

Involvement of Pex13p in Pex14p Localization and Peroxisomal Targeting Signal 2–dependent Protein Import into Peroxisomes

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Abstract. Pex13p is the putative docking protein for peroxisomal targeting signal 1 (PTS1)-dependent protein import into peroxisomes. Pex14p interacts with both the PTS1- and PTS2-receptor and may represent the point of convergence of the PTS1- and PTS2-dependent protein import pathways. We report the involvement of Pex13p in peroxisomal import of PTS2-containing proteins. Like Pex14p, Pex13p not only interacts with the PTS1-receptor Pex5p, but also with the PTS2-receptor Pex7p; however, this association may be direct or indirect. In support of distinct peroxisomal binding sites for Pex7p, the Pex7p/Pex13p and Pex7p/Pex14p complexes can form independently. Genetic evidence for the interaction of Pex7p and Pex13p is provided by the observation that overexpression of Pex13p suppresses a loss of function mutant of Pex7p. Accord-

ingly, we conclude that Pex7p and Pex13p functionally interact during PTS2-dependent protein import into peroxisomes. NH₂-terminal regions of Pex13p are required for its interaction with the PTS2-receptor while the COOH-terminal SH3 domain alone is sufficient to mediate its interaction with the PTS1-receptor. Reinvestigation of the topology revealed both termini of Pex13p to be oriented towards the cytosol. We also found Pex13p to be required for peroxisomal association of Pex14p, yet the SH3 domain of Pex13p may not provide the only binding site for Pex14p at the peroxisomal membrane.

Key words: peroxisome • peroxin • Pex13p • Pex14p • protein import

PEROXISOMAL matrix proteins are synthesized on free polyribosomes and imported after translation into preexisting organelles (Lazarow and Fujiki, 1985). The presence of two distinct peroxisomal targeting signals (PTSs)¹ indicates the involvement of two pathways in the sorting process of peroxisomal matrix proteins. PTS1, present in the majority of peroxisomal matrix proteins, comprises the three COOH-terminal amino acids with the sequence SKL or conserved variants (for review see McNew and Goodman, 1996). Only one known peroxisomal

matrix protein in *Saccharomyces cerevisiae*, thiolase, targets the peroxisomal lumen by the PTS2, which is typically localized close to the NH₂ terminus of a protein, and consists of a conserved nonapeptide with the consensus sequence RLX₅H/QL (for review see De Hoop and Ab, 1992).

Recognition of PTS1 and PTS2 targeting signals is performed by the PTS specific import receptors, Pex5p and Pex7p, respectively (for review see Subramani, 1996; Erdmann et al., 1997). Cells deficient in either protein display partial import deficiencies. *pex5Δ* cells correctly localize PTS2 proteins, but are deficient in the targeting of PTS1 proteins. *pex7Δ* cells exhibit the reverse phenotype (for review see Elgersma and Tabak, 1996). The intracellular localization of both targeting signal receptors is still a matter of debate. A predominantly cytosolic, membrane-bound, and even intraperoxisomal localization have been reported for both receptors (for review see Rachubinski and Subramani, 1995).

An attractive model to reconcile the different localization of the import receptors is the extended shuttle hypothesis (Dodt and Gould, 1996; van der Klei and Veenhuis, 1996; Erdmann et al., 1997), which is a modification

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1. **Abbreviations used in this paper:** Fbp1p, fructose 1,6 bisphosphatase; Fox3p, antithiolase; MBP-SH3, maltose-binding protein SH3 domain fusion protein; nt, nucleotide; ORF, open reading frame; PTS, peroxisomal targeting signal; RT loop, reverse transcriptase loop; SD, minimal media; YPD, yeast complete media.

of the original hypothesis of shuttling receptors (Marzioch et al., 1994). The extended shuttle suggests that the import receptors Pex5p and Pex7p bind cargo proteins in the cytosol, dock to specific proteins at the periphery of the peroxisomal membrane, subsequently enter the peroxisome, release their cargo in the lumen of the peroxisome, and shuttle back to the cytoplasm. There is no experimental evidence for this model, but it is consistent with the observation that peroxisomes are able to import both folded and oligomeric proteins (for review see McNew and Goodman, 1996). However, the mechanism of protein translocation across the peroxisomal membrane remains unclear.

These shuttle models predict the existence of docking sites at the peroxisomal membrane for cargo-loaded PTS receptors. To date, two peroxisomal membrane proteins have been described which display the necessary properties to serve as docking sites for PTS receptors at the organelle. Pex13p, an integral peroxisomal membrane protein, specifically binds by means of its cytosolic SH3 domain to the PTS1 receptor Pex5p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996). The second protein, Pex14p, is a membrane protein located at the outer surface of the peroxisome (Albertini et al., 1997; Brocard et al., 1997; Komori et al., 1997; Fransen et al., 1998). Pex14p physically interacts with both receptors, Pex5p and Pex7p, as well as with the peroxisomal membrane proteins Pex13p and Pex17p (Albertini et al., 1997; Brocard et al., 1997; Huhse et al., 1998). Together, these data suggest that the two import pathways are not independent but overlapping, with Pex14p as the point of convergence of the pathways at the peroxisomal membrane (Albertini et al., 1997).

We report that Pex13p directly or indirectly interacts with the PTS2 receptor. In cells lacking Pex14p, Pex13p efficiently coimmunoprecipitates with Pex7p and interacts with Pex7p in the yeast two-hybrid system. In addition,

overexpression of Pex13p suppresses the protein import defect caused by HA-tagged, functionally compromised Pex7p, further suggesting an interaction between the two proteins by genetic means. Regions NH₂-terminal of the COOH-terminal SH3 domain of Pex13p were required for its interaction with Pex7p. Reinvestigation of the membrane topology of Pex13p revealed that both termini of the protein are exposed to the cytosol. Pex13p was also required for Pex14p localization at the peroxisomal membrane. However, the peroxisomal targeting of Pex14p did not require interaction with the SH3 domain of Pex13p.

Materials and Methods

Strains and Culture Conditions

S. cerevisiae strains used in this study are listed in Table I. Yeast complete (YPD) and minimal media (SD) have been described previously (Erdmann et al., 1989). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract, and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. When necessary, auxotrophic requirements were added according to Ausubel et al. (1992). For induction of the *CUPI* promoter CuSO₄ was added according to Marzioch et al. (1994).

Plasmids

For construction of pRS-*PEX7*HA₃, the SacI/KpnI fragment of YIp-*PEB*-HA₃ (Zhang and Lazarow, 1995) was subcloned into low copy plasmid pRS316 (Sikorski and Hieter, 1989). YEp351-*PEX14* was constructed by subcloning the SacI/BamHI fragment of pRS-*PEX14* into YEp351 (Hill et al., 1986; Albertini et al., 1997). YEp351-*PEX13* was constructed by subcloning the XhoI/PstI fragment of pRS-*PEX13* into YEp351 (Erdmann and Blobel, 1996).

For construction of Pex14pAXXA and Pex13pE320K, mutations were introduced by PCR using gene splicing by overlap extension (Yon and Fried, 1989). Primers used to construct Pex14pAXXA were: KU107, 5'-(GGAATTCGAGGCCTTATGAGTGACGTGGTCAGT)-3', (nucleotides [nt] 1–18); RE9, 5'-(ATCCCTGTGGGCCAGCGTTGCTGG-CATCGC)-3', (nt 249–278; underlined nt represent introduced mutations); RE8, 5'-(GCGATGCCAGCAACGCTGGCCACAGGGAT)-3', (nt 249–278); and KU109, 5'-(GGGGATCCCGGGATACCTATGGGATG-

Table I. Yeast Strains Used in This Study

Name	Genotype	Source or reference
UTL-7A	<i>MATa, ura3-52, trp1, leu2-3/112</i>	W. Duntze (Bochum)
<i>pex5Δ</i>	<i>MATa, ura3-53, trp1, prb1-112, prp4-3, pex5::LEU2</i>	(Van der Leij et al., 1993)
<i>pex5/14Δ</i>	<i>MATa, pex5::kanMX, pex14::loxP, ura3-52, trp1, leu2-3/112</i>	This study
<i>pex7Δ</i>	<i>MATa, pex7::LEU2, ura3-52 trp1</i>	(Marzioch et al., 1994)
<i>pex13Δkan</i>	<i>MATa, pex13::kanMX4, ura3-52, trp1, leu2-3/112</i>	(Huhse et al., 1998)
<i>pex13/17Δ</i>	<i>MATa, pex13::URA3, pex17::kanMX, trp1, leu2-3/112</i>	This study
<i>pex14Δ</i>	<i>MATa, pex14::LEU2, ura3-52, trp1</i>	(Albertini et al., 1997)
<i>pex14/17Δ</i>	<i>MATa, pex14::LEU2, pex17::kanMX, ura3-52, trp1</i>	This study
<i>pex17Δ</i>	<i>MATa, pex17::LEU2, ura3-52, trp1</i>	(Huhse et al., 1998)
<i>PCY2</i>	<i>MATa, Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801^{amber}, his3-Δ200, trp1-Δ63, leu2 ade2-101^{ochre}</i>	P. Chevray (Baltimore)
<i>PCY2-pex5Δ</i>	as <i>PCY2</i> plus <i>pex5::kanMX4</i>	This study
<i>PCY2-pex13Δ</i>	as <i>PCY2</i> plus <i>pex13::kanMX4</i>	(Huhse et al., 1998)
<i>PCY2-pex14Δ</i>	as <i>PCY2</i> plus <i>pex14::kanMX4</i>	(Huhse et al., 1998)
<i>PCY2-pex17Δ</i>	as <i>PCY2</i> plus <i>pex17::kanMX4</i>	(Huhse et al., 1998)
<i>HF7c</i>	<i>MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3/112, gal4-542, gal80-538, LYS::GAL1-HIS3, URA:::(GAL4 17-mers)₃-CYC1-LacZ</i>	Clontech
<i>HF7c-pex5Δ</i>	as <i>HF7c</i> plus <i>pex5::kanMX4</i>	This study
<i>HF7c-pex13Δ</i>	as <i>HF7c</i> plus <i>pex13::kanMX4</i>	This study
<i>HF7c-pex14Δ</i>	as <i>HF7c</i> plus <i>pex14::kanMX4</i>	This study
<i>HF7c-pex17Δ</i>	as <i>HF7c</i> plus <i>pex17::kanMX4</i>	This study

GAGTCTTC)-3', (nt 1016–1026). pRS-*PEX14* (Albertini et al., 1997) served as template. Primers used to construct Pex13p-E320K were T3, 5'-(AATTAACCTCACTAAAGGG)-3'; RE51, 5'-(CTCTGGG-TTTTTGGGAACAAATC)-3', (nt 946–969); RE50, 5'-(GATTTGT-TCCAAAACCCAGAG)-3', (nt 946–969); and T7, 5'-(GTAATAC-GACTCACTATAGGGC). pRS-*PEX13* (Erdmann and Blobel, 1996) served as template. The *PEX14*-PCR product was digested with EcoRI/BamHI and subcloned into pBluescript SK(+) (Stratagene) resulting in plasmid pWG14/4. The *PEX13*-PCR product was digested with XhoI/PstI and subcloned into pBluescript SK(+) resulting in plasmid pWG13/13.

For complementation studies, pRS-*PEX14* (Albertini et al., 1997) was digested with HpaI/HindIII and the internal fragment of *PEX14* (nt 123–702) was exchanged by a HpaI/HindIII fragment of *PEX14*AXXA from plasmid pWG14/4. The resulting plasmid pWG14/9 contained the *PEX14*AXXA-ORF as well as 601 bp of the 5'- and 628 bp of the 3'-noncoding region of *PEX14*. For complementation studies of *pex13Δ*, the 1.9-kb XhoI/PstI fragment of pWG13/13 containing the *PEX13*E320K-ORF, as well as 356 bp 5'- and 381 bp 3'-noncoding region of *PEX13* were subcloned in the yeast CEN-plasmid pRS351 (Sikorski and Hieter, 1989) resulting in pWG13/15.

Two-Hybrid Assay

The two-hybrid assay was based on the method of Fields and Song (1989). The ORFs of selected *PEX* genes were fused to the DNA-binding domain or transcription-activating domain of *GAL4* in the vectors pPC86 and pPC97 (Chevray and Nathans, 1992). To construct the Gal4p-(BD)-Pex13p fusion, the EcoRI/SpeI fragment of plasmid pSP43D (Erdmann and Blobel, 1996) containing the complete ORF of *PEX13* was subcloned into pPC86 resulting in pPC86-*PEX13*. To construct the Gal4p-(DB)-Pex14pAXXA fusion, the 1.1-kb EcoRI/SacII fragment from pWG14/4, containing the complete *PEX14*AXXA-ORF was subcloned into EcoRI/SacII-digested pPC86. The resulting plasmid was designated pWG14/6. The SalI/SacII of pWG14/6 contained *PEX14*AXXA and was subcloned into SalI/SacII digested pPC97, resulting in plasmid pWG14/8.

The *PEX13*E320K ORF was amplified from plasmid pWG13/13 by PCR, using oligonucleotides 43fus1 5'-(GTGAATTCGGATCCATAT-GTCATCCACAGCAGTA)-3' and the T7-primer (see above). The resulting PCR fragment was subcloned into a EcoRI/SpeI digested pPC86, resulting in plasmid pWG13/18. Amino acids 286–386 of Pex13pE320K were amplified by PCR using primers 37hyb1 5'-(TCCAGAATTCG-GATCCTACAGACCTCTGGAACATA)-3', T7, and pWG13/13 as templates. The PCR product was subcloned with EcoRI/SpeI into pPC86, resulting in plasmid pWG13/16. Subsequently, pWG13/16 was digested with SmaI/SpeI, and the excised *PEX13*E320K fragment was subcloned into SmaI/SpeI digested pPC97, resulting in plasmid pWG13/19.

Constructions of the Gal4p-AD-Pex5p, Gal4p-DB-Pex7p, and Gal4p-AD-Pex14p fusion proteins have been described previously (Erdmann and Blobel, 1996; Rehling et al., 1996; Albertini et al., 1997). Cotransformation of two-hybrid vectors into the PCY2 and HF7c was performed according to Gietz and Sugino (1988). Double transformants were selected on SD synthetic medium without tryptophane and leucine. β -galactosidase activity of transformed cells was determined by a filter assay described by Rehling et al. (1996), using X-Gal (GIBCO BRL) as substrate. His auxotrophy of transformed HF7c was determined by growth on selective plates lacking leucine, tryptophane, and histidine, but containing 10 mM 3-aminotriazole.

Construction of Knockout Strains

To delete *PEX5*, *PEX13*, *PEX14*, and *PEX17* in the yeast two-hybrid reporter strains *PCY2* and *HF7c*, as well as the deletion of *PEX7* and *PEX13* in wild-type *UTL7A*, *PEX14* in *pex17Δ*, *PEX17* in *pex13Δ*, and *PEX5* in *pex14Δ*, the *kanMX4* gene was used as a selective marker for insertion into the genomic locus (Wach et al., 1994). Deletion cassettes containing the *kanMX4* gene and the 5' and 3' untranslated regions of the corresponding ORFs were constructed by PCR using pFA6a-*kanMX4* (Wach et al., 1994) as a template. To generate the deletion cassettes for the *PEX5* gene deletion, the primer sets KU301, 5'-(TATACATCAA-TAAACAATATATACATAACACATGGACGTACGTACGCTGCAG-GTGGAC)-3' and KU302, 5'-(TGATGCGAGAGATAAAAATTGCG-GAGAACCATATCAATCGATGAATTCGAGCTCG)-3' were used. For the *PEX13* gene deletion, the primer sets KU274, 5'-(TATCT-ATAAATATCAAGGGGATTCTATACATAACAATACCTGCGC-GTACGCTGCAGGTGCAG)-3' and KU275, 5'-(TTTACTATATAT-

ATATGCGAATATATGTGTGCAAATATTGATGCAATCGATGA-ATTTCGAGCTCG)-3' were used. For the *PEX7* gene deletion, KU371, 5'-(GTTAACGGACTCATCTAACATTTTTGCGATAATTTATACAA-CATCGCTAGCTGCAGGTGCAG)-3' and KU372, 5'-(GTTTAAAT-ATATGCAAAAATTTGTGTAAAAAGAATATGTGTCAACATC-GATGAATTCGAGCTCG)-3' were used. For *PEX14* gene deletion, the primer sets KU289, 5'-(GAAAACCTCAAGTAAAACAGAGAAAGTTG-TAAGGTGAATAAGGACGTACGTACGTGCAGGTGCAG)-3' and KU290, 5'-(AATTACAATTTCCGTTAAAAAATAATTACTTACATAGAA-TTGGGATCGATGAATTCGAGCTCG)-3' were used. Finally, for a *PEX17* gene deletion the primer sets KU251, 5'-(TCCATCATT-CTGATAAGCAGAACCACGTAAGGACAGATAAAATCCGATC-GTGCAGGTGCAG)-3' and KU273, 5'-(ACGTGCACTAGAGCG-TTTTAAATCAATGCTATTATTTTATTGATTGATCGATGAATTCG-AGCTCG)-3' were used.

For construction of the double knockout strain *pex5/14Δ*, the *PEX14* locus was replaced by the *loxP-kanMX4-loxP* cassette (Güldener et al., 1996). The *PEX14* deletion cassette was constructed by PCR with the primer set KU289, 5'-(GAAAACCTCAAGTAAAACAGAGAAAGTTG-TAAGGTGAATAAGGACGTACGTGCAGGTGCAG)-3' and RE24, 5'-(CAATTTCCGTTAAAAAATAATTACTTACATAGAAATTCG-GATAGCCACTAGTGGATCTG)-3' with plasmid pUG6 as template (Güldener et al., 1996).

The *loxP-kanMX4-loxP* cassette was integrated into the *PEX14* locus and excised afterwards by the CRE recombinase expressed from plasmid pSH47 (Güldener et al., 1996). Subsequently, *PEX5* was deleted as described above.

PCRs and selection for geneticin resistance of transformants were performed according to Wach et al. (1994). Authenticity of each gene deletion by integration of the *kanMX4* gene was confirmed by PCR.

Cell Fractionation

Spheroplasting of yeast cells, homogenization, and differential centrifugation at 25,000 *g* of homogenates were performed as described previously (Erdmann et al., 1989).

For subfractionation by flotation gradient centrifugation, solid sucrose was added to a cell free extract, for a final concentration of 50% (wt/wt). 7 ml of this extract was overlaid with 5 ml 46% (wt/wt), 15 ml 38% (wt/wt), and 3 ml 25% (wt/wt) sucrose in gradient buffer (5 mM MES, 1 mM EDTA, 1 mM KCl, 0.1% [vol/vol] ethanol). Gradients were centrifuged in a Sorvall-SV288 rotor at 20,000 rpm for 4 h. 27 fractions were collected from the bottom and processed for enzyme measurement and Western blotting.

Enzyme Assays

3-oxoacyl-CoA thiolase (EC 2.3.1.16), catalase (EC 1.11.1.6), and fumarate hydratase (fumarase; EC 4.2.1.2) were assayed according to published procedures (Moreno de la Garza et al., 1985; Veenhuis et al., 1987).

Protease Protection Assay

The peroxisomal peak fractions of a sucrose density gradient were pooled and subsequently diluted fivefold in gradient buffer (Erdmann and Blobel, 1995). Peroxisomes were concentrated by centrifugation at 25,000 *g* for 30 min. The resultant pellet was resuspended in homogenization buffer (Erdmann et al., 1989) supplemented with 50 mM KCl, but without protease inhibitors. Equal amounts of isolated peroxisomes were incubated for 10 min on ice with increasing amounts of proteinase K. The protease was inhibited by the addition of 4 mM PMSF. The proteins were then precipitated with TCA and the samples were processed for SDS-PAGE.

Antibodies, Western Blotting and Coimmunoprecipitation

The protein fusion and purification system of New England Biolabs was used for overexpression of a maltose-binding protein-SH3 domain fusion protein (MBP-SH3). Amino acids 286–386 of Pex13p comprising the SH3 domain of Pex13p were fused to the COOH terminus of the *Escherichia coli* MBP (Erdmann and Blobel, 1996). MBP-SH3 was expressed from pMAL-SH3 in *E. coli* strain TG1. The expression was induced with 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and affinity purification of the MBP-SH3 fusion protein on amylose resin was performed according

to the manufacturer's protocol. Polyclonal antibodies were raised against the fusion protein (Eurogentec).

Rabbit polyclonal anti-Pex11p antibodies were raised against a synthetic peptide (KAKSQSQGDEHEDHK) corresponding to amino acids 162–176 generated by Eurogentec.

Antithiolase (Fox3p; Erdmann and Kunau, 1994), anti-Pcs60p (Blobel and Erdmann, 1996), anti-Pex3p (Höhfeld et al., 1991), anti-Pex5p (Albertini et al., 1997), anti-Pex14p (Albertini et al., 1997), anti-Pex17p (Huhse et al., 1998), and anti-fructose 1,6 bisphosphatase (anti-Fbp1p; Bigl and Escherich, 1994) have been described previously. Anti-rabbit or anti-mouse IgG-coupled HRP (Nycomed Amersham) was used as a secondary antibody, and blots were developed using the ECL-system (Nycomed Amersham). Western blot analyses were performed according to standard protocols (Harlow and Lane, 1988).

Coimmunoprecipitation experiments were performed as described in Rehling et al. (1996) with the exception of omitting the 35,000 *g* sedimentation step.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed according to the procedure of Rout and Kilmartin (1990), with modifications previously described in Erdmann (1994). Rabbit antisera against thiolase were used at dilutions of 1:3,000. For detection, 6 μ g/ml solution of CY3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories) was used. HA-tagged Pex11p was detected with monoclonal 12CA5 antiserum (BAbCO; dilution of 1:1,000).

Membrane Sedimentation

Yeast cells were grown on 0.3% SD medium to late log phase and subsequently for 15 h in YNOD (0.1% dextrose, 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract, and 0.67% yeast nitrogen base). Cells were washed with dH₂O and 1 g was used per sedimentation. 3 ml of buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl), protease inhibitors (0.02% PMSF [Serva], 15 μ g/ml bestatin, 1.5 μ g/ml pepstatin, 1 μ g/ml leupeptin, 0.1 μ g/ml chymostatin [Boehringer Mannheim], 0.21 mg/ml NaF), and 3 g glass beads (0.5 mm) were added to the cells. Breakage was achieved by vortexing for 4 min (8 \times 30 s, with breaks \geq 30 s on ice; Lamb et al., 1994). Samples were filtered through cotton wool, and the filtrate was transferred to Corex tubes and centrifuged at 1,000 *g* for 30 min. Supernatants were normalized for protein and volume, and membranes were sedimented at 200,000 *g* for 30 min (Sorvall AH650, 40,850 rpm) through a cushion of 0.25 M sucrose in buffer A. The resulting pellet was resuspended in buffer A plus protease inhibitors corresponding to the volume of the supernatant. Aliquots of the samples were analyzed by SDS-PAGE.

Results

Interaction of Pex7p with Pex5p Depends on the Presence of Pex14p

It has been reported that the import receptors Pex5p and Pex7p interact with each other in the yeast two-hybrid system, which opened the possibility that both proteins may form a heteromeric cytosolic signal recognition complex (Rehling et al., 1996). However, the yeast two-hybrid system does not necessarily distinguish between a direct and indirect binding of two *S. cerevisiae* proteins, as endogenous proteins may contribute to the observed interaction. As Pex14p can bind both import receptors, we investigated whether the Pex5p/Pex7p interaction is still observed in a yeast two-hybrid reporter strain deleted for the genomic copy of *PEX14* (Huhse et al., 1998). As shown in Fig. 1, the interaction of the two import receptors strictly depended on the presence of Pex14p which may be required for the activation of either receptor, or it may function as a bridging molecule between Pex5p and Pex7p.

Association of Pex7p with Membranes in the Absence of Pex14p

Deletion of genes encoding the three components of the docking machinery known to date, Pex13p, Pex14p, or Pex17p, results in an import defect for both PTS1 and PTS2 proteins. One possible explanation could be a functional overlap of the proteins in PTS1- and PTS2-dependent protein import into peroxisomes. This prediction prompted us to search for additional Pex7p-binding proteins at the peroxisomal membrane. We expressed a functional mycPex7p fusion protein (Marzioch et al., 1994) in wild-type, *pex17 Δ* , *pex14 Δ* , and *pex13 Δ* cells, and determined the localization of the protein by subcellular fractionation studies. Fractions were analyzed for the presence of mycPex7p and for peroxisomal membrane markers

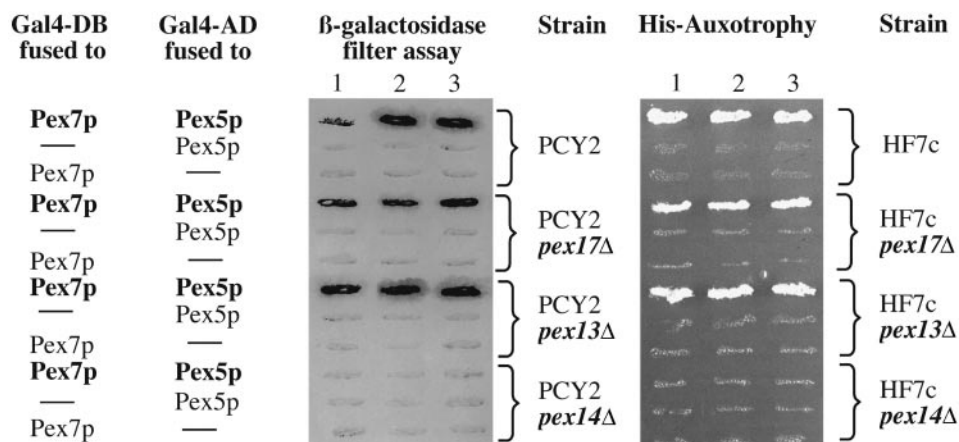


Figure 1. The two-hybrid interaction of the PTS receptors Pex5p and Pex7p requires the presence of Pex14p. Two-hybrid interactions between Pex5p and Pex7p were monitored by a filter assay for β -galactosidase activity and test for His auxotrophy in wild-type strains PCY2 and HF7c, respectively, or isogenic strains deleted for *PEX17*, *PEX13*, and *PEX14*. The Pex5p/Pex7p interaction is still observed in the strains lacking either Pex17p or Pex13p, but not in strains lacking Pex14p. Pex14p may be required for the activation of either receptor, or it may function as a bridging molecule between Pex5p and Pex7p. Three representative independent transformants are shown (1–3).

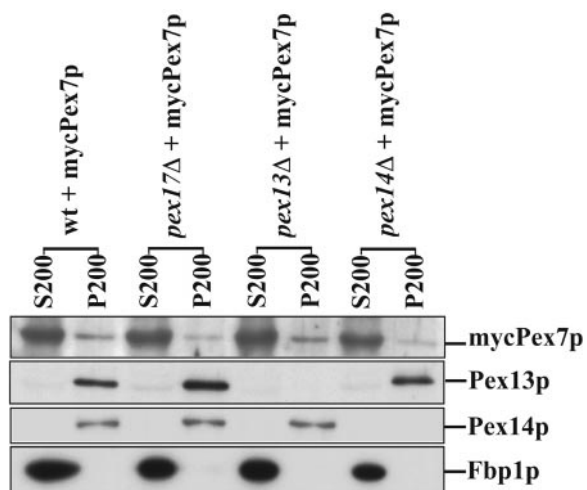


Figure 2. A portion of mycPex7p is associated with a membrane sediment in *pex14Δ* cells. Immunological detection of mycPex7p and peroxisomal membrane markers Pex13p and Pex14p in 200 K supernatant and pellet fractions of homogenates from indicated *pex* mutants. The cytosolic marker Fbp1p served as a control for nonspecific presence of proteins in the membrane sediment. Note the residual amount of Pex7p in the membrane pellet of *pex14Δ* cells. Equal portions of membrane and supernatant fractions were analyzed.

Pex14p and Pex13p, as well as for cytosolic Fbp1p (Entian et al., 1988) as a control for proper separation. Pex14p and Pex13p, but not Fbp1p, pelleted, indicating the complete sedimentation of cytosol-free peroxisomal membranes (Fig. 2). As reported previously (Rehling et al., 1996), mycPex7p was predominantly found in the soluble fraction in wild-type cells, while a low but significant amount was detected in the membrane fraction. A decrease of mycPex7p in the pellet fraction of *pex14Δ* cells (Fig. 2) suggests that the majority of sedimentable Pex7p associates with membranes in a Pex14p-dependent manner. However, in *pex14Δ* cells a significant amount of mycPex7p was detected in the membrane pellet fraction (Fig. 2), indicating that next to Pex14p additional binding factors for Pex7p exist at the peroxisomal membrane.

Coimmunoprecipitation of Pex13p and Pex7p in the Absence of Pex14p and Pex5p

To determine the binding partners of Pex7p, we isolated mycPex7p from wild-type and various *pex* mutant strains by immunoprecipitation, and immunoblotted for the presence of selected peroxins. As reported previously, we found Pex5p, Pex13p, Pex14p, and Pex17p associated with mycPex7p when the receptor was precipitated from wild-type or complemented *pex7Δ* cells (Albertini et al., 1997; Huhse et al., 1998). Comparison of the constituents of the precipitates revealed five interesting observations.

First, in *pex14Δ* and *pex5Δ/pex14Δ* strains, Pex13p still coimmunoprecipitated with mycPex7p (Fig. 3), suggesting that Pex13p associates directly or indirectly with Pex7p. Moreover, this result indicated that neither Pex14p nor Pex5p is required for the formation of this subcomplex of

Pex13p and Pex7p. Second, the amount of Pex5p in the precipitate from *pex14Δ* cells was drastically reduced, while the amount in Pex13p remained essentially unchanged (Fig. 3, lane *pex14Δ*). This result supports the notion that the amount of Pex5p bound to Pex13p does not determine the stoichiometry of the Pex13p-Pex7p subcomplex. However, it also suggests that Pex13p may not bind both import receptors equally at the same time. Third, Pex13p, Pex14p, and Pex5p still coimmunoprecipitated with Pex7p in *pex17Δ* cells (Fig. 3, lane *pex17Δ*). Obviously, Pex7p is associated with components of the peroxisomal translocation machinery in the absence of Pex17p, suggesting that the presence of Pex17p is not a prerequisite for docking of Pex7p to the peroxisomal membrane. Fourth, the lack of Pex17p in the coimmunoprecipitate from *pex14Δ* cells (Fig. 3, lane *pex14Δ*), suggests that Pex14p is required for the association of Pex17p with the complex, and is consistent with the assumption that Pex17p binding to the complex may be via Pex14p. How-

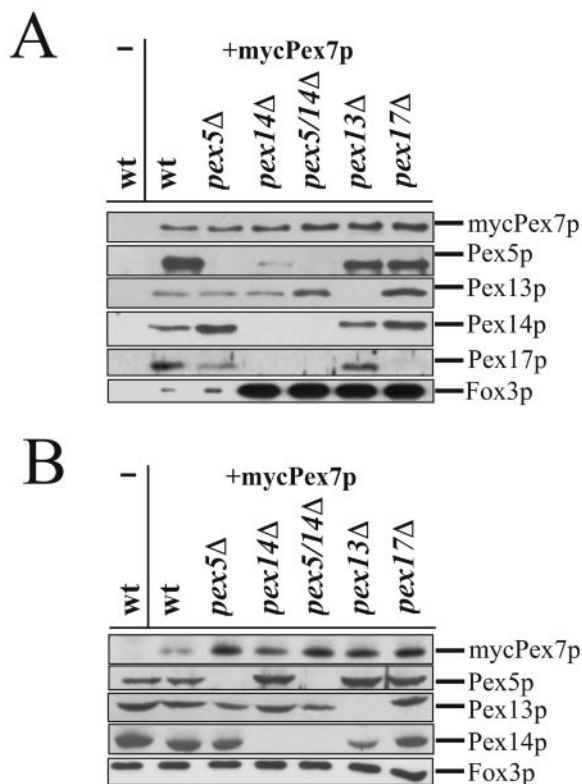


Figure 3. Pex7p is associated with Pex13p in the absence of Pex5p and Pex14p. (A) Whole cell extracts of wild-type (UTL-7A) cells, and of wild-type, *pex5Δ*, *pex14Δ*, *pex5/14Δ*, *pex13Δ*, and *pex17Δ* cells expressing mycPex7p, were immunoprecipitated using antibodies against the c-myc epitope. A significant amount of Pex13p coimmunoprecipitated with Pex7p in the absence of Pex5p and Pex14p. Note the decreased amount of Pex5p in the precipitate from *pex14Δ* cells while the amount of Pex13p remained nearly unchanged. Equal amounts of immunoprecipitates (10% of total) were separated by SDS-PAGE and subjected to immunoblot analysis using antibodies against c-myc, Pex5p, Pex13p, Pex14p, and Pex17p. (B) Immunoblot of equal portions of the whole cell extracts (1% of total) for mycPex7p, Pex13p, Pex14p, Pex5p, and Fox3p.

ever, this observation must be interpreted with care since the *pex14Δ* cells contain much less immunologically detectable Pex17p (Huhse et al., 1998). Finally, the amount of Fox3p that coimmunoprecipitates with Pex7p drastically increases in mutants with an import defect for PTS2 proteins (*pex17Δ*, *pex13Δ*, *pex14Δ*, and *pex5Δ/pex14Δ*) relative to the strains unaffected in this pathway (wild-type and *pex5Δ*). Since the total amount of both proteins is similar in all strains (Fig. 3 B), it seems unlikely that the observed Pex7p/Fox3p complex has formed in vitro after cell disruption. A simple explanation for this may be that the high cytosolic concentration of thiolase in the import mutants results in greater occupation of the PTS2 receptor.

To exclude the possibility of nonspecific coprecipitation of proteins, we checked the precipitates for the presence of peroxisomal membrane proteins Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995) and Pmp35p (putative peroxisomal ATP-transporter; Erdmann, R., unpublished observations). These proteins were not detected in any of the samples, indicating the specificity of the observed interactions (data not shown).

Pex13p Interacts with the PTS2 Receptor Pex7p in the Yeast Two-Hybrid System

The observed in vivo association of Pex7p with Pex13p in cells lacking Pex14p and Pex5p encouraged us to analyze the interaction of these proteins in more detail. In previous experiments, only fragments of Pex13p were used to address whether Pex13p binds to Pex7p, and so far they have not indicated an interaction between these two proteins (Erdmann and Blobel, 1996). To revisit this possibility, we analyzed the interaction of the full length Pex13p with Pex7p in the yeast two-hybrid system. The results shown in Fig. 4 A reveal that the full length Pex13p is in-

deed able to interact with the PTS2-receptor Pex7p. The controls included show that coexpression of either of the fusion proteins alone did not support transcription activation of the reporter genes.

To analyze whether the observed Pex13p/Pex7p two-hybrid interaction depends on known binding partners for Pex13p, tests were also performed in isogenic *pex5Δ* and *pex14Δ* strains (Fig. 4 A). Furthermore, we analyzed the association of Pex7p with a mutated Pex13pE320K in a *pex5Δ* mutant (Fig. 4 B). Because Pex13pE320K lost the ability to interact with Pex14p in the yeast two-hybrid system (Fig. 4 B, see also Fig. 8), this experiment was expected to monitor the Pex13p/Pex7p interaction upon simultaneous elimination of the Pex14p and Pex5p influence. As shown in Fig. 4, these two-hybrid analyses did not reveal an influence of Pex5p or Pex14p on the Pex13p/Pex7p interaction. No difference was observed independent of whether the Pex7p/Pex13p interaction was analyzed in wild-type, *pex5Δ*, or *pex14Δ* strains (Fig. 4 A), or for the Pex7p/Pex13pE320K interaction in *pex5Δ* cells (Fig. 4 B). These results indicate that neither Pex14p nor Pex5p is required for the in vivo interaction of Pex7p with Pex13p, and therefore are in agreement with results obtained in the coimmunoprecipitation experiment (Fig. 3).

The two-hybrid interaction of the complete Pex13p with Pex14p is only detected by histidine prototrophy (Fig. 4 B), indicating that regions NH₂-terminal of the SH3 domain of Pex13p may weaken the interaction of these proteins in the two-hybrid system.

PEX13 Suppresses a Defect in Pex7p Function

Mutant cells lacking Pex7p are characterized by their inability to grow on oleic acid as the sole carbon source (Fig. 5 A) and by mislocalization of peroxisomal thiolase to the

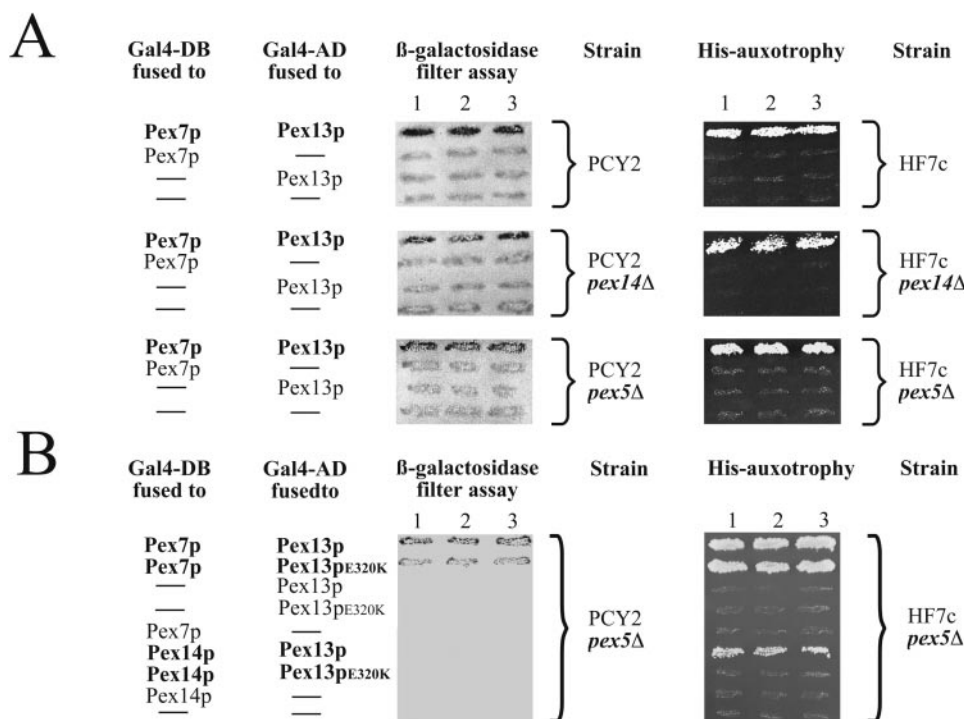


Figure 4. Pex13p interacts with Pex7p in the yeast two-hybrid system independent of Pex5p or Pex14p. Interaction of (A) Pex13p with Pex7p and (B) Pex13pE320K with Pex7p and Pex14p. An interaction of Pex13p and Pex7p is indicated by the β-galactosidase expression and the histidine prototrophy of the transformants and is still observed in strains lacking either Pex14p or Pex5p. Note that Pex13pE320K lacks an interaction with Pex14p, but still interacts with Pex7p in strains lacking Pex5p. Assays were carried out in the yeast reporter strain PCY2 and HF7c, or isogenic strains deleted for *PEX14* or *PEX5* as indicated. Three representative independent transformants are shown (1–3).

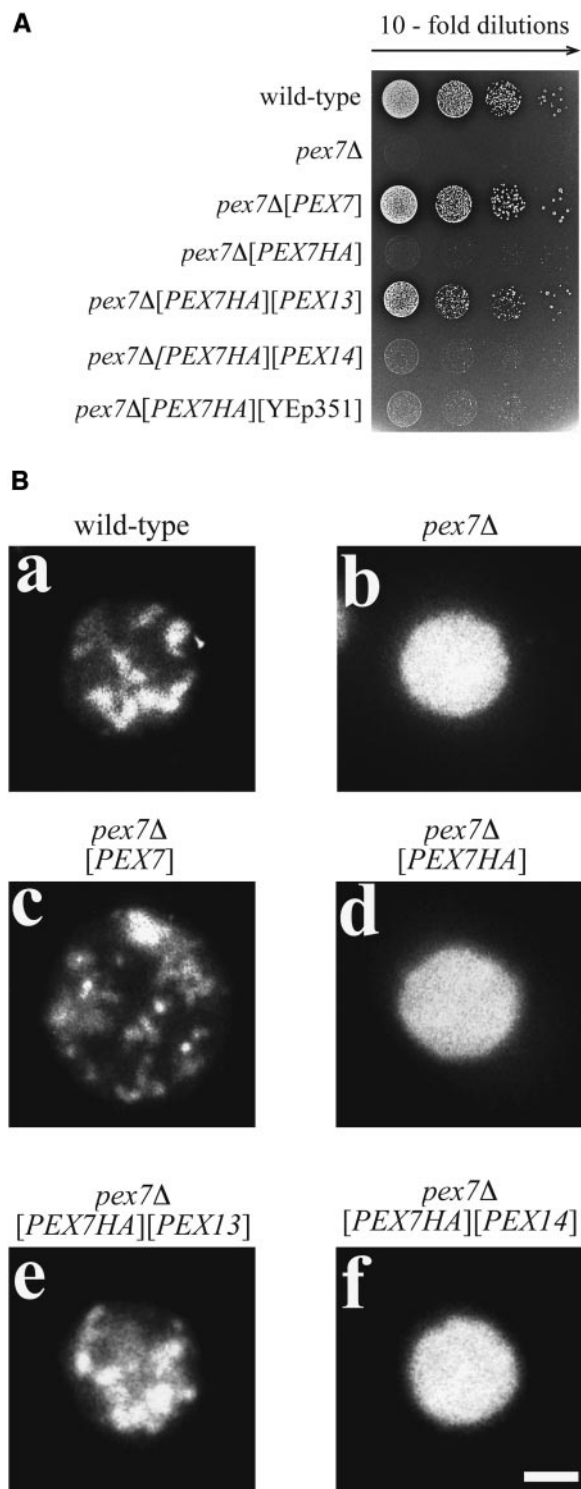


Figure 5. Suppression of the reduced complementing activity of an HA-tagged Pex7p by overexpression of Pex13p. Expression from a low copy plasmid of a Pex7p that is COOH-terminally fused to a triple hemagglutinin tag (*PEX7HA*) results in only partial complementation of the *pex* phenotype of *pex7Δ* cells. Upon overexpression, the inability of Pex7p-HA₃ to fully complement the *pex7Δ* phenotype is suppressed by Pex13p, but not by Pex14p. (A) Growth behavior of wild-type and *pex7Δ* mutant cells, as well as indicated single and double transformants on oleic acid medium. Note the weak growth of *pex7Δ* which express the *PEX7HA* in comparison to the nearly normal growth behavior

of these cells upon additional overexpression of Pex13p. The spots to the left correspond to 2×10^4 cells. (B) Immunofluorescence microscopical localization of thiolase (Fox3p) in wild-type and *pex7Δ* mutant cells, as well as indicated single and double transformants. The import defect for PTS2 proteins in *pex7Δ* cells, *pex7Δ* cells (b) expressing *Pex7p*-HA₃ from pRSPEX7HA alone (d) or in conjunction with *PEX14* (f) is indicated by overall cell labeling. Suppression of the import defect for PTS2 proteins by overexpression of Pex13p in *pex7Δ* cells expressing pRSPEX7HA is indicated by the punctate fluorescence pattern (e) that is comparable to the pattern observed in wild-type cells (a) or fully complemented *pex7Δ* cells (c). Bar, 5 μ m.

The NH₂ Terminus and the COOH-terminal SH3 Domain of Pex13p Face the Cytosol

Pex13p is an integral peroxisomal membrane protein and the cytosolic orientation of the COOH-terminal SH3 domain was shown previously in human fibroblasts (Elgersma et al., 1996) and *Pichia pastoris* (Gould et al., 1996). However, the COOH-terminal SH3 domain alone is not sufficient to interact with Pex7p, suggesting that regions NH₂-terminal to the SH3 domain are involved in this association. To address whether the NH₂ terminus of Pex13p is localized to the lumen of peroxisomes or to the cytosol, we analyzed the accessibility of an NH₂-terminally myc-tagged Pex13p to exogenously added protease K. The tag has been shown previously not to affect the function of Pex13p (Erdmann and Blobel, 1996). Thus, the topology of the myc-tagged Pex13p is likely to reflect the in vivo situation for the wild-type protein. As judged by immunoblot analysis, both the NH₂-terminal myc-tag as well as the SH3 domain of Pex13p were rapidly degraded by the protease (Fig. 6). Intraperoxisomal thiolase remained stable under these conditions and was only degraded in the presence of detergents (data not shown). From this data, we conclude

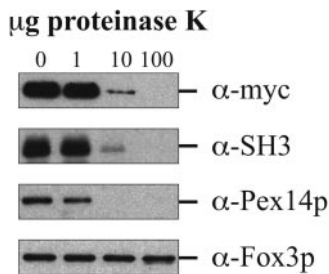


Figure 6. The NH₂ terminus and the COOH-terminal SH3 domain of Pex13p are exposed to the cytoplasm. Protease protection analysis of isolated peroxisomes. Peroxisomes were prepared from *pex13Δ* cells expressing myc-Pex13p (see Materials and Methods). Equal amounts of the organelles were incubated

with increasing concentrations of proteinase K in the absence of detergent for 10 min on ice. Equal amounts were separated by SDS-PAGE and analyzed by immunoblotting using specific antibodies for the SH3 domain of Pex13p, the myc-epitope, Pex14p, and the peroxisomal matrix protein thiolase (Fox3p). While the intraperoxisomal thiolase was stable throughout the experiment, the COOH and NH₂ termini of Pex13p, as well as Pex14p, were rapidly degraded.

that both the NH₂ terminus and the COOH-terminal SH3 domain are exposed to the cytosol. This result also implicates the presence of an even number of transmembrane spans within Pex13p.

Pex13p Is Required for Association of Pex14p with Peroxisomal Membranes

Pex13p interacts with Pex14p via its COOH-terminal SH3 domain (Albertini et al., 1997; Brocard et al., 1997); however, both proteins can interact with Pex7p independently. The latter is in agreement with the assumption that Pex13p and Pex14p contribute to distinct docking sites for Pex7p at the peroxisomal membrane. Since Pex14p is a peripheral membrane protein, two questions arise: How is it associated with the peroxisomal membrane, and Does Pex13p contribute to its localization? In an attempt to identify proteins required for the targeting and binding of Pex14p to the peroxisomal membrane, we analyzed the subcellular localization of Pex14p in different *pex*-mutant cells (Fig. 7 A). The congruent fluorescence patterns for Pex14p and the peroxisomal membrane marker Pex11p in *pex17Δ* cells indicate a peroxisomal localization of Pex14p. This observation suggests that Pex17p is not required for the targeting of Pex14p to the peroxisomal membrane. In contrast, no congruent fluorescence patterns were observed in *pex13Δ* cells. Since the HA-tagged Pex11p is known to be targeted to peroxisomal membrane ghosts in *pex13Δ* cells (Erdmann and Blobel, 1996), the lack of congruence suggests that the majority of Pex14p is mislocalized. To confirm this result by independent means, we performed a flotation of wild-type, *pex13Δ*, and *pex17Δ* homogenates in sucrose gradients (Fig. 7 B). Gradients were designed such that peroxisomal membrane ghosts would float to the upper fractions of the gradient, whereas intact peroxisomes would predominantly remain in the loading zone. In *pex13Δ* cells, the peroxisomal membrane markers Pex3p and Pex11p were predominantly found in fractions that correspond to the peroxisomal membrane ghosts. However, Pex14p was not detected in these fractions, but was found to cosegregate with mitochondrial

fumarase. These data suggest that the peroxisomal membrane ghosts in *pex13Δ* cells lack Pex14p. Thus, the presence of Pex13p is a prerequisite for peroxisomal membrane association of Pex14p. Pex13p could be involved in targeting, or it could be required for binding or retention of Pex14p at the peroxisome.

Binding to the SH3 Domain of Pex13p Is Not Essential for the Peroxisomal Membrane Association of Pex14p

To clarify whether binding of Pex14p to the SH3 domain is a prerequisite for the peroxisomal targeting of Pex14p, we analyzed the subcellular localization of Pex14p under conditions in which this interaction is blocked. A proline-rich sequence which corresponds to a type II SH3 domain binding motif is present within the primary sequence of Pex14p (Albertini et al., 1997; Brocard et al., 1997). We substituted Pro87 and Pro90 of this putative binding motif (PPTLPHRDW) by two alanines (PATLAHRDW). Remarkably, the mutated Pex14pAXXA still complemented the peroxisome biogenesis defect of *pex14Δ* cells (data not shown). We also introduced an E320K mutation in the reverse transcriptase loop (RT loop) of the SH3 domain of Pex13p. This mutation has been reported to result in the inactivation of Pex13p function (Elgersma et al., 1996). As shown in Fig. 8, the mutated Pex14pAXXA had lost the ability to bind Pex13p in the yeast two-hybrid system while binding to Pex5p, Pex7p, and oligomerization of the protein was unchanged. Also the E320K mutation of Pex13p abolished the two-hybrid interaction of the SH3 domain of Pex13p with Pex14p (Fig. 8). These results suggest that strong interactions between Pex14p and the SH3 domain of Pex13p are dependent on the PXXP motif within Pex14p, as well as on the RT loop of the SH3 domain of Pex13p.

Next, we analyzed the Pex14pAXXA (Fig. 9 A) association with peroxisomal membrane ghosts of *pex14Δ/pex17Δ* double mutants which were predicted to contain peroxisomal membrane ghosts even upon complementation of the *pex14Δ* mutation. In addition, we analyzed whether peroxisomal membrane ghosts that harbor mutated Pex13p-E320K still contain Pex14p. The subcellular localization of Pex14p, Pex14pAXXA, and peroxisomal membrane markers was analyzed by double immunofluorescence microscopy and flotation analysis. Colocalization was observed for HA-Pex11p and Pex14pAXXA in *pex14Δ* cells, as well as for HA-Pex11p and Pex14p in *pex13Δ* cells expressing Pex13pE320K, indicative of peroxisomal membrane association of these proteins (Fig. 9 A). These results were corroborated by flotation analysis which revealed that Pex14pAXXA was associated with the fraction containing the peroxisomal membrane ghosts of *pex14Δ/pex17Δ*, as were Pex14p in *pex13Δ/pex17Δ* cells expressing Pex13p-E320K (Fig. 9 B). These observations suggest that Pex14p is associated with peroxisomes and peroxisomal membrane ghosts independent of interaction between the proline-rich motif of Pex14p and the RT loop in the SH3 domain of Pex13p. Interestingly, the fractionation of *pex13Δ/pex17Δ*[PEX13E320K] shows that, although the RT loop of the SH3 domain of Pex13p is not absolutely required for the targeting of Pex14p to the membrane of peroxisomal ghosts, it appears to enhance or stabilize the targeting,

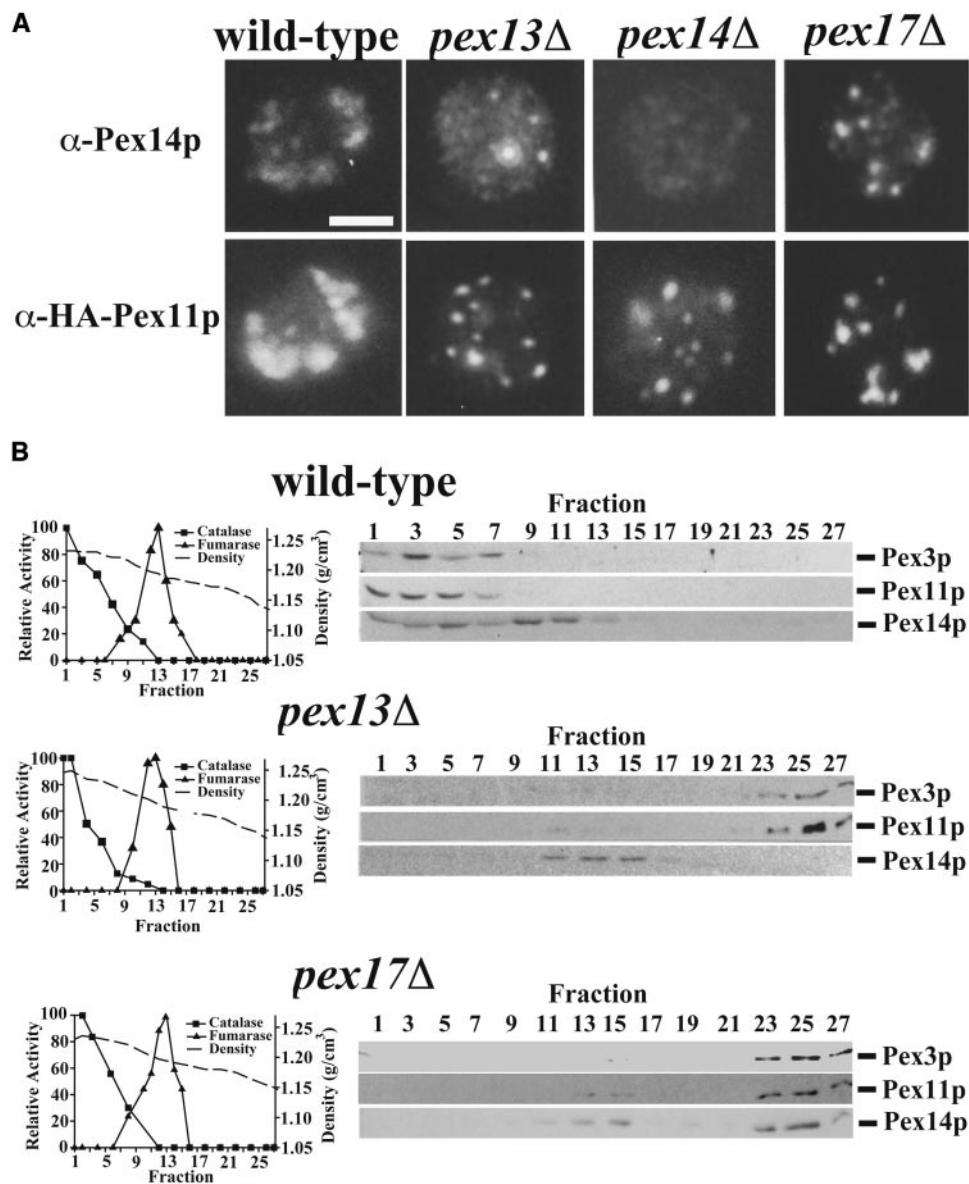


Figure 7. Pex13p is required for the peroxisomal membrane targeting of Pex14p. (A) Oleic acid-induced wild-type, *pex13* Δ , *pex14* Δ , and *pex17* Δ cells, expressing HA-tagged Pex11p were processed for double immunofluorescence microscopy localization of Pex14p and HA-Pex11p. The congruent fluorescence patterns in wild-type and *pex17* Δ cells indicate a peroxisomal localization of Pex14p. No congruent fluorescence pattern was observed in *pex13* Δ cells, suggesting that the majority of Pex14p is mislocalized. Detection was performed with rabbit polyclonal antibodies against Pex14p and mouse mAb against the HA-epitope. Secondary antibodies were FITC-conjugated anti-mouse IgG and CY3-conjugated anti-rabbit IgG. Bar, 5 μ m. (B) Flotation of peroxisomal membranes in wild-type, *pex13* Δ , and *pex17* Δ cells. Cell-free extracts were separated on 50–25% (wt/wt) sucrose step gradients. Localization of Pex14p as well as peroxisomal membrane markers Pex3p and Pex11p, and peroxisomal catalase and mitochondrial fumarase in fractions were monitored by immunoblot analysis and enzyme activity measurements. The peroxisomal membrane ghosts were detected at the top of the gradient while intact peroxisomes and cytosolic proteins predominantly remained in the loading zone. In *pex13* Δ cells, no Pex14p was detected in the ghost fractions, but was found mislocalized to fractions of lighter density.

as only Pex14p trails through the gradients of this mutant strain (Fig. 9 B).

Discussion

The peroxisomal membrane protein Pex14p has been reported to bind both the PTS1 and the PTS2 receptor, which led Albertini et al. (1997) to the conclusion that Pex14p may represent the point of convergence of the PTS1- and PTS2-dependent protein import pathways at the peroxisomal membrane. Here, we report that Pex13p is also involved in both the PTS1- and PTS2-dependent protein import into peroxisomes. Pex13p interacts directly or indirectly with the PTS2 receptor Pex7p and overexpression of Pex13p suppresses the protein import defect caused by a functionally impaired epitope-tagged Pex7p. Pex13p is also shown to be required for the peroxisomal

association of Pex14p; however, evidence is provided that the SH3 domain of Pex13p may not represent the only binding site for Pex14p at the peroxisomal membrane.

Involvement of Pex13p in Targeting of Pex14p to the Peroxisomal Membrane

The SH3 domain of Pex13p has been reported to interact with the PTS1 receptor Pex5p and with Pex14p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996; Albertini et al., 1997; Fransen et al., 1998; Fig. 8). A mutation in the RT loop of the SH3 domain of Pex13p, as well as a mutation of a putative class II SH3 ligand motif of Pex14p abolished the two-hybrid interaction of both proteins (Fig. 8), supporting the notion of a typical SH3 domain-ligand interaction between Pex13p and Pex14p. Interestingly, although the E320K mutation of the RT loop

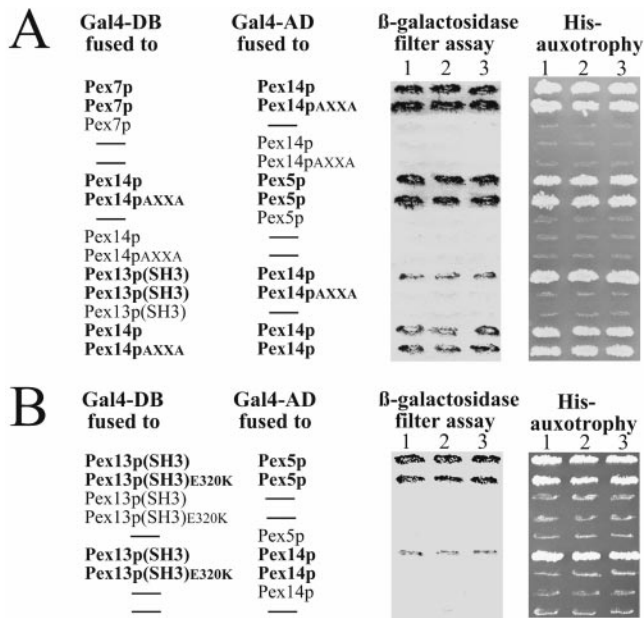


Figure 8. The Pex13p/Pex14p interactions require a proline-rich motif in Pex14p, as well as the RT loop of Pex13p. (A) Two-hybrid interaction of Pex14p and Pex14p^{AXXA} with Pex5p, Pex7p, Pex13p, and Pex14p. Pex14p^{AXXA} contains the mutation of Pro87 and Pro90 of a putative SH3 ligand motif of Pex14p. The two-hybrid interactions were monitored by determination of the β-galactosidase expression and of the histidine prototrophy of *pex14Δ* deletion strains of PCY2 and HF7c, respectively. The Pex14p^{AXXA} still interacted with Pex5p, Pex7p, and Pex14p, but an interaction with the SH3 domain of Pex13p is no longer observed. (B) The SH3 domain of Pex13p^{E320K} still interacts with the PTS1 receptor Pex5p but not with Pex14p. The Pex13p^{E320K} contains the substitution of Glu320 by Lys320 within the RT loop of the SH3 domain. The interaction of Pex13p(SH3) and Pex13p(SH3)^{E320K} with Pex5p and Pex14p was analyzed by the β-galactosidase expression and the histidine prototrophy in PCY2 and HF7c, respectively. Three representative independent transformants are shown (1–3).

of the SH3 domain of Pex13p abolishes its two-hybrid interaction with Pex14p, the mutated SH3 domain still interacts with Pex5p (Fig. 8 B). Accordingly, we conclude that there are distinct binding sites for both Pex5p and Pex14p within this domain or adjacent regions contained within the construct used for the assay.

Remarkably, neither the E320K mutation of the SH3 domain of Pex13p nor the mutation of the proline-rich motif of Pex14p prevented the peroxisomal localization of Pex14p (Fig. 9). This observation suggests that the binding of Pex14p to the SH3 domain of Pex13p is not absolutely required for the targeting and binding of Pex14p to peroxisomes. Why then does the absence of Pex13p lead to the mistargeting of Pex14p (Fig. 7)? One possibility is that Pex13p is a component of a protein complex at the peroxisomal membrane that may disintegrate in the absence of the entire protein, but remains stable without the SH3-dependent interaction between Pex13p and Pex14p. Association of Pex14p with the complex may not require a direct interaction with Pex13p, but may be mediated by

other components of the complex. The simplest explanation for our observations on the Pex13p/Pex14p interaction is the existence of an as yet unrecognized binding partner for Pex13p that may also provide the binding site for Pex14p at the peroxisomal membrane. This missing link, however, is not Pex17p. It is true that Pex17p is another binding partner of Pex14p, but our data suggest that Pex17p is not required for association of the Pex13p/Pex14p/Pex5p/Pex7p complex, as all these components can efficiently coprecipitate in the absence of Pex17p (Fig. 3). Moreover, we found no Pex17p in a precipitate from *pex14Δ* cells that still contains Pex13p and Pex7p (Fig. 3), leading to two conclusions. First, a subcomplex of Pex13p and Pex7p can form in the absence of Pex14p and Pex17p, and second, Pex14p is required for the association of Pex17p with the complex. The latter may be explained by the assumption that Pex17p is bound to the complex via Pex14p.

Pex7p/Pex13p Interaction

The amount of Pex7p in the membrane sediment of *pex14Δ* cells is significantly lower than in wild-type or *pex13Δ* cells (Fig. 2), suggesting that Pex14p may contribute to the majority of the total binding capacity of the peroxisomal membrane for the PTS2 receptor. However, a significant amount of Pex7p was sedimented in the absence of Pex14p (Fig. 2, lane *pex14Δ*). Interestingly, in cells lacking both Pex13p and Pex14p, no Pex7p was found in the membrane pellet, which suggests that Pex13p contributed to the remaining Pex7p associated with peroxisomal membranes of *pex14Δ* cells (data not shown). This result, however, has to be interpreted with care since the double deletion of *PEX13* and *PEX14* did result in a significant decrease in immunologically detectable Pex7p (Girzalsky, W., and R. Erdmann, unpublished observations).

The observations that Pex13p and Pex7p interact in the two-hybrid system and can be efficiently coimmunoprecipitated indicate that the proteins interact in vivo (Figs. 3 and 4). Whether Pex13p directly binds Pex7p remains to be shown. Attempts to demonstrate direct binding of the proteins by coimmunoprecipitation of in vitro translated proteins were unsuccessful (data not shown). Our data do not exclude the existence of a bridging protein which would directly interact with both Pex13p and Pex7p, a requirement fulfilled by Pex14p. However, two observations indicate that the hypothetical bridging protein is not one of the known binding partners for Pex13p. First, the Pex7p/Pex13p interaction is also observed in the absence of these proteins (Figs. 3 and 4), and second, the COOH-terminal SH3 domain alone is sufficient for the Pex13p/Pex14p and Pex13p/Pex5p two-hybrid interaction, but not for the interaction of Pex13p with Pex7p (Erdmann and Blobel, 1996). A direct interaction of Pex13p and Pex7p is further suggested by the genetic suppression of the defect caused by a functionally compromised HA-tagged Pex7p by overexpression of Pex13p (Fig. 5).

As discussed above, a Pex5p/Pex7p two-hybrid interaction is not observed in *pex14Δ* (Fig. 1). At first, this observation seems rather surprising, since both Pex5p and Pex7p independently interact with Pex13p in the two-

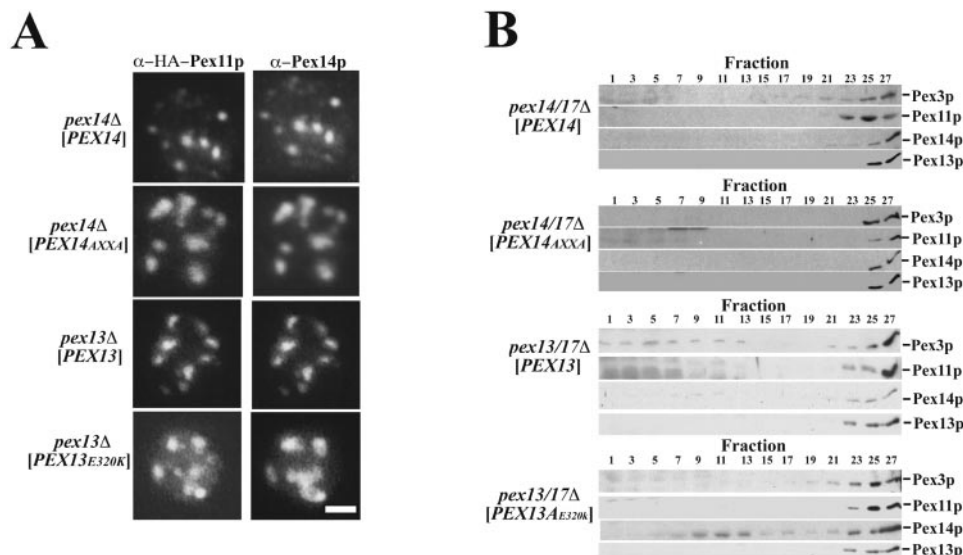


Figure 9. Binding to the SH3 domain of Pex13p does not seem to be required for the peroxisomal membrane targeting of Pex14p. (A) Double immunofluorescence microscopy localization of Pex14p and HA-Pex11p. Oleic acid-induced *pex13 Δ* cells coexpressing HA-tagged Pex11p either with Pex13p or Pex13pE320K, as well as oleic acid-induced *pex14 Δ* cells coexpressing HA-tagged Pex11p either with Pex14p or Pex14pAXXA, were processed for double immunofluorescence microscopy localization of Pex14p and HA-Pex11p. Rabbit antibodies against Pex14p and mouse antibodies against the HA-epitope were used for the detection.

Neither mutation in the proline-rich motif of Pex14p nor mutation of the RT loop of the SH3 domain of Pex13p resulted in the mislocalization of Pex14p. Secondary antibodies were FITC-conjugated anti-mouse IgG and CY3-conjugated anti-rabbit IgG. Bar, 5 μ m. (B) Flotation of peroxisomal membranes in *pex13/17 Δ* cells expressing wild-type or mutated Pex13p and *pex14/17 Δ* cells expressing wild-type or mutated Pex14p. Cell-free extracts were separated on 50–25% (wt/wt) sucrose step gradients and localization of Pex3p, Pex11p, and Pex14p was monitored by immunoblot analysis. Peroxisomal membrane ghosts were detected at the top of the gradient. A colocalization of Pex14p with the peroxisomal membrane markers suggests the association of the protein with peroxisomal membrane ghosts independent of the interaction of Pex14p with the SH3 domain of Pex13p.

hybrid system (Fig. 4). One could imagine that Pex13p may serve as a bridging molecule between the import receptors to mediate an indirect binding which could have emerged in the two-hybrid system. However, the amount of Pex5p simultaneously associated with the Pex7p-Pex13p complex may be too low to give a positive response. In support of this assumption, the amount of Pex5p coimmunoprecipitating with Pex7p in the absence of Pex14p is extremely reduced, despite the presence of significant amounts of Pex13p (Fig. 3, lane *pex14 Δ*). Perhaps Pex13p does not usually associate simultaneously with both of the import receptors, or association is transient.

The domain of Pex13 that interacts with Pex7p, as well as the side of the peroxisomal membrane where the interaction occurs, remains unknown. Furthermore, the intracellular localization of Pex7p in yeast is still a matter of debate. One group has reported that the protein is exclusively localized in the peroxisomal lumen (Zhang and Lazarow, 1995, 1996), whereas others found the protein to be predominantly localized in the cytosol with a small amount associated with the peroxisomal membrane (Marzioch et al., 1994; Rehling et al., 1996). Because the SH3 domain alone does not mediate the interaction with Pex7p, we suggest that regions NH₂-terminal of the SH3 domain may be required for the interaction or contribute to the correct conformation of the binding site. Previously, the COOH-terminal SH3 domain has been reported to face the cytosol (Elgersma et al., 1996; Gould et al., 1996), and we found that both the NH₂ terminus and the COOH terminus of Pex13p are exposed to the cytosol (Fig. 6), suggesting that the protein traverses the membrane with an even number of membrane spans. In this respect, it is in-

teresting to note that two regions which would fulfill the requirement for α -helical transmembrane segments are present in Pex13p (Elgersma et al., 1996).

The interaction of Pex13p with Pex7p has far reaching implications for our understanding of protein import into the peroxisomal matrix. Why are there several binding sites for the import receptors at the peroxisomal membrane? One hypothesis suggests that the multiple interactions reflect the existence of an import cascade in which the cargo-loaded import receptors are transferred from one component of the import complex to the next (Erdmann et al., 1997). Our confirmation that at least two peroxisomal membrane proteins bind the receptors raises concerns about which functions as the docking protein for each of the import pathways. Experimental evidence that Pex13p may be the docking protein for the PTS1 receptor has been provided (Gould et al., 1996), but the unsolved questions stress the need for reliable in vitro systems to study the order of interactions during the process of peroxisomal protein import.

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