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

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Abstract. We identified a *Saccharomyces cerevisiae* peroxisomal membrane protein, Pex13p, that is essential for protein import. A point mutation in the COOH-terminal Src homology 3 (SH3) domain of Pex13p inactivated the protein but did not affect its membrane targeting. A two-hybrid screen with the SH3 domain of Pex13p identified Pex5p, a receptor for proteins with a type I peroxisomal targeting signal (PTS1), as its ligand.

Pex13p SH3 interacted specifically with Pex5p in vitro. We determined, furthermore, that Pex5p was mainly present in the cytosol and only a small fraction was associated with peroxisomes. We therefore propose that Pex13p is a component of the peroxisomal protein import machinery onto which the mobile Pex5p receptor docks for the delivery of the selected PTS1 protein.

SELECTIVE protein sorting in eukaryotic cells requires (*a*) specific signals in the transported proteins, (*b*) cellular components that recognize and bind these signals, and (*c*) membrane-associated protein complexes to carry out the actual translocation step. Two types of targeting signals have been described that function in protein sorting to peroxisomes. The peroxisomal targeting signal type 1 (PTS1)[1], which is present in the majority of the peroxisomal matrix proteins, consists of a tripeptide at the extreme carboxy terminus of a protein and was first identified in luciferase (Gould et al., 1987; Keller et al., 1987). The second peroxisomal targeting signal (PTS2) resides at the NH_2 terminus of a protein, and was first delineated in thiolase (Osumi et al., 1991; Swinkels et al., 1991). Only a limited number of peroxisomal matrix proteins is imported via this signal.

To identify components involved in peroxisomal protein import several groups have set out to isolate peroxisome assembly mutants in various yeast species (Erdmann et al., 1989; Cregg et al., 1990; Gould et al., 1992; Liu et al., 1992; Van der Leij et al., 1992; Elgersma et al., 1993; Nuttley et al., 1993). These peroxisome assembly mutants, formerly described as *pas*, *per*, *peb*, or *pay* have now been collec-

tively renamed *pex* (see accompanying letter in this issue). Among the 15 complementation groups of *pex* mutants in *Saccharomyces cerevisiae*, the phenotypes of the *pex7* (*pas7*) and *pex5* (*pas10*) mutants are very informative since they display selective protein import defects. The *pex7* mutant is specifically disturbed in the import of PTS2-containing proteins, whereas *pex5* does not import PTS1-containing proteins (Van der Leij et al., 1992, 1993; Marzioch et al., 1994; Zhang and Lazarow, 1995). The *PEX5* gene encodes a 69-kD protein with multiple degenerate repeats of a 34–amino acid motif (tetratricopeptide repeat, TPR), linking it to the family of TPR proteins (Goebl and Yanagida, 1991; Van der Leij et al., 1993). In a two-hybrid assay Pex5p specifically interacts with PTS1-containing proteins such as luciferase and peroxisomal malate dehydrogenase (Tabak et al., 1995). A possible homologue of the *PEX5* gene has been cloned from *Pichia pastoris* and more recently from man (McCollum et al., 1993; Dodt et al., 1995; Fransen et al., 1995; Terlecky et al., 1995; Wiemer et al., 1995). Both the human and the *P. pastoris* proteins exhibit binding of PTS1-containing peptides in vitro. Together these results suggest that the *PEX5* gene encodes the receptor for PTS1-containing proteins.

Besides *pex7* and *pex5*, 13 additional *pex* (*pas*) mutants have been isolated in *S. cerevisiae*. The phenotype of most of these mutants is absence of morphologically distinguishable peroxisomes and cytosolic localization of all peroxisomal matrix proteins, which does not reveal any details of the function of the gene product in question, other than that it is essential for peroxisome biogenesis. However, several of the PEX proteins, collectively called peroxins,

1. *Abbreviations used in this paper.* DB, DNA-binding; DHFR, dihydrofolate reductase; NH, hemagglutinin epitope; *pex*, peroxin; PTS1, peroxisomal targeting signal type 1; SH3, Src homology 3; TA, transcriptional activation.

contain domains which are known to be involved in protein–protein interaction, such as Zn-fingers (Pas4p, Pas5p, [Erdmann and Kunau, 1992]), tetratricopeptide repeats (Pex5p [Van der Leij et al., 1993]), or WD-40 (β-transducin) repeats (Pex7p [Marzioch et al., 1994; Zhang and Lazarow, 1995]). Further analysis of these domains could, therefore, shed light on protein–protein interactions between components of the peroxisomal protein import machinery and help to identify novel partners of peroxins.

In this paper, we report the cloning of the *PEX13* (*PAS20*) gene and show that it encodes a peroxisomal membrane protein with an Src-homology 3 (SH3) domain in its carboxy-terminal region. SH3 domains are composed of ~60 amino acids and form structurally independent modules allowing their incorporation in various regions of the protein (Musacchio et al., 1992). The presence of these domains in a wide variety of unrelated proteins, many of which are involved in signal transduction, led to the proposal that this motif plays an important role in protein–protein interactions (Musacchio et al., 1994; Cohen et al., 1995; Pawson, 1995). The small size of the SH3 domain and its ability to fold independently allowed the determination of its three-dimensional structure, both by solution NMR and X-ray crystallography (for review see Kuriyan and Cowburn, 1993; Musacchio et al., 1994). This revealed that SH3 domains form a hydrophobic binding pocket, consisting of 5–6 β sheets arranged in anti-parallel configuration. Screening of random peptide libraries led to the identification of proline-rich peptides as ligands for SH3 domains (for review see Musacchio et al., 1994). However, the natural partners of most SH3 domain–containing proteins remain to be discovered. Here we report the identification of Pex5p as a ligand for the SH3 domain of Pex13p and show that the SH3-Pex5p interaction is direct. We also show that Pex5p is mainly a cytosolic protein and we discuss a model for PTS1 protein import.

Materials and Methods

Strains and Culture Conditions

Strains used in this study were *S. cerevisiae*: BJ1991 (*MAT α, leu2, trp1, ura3-52, prb1-1122, pep4-3*); HR3 (*MAT a, leu2, his4, URA3::TRP1*) (used for back-crossing of *pex13.1*); *pex13Δ* (*PEX13::LEU2, MAT α, leu2, trp1, ura3-52, prb1-1122, pep4-3*); *pex13.1* (Y5)(*pex13, MAT a, leu2, trp1, ura3-52, prb1-1122, pep4-3*); *pex13.2* (Y56)(*pex13*E₃₂₀K, *MAT a, leu2, trp1, ura3-52, prb1-1122, pep4-3*); *pex13.1-3a* (*pex13, MAT α, his4, leu2, ura3, trp1*) (used for cloning of the wild-type gene); *pex13.1-4a* (*pex13, MAT a, his4, ura3, leu2*) (used for crossing with *pex13Δ*); HF7c (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, gal4-542, gal80-538, LYS2::GAL1*ᵤₐₛ-*GAl1*ₜₐₜₐ-*HIS3 URA3::GAL4*₁₇ₘₑᵣₛ₍ₓ₃₎-*CYC1*ₜₐₜₐ-lacZ) (for 2-hybrid screen); PCY2 (*MATα Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801, his3-Δ200, trp1-Δ63, leu2, ade2-101*) (for 2-hybrid assays); and *Escherichia coli* DH5α (*recA, hsdR, supE, endA, gyrA96, thi-1, relA1, lacZ*).

All media and growth conditions for *S. cerevisiae* are described by Elgersma et al. (1993).

Cloning Procedures

Pex13.1-3a was used for transformation with a genomic single-copy library (YCp50) and a genomic multi-copy library (YEp13) (Nasmyth and Tatchell, 1980; Rose et al., 1987). From each library one clone was isolated that grew on oleate. Restriction analysis of the complementing plasmids of these clones revealed that the inserts of the YCp50-based plasmid (p20.4) and the YEp13-based plasmid (p20.2) were ~12 kb, and the inserts almost

entirely overlapped. A Sau3A subclone (p20.7) of p20.4 with a 1,775 bp insert was still able to complement *pex13.1-3a*. Both strands of this insert were entirely sequenced using the T7 DNA polymerase sequencing kit (Pharmacia Biotech, Uppsala, Sweden).

The insert of p20.7 was cloned between the EcoRI and HindIII sites of pUC19 from which the NdeI and NarI sites had been removed. A major part of the *PEX13* gene was then replaced by the *LEU2* gene, by ligating the *LEU2* gene between the KpnI–NdeI sites of the *PEX13* gene. This disruption resulted in the deletion of the *PEX13* gene starting from amino acid 69. The insert of this plasmid (p20.12) was amplified by PCR using universal sequencing primers, and this DNA was used to transform BJ1991 cells. Leu⁺ transformants were analyzed by Southern blotting for correct integration in the *PEX13* gene.

A BamHI site was introduced just in front of the start codon by PCR on p20.7 with the oligonucleotides 5'-TTTGGATCCATGTCATCCA-CAGCAGTACCAC-3' and 5'-TTTCTGCAGGTGTGTACGCGTTTCA-TCATC-3' as primers. This PCR fragment was cut with BamHI and PstI and cloned between the BamHI and PstI sites of pUC19 (p20.18). The BamHI-HindIII insert of p20.18 containing the entire *PEX13* gene was placed behind the NH-tag (MQDLPGNDNSTAGGT), under the control of the CTA1 promoter (NH-PEX13/CEN, p20.19; NH-PEX13/2 μm, p20.20) (expression plasmids are described in Elgersma et al. [1993]). The PEX13ΔSH3 was constructed by digesting p20.18 with BamHI and SspI and by ligating this fragment in the BamHI–SphI (blunt) sites of the CTA1 promoter expression plasmids. This resulted in the replacement of the COOH-terminal 28 amino acids by the dipeptide Gln-Ala. For expression in mammalian cells, NH-PEX13 was subcloned in pcDNA (Invitrogen, San Diego, CA), under the control of the CMV promoter.

The *pex13.2* allele was rescued by the gap-repair method (Rothstein, 1991). Two gaps were introduced in the *PEX13* gene by cutting plasmid p20.11 (containing a 1,950-bp insert with the *PEX13* gene) with PstI-SalI (bp −362 to +429) or with KpnI (bp +204 to +739). The vector bands were gel purified and transformed to the *pex13.2* mutant. 85% of the Ura⁺ transformants obtained with the PstI-SalI-digested plasmid were able to grow on oleate, whereas only 35% of the transformants obtained with the KpnI-digested plasmid grew on oleate. From this we concluded that the actual mutation was further downstream of base +739. Therefore, the plasmids of two transformants that were unable to grow on oleate, were rescued and sequenced from the 5' end until the BstBI site. The remainder of the gene was replaced by the authentic wild-type *PEX13* gene.

Antibodies

The SH3 domain of PEX13 (amino acids 284-386) was obtained by PCR using the oligonucleotides 5'-TTTGGATCCACTAAACTACAGACCT-CTGG-3' and 5'-TTTCTGCAGGTGTGTACGCGTTTCATCATC-3' as primers. The PCR product was cloned in frame with the DHFR gene of pQE13 (Qiagen, Inc., Chatsworth, CA) (p20.24). The resulting 6× His-tagged DHFR-SH3 fusion protein was subsequently expressed in *E. coli* SG13009 and purified by Ni-chelating chromatography under denaturing conditions according to the manual provided by Qiagen. The partially purified protein was subjected to SDS-PAGE; the DHFR-SH3 band was excised, eluted, and used to immunize rabbits.

Similarly, thiolase (amino acids 17-215) was fused in-frame to the 6× His-tag and expressed in *E. coli*. Purification and immunization was done as described above.

The antibody raised against Pex5p (amino acids 45-525) and Pat1p (peroxisomal ABC transporter) will be described elsewhere. The NH-antibody was kindly provided by P. van der Sluijs (University of Utrecht, Utrecht, The Netherlands), and ISP42-antibody was a kind gift of M. Meijer (University of Amsterdam, Amsterdam, The Netherlands).

Western Blot Analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose filters and processed as described by Distel et al. (1992), except that 2% nonfat dry milk was used as a blocking agent. Antigen-antibody complexes were visualized with goat anti–rabbit IgGs conjugated to alkaline phosphatase as recommended by the manufacturer (Boehringer Mannheim GmbH, FRG).

Ligand Blot Assay

The *PEX5* coding sequence was cloned into pBluescriptII KS and transcripts were generated from linearized DNA using the T7 RNA poly-

merase (Promega, Madison, WI). Radioactively labeled Pex5p was produced in vitro in a reticulocyte lysate translation reaction (Promega) using a mixture of ^{35}S-labeled methionine and cysteine (Amersham International, Little Chalfort, UK). The 6×His-tagged DHFR-SH3 fusion protein was purified as described above. As a control we purified the nonfused 6xHis-tagged DHFR protein using the empty pQE13 vector. The different His-tagged proteins were separated on a 12% SDS-polyacrylamide gel and blotted onto a PVDF membrane (BioRad Laboratories, Richmond, CA). One part of the membrane was stained with Poinceau S to confirm proper transfer of the proteins, the other part of the membrane was blocked in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween-20) with 10% nonfat dry milk, 100 mM methionine, and 0.02% NaN$_3$ for 2 h at 4°C. The blocked membrane was then incubated overnight at 4°C in 3× diluted blocking mixture containing 100 μl of in vitro translation product, washed once in TBS, and exposed to XAR-5 film (Eastman Kodak, Rochester, NY) to visualize bound Pex5p.

Subcellular Fractionation and Nycodenz Gradient Analysis

Subcellular fractionations of oleate-grown cells and Nycodenz gradients were performed as described by Elgersma et al. (1995). Gradient fractions were used for enzyme measurements. The remainder of the fractions was precipitated with 2 vol of 10% trichloric acetic acid (TCA) in the presence of 10 μg/ml of cytochrome C oxidase as carrier, and analyzed by SDS-PAGE and Western blotting.

Carbonate Extraction

An organellar pellet (25,000 g) prepared from wild-type cells was resuspended in 100 mM Tris-buffer (pH 8.0). After freezing and centrifugation (233,000 g for 60 min), the pellet was extracted with 100 mM Tris-buffer (pH 8.0), 1 M NaCl. After another centrifugation step, the pellet was extracted with 100 mM sodium carbonate (pH 11.5). This was centrifuged once more and equivalent portions of pellet (P) and supernatant fractions (S) were layered on an SDS-polyacrylamide gel, blotted onto nitrocellulose, and probed with different antibodies. All extraction steps were performed at 4°C, in the presence of protease inhibitors (1 mM phenylmethylsulfonylfluoride [PMSF] and 2 μg/ml of chymostatin, leupeptin, antipain, and pepstatin).

Alternatively, a freshly prepared organellar pellet was directly used for carbonate extraction, and further treated as described above. This procedure gave the same result with respect to the distribution of the membrane proteins.

Digitonin Titration

Cells grown on oleate were converted to spheroplasts and progressively permeabilized with digitonin according to Zhang and Lazarow (1995), except that digitonin incubations were performed at 30°C for 10 min. After quickly cooling the samples on ice-water, they were spun for 5 min at 11,000 g at 4°C. The supernatants were collected and used for SDS-PAGE or enzyme measurements.

Microinjection and Immunofluorescence

Cultured, primary skin fibroblasts used for microinjection experiments were derived from a control subject HENG90AD. Cells were grown and microinjected essentially as described (Motley et al., 1994), except that the DNA was injected at a concentration of 0.25 mg/ml. Cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (pH 7.5) for 20 min at room temperature and further processed as described by Motley et al. (1994). Mouse monoclonal anti-human catalase (Wiemer et al., 1992) was visualized with Cy3-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, CA), and rabbit polyclonal anti-SH3 was visualized with FITC-conjugated goat anti–rabbit IgG (Sigma Chemical Co., Poole, UK).

Two-Hybrid Methodologies

To construct the GAL4(DB)-DHFR-SH3 fusion protein, p20.24 was digested with BamHI, the ends were filled in with Klenow polymerase followed by SpeI digestion. The fragment encompassing DHFR-SH3 was then inserted in the SalI site, the ends of which were made blunt with Klenow polymerase, and the SpeI site of pPC86DB. pPC86DB was made by

inserting the KpnI–SacI fragment of pPC97 (Chevray and Nathans, 1992) encompassing the *ADC1* gene promoter and GAL4 DNA-binding (DB) domain in pPC86 digested with KpnI and SacI. The GAL4(DB)-DHFRΔSH3 was constructed by cutting p20.24 with BamHI and SspI, filling the ends with Klenow polymerase and ligating this fragment between the blunted SalI and SpeI sites of pPC86DB. The GAL4(TA) fusion library contains genomic fragments (mean insert size ∼1 kb) fused to the GAL4 transcription activation (TA) domain in plasmid pACT (Durfee et al., 1993; kindly provided by S. Elledge, Baylor College of Medicine, Houston, TX). Strain HF7c expressing the GAL4(DB)-DHFR-SH3 fusion protein was transformed with the genomic library according to the method of Schiestl and Gietz (1989). Approximately 6.10^5 transformants were plated on minimal glucose medium lacking tryptophan, leucine, and histidine, and His$^+$ colonies were picked after 3 d of incubation at 30°C. The library plasmids of these colonies were rescued, transformed to strain PCY2 expressing GAL4(DB)-DHFR-SH3, and scored for lacZ expression using a filter assay for β-galactosidase activity (Breeden and Nasmyth, 1985). Clones that activated both reporter genes were further analyzed to determine whether the library encoded proteins interacted specifically with SH3 portion of the GAL4(DB)-DHFR-SH3 fusion protein.

Enzyme Assays

Published procedures were used for measuring 3-hydroxyacyl-CoA dehydrogenase activity (an activity of multifunctional enzyme) (Wanders et al., 1990), succinate dehydrogenase activity (Munujos et al., 1993), catalase activity, (Lücke, 1963), and PGI activity (Bergmeyer et al., 1983).

Electron Microscopy

Oleate-induced cells were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Ultra-thin sections were prepared as described by Gould et al. (1990).

Results

Cloning of the PEX13 Gene and Analysis of the Protein Sequence

The *pex13.1* (*pas20.1*) mutant which was previously isolated using a positive selection procedure (Elgersma et al., 1993), was used to clone the corresponding *PEX13* wild-type gene by functional complementation with a genomic library. Nucleotide sequencing of the smallest complementing insert (1,775 bp) revealed an open reading frame of 1,158 bp encoding a protein with a calculated mass of 42.7 kD (Fig. 1 A). More recently, this gene has also been sequenced in the Genome Sequencing Project of *S. cerevisiae* (accession number L94701).

To construct a *pex13* disruption mutant (*pex13Δ*), we replaced the *PEX13* gene by the *LEU2* gene. The *pex13Δ* mutant was crossed with the original (back-crossed) *pex13.1* mutant. The resulting diploids were unable to grow on oleate, indicating that the authentic *PEX13* gene, and not a suppressor, had been cloned and deleted. Furthermore, *pex13Δ* regained its ability to grow on oleate when transformed with the *PEX13* gene, indicating that the growth defect of the *pex13Δ* mutant on oleate is reversible.

Examination of the deduced amino acid sequence of Pex13p revealed that it contained three distinct domains. The NH$_2$-terminal part (amino acids 69-142) of the protein was rich in glycine, tyrosine, asparagine, and serine. A similar type of domain was present in proteins of the nuclear pore complex, some of which participate directly in nuclear transport (Rout and Wente, 1994). The glycine/tyrosine stretch was followed by three rather hydrophobic
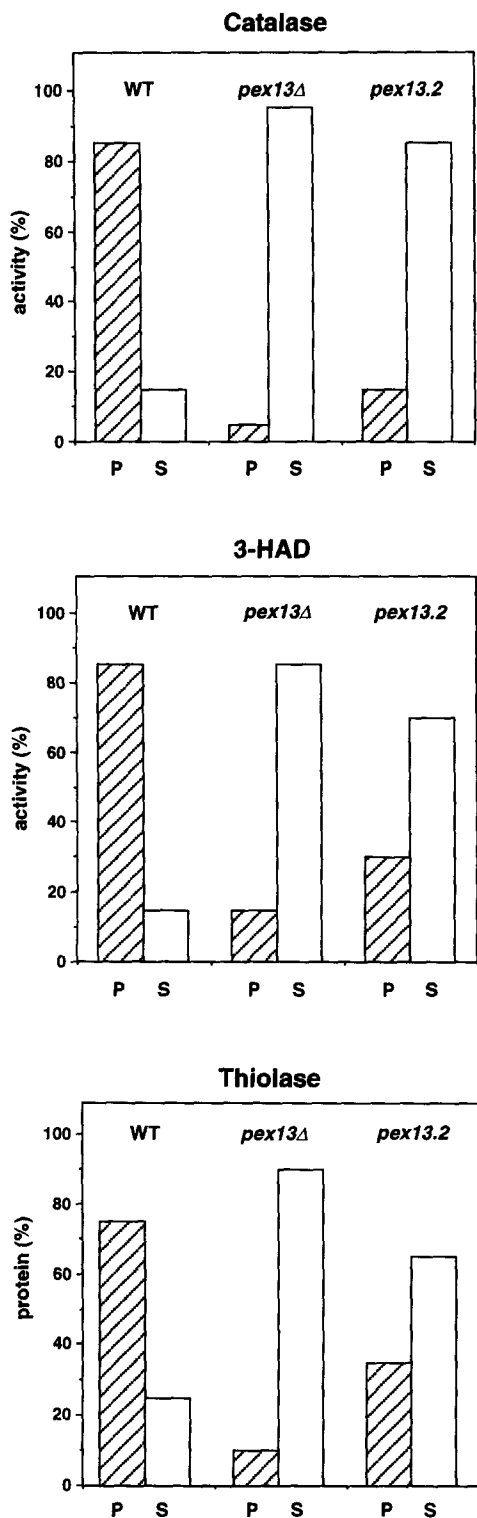
A

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-228                TAAATACATTAATTACCAAAAAAAATGATATAATAAAGTCTTCTTTTA -181
-180 TCTGATTAATGACACCTTTTTCAGCGGGCCAAATTTTTTTTATTTCCGAAGCCCGCCGGA -121
-120 CTAATATTAATTATATCAGATAGTTGGTGAGCAAACGCATGTGGAAATGAACAACAATTT  -61
 -60 CGAAAGTGCATATACTATCTATAAATATCAAGGGGATTCTATACTATAACAATACCTGCG   -1
   1 ATGTCATCCACAGCAGTACCACGACCTAAACCTTGGGAGACCAGTGCTTCGTTAGAGGAG   60
   1   M   S   S   T   A   V   P   R   P   K   P   W   E   T   S   A   S   L   E   E    20
  61 CCACAGCGTAATGCTCAGTCTTTAAGTGCGATGATGACCTCGAATCAGCAGGATTCCAGG  120
  21   P   Q   R   N   A   Q   S   L   S   A   M   M   T   S   N   Q   Q   D   S   R    40
 121 CCAACTGAAGAAAGCAATAACAGTAATAGTGCAAGTGAGTCTGCTCCCGAAGTTTTGCCG  180
  41   P   T   E   E   S   N   N   S   N   S   A   S   E   S   A   P   E   V   L   P    60
 181 CGGCCTGCGGCACTTAACTCCTCTGGTACCTACGGCGAATCGAATACAATACCGGGAATA  240
  61   R   P   A   A   L   N   S   S   G   T   Y   G   E   S   N   T   I   P   G   I    80
 241 TACGGTAATAGCAATTATGGGATACCCTATGATAATAATCCGTATAGTATGAACTCTATA  300
  81   Y   G   N   S   N   Y   G   I   P   Y   D   N   N   P   Y   S   M   N   S   I   100
 301 TACGGAAACAGCATAGGGAGGTATGGCTATGGAGGATCGTACTATGGCAACAATTATGGA  360
 101   Y   G   N   S   I   G   R   Y   G   Y   G   G   S   Y   Y   G   N   N   Y   G   120
 361 TCGTTTTATGGAGGAGGGTACGGTGCAGGAGCGGGATATGGAATGAATAATGGAAGTGGA  420
 121   S   F   Y   G   G   G   Y   G   A   G   A   G   Y   G   M   N   N   G   S   G   140
 421 CTAGGTGAGTCGACGAAGGCCACCTTTCAGTTAATAGAAAGTTTGATAGGCGCTGTTACT  480
 141   L   G   E   S   T   K   A   T   F   Q   L   I   E   S   L   I   G   A   V   T   160
 481 GGATTTGCACAAATGCTGGAATCTACTTATATGGCCACACACAACTCGTTTTTCACCATG  540
 161   G   F   A   Q   M   L   E   S   T   Y   M   A   T   H   N   S   F   P   T   M   180
 541 ATATCCGTCGCAGAACAGTTTGGTAATTTGAAGGAAATGCTAGGTTCATTTTTTGGTATC  600
 181   I   S   V   A   E   Q   F   G   N   L   K   E   M   L   G   S   F   F   G   I   200
 601 TTTGCTATAATGAAGTTTCTTAAAAAAATACTCTATCGTGCTACTAAGGGGAGACTAGGA  660
 201   F   A   I   M   K   F   L   K   K   I   L   Y   R   A   T   K   G   R   L   G   220
 661 ATACCACCAAAGAATTTCGCAGAATCTGAAGGAAGCAAAAATAAACTAATTGAAGATTTT  720
 221   I   P   P   K   N   F   A   E   S   E   G   S   K   N   K   L   I   E   D   F   240
 721 CAAAAGTTTAATGATAGTGGTACCATAAATTCTAATGAGAAAGCCACAAGAAGGAAAATT  780
 241   Q   K   F   N   D   S   G   T   I   N   S   N   E   K   A   T   R   R   K   I   260
 781 TCATGGAAACCGCTACTATTTTTTTTTGATGGCAGTATTTGGTTTTCCATATCTACTAAAC  840
 261   S   W   K   P   L   L   F   F   L   M   A   V   F   G   F   P   Y   L   L   N   280
 841 AAATTTATTACTAAACTACAGACCTCTGGAACCATACGAGCATCGCAAGGAAATGGTAGC  900
 281   K   F   I   T   K   L   Q   T   S   G   T   I   R   A   S   Q   G   N   G   S   300
 901 GAGCCTATTGATCCTTCGAAGTTAGAATTTGCAAGAGCGTTATATGATTTTGTTCCAGAA  960
 301   E   P   I   D   P   S   K   L   E   F   A   R   A   L   Y   D   F   V   P   E   320
 961 AACCCAGAGATGGAAGTTGCTTTAAAAAAGGGCGACTTGATGGCAATTTTGAGTAAGAAA 1020
 321   N   P   E   M   E   V   A   L   K   K   G   D   L   M   A   I   L   S   K   K   340
1021 GACCCTCTTGGGAGGGATTCTGACTGGTGGAAAGTGAGGACAAAGAACGGCAATATTGGC 1080
 341   D   P   L   G   R   D   S   D   W   W   K   V   R   T   K   N   G   N   I   G   360
1081 TACATTCCGTATAACTATATTGAGATCATAAAAAGACGGAAGAAAATTGAGCATGTTGAT 1140
 361   Y   I   P   Y   N   Y   I   E   I   I   K   R   R   K   K   I   E   H   V   D   380
1141 GATGAAACGCGTACACACTAGTGCATCAATATTTGCACACATATATTCGCATATATATAT 1200
 381   D   E   T   R   T   H   *                                             387
1201 AGTAAAATCTAAAGTAATGTAAATATTCCAGGGAGAATCAGCAGAAACGAGACGAAGTAC 1260
1261 TTCTTAACAAAAAGCCAGATAACGGTGCAAAAACACATTATATTCTCCATCTTGAAAACA 1320
1321 CAATCTCCTACGTACACATATGCGTATTTATTTTTATATGAATGAAACGCCTATGGATAT 1380
1381 ATATATGGGAATATAGACATTCACAATTTCAGGACAATTGTGATATGGCAATATCCAAGG 1440
1441 CCTGCGGTGCTGGGCTTCGCTTTTTTTCACCAACAAAATGAGATGGACAAGTTTATCATA 1500
1501 GCAAAGAAACAATAAGCAGATTACATAAAGTCGTCATCACCGAATTC              1547
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B

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loop:                    RT                   N-src          distal
consensus:     ALYDY      Eh      GD   hh          WW          GahP    Yh
                 F F       D       E               Y                   F

Sc PEX13     FARALYDFVPENPEMEVALKKGDLMAILSKKDPLGR   DSDWWKVRTKN  GNIGYIPYNYIEII
C csrc       TFVALYDYESRT ETILSFKKGERLQIVNNT         EGDWWLAHSLTTGQTGYIPSNYVAPS
C speca      LVLALYDYQEKS PREVTMKKGDILTLLNST          NKDWWKVEV  NDRQGFVPAAYVKKL
H fyn        LFVALYDYEART EDILSFHKGEKFQILNSS          EGDWWEARSLTTGETGYIPSNYVAPV
H csk        ECIAKYNFHGTA EQILPFCKGDVLTIVAVTK         DPNWYKAKNKV GREGIIPANYVQKR
H PLCγ       AVKALFDYKAQR EDELTFIKSAIIQNVEKQ          EGGWWRGDYGGKKQL WFPSNYVEEM
RasGAP       RVRAILPYTKVPDTDEISFLKGDMFIVHNE           EDGWMWVTNLRTDEQGLIVEDLVEEV
H PI3K       QYRALYDYKKER EEDIDLHLGDILTVNKGS/..../EIGWLNGYNETTGERGDFPGTYVEYI
Ce Sem5C     FVQALFDFNPQE SGELAFKRGDVITLINKD          DPNWWEGQLNNRR GIFPSNYVCPY
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Figure 1. PEX13 encodes an SH3 domain–containing protein. (A) Sequence of the PEX13 gene and protein. The start codon is underlined, and the stop codon is marked by an asterisk. Putative transmembrane segments in Pex13p are also underlined, and the SH3 domain is highlighted by boldface type. These sequence data are available from GenBank/EMBL/DDBJ under accession number U17246. (B) Alignment of SH3 domain amino acid sequences. The positions of the RT, N-Src, and distal loops are indicated. Symbols used for the consensus are h, hydrophobic amino acid; a, aromatic amino acid. Residues that fit the consensus are highlighted by boldface type. The Pex13p SH3 is shown in the top line. The E_{320} mutation in the RT loop of Pex13.2p is underlined. SH3 domains were selected because their 3D-structure has been resolved. The sequences were taken from the SwissProt Database. Prefixes: Sc, S. cerevisiae; Ce, C. elegans; H, human; C, chicken.

stretches. Using the algorithm of Klein et al. (1985), one putative transmembrane domain was predicted in this region (amino acids 264-280). The algorithm of Rao and Argos (1986) predicted an additional transmembrane domain (amino acids 151-166). The most striking feature of the primary structure of Pex13p was the Src-homology 3 (SH3) domain in the carboxy terminus of the protein. The SH3 domain of Pex13p differs from most other SH3 domains by the presence of an additional amino acid in the RT loop (Fig. 1 B).

The pex13Δ Mutant Is Disturbed in Import of PTS1- and PTS2-containing Proteins

The phenotype of pex13Δ was studied by subcellular fractionation. We found that the import of matrix enzymes containing a PTS1 or PTS2 was disturbed in pex13Δ (Fig. 2). In addition, no peroxisomes were found in pex13Δ by electron microscopy. Despite the absence of detectable peroxisomes, a considerable amount of the peroxisomal membrane proteins Pat1p (peroxisomal ABC transporter,

Catalase

activity (%)

| | WT | | pex13Δ | | pex13.2 | |

100
80
60
40
20
0

P S   P S   P S

3-HAD

activity (%)

| WT | pex13Δ | pex13.2 |

100
80
60
40
20
0

P S   P S   P S

Thiolase

protein (%)

| WT | pex13Δ | pex13.2 |

100
80
60
40
20
0

P S   P S   P S

Figure 2. Distribution of peroxisomal matrix enzymes in wild-type (WT), *pex13Δ*, and *pex13.2* cells. The organellar pellet (*P*) and cytosol (*S*) fraction obtained after subcellular fractionation of oleic-acid induced strains were assayed for different marker enzymes. Catalase and 3-hydroxyacyl-CoA dehydrogenase (3-HAD) (an activity of multi-functional enzyme) as markers for PTS1-mediated protein import, thiolase as marker for PTS2-mediated protein import. Thiolase was quantified after Western blotting. The bars indicate the average of at least two experiments. The recoveries of the enzymes varied between 80 and 120%.

Hettema et al., 1996) and Pas21p (Elgersma, Y., unpublished data) was present in the organellar pellet fraction obtained from *pex13Δ* cells, suggesting that peroxisomal remnants might still be present in these cells (data not shown).

Subcellular fractionation studies revealed a difference between *pex13Δ* and one of the two isolated *pex13* mutants: in *pex13Δ* 5–15% of PTS1- or PTS2-containing proteins was associated with the organellar pellet fraction, whereas in *pex13.2*, these numbers were consistently higher (15–35%) (Fig. 2). Occasionally, we observed small peroxisomes in *pex13.2* by electron microscopy, that could be immunocytochemically labeled using anti-thiolase antibodies (Fig. 4 *C*). These results indicate that *pex13.2* has a residual level of peroxisomal protein import suggesting the presence of a missense mutation in the *PEX13.2* gene. We therefore mapped the position of the *pex13.2* mutation, using the gap-repair method (Rothstein, 1991; see Materials and Methods). This procedure indicated that the mutation was in the 3' end of the mutant gene. The plasmids bearing the *pex13.2* allele were rescued and the 3' end of the mutant gene was sequenced. We found a single mutation, resulting in the $Glu_{320} \rightarrow Lys$ substitution in the RT loop of the SH3 domain (see Fig. 1 *B*). To confirm that this mutation was responsible for inactivation of the *PEX13.2* gene, we replaced the unsequenced 5' section of the *PEX13.2* gene with the corresponding part of the wild-type gene. This "hybrid" gene could not complement the *pex13Δ* mutant, indicating that the $Glu_{320} \rightarrow Lys$ mutation indeed inactivated the Pex13p.

Pex13p Is a Peroxisomal Membrane Protein

The COOH terminus of Pex13p (residues 284-386) was expressed in *Escherichia coli* and used to raise rabbit antibodies. This antibody reacted with only one polypeptide of the predicted size in immunoblots of extracts of wild-type cells which was not present in extracts of *pex13Δ* cells (Fig. 3 *A*).

The localization of Pex13p was studied by subcellular fractionation. Western blot analysis of the fractions revealed that Pex13p was exclusively present in the organellar pellet obtained from wild-type cells (Fig. 3 *B*). Further fractionation of this pellet on a Nycodenz gradient showed that Pex13p colocalized with a peroxisomal marker enzyme (multifunctional enzyme), indicating that Pex13p is associated with peroxisomes (Fig. 3 *C*).

The subcellular localization of Pex13p was also analyzed by electron microscopy using a tagged (NH epitope) version of Pex13p, expressed under the control of the *PEX13* promoter. This NH-tagged protein could fully restore the growth defect of the *pex13Δ* mutant on oleate, indicating that the tag did not interfere with protein function (data not shown). However, expression was too low to detect the protein by immunoelectron microscopy. Therefore, we placed NH-PEX13 under the control of the catalase (CTA1) promoter. This plasmid could partially restore the growth defect of *pex13Δ* on oleate, and normal peroxisomes were present as judged by electron microscopy (Fig. 4 *B*). In these cells Pex13p could easily be detected with both the NH antibodies and the antibodies against Pex13p. We observed specific labeling of peroxisomal membranes (Fig. 4, *A* and *B*).
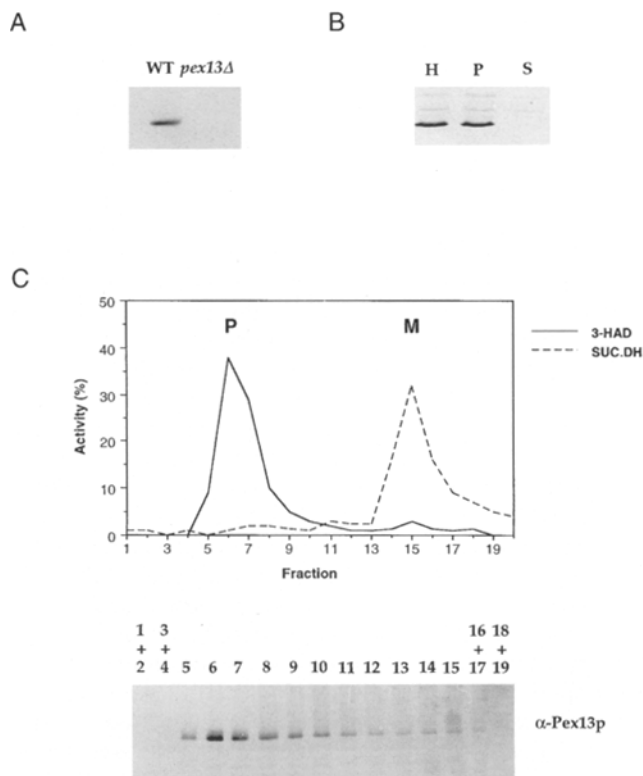
Figure 3. Subcellular localization of Pex13p. (A) Western blot of TCA-lysates of wild-type (wt) and pex13Δ cells grown on oleate. The membrane was probed with anti-Pex13p antibody. (B) Subcellular fractionation of wild-type cells. Comparable volumes of the homogenate (H), pellet (P), and supernatant (S) were used for Western blotting with anti-Pex13p. (C) Nycodenz density gradient of the organellar pellet obtained from wild-type cells. Activity (%) is the relative amount of activity in each fraction compared to the total amount of activity which was present in the organellar pellet. Fraction 1 corresponds to the bottom fraction, while fraction 20 is the top fraction. Succinate dehydrogenase (SUC.DH) was used as mitochondrial marker enzyme, and 3-hydroxyacyl-CoA dehydrogenase (3-HAD) was used as peroxisomal marker enzyme. Equal volumes of the gradient fractions were analyzed by SDS-PAGE and Western blotting using anti-Pex13p.

The primary sequence of Pex13p strongly suggests that Pex13p is an integral membrane protein. This was verified by carbonate-extraction analysis, a treatment that removes peripheral but not integral proteins from membranes (Fujiki et al., 1982). A crude organellar (25,000 g) pellet derived from oleate-grown wild-type cells was prepared and used for subsequent extractions with Tris (pH 8.0), Tris (pH 8.0)/1 M NaCl, and sodium carbonate (pH 11.5) (Fig. 4 E). Pex13p could not be extracted with Tris (pH 8.0) or Tris (pH 8.0)/1 M NaCl but reproducibly showed a bimodal distribution with respect to carbonate treatment: ~50% was resistant to carbonate extraction. A similar distribution was also observed for Pat1p, a peroxisomal ABC transporter which has 6–7 membrane-spanning domains (Hettema et al., 1996), and Pas21p which has one putative transmembrane region (Elgersma, Y., unpublished data). The matrix enzyme thiolase was partially released with Tris (pH 8.0), whereas the remainder could be released with Tris (pH 8.0)/1 M NaCl. The mitochondrial integral

membrane protein Isp42p (used as a control) was present in all the nonextractable pellet fractions. These results strongly suggest that Pex13p is an integral peroxisomal membrane protein.

SH3 domains are often involved in targeting proteins to specific subcellular locations, either to membranes by binding to integral membrane proteins, or to the cytoskeleton by binding to cytoskeletal proteins. The results obtained with the carbonate-extraction procedure already indicated that the SH3 domain of Pex13p is probably not responsible for membrane targeting. This was further substantiated by expressing the PEX13.2 open reading frame (Pex13p-SH3^{E320K}) tagged at its NH$_2$ terminus with the NH epitope in wild-type cells. Immunoelectron microscopic analysis of the transformed cells showed that targeting of the mutant protein was unaffected, since the observed labeling was similar to the labeling obtained with cells expressing wild-type NH-Pex13p (compare Fig. 4, A and D). These results indicate that the SH3 domain is required for Pex13p function, but not for directing Pex13p to the peroxisomal membrane. They also support the conclusion that Pex13p is itself an integral membrane protein.

The SH3 Domain of Pex13p Faces the Cytosol

We expressed NH-tagged Pex13p in human fibroblasts to study both the targeting and the topology of Pex13p. A clear, punctate immunofluorescence pattern was detected with antibodies directed against the SH3 domain (Fig. 5 A). The pattern coincided with the pattern obtained using anti-catalase antibodies (Fig. 5, A and B). No signal was observed with anti-SH3 antibodies in control cells, indicating that these antibodies do not cross react with human proteins (Fig. 5 A). These results indicate that NH-Pex13p is targeted to peroxisomes in higher eukaryotic cells.

The proper targeting of Pex13p in human fibroblasts and the availability of antibodies directed against the SH3 domain enabled us to determine the topology of this domain using the differential permeabilization procedure described by Motley et al. (1994). The plasma membrane of cells expressing NH-Pex13p was selectively permeabilized with low concentrations of digitonin. With anti-SH3 antibodies, a punctate staining was observed (Fig. 5 C), whereas only diffuse background labeling was manifest with antibodies directed against catalase (a peroxisomal matrix protein)(Fig. 5 D). When Triton X-100 was included in the permeabilization buffer, both the plasma membrane and the peroxisomal membrane were permeabilized. Under these conditions particulate catalase could be detected (Fig. 5 B). The fact that punctate labeling could be detected after permeabilization of only the plasma membrane but not the peroxisomal membrane indicates that the SH3 domain faces the cytosol. Considering the proper targeting of yeast Pex13p in fibroblasts, we interpret the data as support for the cytosolic orientation of the SH3 domain in yeast. The NH$_2$ terminus of Pex13p either sticks into the peroxisomal matrix or faces the cytosol depending on the number of transmembrane domains present in the protein.

Isolation and Characterization of Proteins Interacting with the SH3 Domain

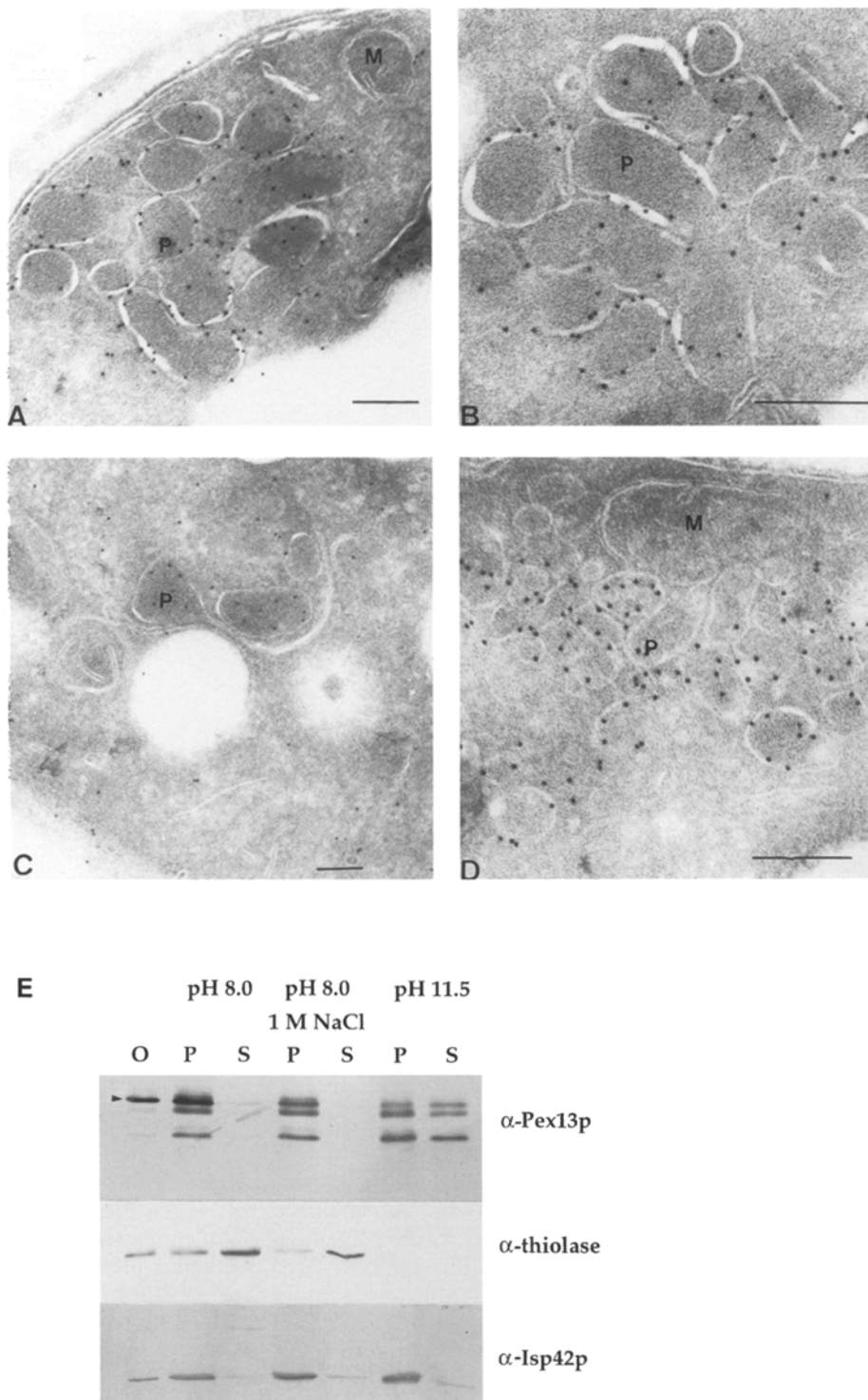To identify proteins that are capable of interacting with

Figure 4. Pex13p is tightly associated with peroxisomal membranes. Electron microscopic analysis of the intracellular localization of Pex13p in: (*A*) Wild-type cells expressing NH-PEX13 from the CTA1 promoter (anti-NH), (*B*) *pex13Δ* cells expressing NH-PEX13 from the CTA1 promoter (anti-NH), (*C*) *pex13.2* cells (anti-thiolase) and (*D*) wild-type cells expressing NH-PEX13.2 from the CTA1 promoter (anti-NH). All cells were induced on oleate. (*E*) Resistance of Pex13p to carbonate extraction. A portion of the organellar pellet was extracted with Tris-buffer (pH 8.0). After centrifugation the pellet was extracted with Tris-buffer (pH 8.0)/1 M NaCl, and after centrifugation, the resulting pellet was extracted with 100 mM sodium carbonate (pH 11.5). This was centrifuged once more, and equivalent portions of pellet (*P*) and supernatant (*S*) were layered on an SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with the antibodies indicated. *O* is start material of the organellar pellet. The arrow indicates the full-length Pex13p. The faster migrating bands are breakdown products of Pex13p. Bar, 0.2 μm.

the SH3 domain of Pex13p, we applied the yeast two-hybrid screen (Fields and Song, 1989). A fusion protein consisting of the GAL4 DNA-binding (DB) domain (residues 1-147) and the PEX13 SH3 domain (residues 284-386), separated by mouse dihydrofolate reductase (DHFR) as a spacer, was used as "bait" in this screen.

The GAL4(DB)-DHFR-SH3 fusion protein was unable to activate transcription in a yeast reporter strain expressing only the GAL4 transcriptional activation (TA) domain (Table I). To identify proteins that interact with the SH3 domain of Pex13p, we transformed the reporter strain expressing the GAL4(DB)-DHFR-SH3 fusion protein with a DNA library in which yeast genomic DNA fragments were fused to the GAL4(TA) domain (Durfee et al., 1993). From approximately 6.10^5 double transformants (representing five times the genome size), 19 colonies both grew in media lacking histidine and activated the lacZ reporter. For additional controls, the recovered plasmids of these 19 clones were transformed to strains expressing either GAL4(DB) or GAL4(DB)-DHFRΔSH3 in which the last
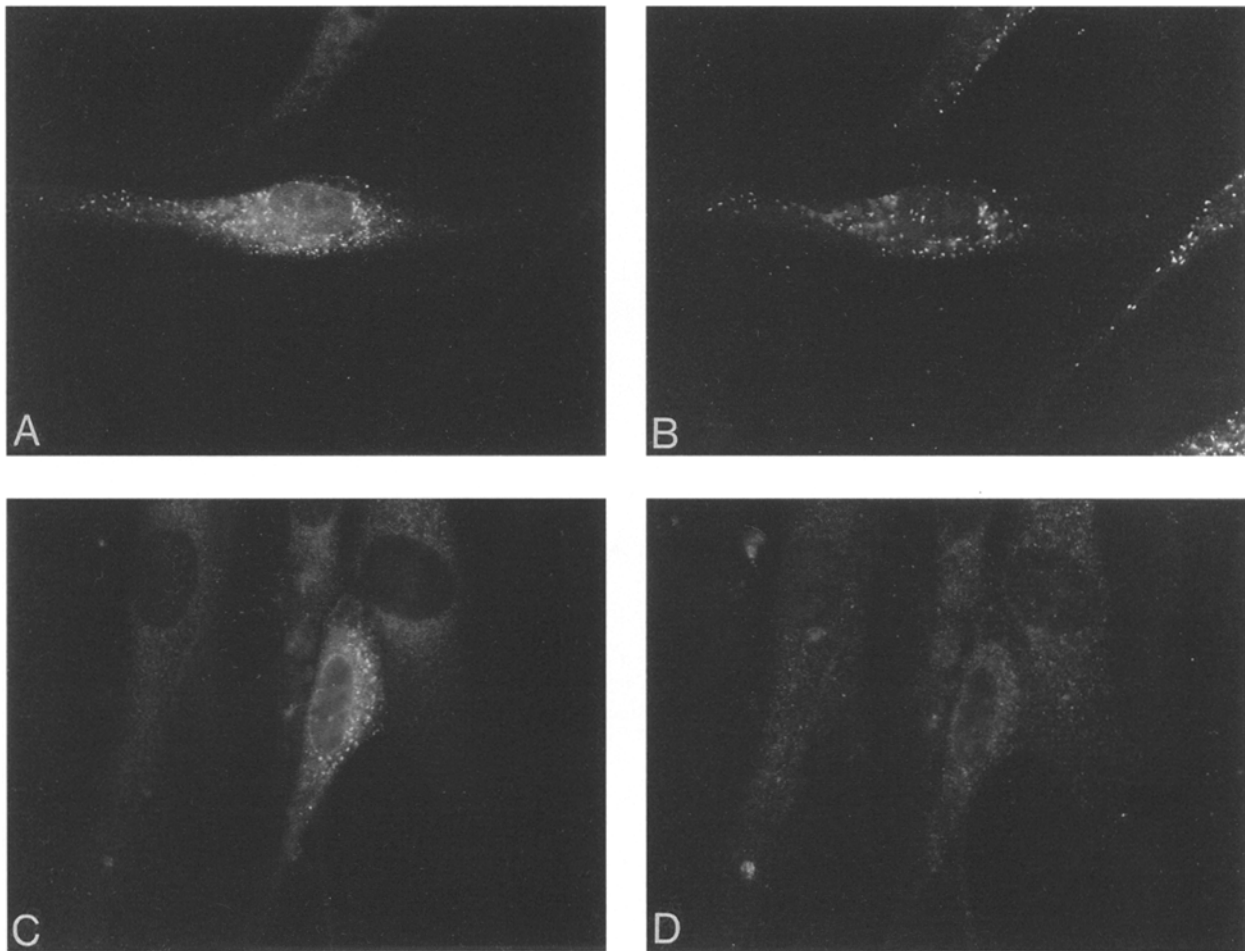
Figure 5. Localization of Pex13p and topology of the SH3 domain in human fibroblasts. Human fibroblasts microinjected with NH-PEX13 expression plasmid were processed for double-labeling of Pex13p (*left panels*) and catalase (*right panels*) using polyclonal anti-SH3 and monoclonal anti-catalase antibody, respectively. Pex13p (*A*) colocalizes with the peroxisomal matrix protein catalase (*B*) in cells permeabilized with digitonin plus Triton X-100 (permeabilizing both the plasma membrane and the peroxisomal membrane). When only the plasma membrane was permeabilized with digitonin a particulate pattern of labeling was detected with anti-SH3 (*C*), whereas a diffuse pattern of labeling was seen with anti-catalase (*D*).

nine amino acids of the SH3 domain were deleted. This deletion inactivated Pex13p, since a construct with the same deletion in the *PEX13* gene (PEX13ΔSH3) was unable to complement the *pex13Δ* mutant (data not shown). 5 of the 19 clones were unable to activate the lacZ gene with either of the above fusion proteins, suggesting that these library-encoded proteins interacted specifically with the SH3 domain (Table I). Sequence analysis revealed that four of the five clones encoded Pex5p. All four *PEX5*

Table I. Reconstitution of GAL4 Activity by Hybrid GAL4 Proteins

GAL4(DB) fusion	GAL4(TA) fusion	Colony color
—	—	white
DHFR-SH3	—	white
—	PEX5	white
DHFR-SH3	PEX5	blue
DHFR-ΔSH3	PEX5	white

β-Galactosidase activity was determined by a filter assay for yeast strains containing the indicated plasmids.

clones were fused in-frame to GAL4(TA) at their NH_2 termini (one clone at amino acid residue 11, three clones at residue 43) and contained the rest of the Pex5p open reading frame. Pex5p was previously identified as a protein required for import of PTS1-containing proteins and belongs to the tetratricopeptide repeat (TPR) family of proteins (Van der Leij et al., 1992, 1993). More recently, we have demonstrated that this protein is able to bind PTS1-containing proteins, suggesting that Pex5p is the PTS1-binding receptor (Tabak et al., 1995). Together, these results indicate that interaction between Pex5p and the SH3 domain of Pex13p is required for import of peroxisomal matrix enzymes.

To test whether the interaction between Pex13p SH3 and Pex5p was direct or mediated by a third protein, we examined their association in vitro using a ligand blot assay. In this assay *E. coli* purified DHFR-SH3 fusion protein and wild-type DHFR were separated by SDS-PAGE and transferred to a PVDF membrane. One part of the membrane was stained for protein (Fig. 6 *A*), whereas the other part was incubated in blocking buffer to allow rena-

Figure 6. In vitro binding of Pex5p to the Pex13p SH3 domain. Purified protein (lanes *1* and *3*) and whole bacterial lysates (lanes *2*) were separated by SDS-PAGE and transferred to PVDF membrane. One part of the membrane was stained for protein (*A*), the other part was probed with ^{35}S-labeled Pex5p (*B*) (see Materials and Methods). Lane *1*, 6xHis DHFR-SH3 (± 5 μg); lane *2*, 6xHis DHFR-SH3 (lysate); lane *3*, 6xHis DHFR (± 10 μg).

turation of the blotted proteins, and probed with ^{35}S-labeled, in vitro translated, Pex5p (Fig. 6 *B*). We found that only DHFR-SH3 (lanes *1*) but not wild-type DHFR (lanes *3*) bound Pex5p, although twice as much wild-type DHFR was loaded onto the gel. Furthermore, the DHFR-SH3 fusion protein was easily detected in a whole bacterial lysate of a DHFR-SH3–expressing strain (lanes *2*). These data indicate that binding between Pex13p SH3 and Pex5p was specific and direct.

Pex5p Is Primarily Located in the Cytosol

Given the Pex13p–Pex5p interaction and the fact that Pex5p is a PTS1-binding protein without any obvious domains that could anchor the protein to the peroxisomal membrane, it is conceivable that Pex5p is bound to the peroxisomal membrane via interaction with the SH3 domain of Pex13p.

Therefore, we investigated the localization of Pex5p by various techniques. Surprisingly, Western blot analysis of the fractions obtained by subcellular fractionation of wild-type cells showed that Pex5p was primarily present in the (cytosolic) supernatant fraction, and only a small amount was found in the organellar pellet fraction (Fig. 7 *A*). The organellar pellet fraction was further analyzed by Nycodenz equilibrium density gradient centrifugation followed by Western blotting of the obtained fractions. Pex5p coincided with the peroxisomal marker enzyme, indicating that the small sedimentable fraction of Pex5p is specifically associated with peroxisomes (Fig. 7 *B*).

To confirm the predominantly cytoplasmic location of Pex5p by an independent biochemical method, we selectively permeabilized spheroplasts with digitonin and followed the subsequent leakage of Pex5p and various marker proteins from the cells (Fig. 7 *C*). Pex5p was released at low concentrations of digitonin as was the cytosolic marker phosphoglucose isomerase (PGI). In contrast, the peroxisomal matrix proteins catalase and thiolase (data not shown) were released at much higher concentrations of digitonin. Pas21p, an integral membrane protein, was only partly released at very high digitonin concentrations. These results indicate that the majority of Pex5p resides in
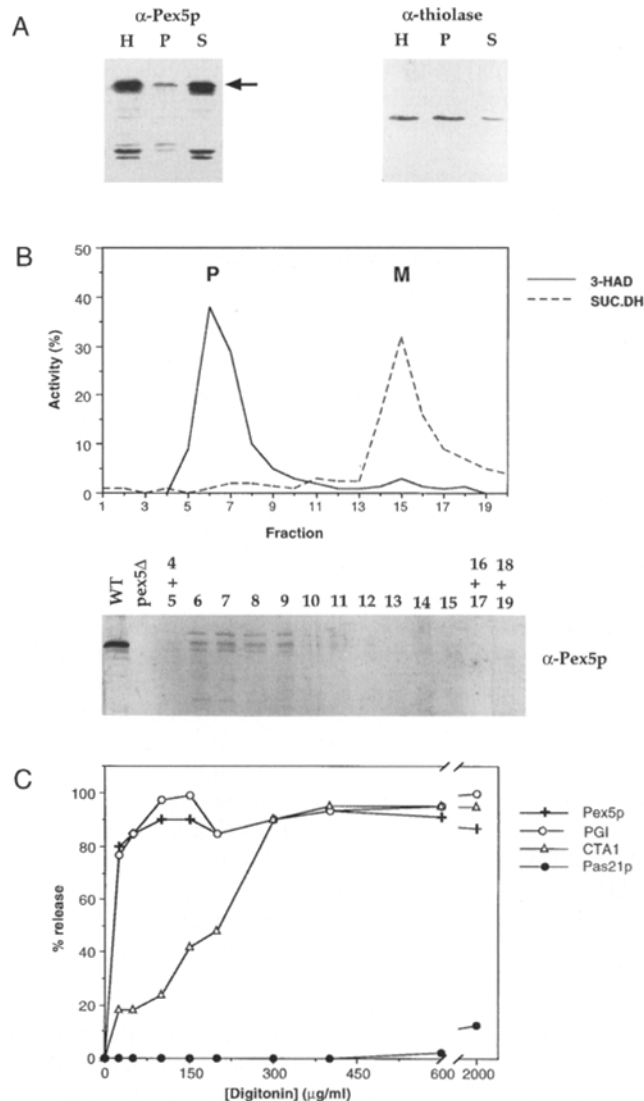
Figure 7. Subcellular localization of Pex5p. (*A*) Subcellular fractionation of oleate-induced wild-type cells resulting in an homogenate fraction (*H*), an organellar pellet fraction (*P*), and a supernatant fraction (*S*) containing cytosolic proteins. Comparable volumes of the fractions were used for Western blotting with anti-Pex5p and anti-thiolase antibodies. The arrow indicates the size of the full-length Pex5p. (*B*) Density gradient centrifugation on Nycodenz of the crude organellar pellet. The obtained fractions were used both to measure the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase (3-HAD) and the mitochondrial marker enzyme succinate dehydrogenase (SUC.DH), and for Western blotting to detect Pex5p. The first two lanes are TCA-lysates of wild-type and *pex5Δ* cells grown on oleate. (*C*) Release of proteins by digitonin titration of oleate grown wild-type cells. Spheroplasts were incubated for 10 min at 30°C with increasing amounts of digitonin. After quickly cooling the samples, the spheroplasts were pelleted and the supernatants were used for enzyme assays or for Western blotting. Phosphoglucose isomerase (PGI) was used as cytosolic marker enzyme, catalase (CTA) was used as marker enzyme for peroxisomal matrix proteins, whereas Pas21p was used as marker for peroxisomal membrane proteins. Pex5p and Pas21p were quantified after Western blotting.

the cytoplasm. The localization of Pex5p was also studied by immunoelectron microscopy using an NH-tagged Pex5p version and NH-antibodies. The results obtained confirmed the biochemical data: almost exclusive cytosolic labeling was observed (data not shown).

Discussion

Pex13p Is an SH3-containing Membrane Protein

We report here the molecular characterization of a novel SH3-containing protein, Pex13p, that is essential for peroxisomal protein import in *S. cerevisiae*. Biochemical and immuno-electronmicroscopic analysis showed that Pex13p is a peroxisomal membrane protein. A two-hybrid screen with the SH3 domain of Pex13p identified Pex5p, the PTS1 receptor, as a partner of Pex13p. Pex5p bound directly to Pex13p SH3 in a ligand blot assay, which identified Pex5p as a bona fide SH3 ligand. In addition, we showed that Pex5p is predominantly a cytosolic protein.

Several independent lines of evidence proved that the SH3 domain is essential for Pex13p function and for the interaction with Pex5p. First, a point mutation in the RT loop of the SH3 domain (PEX13-SH3^{E320K}) resulted in a nonfunctional protein. Second, the *PEX13ΔSH3* gene (lacking the carboxy-terminal 28 amino acids, including at least 9 amino acids of the SH3 domain) could not complement the *pex13Δ* mutant. Third, the small COOH-terminal deletion in the SH3 domain (Pex13pΔSH3) abrogated the two-hybrid interaction with Pex5p. Interestingly, the SH3 domain of Pex13p is not required for localization of Pex13p itself, since neither the peroxisomal targeting of the nonfunctional SH3-domain mutant Pex13p-SH3^{E320K} in yeast nor that of the SH3 deletion mutant (Pex13pΔSH3) in human fibroblasts (data not shown) was affected. Many SH3-containing proteins are found in the proximity of membranes or the cytoskeleton. For a number of these proteins it has been demonstrated that the SH3 domains mediate localization to these regions (Bar-Sagi et al., 1993; De Mendez et al., 1994; Rotin et al., 1994). The SH3 domain of Pex13p does not determine its subcellular location, but it is probably involved in recruiting another protein, Pex5p, to the peroxisomal membrane. A similar function was proposed for the transmembrane protein Sho1p, which binds with its SH3 domain to a proline-rich sequence in Pbs2p, a mitogen-activated protein kinase kinase (Maeda et al., 1995). A function in localizing proteins to specific sites in the cell is found for many other SH3-containing proteins, although in most cases the SH3 domain is present on the cytosolic and not on the membrane-associated protein.

Only a few natural SH3-binding proteins have been identified so far and the binding sites in these proteins have been mapped to proline-rich sequences (Ren et al., 1993; Finan et al., 1994; Musacchio et al., 1994). Other SH3-binding sequences have been delineated by screening of combinatorial peptide (Yu et al., 1994) and phage display libraries (Rickles et al., 1994). From these experiments, a PxxP motif has evolved as a consensus for SH3-binding sequences. So far, only the epithelial Na$^+$ channel, αrENaC, appears to contain an SH3-binding site which does not conform to the PxxP consensus (Rotin et al.,

1994). A PxxP motif is also not present in the Pex5p primary sequence, but the protein contains one "proline-rich" sequence (PSSGRLPP, residues 88 to 95) with a spacing of the prolines identical to that of the SH3-binding sequence in αrENaC. Whether this sequence motif is indeed involved in Pex13p SH3-binding remains to be tested. It is noteworthy that only near full-length *PEX5* genes (≈2 kb inserts) were isolated in the two-hybrid screen, since the average insert length of this library is only 1 kb. Deletion analysis of *PEX5* confirmed that indeed nearly full-length Pex5p was required for interaction with Pex13p SH3 in a two-hybrid assay (data not shown). Recently, a similar finding has been reported for the interaction between the human immunodeficiency virus type 1 (HIV-1) Nef protein and the SH3 domain of Hck, a member of the Src family of protein kinases (Lee et al., 1995). High-affinity binding to the Hck SH3 was only observed for the intact Nef protein, but not for peptides containing the Nef SH3-binding motif. These observations suggest that SH3-binding motifs may be presented in a distinct conformation in the context of the intact protein. Alternatively, additional contacts between the ligand and the SH3 domain may contribute to the affinity and the specificity of the interaction.

A Role for Pex13p and Pex5p in Peroxisomal Protein Import

We showed previously that the *pex5Δ* mutant is specifically deficient in the import of proteins with a type I peroxisomal targeting signal (PTS1) (Van der Leij et al., 1993). In a yeast two-hybrid assay Pex5p interacts with the PTS1-containing protein luciferase (Tabak et al., 1995). A single amino acid substitution in the PTS1 that abrogates the import of the mutant luciferase in vivo also prevents interaction with Pex5p in a two-hybrid assay (Distel et al., 1992; Elgersma et al., 1993; Tabak et al., 1995). In this respect, Pex5p conforms to the definition of a receptor, with a PTS1 as a ligand. Interestingly, our subcellular fractionation result indicates that the majority of Pex5p (~85%) was located in the cytosol. Only a small amount of Pex5p is associated with peroxisomes and copurified with the organelles upon equilibrium density centrifugation in a Nycodenz gradient. An independent biochemical method, based on the release of proteins after differential permeabilization of yeast spheroplasts, confirmed the predominant cytosolic localization of the protein. The absence of membrane-spanning regions in the primary sequence of Pex5p is in line with these observations. Based on these results we propose that Pex5p selects PTS1-containing proteins in the cytosol and delivers them to the peroxisomal membrane for further transport into the matrix space (Fig. 8). Our two-hybrid analysis and the observed direct interaction between Pex5p and Pex13p SH3 suggest that Pex13p is the membrane protein involved in peroxisome-specific docking of PTS1-loaded receptor. To be able to function as a docking protein for a cytosolic receptor complex, the COOH terminus of Pex13p containing the SH3 domain must be accessible from the cytosol. We obtained evidence for this by targeting Pex13p to peroxisomes in human fibroblasts. When the plasma membrane of Pex13p-expressing cells was selectively permeabilized with digitonin, the
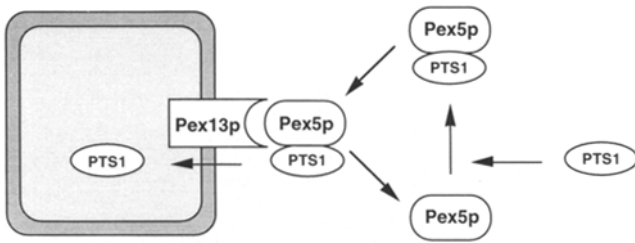
Figure 8. Model for the import of PTS1-containing proteins. Pex5p binds PTS1-containing proteins in the cytosol, which is followed by docking at the peroxisomal membrane by binding the SH3 domain of Pex13p. After release and import of the PTS1-containing protein, Pex5p is able to recycle to do other rounds of binding and delivery.

SH3 domain was labeled by the anti-SH3 antibody, indicating that it is facing the cytosol. Although we still lack data such as pulse-chase experiments to formulate a more dynamic model, the predominantly cytosolic localization of Pex5p suggests that the protein shuttles between the cytosol and the peroxisomal membrane as escort of PTS1-containing proteins. During these cycles, PTS1-proteins are released and transferred to a peroxisome-associated protein translocation complex.

Surprisingly, different subcellular locations have been reported for homologues of *S. cerevisiae* Pex5p in other species: primarily cytosolic (human Pex5p{PxR1p} [Dodt et al., 1995; Wiemer et al., 1995]), exclusively associated with peroxisomes and firmly attached to the cytosolic face of the membrane (human Pex5p [Fransen et al., 1995]; *Pichia pastoris* Pex5p{Pas8p} [McCollum et al., 1993; Terlecky et al., 1995]), and intraperoxisomal (*Hansenula polymorpha* Pex5p{Per3p} [Van der Klei et al., 1995]; *Yarrowia lipolitica* Pex5p{Pay32p} [Szilard et al., 1995]). Why the PTS1 receptors are found in such different locations is still a matter of debate (see also Rachubinski and Subramani, 1995). One possible explanation is that the PTS1 receptors not only shuttle between the cytosol and the peroxisomal membrane, but that, under certain physiological conditions, they can be coimported with the selected PTS1 proteins. A prerequisite for coimport is that protein complexes can pass the membrane. Indeed, oligomeric forms of proteins and even gold particles coated with PTS1 peptides can be translocated into peroxisomes (Glover et al., 1994; McNew and Goodman, 1994; Walton et al., 1995). Alternatively, the PTS1 receptors do not perform the same function. Despite extensive sequence similarity, PTS1 receptor genes fail to functionally complement PTS1 receptor mutants in other species (Dodt et al., 1995; Szilard et al., 1995; Van der Klei et al., 1995). Some of the PTS1-binding proteins, therefore, might not shuttle but only function inside peroxisomes as components of the protein translocation machinery (Szilard et al., 1995). Further analysis is required to distinguish between these possibilities.

Our subcellular fractionation studies showed that *pex13Δ* is disturbed in import of both PTS1- and PTS2-containing proteins. This is a puzzling observation since only the PTS1 receptor was found in a two-hybrid screen with Pex13p SH3 as a bait. However, it is possible that Pex13p is able to bind the PTS2 receptor (Pex7p), but that this interaction has gone undetected in our two-hybrid screen. Alternatively, Pex13p is not just a docking protein for Pex5p, but rather a structural component of a translocation complex common to PTS1- and PTS2-containing proteins. Further investigation is required to resolve this issue.

The use of a cytosolic receptor and a membrane-associated docking protein is a recurring theme in protein targeting to cellular compartments. Most proteins destined for the ER are first bound in the cytosol by the signal recognition particle (SRP) before associating with the ER membrane-located docking proteins (Walter and Johnson, 1994). Protein import into the nucleus also begins in the cytosol with binding of the import substrate by a receptor complex consisting of two subunits, importin-α (karyopherin-α) and importin-β (karyopherin-β). This is followed by docking of receptor-substrate complexes at the cytoplasmic face of the nuclear pore complex (NPC) (Sweet and Gerace, 1995). Similarly, the initial step of mitochondrial protein import is mediated by a cytosolic factor MSF (mitochondrial import simulating factor) that selectively binds the amino-terminal targeting sequence of (most) mitochondrial precursors (Hachiya et al., 1995). Subsequently, the precursors are handed over to the mitochondrial outer membrane complex for translocation across the mitochondrial membranes (Lithgow et al., 1995). Our results show that the mechanistic principles used in targeting to ER, nucleus, and mitochondria also apply to peroxisomal protein import: a cytosolic receptor recognizes the protein to be imported and directs it to the organellar membrane.

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