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. Accordingly, 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) 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 R LX₅ H/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 Egersma 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).
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 McNee 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 (A Ibertini 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 (A Ibertini 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 (A Ibertini 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 NH$_2$-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). Y NO 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 A usubel et al. (1992). For induction of the CUP1 promoter CuSO$_4$ was added according to M arzioch et al. (1994).

**Plasmids**

For construction of pRS-PEX7 HA, the SacI/KpnI fragment of YIp-PEB-HA$_3$ (Zhang and Lazarow, 1995) was subcloned into low copy plasmid pH 316 (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 Xhol/PsrI fragment of pRS-PEX13 into YEp351 (Erdmann and Blobel, 1996).

For construction of Pex14pAXX and Pex13pE320K, mutations were introduced by PCR using gene splicing by overlap extension (Yon and Fried, 1989). Primers used to construct Pex14pAXX were: KU107, 5'-GAAGATTCGAGGCCTTATGAGTGACGTGGTCAGT-3'; RE8, 5'-CATCGC-(ATCCCTGTGGGC-CAAGCAGTCG)-3', (nucleotides [nt] 1–18); RE9, 5'-GGGGATCCCGGGATACCTATGGGATGGTCACAATC-3', (nt 249–278); underlined nt represent introduced mutations); RE8, 5'-GGCATGCTGAAAACCTGGCCACGGATCC-3', (nt 249–278); and KU109, 5'-GGGGATCCCGGGATACCTATGGGATGGTCACAATC-3'.

### Table I. Yeast Strains Used in This Study

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<th>Name</th>
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The protein fusion and purification system of New England Biolabs was used. For expression of a glutathione S-transferase (GST) fusion protein (MBP-SH3), amino acids 286–386 of Pex13p comprising the SH3 domain of Pex13p were fused to the COOH terminus of the GST gene and the 5′- and 3′-noncoding region of PEX14 in the yeast two-hybrid recombination vector pSH47 (Gündener et al., 1996). Subsequently, the PEX14 gene deletion the primer sets KU251, 5′-(ACGTGCAGTAATTTTCACTTACATGAGAATGTCGAC)-3′ and KU273, 5′-(ACGTGCATCGTCAATTTTCACTTACATGAGAATGTCGAC)-3′ were used. Finally, for a PEX17 gene deletion, the primer sets KU251, 5′-(TCTTAGATTTTTCATACTTATTTGCTGACATATTATAACATGACATCTCAGTCGAC)-3′ and KU372, 5′-(GTTTTAAATAATGAAACATTTTCACTTATTTGCTGACATATTATAACATGACATCTCAGTCGAC)-3′ were used. For PEX14 gene deletion, the primer sets KU289, 5′-(GAAAATCTCAAATGAAACATTTTCACTTATTTGCTGACATATTATAACATGACATCTCAGTCGAC)-3′ and KU290, 5′-(GAAAATCTCAAATGAAACATTTTCACTTATTTGCTGACATATTATAACATGACATCTCAGTCGAC)-3′ were used. For a PEX17 gene deletion, the primer sets KU251, 5′-(TCTTAGATTTTTCATACTTATTTGCTGACATATTATAACATGACATCTCAGTCGAC)-3′ and KU273, 5′-(ACGTGCATCGTCAATTTTCACTTACATGAGAATGTCGAC)-3′ were used. For a PEX17 gene deletion, the primer sets KU251, 5′-(TCTTAGATTTTTCATACTTATTTGCTGACATATTATAACATGACATCTCAGTCGAC)-3′ and KU273, 5′-(ACGTGCATCGTCAATTTTCACTTACATGAGAATGTCGAC)-3′ were used.

To delete the PEX5, PEX13, PEX14, and PEX17 in the yeast two-hybrid reporter strains, PEX14 and PEX17 in wild-type UL71A, PEX14 in pex17A, PEX17 in pex13A, and PEX5 in pex14A, the kanMX4 gene was used as a selective marker for insertion into the genomic locus (Wach et al., 1994). Deletion cassette containing the kanMX4 gene and the 5′ and 3′ untranslated regions of the corresponding ORFs were constructed by PCR using PsaI and KpnI restriction enzymes (Erdmann and Blobel, 1995; Rehling et al., 1996; Libinetti et al., 1997). Southern transfer of two-hybrid vectors into the PCY2 and HFC7, as well as the deletion PEX7 and PEX13 in wild-type UTL71A, PEX14 in pex17A, PEX17 in pex13A, and PEX5 in pex14A, the kanMX4 gene was used as a selective marker for insertion into the genomic locus (Wach et al., 1994). Deletion cassette containing the kanMX4 gene and the 5′ and 3′ untranslated regions of the corresponding ORFs were constructed by PCR using PsaI and KpnI restriction enzymes (Erdmann and Blobel, 1995; Rehling et al., 1996; Libinetti et al., 1997). Southern transfer.
to the manufacturer’s protocol. Polyclonal antibodies were raised against the fusion protein (Eurorgenet).

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

A nuclease (Fex3p; Erdmann and Kunau, 1994), anti-Pcs60p (Bibel and Erdmann, 1996), anti-Pex3p (Höpfel 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. A n-t-Rabbit or anti-mouse IgG-coupled HRP (Nycomed Amersham) was used as a secondary antibody, and plates were developed using the ECL-system (Nycopamed A mersham). Western blot analyses were performed according to standard protocols (Harlow and Lane, 1988).

Immunoprecipitation 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 antiserum against thiolase were used at dilutions of 1:3,000. For detection, 6 µg/ml solution of CY3-conjugated donkey anti-rabbit (Jackson Immunoresearch Laboratories) was used. HA-tagged Pex11p was detected with monoclonal 12CA5 antisera (BAcO; 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 and dH2O), and 3 g glass beads (0.5 mm) were added to the cells. Breakage was achieved by vortexing for 4 min (8 x 30 s, with breaks = 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 A H 650, 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. A aliquots of the samples were analyzed by SD S-PA GE.

**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 (M arzioch et al., 1994) in wild-type, pex17Δ, pex14Δ, and pex13Δ 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.

**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 (Rheling 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.

![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 PCY 2 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).](https://www.jcb.rupress.org/doi/abs/10.1083/jcb.144.4.1154)
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 \( pex14^{\Delta} \) cells (Fig. 2) suggests that the majority of sedimentable Pex7p associates with membranes in a Pex14p-dependent manner. However, in \( pex14^{\Delta} \) cells a significant amount of myc-Pex7p 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 \( pex7^{\Delta} \) cells (Albertini et al., 1997; Huhse et al., 1998). Comparison of the constituents of the precipitates revealed five interesting observations.

First, in \( pex14^{\Delta} \) and \( pex5^{\Delta} \) 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 \( pex14^{\Delta} \) cells was drastically reduced, while the amount in Pex13p remained essentially unchanged (Fig. 3, lane \( pex14^{\Delta} \)). 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 \( pex17^{\Delta} \) cells (Fig. 3, lane \( pex17^{\Delta} \)). 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 \( pex14^{\Delta} \) cells (Fig. 3, lane \( pex14^{\Delta} \)), 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.

Figure 2. A portion of mycPex7p is associated with a membrane sediment in \( pex14^{\Delta} \) 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 \( pex14^{\Delta} \) cells. Equal portions of membrane and supernatant fractions were analyzed.

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, \( pex5^{\Delta} \), \( pex14^{\Delta} \), \( pex5/14^{\Delta} \), \( pex13^{\Delta} \), and \( pex17^{\Delta} \) 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 \( pex14^{\Delta} \) 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 Fox3p. (B) Immunoblot of equal portions of the whole cell extracts (1% of total) for mycPex7p, Pex13p, Pex14p, 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 communoprecipitates 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 indeed 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 communoprecipitation 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**

M utant 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
Expression of a COOH-terminally HA-tagged Pex7p from the low copy plasmid pRSPEx7-HA3 leads only to a partial complementation of the pex7Δ mutant phenotype (Zhang and Lazarow, 1995). This is indicated by the inability of the transformants to grow on oleic acid plates (Fig. 5 A) and a reduced ability to import Fox3p (thiolase) into peroxisomes. The latter is evident by the pronounced cytosolic mislocalization of this protein (Fig. 5 B, panel d). This mutant phenotype of pex7Δ[pRSPEx7-HA3] was employed to investigate whether overexpression of Pex7p-binding partners may suppress a defect in Pex7p function. Cells expressing Pex7p-HA3 were transformed with multicopy plasmids containing either PEX14 or PEX13 under the control of their own promoters. As judged by their growth characteristics on oleic acid medium (Fig. 5 A) and by the fluorescence pattern for thiolase (Fig. 5 B), overexpression of PEX13, but not PEX14, rescued the mutant phenotype caused by the defective Pex7p-HA. Even though the suppression was not as efficient as complementation with the wild-type PEX7, this observation demonstrates that Pex13p can suppress the mutant phenotype of pex7Δ[pRSPEx7-HA3], providing genetic evidence for an interaction between Pex7p and Pex13p.

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 (Eldersma 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 and 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...
that both the NH₂-terminus and the COOH-terminal SH3 domain are exposed to the cytosol. This result also implies 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 (PA TL A H R D W). Remarkably, the mutated Pex14pA AX A still complemented the peroxisomal biogenesis defect of pex14Δ cells (data not shown). We also introduced an E 320K 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 (Egersma et al., 1996). As shown in Fig. 8, the mutated Pex14pA AX A 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. An iso the E 320K 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 Pex14pA AX A (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/E 320K still contain Pex14p. The subcellular localization of Pex14p, Pex14pA AX A, and peroxisomal membrane markers was analyzed by double immunofluorescence microscopy and flotation analysis. Colocalization was observed for HA - Pex14p and Pex14pA AX A in pex14Δ cells, as well as for HA - Pex11p and Pex14p in pex13Δ cells expressing Pex13p/E 320K, indicative of peroxisomal membrane association of these proteins (Fig. 9 A). These results were corroborated by flotation analysis which revealed that Pex14pA AX A was associated with the fraction containing the peroxisomal membrane ghosts of pex14Δ/pex17Δ, as were Pex14p in pex13Δ/pex17Δ cells expressing Pex13p/E 320K (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 exhibited by Pex14p in pex13Δ/pex17Δ cells expressing Pex13p/E 320K (Fig. 9 B) 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,
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 A. Bertini 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 (E. Igersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996; A. Bertini 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), suggesting the notion of a typical SH3 domain-ligand interaction between Pex13p and Pex14p. Interestingly, although the E320K mutation of the RT loop
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 Pex14pAXXA with Pex5p, Pex7p, Pex13p, and Pex14p. Pex14pAXXA 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 Pex14pAXXA 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 Pex13pE320K still interacts with the PTS1 receptor Pex5p but not with Pex14p. The Pex13pE320K 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).

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 Pex14pAXXA with Pex5p, Pex7p, Pex13p, and Pex14p. Pex14pAXXA 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 Pex14pAXXA 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 Pex13pE320K still interacts with the PTS1 receptor Pex5p but not with Pex14p. The Pex13pE320K 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).

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. A 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 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-
Flotation of peroxisomal membranes in even number of membrane spans. In this respect, it is interesting that the protein traverses the membrane with an minus of Pex13p are exposed to the cytosol (Fig. 6), suggesting of Pex14p with the peroxisomal membrane markers suggests the association of the protein with peroxisomal membrane ghosts and Pex14p was monitored by immunoblot analysis. Peroxisomal membrane ghosts were detected at the top of the gradient. A colocalization type or mutated Pex14p. Cell-free extracts were separated on 50–25% (wt/wt) sucrose step gradients and localization of Pex3p, Pex11p, in independent of the interaction of Pex14p with the SH3 domain of Pex13p.

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 μm. (B) Flotation of peroxisomal membranes in pex13/17Δ cells expressing wild-type or mutated Pex13p and pex14/17Δ 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.

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 Lazaro, 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 SH 3 domain alone does not mediate the interaction with Pex7p, we suggest that regions NH2-terminal of the SH 3 domain may be required for the interaction or contribute to the correct conformation of the binding site. Previously, the COOH-terminal SH 3 domain has been reported to face the cytosol (Engersma 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 interesting to note that two regions which would fulfill the requirement for α-helical transmembrane segments are present in Pex13p (Engersma 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.

We are grateful to Ulrike Freimann, Utta Ricken, and Sigrid Wüthrich for technical assistance. We thank Paul Lazarow for the plasmid YIpPEB1-HA3 and William B. Snyder, Gabriele Dodt, Michael Linkert, and M. Michael Schwierskott for reading of the manuscript.

Julia Kipper was supported by a fellowship from the Bochumer Graduiertenkolleg Biogenesis und Mechanismen komplexer Zellfunktionen. This work was supported by grants from the Deutsche Forschungsgemeinschaft to R. Erdmann (Er 178/2-1, Er 178/2-2, and SFB 480) and to W. - H. K. unau (Ku 329/17-3), as well as by the Fonds der Chemischen Industrie.

Received for publication 14 September 1998 and in revised form 10 February 1999.

Figure 9. Binding to the SH 3 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 pex13Δ cells coexpressing HA-tagged Pex11p either with Pex13p or Pex13pE320K, as well as oleic acid-induced pex14Δ cells coexpressing HA-tagged Pex11p either with Pex14p or Pex14pA XXA, 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.
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