *Δpex13* cells (Fig. 4). Punctate staining for both peroxisomal proteins was restored in *Δpex13* cells transformed with plasmids expressing Pex13p (Fig. 4).

Pex13p Is an Integral Peroxisomal Membrane Protein

That Pex13p is indeed an integral membrane protein of the peroxisomal membrane was shown by several means. First, in double immunofluorescence microscopy of *Δpex13* cells that were complemented with the NH_2-terminally myc-tagged Pex13p, there was colocalization with thiolase, indicating that Pex13p is a peroxisome-associated protein (Fig. 5 *A*, note the highly magnified staining pattern of a single cell). Second, sucrose gradient analysis of a homogenate of these cells showed that the catalase enzymatic activity (Fig. 5 *B*, *upper panel*) as well as thiolase and Pex13p (as assayed by immunoblotting, Fig. 5 *B*, *lower panel*) cosedimented at a peroxisome-characteristic density of 1.21 g/ml, well separated from the mitochondrial fu-

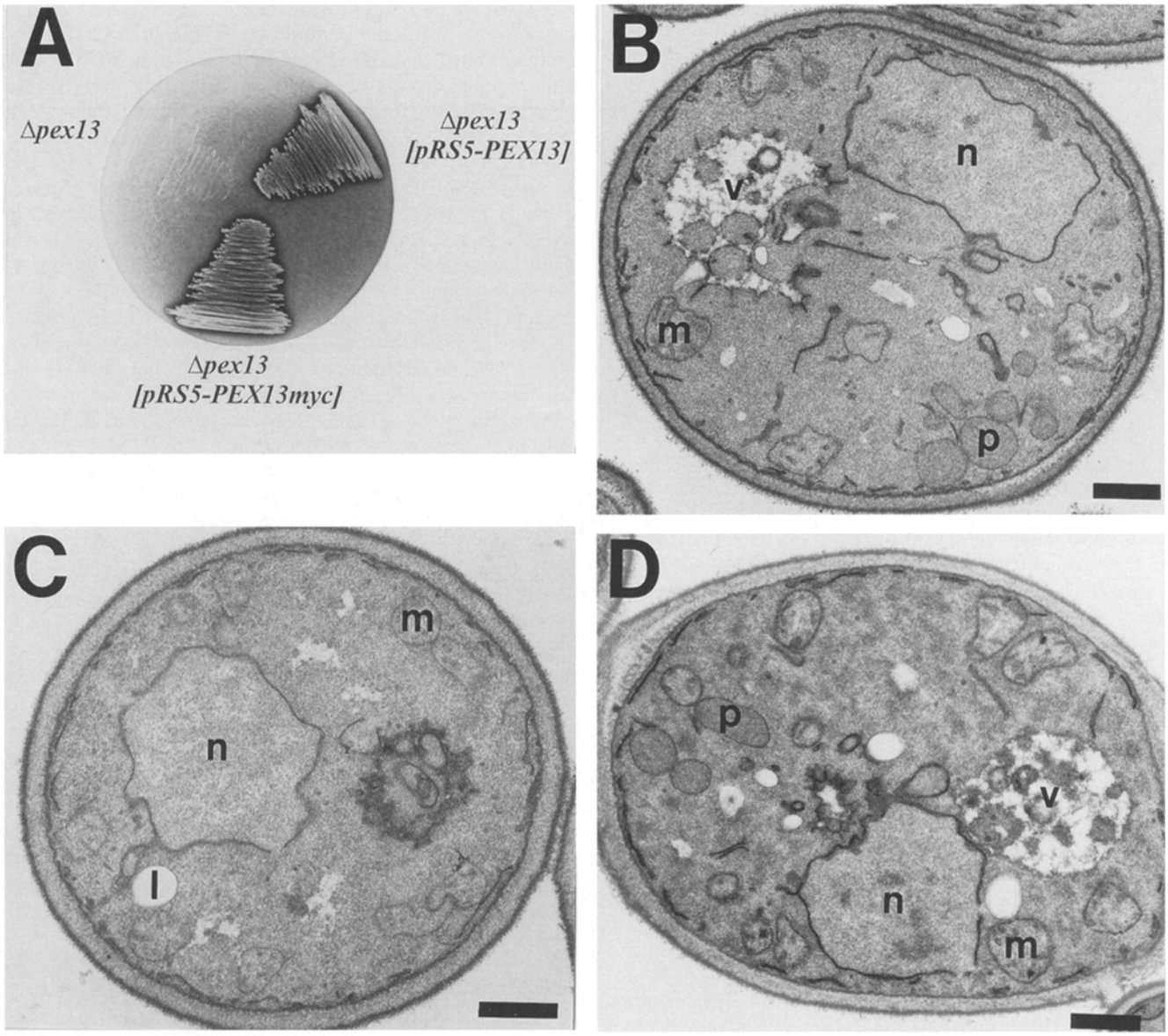

Figure 3. Pex13p is essential for growth on oleic acid and *Δpex13* cells lack morphologically detectable peroxisomes. (*A*) *Δpex13* mutant cells and *Δpex13* cells expressing Pex13p or myc-tagged Pex13p from single copy plasmids were plated on oleic acid medium and incubated for 5 d at 30°C. (*B–D*) Electron micrographs of 12-h oleic acid–induced wild-type (*B*), *Δpex13* mutant (*C*), and complemented *Δpex13* mutant cells (*D*). *p*, peroxisome; *m*, mitochondrion; *n*, nucleus; *v*, vacuole; *l*, lipid droplet. Bars, 1 μm.

marase marker that sedimented at a density of 1.18 g/ml (Fig. 5 *B*, *upper panel*). Third, immunoelectron microscopy of *Δpex13* cells complemented with NH_2-terminally myc-tagged Pex13p showed decoration of the peroxisomal membrane with 10-nm gold labeled anti-myc antibodies (Fig. 6 *A*, panel *a*). Double immunoelectron microscopy with anti-thiolase antibodies showed the classical peroxisomal content labeling for thiolase (5 nm gold) and the peripheral labeling for Pex13p (10 nm gold) (Fig. 6 *A*, panel *b*), as expected for a peroxisomal membrane protein. And fourth, SDS-PAGE and subsequent immunoblot analyses of proteins of an organellar fraction that was subjected to extraction by low salt and high salt, and at pH 11, showed that Pex13p was resistant to extraction at pH 11, as expected for an integral membrane protein (Fig. 6 *B*). Likewise, another integral peroxisomal membrane protein, Pex3p (former Pas3p, Höhfeld et al., 1991), also remained resistant to alkali extraction (Fig. 6 *B*). The peroxisomal membrane-associated protein Pcs60p was extracted at high salt, whereas most of the thiolase, a peroxisomal matrix protein, was already extracted at low salt (Fig. 6 *B*).

Δpex13 Cells Retain Peroxisomal Membranes

If the peroxisomal membrane contains separate machineries for the translocation of peroxisomal matrix proteins and for the integration of peroxisomal membrane proteins, the latter would be expected to be unaffected in *Δpex13* cells. Indeed, in double immunofluorescence mi-

Table I. Distribution Pattern of Peroxisomal and Mitochondrial Marker Enzymes in the 25,000-g Supernatant and Pellet Fraction of Cell Lysates from Oleic Acid Induced Wild-Type, Δpex13, and Complemented Δpex13 Cells

Strain		Enzyme activity (nkat/fraction) Total	Supernatant fraction (A1)	Pellet fraction (A2)	A1/A2
Wild-type	Catalase	16.7×10^4	2.6×10^4	8.4×10^4	0.23
	Thiolase	340	136	184	0.74
	Fumarase	68	23	40	0.58
Δpex13	Catalase	16.1×10^4	18×10^4	0.1×10^4	180
	Thiolase	390	378	5	76
	Fumarase	75	23	48	0.48
Δpex13	Catalase	11.6×10^4	2.6×10^4	7.1×10^4	0.37
[pRS-*PEX13*]	Thiolase	332	106	202	0.52
	Fumarase	67	21	48	0.44

croscopy of *Δpex13* cells, the previously identified peroxisomal membrane protein Pex11p (former Pmp27p; Erdmann and Blobel, 1995; Marshall et al., 1995) still shows the peroxisome-characteristic punctate immunofluorescence (Fig. 7 *A*, panel *b*), whereas the mislocalized thiolase gives diffuse cytosolic immunofluorescence (Fig. 7 *A*, panel *a*).

Double immunoelectron microscopy of *Δpex13* cells confirms the membrane localization of Pex11p (10 nm gold) (Fig. 7 *B*, panels *a* and *b*) and the absence of enclosed content proteins, either of the PTS2 peroxisomal protein thiolase (5 nm gold) (Fig. 7 *B*, panel *a*) or of the PTS2 peroxisomal protein Pcs60p (5 nm gold) (Fig. 7 *B*, panel *b*).

Flotation of *Δpex13* or of control wild-type cell homogenates in sucrose gradients and analysis of the sucrose gradient fractions by enzyme assays (Fig. 7 *C*, *upper panels*) or by SDS-PAGE and subsequent immunoblotting (Fig. 7 *C*, *lower panels*) confirmed the immunofluorescence and immunoelectron microscopy data. As expected, in homogenates of wild-type cells, the peroxisomal matrix proteins thiolase, MFP (multifunctional protein of peroxisomal fatty acid oxidation; Hiltunen et al., 1992), Pcs60p and catalase floated with the peroxisomal membrane protein Pex11p to a density of 1.23 g/ml (Fig. 7 *C*, *left panel*). In contrast, in *Δpex13* cell homogenates, the peroxisomal matrix proteins remained in the load zone of the sucrose gradient, whereas the peroxisomal membrane marker protein Pex11p floated to a density of 1.17 g/ml, slightly lighter than the mitochondrial marker fumarase (Fig. 7 *C*, *right panel*). Together these data indicate that a distinct membrane retaining the integral peroxisomal membrane protein Pex11p but devoid of associated peroxisomal matrix proteins is present in the *Δpex13* cells.

Pex13p Functions as a Receptor for the Signal Recognition Factor PTS1R

SH3 domains are known to mediate specific protein–protein interactions (Cohen et al., 1995). To identify proteins that might be capable of interacting with the SH3 domain of Pex13p, we used the yeast two hybrid methodology (Fields and Song, 1989). Being a peroxin (a protein involved in peroxisome assembly), Pex13p might interact with known peroxins (Erdmann and Kunau, 1992). Therefore, we performed a limited two hybrid screen to test for interactions between the Pex13p SH3 domain and the coding regions of yeast Pex3p (former Pas3p), Pex4p (former Pas2p), Pex5p (former Pas10p, identical to PTS1R; Van der Leij et al., 1993), Pex7p (Pas7p, identical to PTS2R; Marzioch et al., 1994; Zhang and Lazarow, 1995), Pex10p (former Pas4p), Pex12p (former Pas5p), Pex8p (former Pas6p), Pas9p, Pas11p, and Pas12p. To this end the Pex13p SH3 domain was fused to the *GAL4* activation domain and the various peroxins were fused to the *GAL4* DNA-binding domain. Fusion proteins were coexpressed in strain HF7c which contains the *lacZ* and the *HIS3* gene under the control of the *GAL1* promoter. Activation of the *HIS3* and *lacZ* transcription showed that the SH3 domain of Pex13p interacted only with Pex5p and not with any of the other peroxins that were tested (Fig. 8 *A*), i.e., the SH3 domain of Pex13p interacts with PTS1R, but not with PTS2R. To test for a direct biochemical interaction, the SH3 domain of Pex13p was fused to the maltose-binding protein (MBP) and Pex5p was fused to glutathione-*S*-transferase (GST). Both fusion proteins as well as GST and MBP were expressed in *E. coli* (Fig. 8 *B*, lanes *1–4*). The expressed GST or the GST fusion protein were then immobilized on glutathione Sepharose. An *E. coli* extract containing either MBP or MBP-SH3 was incubated with the various matrices and the bound material was eluted with gluthathione. The eluted proteins were subjected to SDS-PAGE and either stained with Coomassie blue (Fig. 8 *B*, lanes *5–10*) or immunoblotted with anti-MBP antibodies (Fig. 8 *C*). It is clear that MBP-SH3 binds to GST-Pex5p (Fig. 8, *B* and *C*, lane *8*). The binding between SH3 and Pex5p (PTS1R) is specific as is evident from various controls shown in lanes *5*, *6*, *7*, *9*, and *10*. Hence, we conclude that Pex13p functions as a peroxisomal membrane receptor for the soluble PTS1 recognition factor (PTS1R).

Discussion

Pex13p is only the third component of the peroxisomal import apparatus to be identified. The other two known components, PTS1R (Pex5p) and PTS2R (Pex7p), serve as cognate recognition factors for the COOH-terminal (PTS1) and the NH_2-terminal (PTS2) signal of peroxisomal matrix proteins, respectively. The COOH-terminal domain of Pex13p contains a src homology 3 (SH3) domain. This domain was found to directly interact with PTS1R and therefore Pex13p is proposed to serve as the peroxisomal membrane's docking site for PTS1R. Deletion of Pex13p abolishes import of PTS1- and, surprisingly, also of PTS2-type peroxisomal matrix proteins but still allows the membrane integration of a bona fide peroxisomal membrane protein, Pex11p.

Structural Domains of Pex13p

Pex13p is among the very minor integral membrane proteins of purified and high salt washed peroxisomal membranes from oleate-induced cells (Fig. 1). Thus, even under conditions where substrate (oleate) utilization requires peroxisomal function and results in a massive induction of peroxisomes, Pex13p, being an integral membrane compo-

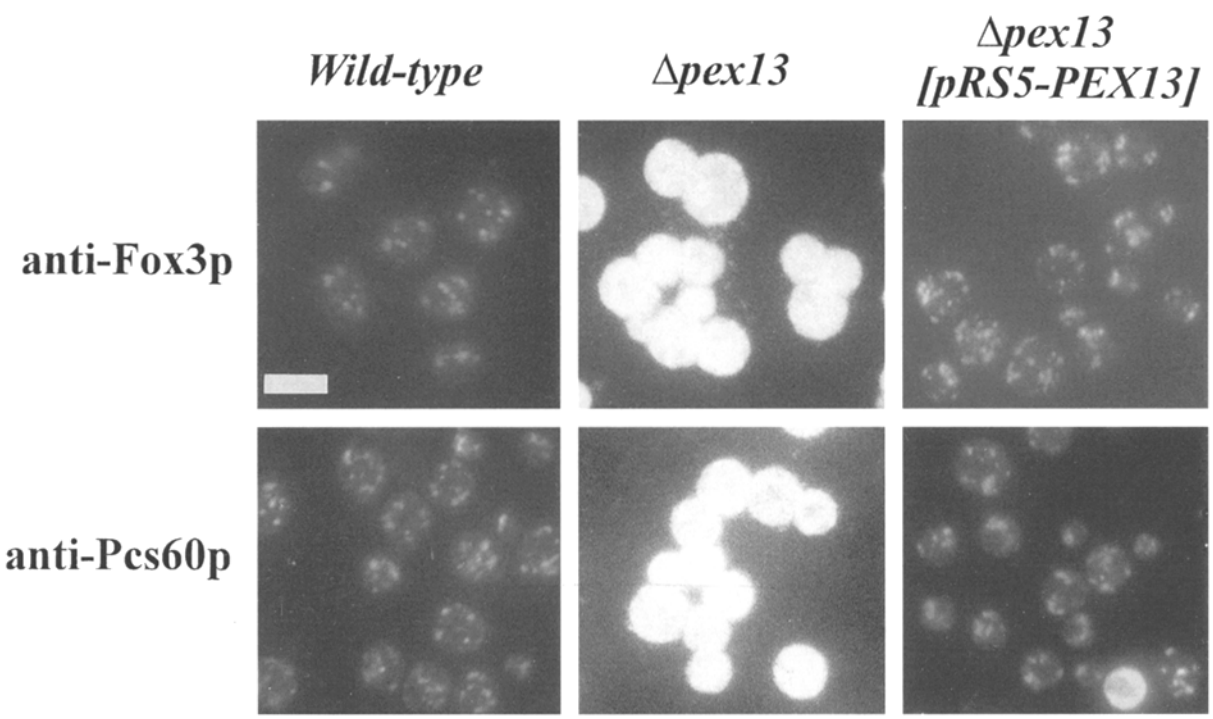

Figure 4. Immunofluorescence localization of PTS2-containing thiolase and PTS1-containing Pcs60p in wild-type, *Δpex13* mutant, and *Δpex13* mutant cells expressing Pex13p. Bar, 5 μm.

nent of the protein import machinery, appears to occupy only a minute fraction of the peroxisomal membrane.

Pex13p contains neither of the known peroxisomal targeting signals for matrix proteins (Subramani, 1992) nor a sequence resembling the only described targeting signal for a peroxisomal membrane protein (McCammon et al., 1994).

Pex13p consists of an NH_2-terminal hydrophilic domain, a central hydrophobic domain and a hydrophilic COOH-terminal domain. The central hydrophobic domain is predicted to contain at least one region which could form a membrane spanning α helix. The topology of Pex13p in the peroxisomal membrane remains to be determined. The most striking feature of the COOH-terminal hydrophilic region is the presence of an SH3 domain which interacts with the PTS1R. As the PTS1R of *S. cerevisiae* predominantly resides in the cytosol (Tabak et al., 1995), Pex13p's SH3 domain is most likely exposed on the cytoplasmic surface of the peroxisomal membrane. Most SH3-containing proteins are soluble cytosolic proteins. There is a precedent for an SH3 containing integral membrane protein, which is the yeast plasma membrane protein Sho1p which functions as an osmosensor (Maeda et al., 1995)

The phenotype of Pex13p deletion in yeast resembles that of certain severe human peroxisomal disorders, characterized by the presence of peroxisomal membrane ghosts and mislocalized peroxisomal matrix proteins (for review see Lazarow and Moser, 1994), and of which eleven complementation groups have yet been characterized (Slawecki et al., 1995). It will be interesting to determine whether the expressed human sequence tags that show homology to Pex13p indeed are part of a human orthologue and whether a wild-type human orthologue (or Pex13p) can complement any of the eleven complementation groups of this peroxisomal disorder.

Pex13p Is Essential for Peroxisomal Protein Import

The mutant phenotype of Pex13p deficient cells is characterized by the inability of the cells to grow on oleic acid as a single carbon source, absence of morphologically detectable peroxisomes, and mislocalization of peroxisomal matrix proteins to the cytosol (Figs. 3, 4, and 7; Table I). This phenotype is characteristic for mutants lacking proteins essential for peroxisome assembly (*pex*-phenotype; Erdmann et al., 1989; Lazarow, 1993). However, not every peroxin necessarily is part of the protein import machinery. Proteins essential for peroxisome-formation, -proliferation, -morphology, or induction of peroxisome proliferation might exhibit such a *pex*-phenotype. Several lines of evidence make an involvement of Pex13p in the above mentioned peroxisomal functions rather unlikely. (*1*) Peroxisomal membrane ghosts were detected in *Δpex13* mutant cells and they were present in amounts comparable to the number of peroxisomes in wild-type cells (Fig. 7 *A*). (*2*) Electron microscopical investigation of the peroxisomal membrane ghosts of Pex13p deficient cells revealed the organelles to be single membrane bound, spherical structures, which except for the lack of an electron dense matrix resemble wild-type peroxisomes (Fig. 7). (*3*) Immunocytochemical, immunofluorescence microscopical, and biochemical investigations revealed that these membrane ghosts are deficient for all of the peroxisomal matrix proteins tested (Figs. 4 and 7; Table I). Consequently, the general peroxisomal import defect for matrix proteins observed for Pex13p deficient cells is most likely due to the

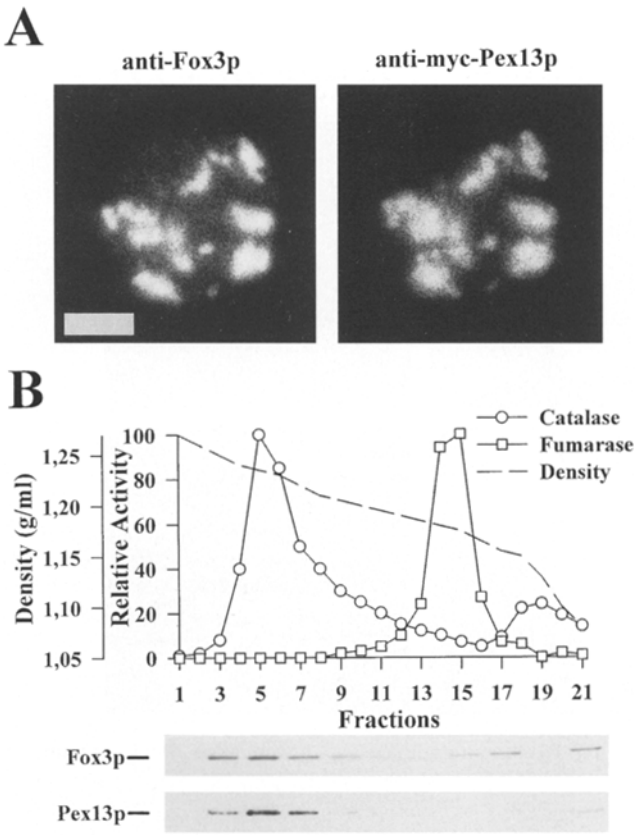

Figure 5. Pex13p is localized in peroxisomes. (*A*) Double immunofluorescence microscopy localization of thiolase and myc-tagged Pex13p. Oleic acid induced *Δpex13* cells expressing the myc-tagged Pex13p were processed for double immunofluorescence microscopy using rabbit antibody against thiolase and mAb against the myc-epitope. Secondary antibodies were FITC-conjugated anti–rabbit IgG and CY3-conjugated anti–mouse IgG. Bar, 2 μm. (*B*) Coenrichment of Pex13p, peroxisomal thiolase, and catalase during peroxisome isolation. The organelles of a 25,000-*g* pellet from *Δpex13* cells expressing myc-Pex13p, were separated on a 36–68% (wt/vol) sucrose gradient. 1.2-ml fractions were collected from the bottom of the gradient. Localization of Pex13p, peroxisomal thiolase, and catalase, as well as mitochondrial fumarase, were monitored by immunoblot analysis and enzyme activity measurements. Peroxisomes peaked in fraction 5 at a density of 1.23 g/ml. Mitochondria peaked in fraction 15 at a density of 1.18 g/ml. Pex13p cosegregated with both peroxisomal markers.

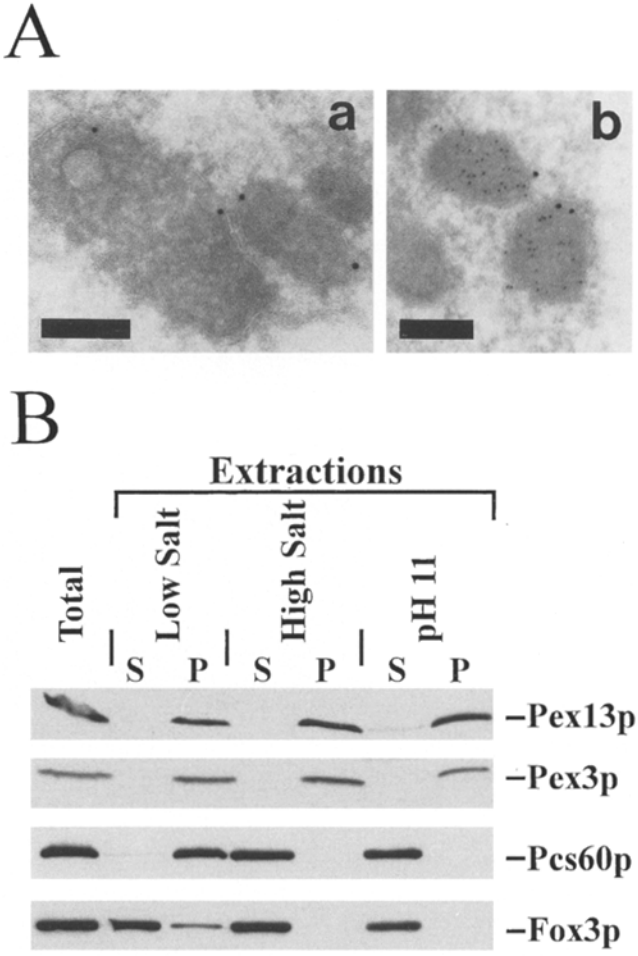

Figure 6. Pex13p is an integral peroxisomal membrane protein. (*A*) Single and double immunogold labeling of peroxisomes in *Δpex13* cells expressing myc-Pex13p. Thin sections of Lowicryl-embedded cells were immunolabeled with antiserum against the myc-epitope (*a* and *b*; 10 nm gold) and thiolase (*b*; 5 nm gold). (*B*) 25,000 *g* organelle pellets were prepared from *Δpex13* cells expressing myc-tagged Pex13p and extracted by low salt, high salt, and pH 11.0 treatments as indicated. Supernatant (*S*) and pellet (P) fractions of a 100,000-*g* sedimentation of each extraction were analyzed. Equal amounts of protein were loaded per lane. Myc-Pex13p, Pex3p (Höhfeld et al., 1991), Pcs60p (Blobel and Erdmann, 1996) and thiolase (Fox3p) amounts in fractions were monitored by immunoblot analysis. Bars: (*A*, *a* and *b*) 0.2 μm.

lack of an essential component of the peroxisomal protein import machinery.

Function of Pex13p

SH3 domains of proteins mediate interactions with other proteins that contain a proline rich region with a PXXP signature motif (Ren et al., 1993; Rickles et al., 1994; Yu et al., 1994). Pex13p's SH3 domain was shown in vivo (yeast two hybrid system) and by in vitro binding experiments to interact with PTSR1 (Fig. 8). Although there are proline rich stretches, there is no PXXP signature motif in *S. cerevisiae* PTS1R (Van der Leij, 1993). However, the mammalian orthologue of PTS1R contains such a signature motif (Dodt et al., 1995; Wiemer et al., 1995; Fransen et al., 1995). The protein–protein interactions mediated by SH3 domains include coupling of intracellular signaling pathways, regulation of catalytic activities of proteins, and localization of proteins to specific subcellular compartments (Cohen et al., 1995). The latter is of special interest for Pex13p function as our results are consistent with the idea that a mobile PTS1R binds PTS1-containing proteins in the cytosol, and delivers them to the peroxisomal membrane via its interaction with the SH3 domain of Pex13p. Recognition of signal sequences by cytosolic factors which subsequently are directed to defined docking sites at the target membrane is a common theme in protein targeting. Examples include SRP/SRP-receptor interaction in the endoplasmic reticulum (Gilmore et al., 1982; Meyer et al., 1982), binding of karyopherin to peptide repeat containing

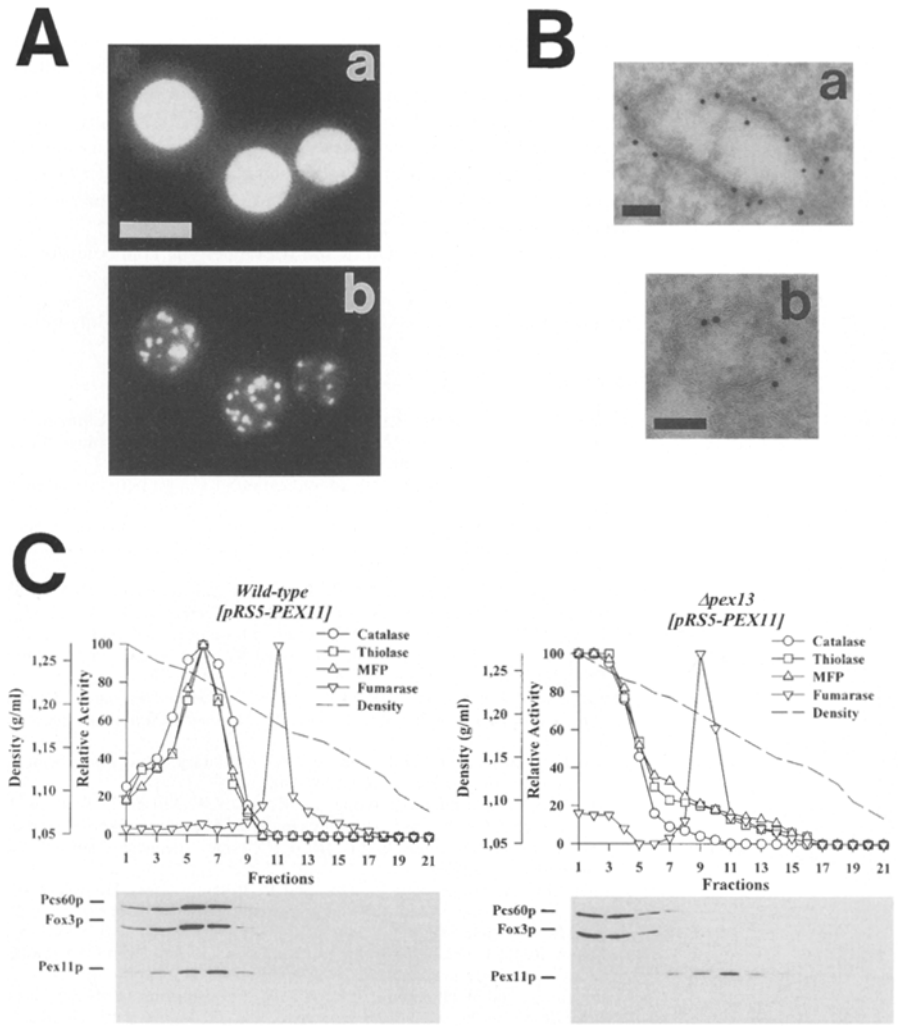

Figure 7. *Δpex13* cells contain peroxisomal membrane ghosts, which lack peroxisomal matrix proteins. (*A*) Double immunofluorescence localization of thiolase (*a*) and the peroxisomal membrane protein Pex11p (*b*) in Pex13p-deficient cells, expressing HA-tagged Pex11p. (*B*) Double immunoelectron microscopy localization of Pex11p (10 nm gold) with thiolase (*a*; 5 nm gold) or Pcs60p (*b*; 5 nm gold). (*C*) Flotation of peroxisomal membranes in wild-type and *Δpex13* cells expressing Pex11p. Cell-free extracts were separated on a 20–54% (wt/vol) sucrose gradient and 1.2-ml fractions were collected from the bottom. Localization of Pex11p, as well as peroxisomal thiolase, catalase, MFP (multifunctional protein of peroxisomal β-oxidation), Pcs60p, and mitochondrial fumarase in fractions was monitored by immunoblot analysis and enzyme activity measurements. Wild-type peroxisomal membranes were predominantly found at a density of 1.23 g/ml (fractions 5-7), cosegregating with the peroxisomal matrix proteins. The peroxisomal membranes of the *Δpex13* mutant were predominantly found in lighter fractions at a density of 1.17 g/ml (fraction 11) whereas the peroxisomal matrix proteins remained in the load zone of the gradient. Bars: (*A*, *a* and *b*) 5 μm; (*B*, *a* and *b*) 0.1 μm.

nucleoporins (Moroianu et al., 1995), the SecB/SecA interaction in prokaryotic protein export (Watanabe and Blobel, 1989) and the interaction of a mitochondrial signal recognition factor (MSF) with the Mas37p-Mas70p receptor in the outer mitochondrial membrane (Hachiya et al., 1995). However, the involvement of an SH3-domain containing protein is a novum to protein import pathways.

Interestingly, *Δpex13* cells are defective in the import of PTS1- and PTS2-containing proteins although Pex13p's SH3 domain interacts with PTS1R but not with PTS2R in the two-hybrid system. We also used the NH_2-terminal region of Pex13p (residues 1 to 250) as a bait in the two-hybrid system but found no interaction with PTS2R (data not shown). These data suggest that Pex13p binds PTS1R but not PTS2R raising the question why the absence of Pex13p affects both import pathways. First of all, this result indicates that the PTS1 and PTS2 pathways are not independent but overlapping. This assumption is also supported by the observation that PTS2R function in mammalian cells depends on the presence of PTS1R (Dodt et al., 1995). However, in *S. cerevisiae*, PTS2 dependent import seems to be functional in the absence of PTS1R (Van der Leij et al., 1993) suggesting the existence of a cognate PTS2R receptor in the peroxisomal membrane. If such a PTS2R receptor were to form a heterodimer with Pex13p, absence of either receptor could abolish both import pathways. Another intriguing possibility is that PTS2R could be part of an heteromeric complex, a peroxisomal SRP, targeting of which to the peroxisomal membrane is mediated by binding of one of the partner proteins to Pex13p. In fact, a PTS2R binding peroxin recently has been identified and its interaction with Pex13p is currently under investigation (Kunau, W.H., personal communication).

In conclusion, we have identified Pex13p, an SH3 domain containing peroxisomal membrane receptor for the peroxisomal signal recognition factor PTS1R. Interaction between these proteins is mediated by Pex13p's SH3 domain. The described interaction makes Pex13p a good candidate to be the docking protein for PTS1 dependent peroxisomal protein import. However, whether Pex13p mediates the initial docking of the PTS1 recognition factor or binds it at a later step of a hypothetical import cascade still has to be elucidated. Furthermore, the data presented provide additional evidence that the import pathways for peroxisomal matrix proteins with different signal sequences are not independent but overlapping. Our results also support the notion that peroxisomal matrix and membrane proteins are imported by different pathways.

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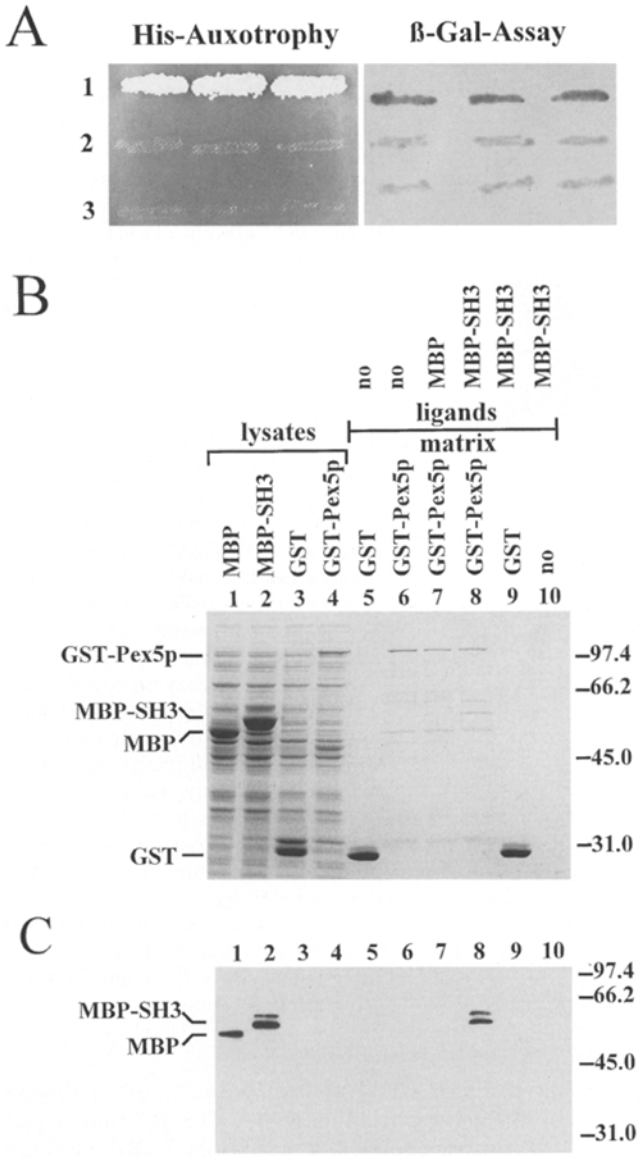

Figure 8. Pex13p physically interacts with the PTS1R (Pex5p) via its SH3 domain. (*A*) Analysis of Pex13p and Pex5p interaction in a two-hybrid system by means of *HIS3* and *lacZ* activation. The SH3 domain of Pex13p was fused to the *GAL4*-DNA–binding domain in pPC97 (pPC-SH3). The entire open reading frame of Pex5p was fused to the *GAL4* activation domain in pPC86 (pPC-Pex5p). Double transformants (*1*) pPC-SH3/pPC-Pex5p (*2*) pPC-SH3/pPC86, and (*3*) pPC97/pPC-Pex5p were selected on SD-plates lacking leucine and tryptophane. To assay His-auxotrophy, cells were replica plated on SD-plates lacking leucine, tryptophane, and histidine but containing 10 mM 3-aminotriazole. Cells were assayed for β-galactosidase activity using a filter assay with X-Gal as substrate. (*B* and *C*) In vitro binding studies using bacterially expressed Pex13p and Pex5p fusion proteins. Fusion proteins GST-Pex5p (Pex5p was fused to the glutathione-*S*-transferase) and MBP-SH3 (the SH3 domain of Pex13p fused to the maltose-binding protein), as well as the unfused GST and MBP were expressed in *E. coli*. First, GST and GST-Pex5p were bound to glutathione sepharose, then, the matrices were incubated with MBP or MBP-SH3 containing extracts as indicated. Whole cell extracts (lanes *1–4*) as well as proteins bound to the gel matrix (lanes *5–10*) were separated on SDS-PAGE, subjected to Coomassie brilliant blue staining (*B*) and immunoblot analysis with antibodies against MBP (*C*). Equal amounts of extracts and eluates were loaded. Molecular weight standards are indicated on the right.

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