Yeast Pex14p Possesses Two Functionally Distinct Pex5p and One Pex7p Binding Sites*

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Current evidence favors a cycling receptor model for the import of peroxisomal matrix proteins. The yeast Pex14 protein together with Pex13p and Pex17p form the docking subcomplex at the peroxisomal membrane and interact in this cycle with both soluble import receptors Pex5p and Pex7p. In a first step of a structure-function analysis of *Saccharomyces cerevisiae* Pex14p, we mapped its binding sites with both receptors. Using the yeast two-hybrid system and pull-down assays, we showed that Pex5p directly interacts with two separate regions of ScPex14p, amino acid residues 1–58 and 235–308. The latter binding site at the C terminus of ScPex14p overlaps with a binding site of Pex7p at amino acid residues 235–325. The functional assessment of these two binding sites of ScPex14p with the peroxisomal targeting signal receptors indicates that they have distinct roles. Deletion of the N-terminal 58 amino acids caused a partial defect of matrix protein import in *pex14Δ* cells expressing the Pex14-(59–341)-p fragment; however, it did not lead to a *pex* phenotype. In contrast, truncation of the C-terminal 106 amino acids of ScPex14p completely blocked this process. On the basis of these and other published data, we propose that the C terminus of Pex14p contains the actual docking site and discuss the possibility that the N terminus could be involved in a Pex5p-Pex14p association inside the peroxisomal membrane.

A crucial step of the receptor cycle, which could provide directionality to the process, is the docking event at the peroxisomal membrane (1, 2). Studies from several laboratories provide evidence that Pex14p is the central component of a docking complex, which also contains Pex17p and Pex13p. As Pex14p interacts with several other membrane-bound proteins in addition to both PTS receptors, it has been proposed to be the point of convergence of the two peroxisomal import pathways (6).

There are conflicting data concerning the nature of the association of Pex14p with the peroxisomal membrane, and the topology of the protein is still not entirely solved. Although we originally report that *Saccharomyces cerevisiae* Pex14p is extractable with carbonate (6), others found that Pex14p from *S. cerevisiae* (7) and other organisms (8–13) behaves as an integral membrane protein. However, there is agreement that the C terminus of the protein is exposed to the cytosol (9, 10, 12, 13). *Rattus norvegicus* Pex14p was found to be tightly associated with a fraction of Pex5p that behaves as an integral membrane protein (13). It was shown that the first 130 amino acids of Pex14p are highly protected from exogenously added protease by the peroxisomal membrane and that this domain is responsible for the strong interaction of Pex14p with the organelle membrane (12, 13).

To explore the function of Pex14p in greater detail, we mapped its binding sites for the two PTS receptors. Unexpectedly, two separate and functionally different binding sites were found for the PTS receptor Pex5p, one in each of the two termini. The C-terminal binding site overlapped with a single binding site determined for Pex7p and was essential for matrix protein import. In contrast, deletion of the N-terminal binding site reduced the efficiency but did not abolish matrix protein import. We proposed a model in which the cytosolically exposed C terminus of Pex14p served as the actual docking site and in which the N terminus could be involved in a Pex14p-Pex5p association inside the peroxisomal membrane.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Culture Conditions**—The *S. cerevisiae* wild-type strain used in this study was *UTL-7A* (14). Yeast complete (yeast extract/peptone/dextrose) and minimal media (SD) have been described previously (15). The yeast knock-out strain *pex14Δ* was a derivate of *UTL-7A*. To delete PEX14, the kanMX4 gene was used as a selective marker for insertion into the genomic locus (16). Deletion cassettes containing the *kanMX4* gene and the 5′ and 3′ flanking regions of the Pex14p-ORF (open reading frame) were constructed by PCR using pFA6a-kanMX4 (16) as a template and Ku 289 (5′-gaaacctcaag-aaaaacagagaagttgtaaggtgaataaggacgtacgctgcaggtcgac-3′) and Ku 290 (5′-aaattacaatttccgttaaaaaactaattacttacatagaattgcgatcgatgaattcgagctcg-3′) as primers. For yeast two-hybrid experiments the yeast strain *PCY2* was used (17).

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3 The abbreviations used are: PTS, peroxisomal targeting signal; GST, glutathione S-transferase; NTA, nitritotriacetic acid; MES, 4-morpholinoethanesulfonic acid; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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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. YNDO medium contained the same components supplemented with 1% glucose. When necessary, auxotrophic requirements were added according to Ref. 18.

Plasmids—For the expression of Myc-Pex7p, Pex14p and Pex14p fragments in a pex14 deletion strain of S. cerevisiae plasmids were cloned using the vectors pRS316, pRS416, and YEp351. For Pex14p and its derivatives, endogenous promoters and terminations were used. The resulting constructs are listed in TABLE ONE. Plasmids used for expressing His\(_6\)-Pex14-\((1–58)-p\), GST-Pex14-\((234–341)-p\), GST, and Pex5p in E. coli are based on pET21d and pGEX-4T-2 vectors and are also listed in TABLE ONE. Detailed cloning strategies are available on request.

Antibodies, Western Blotting—Anti-Pex13p, -Pex14p, -Pex3p, -anti-Pex5p, -Fox1p, -catalase A, -Pcs60p, -Fox3p, -Myc, and anti-Fbp1p have been described previously (6, 19–23). To produce anti-Pex14-\((1–58)-p\)-His\(_6\) was isolated using Ni\(^{2+}\)-NTA-agarose. Pex14-\((1–58)-p\)-His\(_6\) was expressed in BL21(DE3

| Plasmid          | Vector          | Expressed protein | Reference |
|------------------|-----------------|------------------|-----------|
| pRS316-PEX14     | pRS316          | Pex14p           | lc 6      |
| pKN14/21c        | pRS316          | Pex14-(59–341)-p| lc This study |
| pKN14/22c        | pRS416          | Pex14-(1–234)-p  | lc This study |
| pUP14/9          | pPC86           | Pex14-(235–341)-p| lc This study |
| pUP14/18         | pPC86           | Pex14-(250–341)-p| lc This study |
| pUP14/10         | pPC86           | Pex14-(270–341)-p| lc This study |
| pUP14/20         | pPC86           | Pex14-(235–325)-p| lc This study |
| pUP14/19         | pPC86           | Pex14-(235–308)-p| lc This study |
| pPC86-PEX5       | pPC86           | Pex5p            | lc 36     |
| pPC86-PEX14      | pPC86           | Pex14p           | lc 6      |
| pPC97-PEX7       | pPC97           | Pex7p            | lc 31     |
| pPC97-PEX14      | pPC97           | Pex14p           | lc 6      |
| pKN14/11c        | pPC97           | Pex14-(1–58)-p   | lc This study |
| pKN14/21b        | pPC97           | Pex14-(59–341)-p| lc This study |
| pKN14/24b        | pPC97           | Pex14-(59–234)-p| lc This study |
| pKN14/22b        | pPC97           | Pex14-(1–234)-p  | lc This study |
| pKN14/12c        | pPC97           | Pex14-(1–249)-p  | lc This study |
| pKN14/9b         | pPC97           | Pex14-(235–341)-p| lc This study |
| pKN14/18b        | pPC97           | Pex14-(250–341)-p| lc This study |
| pKN14/10b        | pPC97           | Pex14-(270–341)-p| lc This study |
| pKN14/20a        | pPC97           | Pex14-(235–325)-p| lc This study |
| pKN14/19b        | pPC97           | Pex14-(235–308)-p| lc This study |
| pKN5/0–1         | pET21d (+)      | Pex5p            | hc This study |
| pET21d-Pex14-(1–58)-His\(_6\) | pET21d (+) | Pex14-(1–58)-p-His\(_6\) | hc This study |
| pGEX-4T-2-GST-Pex14-(235–341) | pGEX-4T-2 | GST-Pex14-(235–341)-p | hc This study |
| pGEX-4T-3         | pGEX-4T-3       | GST              | hc GE Healthcare, Freiburg |
| pPR7/4           | YEp351          | Myc-Pex7p        | hc 31     |
needed for 2 h at 4 °C. The resin columns were collected in minispin columns (MobiTec). The Ni\textsuperscript{2+}-NTA-agarose was washed 10 times using 200 μl of lysis buffer, followed by 10 times using 100 μl of 200 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 20 mM imidazole, 10 times using 200 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 40 mM imidazole, and 10 times using 200 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 60 mM imidazole. Elution was carried out using 200 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1M imidazole. Every two fractions were pooled before analysis.

The GSH-Sepharose was washed 10 times using 200 μl of lysis buffer. Proteins were eluted with lysis buffer enriched with 10 mM reduced glutathione. Every two fractions were pooled before analysis. As protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 2 μg/ml leupeptin, and 2 μg/ml pepstatin were used.

For the analysis of the binding between GST-Pex14-(235–341)-p and Pex5p, 2 g of cells expressing one or the other fusion protein or GST were used. Lysis buffer did not contain Triton X-100 but 1 mM dithiothreitol. As protease inhibitors, 2 μM leupeptin, 2 μM pepstatin, 200 μM Pefabloc SC, 5 mM benzamidine, and 5 mM NaF were used. Cells were lysed using a French pressure cell press. Soluble fractions were obtained by centrifugation as described above, mixed, and incubated at 4 °C for 1 h before they were subjected to columns with 0.8 ml of GSH-Sepharose. Washing was performed using 32 ml of lysis buffer. Recovered proteins were eluted with 16 ml of lysis buffer enriched with 10 mM reduced glutathione in 0.5-ml fractions.

**Cell Fractionation**—Spheroplasting of yeast cells, homogenization, and differential centrifugation at 25,000 × g of homogenates were performed as described previously (15).

**For separation of cell organelles by density gradient centrifugation, post-nuclear supernatants of wild-type and mutant strains were loaded onto continuous 20–53% (w/v) sucrose density gradients (25 ml). The gradient buffer contained 5 mM MES, 1 mM EDTA, 1 mM KCl, 0.1% (v/v) ethanol/KOH, pH 6.0, at 4 °C. After a centrifugation step of 1.5 h (Sorvall-SV288, 19,500 rpm), ~30 fractions of 1 ml each were collected. 500 μl of each fraction of any gradient were processed for Western blotting using trichloroacetic acid, whereas the other part was used for enzyme measurements.**

The suborganelar localization of proteins was determined by extraction of 25,000 × g organelle pellets by either high salt treatment with buffer containing 10 mM Tris/HCl, pH 8.0, 500 mM KCl, and 1 mM phenylmethylsulfonyl fluoride or using carbonate buffer containing 100

![FIGURE 1. Two-hybrid analysis of the interaction between Pex14p fragments and Pex5p or Pex7p, respectively. PCY2 was co-transformed with plasmids encoding proteins as indicated and tested for β-galactosidase activity using a filter assay and X-gal as a substrate. Three independent double transformants are shown.](image1)

![FIGURE 2. In vitro binding assay. The binding of Pex14-(1–58)-p-His\textsubscript{6} and GST-Pex14-(235–341)-p to Pex5p or Pex7p was studied in vitro by pull-down experiments. Soluble fractions of BL21(DE3) cells heterologously expressing Pex5p, Pex14-(1–58)-p-His\textsubscript{6}, GST-Pex14-(235–341)-p, GST, or no host protein as well as soluble fractions of yeast cells overexpressing Myc-Pex7p were mixed as indicated and incubated with Ni\textsuperscript{2+}-NTA-agarose (A) or GSH-Sepharose (B and C). Recovered proteins of the elution fractions were analyzed by SDS-PAGE and Western blotting using anti-Pex5p and anti-Pex14p antibodies (A), antibodies against Pex5p and GST (B), or anti-Pex14p antibodies and antibodies against the Myc epitope (C). Direct binding of Pex5p to the N and C termini of Pex14p as well as an interaction of Pex7p with the C terminus of Pex14p are shown.](image2)
mM Na2CO3, pH 11.5, and 1 mM phenylmethylsulfonyl fluoride. After incubation for 30 min at 4 °C, the samples were spun for 1 h at 200,000 × g through a cushion of 10 mM Tris/HCl, pH 8.0, and 250 mM sucrose.

Enzyme Assays—Catalase (EC 1.11.1.6) and cytochrome c oxidase (EC 1.9.3.1) were assayed according to published procedures (25–28).

Two-Hybrid Assay—The two-hybrid assay was based on the method of Fields and Song (29). The open reading frames or coding regions of specific fragments of PEX genes were fused to the DNA binding domain or transcription-activating domain of GAL4 into the vectors pPC86 and pPC97 (17) (see TABLE ONE). Co-transformation of two-hybrid vectors into PCK2 yeast cells was performed according to Gietz and Sugino (30). Double transformants were selected on SD synthetic medium and analyzed by SDS-PAGE and Western blotting. For A B, equal fractions of yeast wild-type cells, pex14A cells, as well as mutant cells expressing Pex14-(1–58)-p-His6 antibodies were used, respectively. C and D, cells of indicated yeast strains were grown overnight on glucose minimal media. Subsequently, dilutions were prepared, and 2 μl of each dilution was spotted onto oleate plates. The plates were then incubated for 3–5 days at 30 °C and scored for the appearance of colonies. In comparison to wild-type cells, the cells expressing Pex14-(59–341)-p showed reduced growth on oleate as the sole carbon source, and cells expressing Pex14-(1–234)-p were not able to utilize oleate as did pex14A cells.

FIGURE 3. Expression of Pex14p fragments and phenotypical analysis of cells expressing Pex14-(59–341)-p or Pex14-(1–234)-p by oleate growth test. A and B, equal fractions of yeast wild-type cells, pex14A cells, as well as mutant cells expressing Pex14p full-length and (A) Pex14-(59–341)-p (A) or Pex14-(1–234)-p (B) were analyzed by SDS-PAGE and Western blotting. For immunodecoration, anti-Pex14p antibodies or anti-Pex14-(1–58)-p-His6 antibodies were used, respectively. C and D, cells of indicated yeast strains were grown overnight on glucose minimal media. Subsequently, dilutions were prepared, and 2 μl of each dilution was spotted onto oleate plates. The plates were then incubated for 3–5 days at 30 °C and scored for the appearance of colonies. In comparison to wild-type cells, the cells expressing Pex14-(59–341)-p showed reduced growth on oleate as the sole carbon source, and cells expressing Pex14-(1–234)-p were not able to utilize oleate as did pex14A cells.

RESULTS
Pex5p Binds the N and C Termini of ScPex14p—To understand how ScPex14p facilitates the import of peroxisomal matrix proteins, we first mapped the binding sites of the two PTS assay within this protein. We used the yeast two-hybrid system and first tested N- and C-terminal deletion mutants of Pex14p for the binding of Pex5p. The short N-terminal fragment Pex14-(1–58)-p was chosen because it comprises the most conserved region of Pex14 proteins (data not shown), and Schliebs et al. reports that the analogous region of human Pex14p directly binds to a Partial Import Defect

Deletion of the N-terminal 58 Amino Acid Residues of ScPex14p Leads to a Partial Import Defect—The overall import activity of ScPex14p can be assayed by the ability of pex14A cells expressing wild-type Pex14p to grow on oleate as the sole energy and carbon source or by determining the presence and/or properties of peroxisomes using electron microscopy, immunofluorescence, and cell fractionation. All of these techniques were used to assess the functional significance of the identified PTS receptor binding sites within ScPex14p.
We first tested *pex1Δ* cells expressing Pex14p without the N-terminal 58 amino acid residues under the endogenous promoter of Pex14p. The steady state concentration of this fragment in these cells was similar to that of Pex14p in wild-type cells (Fig. 3A). We observed that *pex1Δ* cells expressing the N-terminal deletion mutant Pex14p-(59–341)-p grew significantly less efficiently on oleate than wild-type cells (Fig. 3B) but that they do contain peroxisomes, which according to their morphology and size, are indistinguishable from those of cells expressing wild-type Pex14p (Fig. 4). As the reduced ability to grow on oleate suggested a partial impairment of the protein import, we explored this possibility in more detail. Immunofluorescence microscopy and cell fractionation studies clearly confirmed this assumption for both the PTS1 and surprisingly the PTS2 import pathway. Antibodies against the PTS2 protein Fox3p as indicated. Secondary antibodies were Cy3-conjugated anti-rabbit IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG. Each frame shows a single cell that is representative of many others. A punctate pattern combined with a background radiance could be observed in *pex14Δ* cells using anti-Fox3p and anti-Pcs60p antibodies; *pex14Δ* cells were characterized by a complete diffuse staining similar to *pex14Δ* cells. The scale bars represent 2.5 μm.

To establish whether Pex14-(59–341)-p localizes to peroxisomal membranes and to compare peroxisomes of *pex14Δ* cells expressing this N-terminal deletion mutant to those of wild-type cells, we analyzed *pex14Δ* cells expressing the N-terminal deletion mutant Pex14p-(59–341)-p with Pex13p, another exclusively membrane-bound peroxin.

All of these findings together led us to conclude that the N-terminal 58 amino acid residues of Pex14p are necessary for the efficiency of peroxisomal protein import but are not essential.

Deletion of the C-terminal 106 Amino Acid Residues of Pex14p Results in a *pex Phenotype*—A similar series of experiments were conducted with *pex14Δ* cells expressing the deletion mutant Pex14p-(1–234)-p, which lacks the two overlapping Pex5p and Pex7p binding sites of the cytosolically exposed C terminus of Pex14p, in amounts comparable with Pex14p in wild-type cells (Fig. 3B). These cells did not grow on oleate (Fig. 3D) and did not possess morphologically recognizable peroxisomes (Fig. 4), and differential centri-
Pex14p Import Receptor Interactions

ugation demonstrated that the matrix proteins catalase, thiolase, and Fox1p were soluble. As in pex14Δ cells, these proteins were found in the 25,000 g supernatant fraction (Fig. 6). Immunofluorescence microscopy corroborated the conclusion of a complete defect of matrix protein import. Antibodies against Pcs60p and thiolase, bona fide PTS1 and PTS2 proteins, respectively, yielded a diffuse immunofluorescence pattern (Fig. 5). Moreover, density gradient centrifugation demonstrated the complete absence of mature peroxisomes at a density of 1.21 g/cm³. The Pex14-(1–234)-p fragment co-migrated together with the integral peroxisomal membrane protein Pex13p near the top of the gradient, indicating the presence of peroxisomal ghosts (Fig. 7D). Pex13p was found in the same gradient fractions as peroxisomal membrane proteins of pex14Δ cells (Fig. 7, C and D).

As all of these different approaches revealed the same properties of cells expressing Pex14-(1–234)-p as those of pex14Δ cells, we conclude that the C-terminal binding sites of Pex5p and Pex7p are essential for matrix protein import into peroxisomes and that their deletion leads to a null mutant phenotype of the corresponding cells.

Pex14-(59–341)-p and Pex14-(1–234)-p Exhibit Different Extraction Properties—After having observed that both Pex14-(59–341)-p and Pex14-(1–234)-p (just as with wild-type Pex14p) co-migrate with bona

FIGURE 7. Subcellular localization of Pex14-(59–341)-p and Pex14-(1–234)-p. Different proteins, as indicated, were analyzed by enzyme activity and Western blotting after centrifugation of post-nuclear supernatants in a density of 20–53% sucrose gradient. Wild-type cells (A) and pex14Δ cells (C) were compared with pex14Δ[Pex14-(59–341)-p] (B) and pex14Δ[Pex14-(1–234)-p] cells (D). Shown are density (●), relative activity of cytochrome c oxidase (Œ), and catalase (⁄).
Pex14p Import Receptor Interactions

Accumulated evidence suggests that Pex14p plays a key role in the docking event of the two soluble import receptors at the peroxisomal membrane (1, 2). Central to this notion are the observations that Pex14p binds both Pex5p and Pex7p. To gain further insight into this important step, we mapped the binding sites of Pex5p and Pex7p on ScPex14p using the yeast two-hybrid system and pull-down experiments. Surprisingly, we found that Pex5p interacts directly and independently with two different regions of ScPex14p, the N-terminal 58 amino acid residues and ~60 amino acids in the C terminus of the protein. The latter region overlaps with the binding site for Pex7p.

The functional assessment of these two different sites of interactions of ScPex14p with the PTS receptors indicates that they have distinct roles. Deletion of the N-terminal 58 amino acids partially reduces matrix protein import; however, it does not lead to a pex phenotype. In contrast, truncation of the C-terminal 106 amino acids of ScPex14p completely blocks matrix protein import. Cells expressing Pex14-(1–234)-p exhibit a phenotype comparable with that of pex14Δ cells. It is important to note that the steady state concentration of both the N- and C-terminal mutant constructs of ScPex14p found in these cells is comparable with that of ScPex14p in wild-type cells, and both mutants localize to peroxisomal membranes. On the basis of these data, we propose that the cytosolically exposed C terminus of ScPex14p is the actual docking site for Pex5p and Pex7p.

What is the role of the Pex5p binding site within the N terminus of ScPex14p? Previous work using rat liver peroxisomes has established that the N terminus of Pex14p is protected from exogenously added proteases by the peroxisomal membrane (8, 12) and that Pex14p forms a tight complex with a membrane-bound fraction of Pex5p that exhibits the properties of an integral membrane protein (13). More recently, two different Pex14p-associated protease-resistant populations of Pex5p could be resolved (34). These data led Azevedo and co-workers to conclude that Pex14p could be more than just a docking protein for Pex5p, perhaps even forming part of a translocation machinery. In accordance with this view, we would like to propose that the interaction between ScPex14p and Pex5p inside the peroxisomal membrane occurs via the N-terminal binding site in Pex14p. This is supported by the presented data regarding the effect the N terminus has on the topology of Pex14p.

Presently, there are no conclusive experimental data to explain why the deletion of the N-terminal binding site of Pex5p in Pex14p also affects the PTS2 pathway. However, it is conceivable that a slightly changed topology of Pex-(59–341)-p has an effect on its interaction with other membrane-bound peroxins of the translocation machinery.

It is interesting to speculate that this interaction between the N terminus of Pex14p and Pex5p could help to form or stabilize the observed Pex14p-Pex5p complex inside the peroxisomal membrane. If such a scenario is true, our present finding that the deletion of the N-terminal 58 amino acid residues of ScPex14p only partially impairs (but not totally blocks) matrix protein import would mean that the formation of the Pex14p-Pex5p complex is important for the efficiency of import under physiological conditions but is not absolutely required for the process to occur. In turn, this conclusion then argues that not Pex14p but Pex5p, perhaps with the help of Pex13p, is the essential component of such a putative import channel. This view would be in agreement with the intriguing finding that, in Hansenula polymorpha, the pex phenotype resulting from the absence of Pex14p can be rescued by an overexpression of Pex5p (35). The question whether Pex14-(1–234)-p in contrast to Pex14-(59–341)-p forms a complex with Pex5p in the peroxisomal membrane of S. cerevisiae will be the subject of future experiments.

Addendum—Since the submission of this manuscript, a publication appeared that also identified two Pex5p binding sites in Pex14p (Williams, C., van den Berg, M., and Distel, B. (2005) FEBS Lett. 579, 3416–3420).

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