Human Pex19p binds a broad spectrum of peroxisomal membrane proteins (PMPs). It has been proposed that this peroxin may: (i) act as a cycling PMP receptor protein, (ii) facilitate the insertion of newly synthesized PMPs into the peroxisomal membrane, or (iii) function as a chaperone to associate and/or dissociate complexes comprising integral PMPs already in the peroxisomal membrane. We previously demonstrated that human Pex19p binds peroxisomal integral membrane proteins at regions distinct from their sorting sequences. Here we demonstrate that a mutant of Pex13p that fails to bind to Pex19p nevertheless targets to and integrates into the peroxisomal membrane. In addition, through in vitro biochemical analysis, we show that Pex19p competes with Pex5p and Pex13p for binding to Pex14p, supporting a role for this peroxin in regulating assembly/disassembly of membrane-associated protein complexes. To further examine the molecular mechanism underlying this competition, six evolutionarily conserved amino acids in the Pex5p/Pex13p/Pex19p binding domain of Pex14p were subjected to site-directed mutagenesis and the corresponding mutants functionally analyzed. Our results indicate that the physically overlapping binding sites of Pex14p for Pex5p, Pex13p, and Pex19p are functionally distinct, suggesting that competition occurs through induction of structural changes, rather than through direct competition. Importantly, we also found that amino acid substitutions resulting in a strongly reduced binding affinity for Pex13p affect the peroxisomal localization of Pex14p.

To date, peroxisome biogenesis studies in a number of evolutionarily diverse organisms have identified 29 gene products (1) called peroxins (abbreviated Pexp1) and including a number corresponding to the order of discovery), which are essential for formation of the organelle. In man, peroxisome biogenesis requires the concerted action of at least 16 of these peroxins (2). Despite the fact that substantial progress has been made in peroxin identification, the function of most of these proteins in the biogenesis process is still only partially understood.

Significant interest has developed in recent years regarding the essentially open question of how PMPs find their way to the organelle (3). In a select few examples, peroxisomal targeting signals (so-called mPTSs) have been identified (4). However, no firm conclusions may yet be drawn about the presence or absence of consensus sequences or common structural properties with respect to these mPTSs (5). The observation that Pex19p interacts with the mPTSs of PpPex3p, Hspex11pβ, Hspex13p, HsPex14p, HsPex16p, HsPMP22, HsPMP34, HsPMP70, HsALDP, and HsALDPR (6–11), and that a portion of cellular Pex19p is found associated with the outer surface of peroxisomes (6, 12), makes this peroxin a reasonable candidate for a cycling PMP receptor protein. However, as shown by multiple independent point mutations, the physically overlapping Pex19p binding domain and the peroxisomal sorting signals of HsPex13p can be functionally separated (10). Moreover, for a number of PMPs including PpPex3p, PpPex13p, PpPex17p, PpPex22p, Hspex3p, HsPex12p, HsPMP70, and RnPex3p, the Pex19p interaction domain and the targeting domain do not overlap at all (7, 10, 13–15). These results argue against a role of Pex19p as a general cycling PMP receptor protein.

Besides being connected with a transport function, Pex19p might function directly (i) in maintaining the solubility of integral PMPs before insertion into the peroxisome membrane (9), (ii) in the insertion process of PMPs into the peroxisome membrane (6) or (iii) in stabilizing PMP interactions within the peroxisomal membrane itself (16). In addition, based on data pointing to a role for Pex19p in interacting with already inserted PMPs, Subramani and coworkers (7) suggested that Pex19p may be functioning as a (dis)assembly factor, or as a chaperone, to regulate membrane-associated protein complexes.

In this study, we employed a Pex13p protein displaying no detectable affinity for Pex19p but still possessing peroxisomal targeting information to demonstrate that Pex19p is not directly implicated in the membrane insertion process of this PMP. In addition, we provide evidence that Pex19p can alter the binding properties of the Pex14p docking complex, supporting a role for this peroxin in regulating assembly/disassembly of membrane-associated protein complexes.
**TABLE I**

| Name            | Oligonucleotide                        |
|-----------------|----------------------------------------|
| Pex13pZomeFW    | 5’-GGGGGATCCATGGCGTCCCAGCCGCCA-3’     |
| Pex13pZomeRV    | 5’-GGGGGATCCAAAAGATCTTGCTTTTCCATC-3’ |
| Pex13-TY1       | 5’-GATCTTGAGGTGCACACCAACCAGGACCCTCAGCA-3’ |
| Pex13-TY2       | 5’-GATCTTGAGGTGCACACCAACCAGGACCCTCAGCA-3’ |
| Pex14.1         | 5’-ACCGTTCAGACACAGCGCTCGATGCCC-3’     |
| Pex14.2         | 5’-ATCCTGAAAGCTCTCCAGACACATTTTC-3’   |
| Pex14.3         | 5’-GGGGGATCCAAAAAGATCTTGCTTTTCCATC-3’ |
| Pex14.4         | 5’-GATCTTGAGGTGCACACCAACCAGGACCCTCAGCA-3’ |
| R25Gfw          | 5’-GATCTTGAGGTGCACACCAACCAGGACCCTCAGCA-3’ |
| R25Grv          | 5’-GATCTTGAGGTGCACACCAACCAGGACCCTCAGCA-3’ |
| R25Gfw          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| F35Sfw          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| F35Srv          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| L36Rfw          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| L36Rrv          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| F52Sfw          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| F52Srv          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| K56Efw          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| K56Erv          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| L58Rfw          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |
| L58Rrv          | 5’-GAAGCATCTCCTAAAGAAGAGGCGTGC-3’   |

* Introduced restriction sites are underlined.

**TABLE II**

| Name            | Protein Cloning vector Insert                  |
|-----------------|-----------------------------------------------|
| pMF792          | HsPex13p-TY-GFP pMF121 Linker ligation (Pex13-TY1, Pex13-TY2) |
| pMF795          | HsPex13p (V178E)-TY-GFP pMF551 Linker ligation (Pex13-TY1, Pex13-TY2) |
| pMF964          | HsPex13p-protein A BamHI digest of PCR product: template pMF121 (Pex13pZomeFW, Pex13pZomeRV) |
| pMF966          | HsPex13p-protein A BamHI digest of PCR product: template pMF551 (Pex13pZomeFW, Pex13pZomeRV) |
| pKG45           | GST-HsPex14p BamHI |
| pMF445          | Biotin-HsPex14p (43–61, 295–377) PinPoint Xa1 BamHI/EcoRV fragment of pMF141 (20) |
| pMF444          | Biotin-HsPex14p (43–61, 295–377) PinPoint Xa1 BamHI/EcoRV fragment of pMF141 (20) |
| pMF999A         | GST-HsPex14p (R25G) SalI/NotI digest of PCR product generated by sequential PCR-steps: 1) Template pKG45 (Pex14.1, R25Gfw) 2) Template pKG45 (R25Gfw, Pex14.2) 3) Template (1) + (2) (Pex14.1, Pex14.2) |
| pMF999B         | GST-HsPex14p (F35S) SalI/NotI digest of PCR product generated by sequential PCR-steps: 1) Template pKG45 (Pex14.1, F35Sfw) 2) Template pKG45 (F35Sfw, Pex14.2) 3) Template (1) + (2) (Pex14.1, Pex14.2) |
| pMF999C         | GST-HsPex14p (L58R) SalI/NotI digest of PCR product generated by sequential PCR-steps: 1) Template pKG45 (Pex14.1, L58Rfw) 2) Template pKG45 (L58Rfw, Pex14.2) 3) Template (1) + (2) (Pex14.1, Pex14.2) |
| pMF999D         | GST-HsPex14p (F52S) SalI/NotI digest of PCR product generated by sequential PCR-steps: 1) Template pKG45 (Pex14.1, F52Srv) 2) Template pKG45 (F52Srv, Pex14.2) 3) Template (1) + (2) (Pex14.1, Pex14.2) |
| pMF999E         | GST-HsPex14p (K56E) SalI/NotI digest of PCR product generated by sequential PCR-steps: 1) Template pKG45 (Pex14.1, K56Efw) 2) Template pKG45 (K56Efw, Pex14.2) 3) Template (1) + (2) (Pex14.1, Pex14.2) |
| pMF999F         | GST-HsPex14p (L58R) SalI/NotI digest of PCR product generated by sequential PCR-steps: 1) Template pKG45 (Pex14.1, L58Rfw) 2) Template pKG45 (L58Rfw, Pex14.2) 3) Template (1) + (2) (Pex14.1, Pex14.2) |
| pMF120          | HsPex14p (WT)-GFP SalI/NotI digest of PCR product: Template pMF101 (Pex14.1, Pex14.2) |
| pMF122          | Biotin-HsPex19p PinPoint Xa3 BamHI/Smal fragment of pMF132 (20)
EXPERIMENTAL PROCEDURES

Plasmids—The plasmids encoding biotin-HsPex5p-(259–639) (pX-aPTS1-BP) (17), His6-MmPex5pL (pKG100) (18), His6-HsPex13p/SH3 (pMF88) (19), HsPex13p(WT)-GFP (pMF121) (10), HsPex13p(V178E)-GFP (pMF551) (10), Gal4pBD-HsPex14p (pMF101) (10), biotin-HsPex14p (pMF42) (19), Gal4pBD-HsPex14p-(22–81, 295–377) (pMF141) (20), Gal4pBD-HsPex19p (pMF132) (10), and DsRed-KSKL (pMF578) (10) are described elsewhere. The plasmid encoding GST-Pex14p (pKG45) was kindly provided by Dr. K. Ghys (Leuven, Belgium). The oligonucleotides (Invitrogen) and plasmids constructed for this study are compiled in Tables I and II, respectively. Cloning vectors were obtained from Amersham Biosciences (pGEX-4T-3 vector), Clontech (pEGFP-N1 vector), Promega (PinPoint Xa vectors), and Euroscarf (pZome-1-C). Standard molecular biology procedures were used (21). PCRs were routinely performed using Pfx DNA polymerase (Invitrogen), and the Escherichia coli strain Top10F (Invitrogen) was used for all DNA manipulations. The identities of essential constructs were confirmed by DNA sequencing.

Cell Culture, Transfections, Fluorescence Microscopy, and Fractionation of CHO Cells—Chinese hamster ovary (CHO) cells were cultured as described elsewhere (10). After transfer to coverslips, the cells were transiently transfected by the polyethylenimine transfection method (22), and processed for (indirect) fluorescence as described (23). The peroxisomal localization of the GFP (direct fluorescence) and protein A (indirect fluorescence) fusion proteins were confirmed by co-localization studies with the peroxisome-targeted DsRed-KSKL reporter protein (10) or endogenous expressed PMP70. Fluorescence was observed under a Leica DMR microscope equipped with FITC/RGFP/Bodipy/Fluo3/DIO and Texas Red filters. To isolate a membranous fraction containing only integral membrane proteins, transfected cells, grown in culture dishes to 90% confluency, were freed from these dishes by scraping, resuspended in 0.1M Na2CO3 (pH 11.5), homogenized (20 strokes) with a Teflon-glass Potter-Elvehjem homogenizer (Kontes), and subjected to a 100,000 × g spin for 1 h. After centrifugation, the pellet was resuspended in 0.1 M Na2CO3 (pH 11.5) and the entire procedure was repeated. This procedure resulted in a combined supernatant fraction yielding soluble and peripheral membrane proteins, and a pellet fraction containing the integral membrane proteins (24). To isolate integral membrane proteins of a purified peroxisomal fraction, the freed cells were resuspended in homogenization buffer (0.25M sucrose, 5 mM MOPS (pH 7.2), 1 mM EDTA (pH 7.2), 1 mM dithiothreitol, 0.1% (v/v) ethanol, and a protease inhibitor mixture (Roche Applied Science) and homogenized (20 strokes) using a stainless steel tissue grinder (Kontes). A postnuclear supernatant resulting from a low speed centrifugation (500 × g, 10 min) was further separated by density gradient centrifugation using a Nycodenz step gradient (25). The obtained fractions were analyzed by Western blotting and the peroxisomal peak fractions were pooled, 10-fold diluted in 0.1 M Na2CO3 (pH 11.5), and processed as described above.

Antibodies—The production and characterization of the rabbit antisera against His6-Pex13p/SH3, His6-Pex14p, His6-Pex19p, and peroxi-
somal matrix proteins is described elsewhere (10, 19). The rabbit and mouse antisera against His6-GFP (encoded by pEGFP-H1, a plasmid kindly provided by Dr. Y. Sakai, Kyoto, Japan) were generated as previously described (10). The rabbit antisera against Pex5p and PMP70 and the mouse monoclonal anti-TY antibody were kindly provided by Dr. M. Baes (Leuven, Belgium), Dr. S. Subramani (San Diego, CA) and Dr. K. Gull (Manchester, United Kingdom), respectively. The mouse monoclonal anti-His6 antibody was purchased from Clontech. The rabbit antibodies raised against catalase (Rockland), cytochrome P450 4A (Affinity Bioreagents Inc.), glutamate dehydrogenase (Rockland), human serum albumin (Sigma), and protein A (Sigma) were also commercially obtained. The fluorescein isothiocyanate-labeled anti-rabbit IgGs were obtained from Sigma.

Expression of Recombinant Proteins and Preparation of Bacterial Lysates—Top10F cells transformed with plasmids encoding the appropriate GST-, biotin-, or His6-tagged fusion proteins were cultured overnight. 5 ml of each culture of E. coli was diluted in 50 ml of yeast extract-tryptone medium containing 50 µg/ml ampicillin. After culture for 1 h at 37 °C, the cells were further grown for 3 h at 33 °C in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (or 2% (w/v) arabinose) to induce the expression of the tagged proteins. The cells were harvested and resuspended in 5 ml of ice-cold binding buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, and a protease inhibitor mixture (1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 µg/ml a2-macroglobulin, and 1 µg/ml chymostatin). Lysates were prepared by sonicating the cells with a Branson Sonifier B15 P cell disrupter, equipped with a microtip (output 5, duty 50%, 10 times 15 s). Cell debris was removed by centrifugation (13,000 × g for 15 min, 4 °C).

In Vitro Binding Assays—The pull-down assays employed to study the interactions between bacterially expressed peroxins were basically performed as described by Fujiki and coworkers (26). Briefly, in a first step glutathione-Sepharose 4B (Amersham Biosciences) or ImmunoPure immobilized streptavidin (Pierce) beads were coated with the appropriate fusion proteins. Therefore, 30–500 l of the bacterial lysates was mixed with 500 l of a 50% slurry of the beads. After being rotated for 1 h at 4 °C, the beads were washed five times with ice-cold binding buffer, and five times with the same buffer minus glycerol. Bound proteins were eluted from the beads by boiling for 5 min in 200 l of SDS-PAGE sample buffer, and, after removing the beads, 20-µl fractions were loaded on SDS-PAGE and analyzed by immunoblotting. To study the interaction between protein A, Pex13pWt/ protein A, Pex13pV178E/Protein A, and Pex19p-GFP, CHO cells co-transfected with plasmids coding for the corresponding fusion proteins (three tissue culture dishes (150 mm ×
20 mm) per condition) were freed from the dishes by scraping, lysed in 1600 μl of ice-cold binding buffer, and homogenized by using a stainless steel tissue grinder (20 strokes). After centrifugation (13,000 × g, 15 min), the supernatant was incubated with 50 μl of a 50% slurry of rabbit IgG-beads (Sigma) and rotated for 1 h at 4 °C. The beads were washed and further processed as described for the bacterially expressed proteins.

RESULTS

A Pex13p Mutant That Fails to Bind to Pex19p Nevertheless Targets to and Integrates into the Peroxisomal Membrane—It has been postulated that Pex19p may be involved in the insertion process of PMPs into the peroxisomal membrane (6). Here we have investigated whether or not Pex13p(V178E), a mutant of Pex13p displaying a peroxisomal distribution pattern but no detectable affinity for Pex19p in the yeast two-hybrid system (10), still achieves stable insertion into the peroxisomal membrane. To accomplish this, first, we confirmed the peroxisomal localization of the employed Pex13p-TY-GFP and Pex13p-protein A fusion proteins in CHO cells by fluorescence microscopy (Fig. 1). As we could not exclude that Pex19p and Pex13p(V178E) do not interact in mammalian cells, we incubated rabbit IgGagarose with cell lysates derived from CHO-cells co-transfected with plasmids coding for GFP-Pex19p and protein A, Pex13p(WT)-protein A or Pex13p(V178E)-protein A. Analysis of the bound proteins revealed that GFP-Pex19p was retained on the beads in the presence of Pex13p(WT)-protein A, but not in the presence of Pex13p(V178E)-protein A (Fig. 2). These observations, which extend our previous findings obtained with the yeast two-hybrid system (10), suggest that Pex19p does also not form a complex with Pex13p(V178E) in mammalian cells. Next, we lysed cells transfected with plasmids encoding GFP, Pex13p(WT)-TY-GFP, and Pex13p(V178E)-TY-GFP in 0.1 M sodium carbonate - a treatment that releases soluble and peripheral membrane proteins (24), and immunoblotted the soluble and insoluble fractions with the anti-TY1 epitope tag monoclonal antibody BB2 (27). Under these conditions, the wild-type and mutated Pex13p-TY-GFP fusion proteins were carbonate-inextractable; that is, were very tightly associated with the organelle membrane (Fig. 3A). Similar results were obtained with extraction with Triton X-114 (data not shown). In another experiment fractions enriched for peroxisomes were employed for carbonate treatment (Fig. 4). These fractions were obtained by density gradient centrifugation of the postnuclear supernatant of cells transfected with Pex13p(WT)-protein A or Pex13p(V178E)-protein A (Fig. 4A). As shown in Fig. 4B, also under these conditions the Pex13p(WT) and Pex13p(V178E) fusion proteins were carbonate-inextractable. Combined with our controls showing that soluble peroxisomal matrix proteins, but not peroxisomal integral membrane proteins, can be completely separated from the membrane fraction (Figs. 3B and 4B), these results indicate that Pex19p is not directly involved in the insertion process of Pex13p into the peroxisomal lipid bilayer.

The Pex5p/Pex19p Binding Site of Pex14p Is Also Essential for Binding to Pex13p—Pex14p functions as a central component of the peroxisomal matrix protein import machinery. In mammals, Pex14p interacts with several peroxins, including Pex5p, Pex13p, itself, and Pex19p (6, 19, 20). Pex5p on the other hand, possesses multiple binding sites for Pex14p (28). Further, Pex13p interacts with Pex14p through its SH3 domain (19), and the coiled-coil region of Pex14p (amino acids 138–200) is sufficient and required for homodimerization (20, 29). Recently, we demonstrated the interesting point that the Pex5p/Pex19p binding sites of Pex14p encompass amino acids 22–81 and cannot be separated physically (20). Here, we extend this observation and now report that this region of Pex14p is also essential for binding to Pex13p (SH3) (Fig. 5). This observation raises the interesting question as to whether or not the binding of Pex14p to Pex5p, Pex13p/SH3, or Pex19p alters its binding properties for the other interaction partners.

Pex5p and Pex19p Compete for Binding to Pex14p—In order to address the important issue of whether or not Pex5p and Pex19p vie for binding to Pex14p, we employed an in vitro competition assay. In this assay, glutathione-Sepharose beads coated with GST-Pex14p were incubated with a fixed amount of a bacterial lysate containing biotinylated Pex19p (or Pex5p- (259–639), a portion of Pex5p containing one Pex14p binding site) and increasing concentrations of biotinylated Pex5p-(259–

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**Fig. 3.** Pex13p(V178E)-TY-GFP, a mutant of Pex13p not bound by Pex19p, is carbonate-inextractable from the peroxisomal membrane in total cell lysates. A, CHO cells were transiently transfected with plasmids encoding GFP, Pex13p(WT)-TY-GFP, or Pex13p(V178E)-TY-GFP. After 24 h, the cells were lysed for 60 min in 0.1 M sodium carbonate (pH 11.5, 4 °C) and separated by centrifugation into soluble (S) and insoluble (I) fractions. Equivalent portions of these fractions were separated by SDS-PAGE and stained with Ponceau S or immunoblotted with the antiserum that specifically recognizes the integral peroxisomal membrane protein Pex14p, and anti-poxmtx, an antiserum that specifically recognizes peroxisomal matrix proteins (19). The migration of the molecular mass markers (expressed in kDa) is indicated.

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FIG. 4. Pex13p(V178E)-protein A, a mutant of Pex13p not bound by Pex19p, is carbonate-inextractable from the peroxisomal membrane in a fraction enriched in peroxisomes. CHO cells were transiently transfected with plasmids encoding Pex13p(WT)-protein A (A) or Pex13p(V178E)-protein A (B) and subjected to a subcellular fractionation procedure (see “Experimental Procedures”) 24 h post-transfection. Equivalent portions of the total cell lysates (L), the nuclear fractions (N), and the post-nuclear fractions (P) and equivalent volumes of the gradient fractions (bottom fraction (B), top fraction (T)) were separated by SDS-PAGE and stained with Ponceau S (detection of total protein profile) or immunoblotted with antisera raised against protein A (detection of Pex13p-protein A fusion proteins), Pex14p (peroxisomal integral membrane protein marker), catalase (peroxisomal matrix protein marker), glutamate dehydrogenase (GDH, mitochondrial protein marker), and cytochrome P450 4A (Cyt P450 4A; microsomal protein marker). The arrow marks the remainder of the bovine serum albumin that is still present after removal of the cell culture medium. The migration of the molecular mass markers (expressed in kDa) is indicated. C, peroxisomal peak fractions (marked with an asterisk) of each gradient were pooled, diluted 10 times in 0.1 M sodium carbonate (pH 11.5, 4 °C) and further processed as described in the “Experimental Procedures.” Equivalent portions of the soluble (S) and insoluble (I) fractions were separated by SDS-PAGE and stained with antisera specific for protein A, Pex14p, and catalase.

FIG. 5. The Pex5p/Pex19p binding site of Pex14p is also essential for binding to Pex13p. Recombinant proteins were expressed in E. coli. Streptavidin beads were coated with biotinylated Pex14p-deletion proteins and incubated with a bacterial lysate containing His<sub>6</sub>-tagged Pex13p/SH3. After thorough washing, the recombinant proteins bound to the streptavidin beads were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by using streptavidin-alkaline phosphatase and antibodies specific for Pex13p. The numbers on the left indicate the amino acid residues present in the corresponding Pex14p deletion mutants. The hydrophobic region and the putative coiled-coil domain of Pex14p are shaded in black and hatched with horizontal lines, respectively. The domain required and sufficient for binding to Pex5p and Pex19p (20) is shaded in gray.
Analysis of recombinant proteins bound to the Sepharose beads revealed that increasing concentrations of Pex5p-(259–639) (Fig. 6A) as well as of Pex19p (Fig. 6B) antagonize binding of the other peroxin to Pex14p. Interestingly, the Pex5p ability to vie for Pex14p binding is PTS1-independent (data not shown). Similar assays were performed with the full-length Pex5p (Fig. 7A). GST-Pex14p, His6-Pex5p, biotin-Pex5p-(259–639), and biotin-Pex19p were recombinantly expressed in E. coli. Glutathione-Sepharose beads were coated with GST-Pex14p and incubated with the indicated amounts (in microliters) of different bacterial lysates. After thorough washing, the recombinant proteins bound to the Sepharose beads were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by using streptavidin-alkaline phosphatase and antibodies specific for Pex5p, Pex14p, or Pex19p. A, increasing concentrations of Pex5p-(259–639) antagonize binding of Pex19p to Pex14p. B, increasing concentrations of Pex19p antagonize binding of Pex5p-(259–639) to Pex14p.
full-length PTS1 receptor, a molecule containing seven Pex14p binding sites. These experiments showed that full-length Pex5p displaced Pex19p from Pex14p more potently than Pex5p-(259–639) (Fig. 7A). Note that His6-Pex5p and biotin-Pex19p were expressed to the same level (Fig. 7B). As these experiments are suggestive, but do not prove that the Pex5p and Pex19p binding sites on Pex14p are the very same, six evolutionarily conserved amino acids in the Pex5p/Pex19p binding domain of Pex14p were mutated and the corresponding mutants were functionally analyzed in vitro (Fig. 8). Surprisingly, none of the mutations abolished the Pex14p-Pex5p interaction. However, four mutations (R25G, F35S, F52S, L58R) resulted in Pex14p molecules displaying a reduced binding affinity for Pex19p. These experiments clearly indicate that the binding sites of Pex14p for Pex5p and Pex19p are not identical.

**Pex13p Competes with Pex19p, but Not with Pex5p for Binding to Pex14p**—In order to investigate whether or not binding of Pex13p/SH3 to Pex14p alters the binding properties of Pex14p for Pex5p and Pex19p, we again employed the in vitro competition assay. This time the glutathione-Sepharose beads coated with GST-Pex14p were incubated with bacterial lysates containing His6-Pex5p, His6-Pex13p/SH3, and/or biotin-Pex19p. Analysis of recombinant proteins bound to the Sepharose beads confirmed our previous observations (20) that Pex5p, Pex13p/SH3, and Pex19p form a ternary complex (Fig. 9A). In addition, this figure shows that Pex13p/SH3 and Pex19p antagonize binding to Pex14p (Fig. 9B). Moreover, mutations within the Pex13p/Pex19p binding site of Pex14p differentially affected the binding of Pex13p/SH3 and Pex19p (Fig. 8), the binding sites for both peroxins appear to be also functionally distinct. Pex13p/SH3 and Pex5p did not affect each other’s binding to Pex14p (Fig. 9A).

**A Strongly Reduced Binding Affinity for Pex13p Affects the Peroxisomal Localization of Pex14p**—It has been proposed that Pex19p may act as a cycling PMP receptor protein (6). Consistent with this hypothesis, it has been reported that the targeting element of Pex14p retains the ability to bind Pex19p (6). By using the Pex14p mutants described above, we investigated whether or not there is a functional link between Pex19p binding and peroxisomal localization of Pex14p. The GFP-tagged Pex14p mutants were expressed in CHO cells, and the localization of the fusion proteins was determined by indirect fluorescence microscopy. Representative pictures of transfected CHO cells that illustrate the observed staining patterns of the GFP-fusion proteins are shown in Fig. 10. These results provide evidence that for Pex14p Pex19p binding (Fig. 8) and

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**Fig. 8. Site-directed mutagenesis analysis of the Pex5p/Pex13p/Pex19p binding site of Pex14p.** GST-Pex14p fusion proteins, His6-Pex5p, His6-Pex13p/SH3, and biotin-Pex19p were recombinantly expressed in E. coli. Glutathione-Sepharose beads were coated with equal amounts of the GST-Pex14p fusion proteins and incubated with bacterial lysates containing His6-Pex5p, His6-Pex13p/SH3, or biotin-Pex19p. After thorough washing, the recombinant proteins bound to the Sepharose beads were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by using streptavidin-alkaline phosphatase and antibodies specific for Pex5p and Pex19p. GST alone did not bind to His6-Pex5p, His6-Pex13p/SH3, and biotin-Pex19p (not shown).

**Fig. 9. Pex13p/SH3 can compete with Pex19p, but not with Pex5p for binding to Pex14p.** GST-Pex14p, His6-Pex13p/SH3, and biotin-Pex19p were recombinantly expressed in E. coli. Glutathione-Sepharose beads were coated with GST-Pex14p and incubated with the indicated amounts (in microliters) of different bacterial lysates. After thorough washing, the recombinant proteins bound to the Sepharose beads were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by using streptavidin-alkaline phosphatase and antibodies specific for Pex13p and Pex14p. A, Pex5p, Pex13p/SH3, and Pex14p can form a ternary complex. B, Pex13p/SH3 and Pex19p both antagonize binding of the other peroxin to Pex14p.
Pex14p Competes with Pex5p and Pex13p for Binding to Pex19p

Fig. 10. Targeting of Pex14p-GFP fusion proteins in CHO cells. CHO cells were transiently transfected with plasmids coding for Pex14pWT (WT), Pex14pR25G (R25G), Pex14pF35S (F35S), Pex14pL36R (L36R), Pex14pF52S (F52S), Pex14pK56E (K56E), or Pex14pL58R (L58R) N-terminally fused to GFP. After 24 h, the cells were processed for indirect immunofluorescence using mouse antibodies specific for GFP and rabbit antibodies specific for endogenous expressed PMP70. Bar, 10 μm.

Pex19p competes with Pex5p and Pex13p for binding to Pex14p. In this context, it is important to mention that these Pex19p binding and peroxisomal localization of Pex14p. This observation is difficult to reconcile with the hypothesis that Pex19p directly mediates the targeting of these PMPs to the peroxisome membrane. Surprisingly, amino acid substitutions resulting in a strongly reduced binding affinity for Pex13p (F35S, L36R, F52S, K56E, L58R) did affect the peroxisomal localization of Pex14p. In this context, it is interesting to point out that Pex13p was also found to be required for peroxisomal association of Pex14p in Saccharomyces cerevisiae (30). Note that two amino acid substitutions (K56E, L58R) did not result in a complete mislocalization of the corresponding Pex14p-GFP fusion proteins (Fig. 10).

DISCUSSION

Based on the observations that (i) Pex19p binds a broad spectrum of PMPs, and (ii) cells deficient in this peroxin lack peroxisomal membrane structures, Pex19p has been implicated in the peroxisome membrane assembly process (6, 12). Although several hypotheses have been put forward (see Introduction), there is currently no consensus as to the exact function of this peroxin. Recently, we and others (7, 10) have demonstrated that Pex19p does not function as a general mPTS receptor. In an attempt to elucidate a non-mPTS receptor role for Pex19p, we have investigated whether or not the protein is involved in the membrane insertion of newly synthesized PMPs. To accomplish this, we employed human Pex13pV178E, a molecule that displays no detectable affinity for Pex19p but that is still associated with peroxisomes (10, this study). Our results show that human Pex13pV178E, expressed in CHO cells remained precipitable after carbonate extraction, a widely used procedure for determining if an integral membrane protein has achieved stable insertion into the lipid bilayer (24). Thus, it appears unlikely that Pex19p functions as a membrane insertion factor for the integral membrane peroxin Pex13p.

Another potential non-mPTS receptor role for Pex19p is that the peroxin functions as an assembly or disassembly factor of peroxisomal membrane protein complexes (7). Accumulating evidence points to the existence of multiple, dynamic complexes of peroxins in vivo; however, at this point very little is known about the extent of these complexes and how the peroxisomal protein translocation apparatus is regulated (26, 29, 31–34). Recently, we and others (6, 10) reported that human Pex19p interacts with the critically important peroxisomal integral membrane protein Pex14p, a pivotal component of the docking complex for the functional peroxisomal native protein import receptors Pex5p and Pex7p. In order to investigate whether or not Pex19p is involved in the regulation of the Pex14p docking complex, we employed in vitro biochemical assays. Specifically, we examined the effect of Pex19p on the Pex5p and Pex13p (another component of the docking complex) binding properties of Pex14p. Our studies revealed that increasing concentrations of Pex19p antagonize binding of Pex5p and Pex13p to Pex14p. Further, in experiments in which increasing concentrations of Pex5p and Pex13p were used, binding of Pex19p to Pex14p was antagonized. As expected, the full-length PTS1-receptor, a molecule containing seven Pex14p binding sites, was a more effective competitor for the Pex14p-Pex19p interaction than Pex5p-(259–639), a molecule containing only one Pex14p binding site. In addition, Pex19p appears to be a more effective competitor for the Pex14p-Pex13p interaction than for the Pex14p-Pex5p interaction, although the basis for this apparent selectivity is unclear. Summarized, these results show that there is a dynamic interplay between Pex14p and its interaction partners Pex5p, Pex13p, and Pex19p.

Recently, we showed that the Pex5p/Pex19p binding sites of Pex14p encompass amino acids 22–81 and cannot be separated physically (20). In this study we provided evidence that this region of Pex14p is also essential for binding to Pex13p/SH3. In addition, site-directed mutagenesis and functional analysis of six evolutionarily conserved amino acids in this domain of Pex14p identified five point mutations (F35S, L36R, F52S, K56E, L58R) that resulted in a strongly reduced binding affinity for Pex13p. The observation that (i) all these mutants fully retained the ability to bind Pex5p, and (ii) three mutations (F35S, F52S, L58R) were selectively affected in Pex19p binding, indicate that the physically overlapping binding sites of Pex14p for Pex5p, Pex13p, and Pex19p are functionally distinct. This suggests that competition occurs through induction of structural changes, rather than through direct competition for binding. We are currently exploring the nature of these structural changes in greater detail.

Interestingly, we observed no functional link between Pex19p binding and peroxisomal localization of Pex14p. This result argues against a role of Pex19p as a cycling receptor protein for Pex14p. Remarkably, we observed that all amino acid substitutions resulting in a strongly reduced binding affinity for Pex13p did affect the peroxisomal localization of Pex14p. In this context, it is important to mention that these
results support the observation in *S. cerevisiae* that the presence of Pex13p is a prerequisite for peroxisomal membrane association of Pex14p (30). Whether or not Pex13p in this organism is involved in targeting or required for binding or retention of Pex14p at the peroxisome is not clear yet (30). In addition, it has been suggested that the SH3 domain of Pex13p may not provide the only binding site for Pex14p at the peroxisomal membrane (34).

Our observations that Pex19p can modulate the Pex5p and Pex13p binding properties of Pex14p in vitro suggest that Pex19p may play an important and direct role in the import of peroxisomal matrix proteins through a regulation of the Pex14p docking complex. In addition, our previous and current findings indicate that Pex19p does not serve a role as a general PMP-import receptor or Pex13p membrane insertion factor.

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