Pex19p-dependent Targeting of Pex17p, a Peripheral Component of the Peroxisomal Protein Import Machinery*

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Pex19p is required for the topogenesis of peroxisomal membrane proteins (PMPs). Here we have demonstrated that Pex19p is also required for the peroxisomal targeting and stability of Pex17p, a peripheral component of the docking complex of the peroxisomal protein import machinery. We have demonstrated that Pex17p is associated with the peroxisomal Pex13p-Pex14p complex as well as with Pex19p. We have identified the corresponding binding sites for Pex14p and Pex19p and demonstrated that a specific loss of the Pex19p interaction resulted in mistargeting of Pex17p. We have shown that a construct consisting only of the Pex19p- and Pex14p-binding sites of Pex17p is sufficient to direct an otherwise cytosolic reporter protein to the peroxisomal membrane in a Pex19p-dependent manner. Our data show that the function of Pex19p as chaperone or import receptor is not restricted to integral membrane proteins but may also include peripheral PMPs. As a consequence of our data, the previous definition of a targeting signal for PMPs (mPTS) as a Pex19p-binding motif in conjunction with a transmembrane segment should be extended to regions comprising a Pex19p-binding motif and a peroxisomal anchor sequence.

The maintenance of peroxisome function depends on the formation of the peroxisomal membrane and the subsequent import of both membrane and matrix proteins. The matrix protein import occurs in a post-translational manner (1) and uses either one of the two well characterized peroxisomal targeting signals, PTS1 or PTS2, that are recognized and bound by specific receptor proteins Pex5p and Pex7p, respectively. Subsequently, the receptor-cargo complexes dock to distinct proteins accessible at the cis-side of the peroxisomal membrane. Pex13p and Pex14p physically bind both import receptors Pex5p and Pex7p, whereas Pex14p also interacts with Pex17p (2–9). These three peroxins are thought to form the receptor-docking complex (for review, see Refs. 10–12), being responsible for the initial binding of the receptor-cargo complex at the peroxisomal membrane. According to the widely accepted model of cycling receptors, the receptor-cargo complex is supposed to dissociate at the peroxisomal membrane in an entirely unknown manner, resulting in the sorting of the cargo protein to the peroxisomal matrix and the release of free receptors to the cytosol, where they are available for another round of import (13, 14).

In contrast to the increasing knowledge on the peroxisomal matrix protein import, our understanding of the topogenesis of peroxisomal membrane proteins (PMPs)2 is rather scarce. Most of the mutants lacking one of the known peroxins are characterized by a severe defect in matrix protein import, whereas the targeting of peroxisomal membrane proteins is not affected, indicated by the presence of peroxisomal remnant structures that contain a number of PMPs but no or only residual amounts of matrix proteins (15). These and other findings led to the conclusion that PMP targeting occurs independently of the import of peroxisomal matrix proteins (5, 6). Among the 32 known proteins involved in peroxisome biogenesis, only Pex3p, Pex16p, and Pex19p are required for the biogenesis of the peroxisomal membrane. Cells lacking either a functional copy of PEX3, PEX16, or PEX19 contain neither peroxisomes nor obvious peroxisomal remnants (for review, see Ref. 16), and the PMPs are rapidly degraded or mislocalized to other locations such as the endoplasmic reticulum and mitochondria (17–25). The observation that most PMPs are recognized by Pex19p triggered the idea that this predominantly cytosolic protein might function as a chaperone and/or import receptor for peroxisomal membrane proteins (17, 22, 24, 25). This, however, is still a matter of debate (26). PMPs use neither one of the two targeting signals, PTS1 and PTS2, that direct proteins to the peroxisomal matrix (for review, see Ref. 16). The targeting signal for peroxisomal membrane proteins (mPTS) has been identified for several proteins, including Pmp47p of Candida boidini, Pmp34p, Pmp22p from mammals, and Pex3p from various species (21, 27). In support of an import receptor function of Pex19p, most PMPs contain conserved binding sites for Pex19p that share a common motif that is essential and in conjunction with a transmembrane segment sufficient for peroxisomal targeting and membrane insertion (28).

Here we have analyzed the peroxisomal targeting and membrane association of Pex17p, a peripheral component of the docking complex of the peroxisomal protein import machinery.

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2 The abbreviations used are: PMP, peroxisomal membrane protein; MBP, maltose-binding protein; GFP, green fluorescent protein; ProtA, protein A; TEV, tobacco etch virus.
Peroxisomal Targeting of Pex17p

TABLE 1
S. cerevisiae strains used

| Strain | Description | Source or reference |
|--------|-------------|---------------------|
| UTL-7A | MATα leu2-3, 112 ura3-52 trp1 | This study |
| pex12Δ | MATα leu2-3, 112 ura3-52 trp1, pex17::LEU2 | This study |
| pex17-1 | MATα leu2-3, 112 ura3-52 trp1, PEX17-TEV-ProtA-kanMX6 | This study |
| pex17Δ | MATα, leu2-3, 112 ura3-52 trp1, pex12::LEU2, PEX17-TEV-ProtA-kanMX6 | This study |
| pex34Δ | MATα, leu2-3, 112 ura3-52 trp1, pex3::LEU2, PEX17-TEV-ProtA-kanMX6 | This study |
| pex19Δ | MATα, leu2-3, 112 ura3-52 trp1, pex19::LEU2, PEX17-TEV-ProtA-kanMX6 | This study |
| PCY2 | MATα, gal4Δ, gal80Δ, URA3-GAL1-lacZ, his2-8, 200, trp1-200, leu2 ade2-101Δ | 7 |

We have defined the binding regions of Pex17p for other peroxins and demonstrated that the peroxisomal targeting and stability of Pex17p requires Pex19p as a chaperone or import receptor as well as Pex14p for a stable anchoring at the peroxisomal membrane.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—Saccharomyces cerevisiae** strains used in this study are listed in Table 1. Strains in which the genomic copy of PEX17 contains epitope tags were generated as described by (29) using primers KU1007 and KU1008 for PCR reactions. Transformants were selected for appropriate marker and proper integration was confirmed by PCR.

Localization of GFP-fusion proteins was analyzed in strain yHPR251, which expresses PTS2-DsRed from an integrated plasmid in strain UTL-7A (30). Yeast complete (YPD) and minimal media (SD) have been described previously (31). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. When necessary, auxotrophic requirements were added according to (32).

**Oligonucleotides and Plasmids**—The plasmids and oligonucleotides used are listed in Tables 2 and 3. To generate a maltose-binding protein-Pex17 fusion, the coding region of PEX17 was amplified by PCR using primers KU129 and KU130 as well as prSPEX17 (7) as the template. The PCR product was digested with EcoRI/NotI and introduced into an EcoRI/NotI-digested pMal-C2 (New England Biolabs) resulting in plasmid pMal-PEX17. For the construction of Pex17pI80P, the mutation was introduced by PCR using gene splicing by overlap extension (33) with primers RE918/RE919 and prSPEX17 as the template. For GFP fusion, mutated or wild-type PEX17 was amplified by PCR with primers RE1006/RE1007. The PEX17- and PEX17I80P-PCR products were digested with BamHI/EcoRI and subcloned into pUG36, resulting in plasmids pH8 and pH9, respectively.

**Yeast Cell Extracts**—Yeast cells were grown on 0.3% SD medium to late log phase and subsequently for 15 h in YNO (0.1% dextrose, 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast

**TABLE 2**
Plasmids used

| Plasmid | Description | Source or reference | Oligonucleotides |
|---------|-------------|---------------------|------------------|
| pPC97-PEX17 | GAL4-PEX17-(1–199) | This study | KU129/KU133 |
| pPC97-PEX17II | GAL4-PEX17-(1–167) | This study | KU129/KU132 |
| pPC97-PEX17III | GAL4-PEX17-(1–125) | This study | KU129/KU131 |
| pPC97-PEX17IV | GAL4-PEX17-(1–88) | This study | KU129/KU130 |
| pPC97-PEX17V | GAL4-PEX17-(1–51) | This study | KU126/KU130 |
| pPC97-PEX17VI | GAL4-PEX17-(52–199) | This study | KU127/KU130 |
| pPC97-PEX17VII | GAL4-PEX17-(89–199) | This study | KU128/KU133 |
| pPC97-PEX17VIII | GAL4-PEX17-(126–199) | This study | KU126/KU132 |
| pPC97-PEX17IX | GAL4-PEX17-(52–125) | This study | KU127/KU132 |
| pPC97-PEX17X | GAL4-PEX17-(52–88) | This study | KU183/KU130 |
| pPC97-PEX17XI | GAL4-PEX17-(89–125) | This study | KU184/KU130 |
| pPC97-PEX17XII | GAL4-PEX17-(145–199) | This study | KU184/KU130 |
| pPC97-PEX17XIII | GAL4-PEX17-(167–199) | This study | KU184/KU130 |
| pPC86-PEX19 | GAL4-PEX19 | 56 | |
| pPC86-PEX14 | GAL4-PEX14 | 2 | KU129/KU130 |
| pMal-PEX17 | MBP-PEX17 | This study | RE920/RE921 |
| pH4 | GFP-PEX17-(52–88) | This study | RE1006/RE1007 |
| pH7 | GFP-PEX17 | This study | RE1006/RE1007 |
| PH8 | GFP-PEX17 | This study | RE1006/RE1007 |
| PH9 | GFP-PEX17I80P | This study | RE920/1007/1084/1085 |
| PH10 | GFP-PEX17-(52–88/167–199) | This study | RE920/RE1007 |
| PH11 | GFP-PEX17-(167–199) | This study | RE920/RE1007 |

**TABLE 3**
Oligonucleotides used

| Oligonucleotide | Description |
|-----------------|-------------|
| KU126 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU127 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU128 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU129 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU130 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU131 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU132 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU133 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU134 | 5’-CCGGATCTGAATTTTCTAGGACAAACG-3’ |
| KU1007 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
| KU1008 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
| RE918 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
| RE919 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
| RE1006 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
| RE1007 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
| RE1084 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
| RE1085 | 5’-AGAATTACGTAGGATTTTCTAGGACAAACG-3’ |
Cells were harvested and aliquots of 30 mg of cells were resuspended in 300 μl of potassium-phosphate buffer, pH 7.4, containing 20% trichloroacetic acid. The samples were frozen at −80 °C for at least 30 min. Samples were sedimented, washed twice with ice-cold 50% acetone, and resuspended in 80 μl of 10% SDS/0.1 mM NaOH and 20 μl of SDS loading buffer (5% β-mercaptoethanol, 15% glycerol, 0.01% bromphenol blue).

Two-hybrid Analysis—The two-hybrid assay was based on the method of Fields and Song (34). Open reading frames were fused to the DNA-binding domain or trans-activating domain of GAL4 in the vectors pPC86 and pPC97 (35). To generate PEX17 and PEX17Δ constructs in pPC97, PEX17 fragments were amplified by PCR using pPC97-PEX17 (7) or pLH9 as a template. The PCR fragments were first cloned into EcoRI/SacI-prepared pPC86 and subsequently into Sall/Sacl-digested pPC97. Co-transformation of two-hybrid vectors into strain PCY2 was performed according to Gietz and Woods (36). Transformed yeast cells were plated onto SD medium without tryptophan and leucine. β-Galactosidase filter assays were performed according to Rehling et al. (37).

In Vitro Binding Assays—For in vitro binding of Pex17p to Pex14p, the maltose-binding protein (MBP) and MBP-Pex17p of soluble fractions obtained from pMal-C2- and pMal-PEX17-transformed Escherichia coli B121, respectively, were bound for 2 h at 4 °C to amylose resin (New England Biolabs). After washing the matrix with buffer A (50 mM NaCl, 50 mM HEPES, pH 7.4), the soluble fractions of bacteria-expressed Pex14pHis6 were added and incubated with the matrix for 2 h at 4 °C with gentle rotation. After washing the matrix with buffer A, bound proteins were eluted with 10 mM maltose in 50 mM Tris-HCl, pH 7.4. The eluted samples were analyzed by immunoblot analysis.

Antibodies and Western Blotting—Anti-Pex3p, -Pex5p, -Pex10p, -Pex12p, -Pex13p, -Pex14p, -Pex17p, and -Fbp1p (fructose-1,6-bisphosphatase) have been described previously (2, 7, 38–41). Anti-rabbit IgG-coupled horseradish peroxidase (Amersham Biosciences) was used as the secondary antibody. Immunoreactive complexes were visualized using anti-rabbit IgG-coupled horseradish peroxidase in combination with the ECL™ system from Amersham Biosciences.

Miscellaneous Methods—Analysis of live cells for DsRed and GFP fluorescence was performed with a Zeiss Axioplan microscope and AxioVision version 4.1 software (Zeiss, Jena, Germany). Immunopurification of native complexes using hslgG-coupled Sepharose was performed as described previously (42).

RESULTS

Genomically Tagged Pex17p-Protein A (ProtA) Is Functional—To isolate and characterize Pex17p, we constructed strains expressing this peroxin fused to two IgG-binding domains derived from Staphylococcus aureus ProtA, with a cleavage site for the tobacco etch virus (TEV) protease inserted between Pex17p and the ProtA tag (29). The corresponding wild-type strain was initially tested for growth on plates containing oleic acid as the sole carbon source, which will support cell growth only if peroxisomal β-oxidation is functional. The pex17Δ
subjected to a rapid turnover in these mutant cells, we investigate whether the membrane-associated Pex17p is also that rely on these PMPs are strongly reduced (17, 43, 44). To Pex19p, the levels of integral peroxisomal membrane proteins have demonstrated that, in cells lacking either Pex3p or Pex19p. Whole cell trichloroacetic acid lysates of the strains indicated with or without expression of Pex17p-ProtA were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of Pex17p-ProtA, Pex14p, and cytosolic fructose-1,6-bisphosphatase (Fbp1p) as the loading control. A, Pex17p co-precipitation study. Pex17p-TEV-ProtA-containing protein complexes were isolated from solubilized membranes by affinity chromatography with IgG-Sepharose and subsequent TEV protease cleavage. As a control, wild-type cells expressing no protein A fusion were treated equally. Equal portions of total unbound and washed fractions and 5-fold amounts of eluate fractions were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of peroxisomal membrane proteins Pex17p, Pex14p, and Pex13p and the PTS1-receptor Pex5p. Detection of Pex11p and mitochondrial Tom40p and Tim22p served as the control for the specificity of the isolation procedure. * indicates a slower migrating variant of Pex17p because of the addition of 14 amino acids of a linker sequence and of the TEV protease cleavage site.

In vivo association of Pex17p with the docking complex and Pex19p. A, reduced steady-state level of Pex17p in cells deficient in either Pex3p or Pex19p. Whole cell trichloroacetic acid lysates of the strains indicated with or without expression of Pex17p-ProtA were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of Pex17p-ProtA, Pex14p, and cytosolic fructose-1,6-bisphosphatase (Fbp1p) as the loading control. B, Pex17p co-precipitation study. Pex17p-TEV-ProtA-containing protein complexes were isolated from solubilized membranes by affinity chromatography with IgG-Sepharose and subsequent TEV protease cleavage. As a control, wild-type cells expressing no protein A fusion were treated equally. Equal portions of total unbound and washed fractions and 5-fold amounts of eluate fractions were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of peroxisomal membrane proteins Pex17p, Pex14p, and Pex13p and the PTS1-receptor Pex5p. Detection of Pex11p and mitochondrial Tom40p and Tim22p served as the control for the specificity of the isolation procedure. * indicates a slower migrating variant of Pex17p because of the addition of 14 amino acids of a linker sequence and of the TEV protease cleavage site.

FIGURE 2. in vivo association of Pex17p with the docking complex and Pex19p. A, reduced steady-state level of Pex17p in cells deficient in either Pex3p or Pex19p. Whole cell trichloroacetic acid lysates of the strains indicated with or without expression of Pex17p-ProtA were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of Pex17p-ProtA, Pex14p, and cytosolic fructose-1,6-bisphosphatase (Fbp1p) as the loading control. B, Pex17p co-precipitation study. Pex17p-TEV-ProtA-containing protein complexes were isolated from solubilized membranes by affinity chromatography with IgG-Sepharose and subsequent TEV protease cleavage. As a control, wild-type cells expressing no protein A fusion were treated equally. Equal portions of total unbound and washed fractions and 5-fold amounts of eluate fractions were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of peroxisomal membrane proteins Pex17p, Pex14p, and Pex13p and the PTS1-receptor Pex5p. Detection of Pex11p and mitochondrial Tom40p and Tim22p served as the control for the specificity of the isolation procedure. * indicates a slower migrating variant of Pex17p because of the addition of 14 amino acids of a linker sequence and of the TEV protease cleavage site.

FIGURE 3. Pex17p interacts directly with Pex14p. MBP or MBP fused to Pex17p and expressed in E. coli were bound to amylose columns and then incubated with recombinant Pex14pHis6. After incubation for 2 h at 4 °C, the bound proteins were eluted from the column. Equal portions of lysate and eluate fractions were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of the indicated proteins.

A strain was unable to grow on this medium in line with previous findings (7) and typical for peroxisomal mutant strains of S. cerevisiae (31). The genomically tagged wild-type strain expressing Pex17p-ProtA grew at a rate indistinguishable from the corresponding non-tagged wild type (Fig. 1A). These data are corroborated by electron microscopic analysis of the ultrastructure of the cells. pex17Δ cells are characterized by the mislocalization of peroxisomal matrix proteins to the cytosol, which leads to the absence of morphologically detectable peroxisomes, whereas the strain harboring the ProtA-tagged Pex17p was indistinguishable from the wild-type cells (Fig. 1B). Thus, the genomic tagging did neither interfere with the growth behavior of the cells on oleic acid medium nor with the morphological appearance of peroxisomes, indicating that the TEV-ProtA-tagged Pex17p is functional in vivo.

Steady State Level of Pex17p in pex Mutants—Previous studies have demonstrated that, in cells lacking either Pex3p or Pex19p, the levels of integral peroxisomal membrane proteins that rely on these PMPs are strongly reduced (17, 43, 44). To investigate whether the membrane-associated Pex17p is also subjected to a rapid turnover in these mutant cells, we determined the steady-state level of Pex17p-ProtA in pex3Δ and pex19Δ as well as pex12Δ cells, a mutant strain which is defective only in matrix protein import, whereas the membrane protein transport remains unaffected (38). Yeast strains were grown on oleic acid-containing medium, and cell lysates were subjected to SDS-PAGE and immunoblot analysis. Samples were probed for the presence of Pex17p, its binding partner Pex14p (2, 7, 9, 45), as well as for cytosolic Fbp1p (41) as an internal control (Fig. 2A). In pex12Δ cells, the Pex17p-ProtA steady-state level was the same as in the wild-type cells. Remarkably, the protein abundance of Pex17p was drastically decreased in strains deficient in either PEX3 or PEX19, whereas the steady-state level of Pex14p, a membrane-associated protein (2, 3), was not affected (Fig. 2A). Thus, the lower Pex17p concentration is not due to a lack of Pex14p, the deficiency of which has been demonstrated to cause Pex17p instability in Pichia pastoris (46). However, the instability of Pex17p in these mutants reflects the behavior of an integral membrane protein. As there is no doubt that Pex17p is a peripheral membrane protein that associates with the peroxisomal membrane by binding to Pex14p, these data establish that not only the stability of integral PMPs but also of the membrane-associated Pex17p depends on proteins, which have been shown to be essential for proper targeting of integral peroxisomal membrane proteins.

Both Pex14p and Pex19p Are Part of Pex17p Complexes—The reduced level of Pex17p in cells lacking Pex19p tempted us to analyze whether both proteins are associated in vivo, as previously reported for P. pastoris (9). We performed IgG affinity chromatography with the ProtA-tagged Pex17p as bait to identify associated protein components. Total cell membranes were solubilized with digitonin and subjected to affinity purification. Pex17p and bound proteins were eluted by cleavage with the TEV protease. Wild-type cells expressing no ProtA fusion served as the control. Aliquots of the TEV protease eluates, representing equal amounts of total membranes from each strain, were separated by SDS-PAGE and subjected to Western blot analysis (Fig. 2B). Pex13p and Pex14p, two docking complex constituents (12, 47–49), the PTS1 receptor Pex5p as well as minor amounts of the proposed PMP receptor Pex19p were present in the eluate from cells harboring the tagged Pex17p. The specificity of the isolation procedure is documented by the
absence of the prominent peroxisomal membrane protein Pex11p (50, 51) and the mitochondrial proteins Tom40p and Tim22p (52) in the eluates and the complete depletion of Pex17p-ProtA from the detergent extract upon incubation with IgG-Sepharose. These data corroborate that Pex17p belongs to the docking complex of the peroxisomal protein import machinery. To determine whether the observed Pex17p interactions with Pex14p are mediated by one of the components identified in the in vivo complex or whether they represent direct protein/protein interactions, in vitro binding assays were performed. MBP and chimeric MBP-Pex17p were immobilized on amylose resin and incubated with lysates containing Pex14p fused to a hexahistidyl tag. As shown in Fig. 3, Pex14pHis6 only bound to the column when the matrices were loaded with MBP-Pex17p, whereas binding to the MBP control was not observed. Interestingly, the same was true for recombinant GST-Pex19p, which was exclusively recovered from the column loaded with MBP-Pex17p, although the amount of Pex19p bound to Pex17p was only just above the detection level (data not shown). Nevertheless, the data support the idea of a specific interaction of Pex17p and Pex19p. In line with this assumption, a small but significant amount of Pex19p was pulled down by Pex17p in vivo. From these findings, we conclude that Pex17p interacts with both Pex14p and Pex19p in a direct manner.

Distinct Binding Sites of Pex17p for Pex14p and Pex19p—We used the two-hybrid system to identify the regions within Pex17p that contribute to the association with Pex14p and Pex19p. Sets of full-length Pex14p or Pex19p fusions with the Gal4p activation domain and Gal4p-DNA-binding domain fusions with progressive carboxyl- and/or amino-terminal deletions of Pex17p were transformed in the S. cerevisiae strain PCY2, and reporter gene expression was analyzed by assaying β-galactosidase activity. As negative controls, co-expression of either of the fusion proteins, together with the corresponding empty vectors, did not support transcription activation of the reporter gene (data not shown). As shown in Fig. 4, co-expression of Gal4p fusions of full-length Pex17p with either Pex14p or Pex19p resulted in considerable β-galactosidase activity, demonstrating the in vivo interaction of Pex17p with Pex14p and Pex19p, respectively. Analysis of the Pex17p/Pex19p interaction in a pex14Δ mutant proved that it is independent of Pex14p (data not shown). As judged by LacZ gene expression,
Pex17p-(167–199) was identified as the smallest fragment that is sufficient and essential for the interaction with Pex14p. This fragment is located at the extreme carboxyl terminus of Pex17p and contains no obvious sequence motifs. Pex17p-(52–88) was identified as the smallest fragment that proved to comprise the binding site for Pex19p (Fig. 4). Pex17p fusion proteins that lacked this region were unable to interact with the Pex19p fusion, indicating that this region is not only sufficient but also required for the two-hybrid signal. Interestingly, Pex17p-(52–88) contains one of the two putative coiled-coil regions (7, 9) and a putative Pex19p-binding sequence (amino acids 73–87), previously identified by peptide scan analysis (28).

To further characterize the binding motif, we introduced a point mutation into Pex17p that leads to a substitution of isoleucine 80 to proline. Mutation of binding peptides from other PMPs at this position has been demonstrated to abolish binding to Pex19p (28). Wild-type and mutated Pex17p (Pex17p-I80P) were tested in the two-hybrid system for their ability to interact with Pex19p and Pex14p. As shown in Fig. 5, the introduction of the I80P substitution within Pex17p resulted in a complete loss of interaction between Pex17p and Pex19p, whereas the interaction of Pex17p with Pex14p remained unaffected. As judged by immunoblot analyses, the Gal4p fusions of Pex17p and Pex17p-I80P were expressed at the same rate (data not shown). These results demonstrate that the Pex17p/Pex19p interaction depends on the predicted and identified Pex19p-binding site and that the interaction can be specifically prevented by the introduction of a point mutation within Pex17p that does not interfere with Pex14p binding.

**FIGURE 5. Specific inhibition of the Pex17p/Pex19p interaction by site-directed mutagenesis.** A yeast two-hybrid assay was performed to study the interaction of wild-type Pex17p in comparison to mutated Pex17p80P with Pex19p (A) and Pex14p (B). β-Galactosidase activity of double transformants expressing the indicated fusion proteins was determined by a filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as substrate. Three representative independent double transformants are shown. DB, DNA-binding domain; AD, activation domain.

**FIGURE 6. Pex19p-dependent targeting of Pex17p.** Plasmids for the expression of GFP fusions of the indicated Pex17p fragments were transformed into wild-type strain PHPR251, which contains peroxisomes labeled by the peroxisomal marker protein PTS2-DsRed. The transformed strains were grown on oleic acid plates for 2 days and examined by fluorescence microscopy. The merged images reveal peroxisomal co-localization of GFP with PTS2-DsRed, and Nomarski optics reveal the position of inspected cells. The gray boxes indicate the predicted Pex19p-binding site. Scale bar = 2.5 μm.
Peroxisomal Targeting of Pex17p Depends on the Pex19p-biding Site—A number of integral membrane proteins have been shown to be targeted to the peroxisomal membrane in a Pex19p-dependent manner (11). To analyze whether this is also true for the peripheral Pex17p (7), we performed fluorescence microscopy experiments. Pex17p fused to GFP was co-expressed in wild-type cells together with the PTS2-DsRed, which served as a peroxisomal marker. GFP-Pex17p exhibited a punctate staining co-localized with PTS2-DsRed, demonstrating the peroxisomal localization of the fusion protein (Fig. 6). To analyze whether the identified Pex19p-binding motif plays a role in the targeting of Pex17p, we introduced the I80P mutation, which impaired Pex19p binding into the GFP-Pex17p fusion. Interestingly, fluorescence microscopy revealed that the GFP-Pex17p-I80P mislocalized to the cytosol with only a very minor portion still being peroxisomal, as judged by the co-localization with the peroxisomal PTS2-DsRed (Fig. 6). These data demonstrate that the prohibition of Pex19p binding correlates with inefficient targeting of Pex17p to the peroxisomal membrane. When Pex17p-(52–88), containing the identified Pex19p-binding site, was expressed in fusion with GFP, a cytosolic staining was observed, indicating that the Pex19p-binding site alone is not sufficient for the peroxisomal targeting of Pex17p. However, the lack of firm association with the peroxisome might be due to the fact that the truncated GFP-Pex17p fusion construct is lacking the binding site for Pex14p. For this reason, we constructed a fusion protein that contains the Pex19p- as well as the Pex14p-binding site of Pex17p. As judged by the observed co-localization of GFP-Pex17p-(52–88/167–199) with PTS2-DsRed, this fragment retained the information for peroxisomal targeting, whereas the Pex14p-binding site of Pex17p alone was not sufficient to direct the fusion construct to peroxisomes. Thus, we conclude that the peripheral Pex17p requires binding to Pex19p for its targeting to peroxisomes, where it is anchored in a Pex14p-dependent manner.

**DISCUSSION**

We investigated the peroxisomal targeting and complex association of *Saccharomyces cerevisiae* Pex17p, a membrane-associated component of the peroxisomal membrane import machinery facing the cytosolic site of peroxisomes. It is still a matter of debate whether Pex17p is required for the import of peroxisomal membrane proteins (9) (46). At least in *S. cerevisiae*, peroxisomal targeting of PMPs seems not to be affected (7, 39). However, there is no doubt that Pex17p is essential for matrix protein import (11). Cells deficient in Pex17p are characterized by the presence of PMP-containing ghosts and the mislocalization of peroxisomal matrix proteins to the cytosol. Furthermore, Pex17p was found to be associated with Pex13p and Pex14p, the other two constituents of the receptor docking complex (42, 44). Yeast two-hybrid studies of a Pex17p/Pex14p interaction (7, 9), the observation that both proteins form a tight core complex even in the presence of 0.5% Triton X-100, and the absence of Pex17p from the docking complex in the Pex14p-deficient cells (42) were the first hints that Pex14p plays a crucial role in Pex17p attachment to the docking complex. Several lines of evidence indicate that Pex17p is anchored to the docking complex at the peroxisomal membrane via Pex14p. First, with Pex17p as bait, Pex14p is pulled down by precipitation (Fig. 2); second, Pex17p binds directly to Pex14p (Fig. 3); and third, a Pex17p-fragment is only stably localized to peroxisomes in the presence of the Pex14p-binding site (Fig. 6). Moreover, in the absence of Pex14p, the intracellular level of Pex17p is below detection level (data not shown), indicating that the association with Pex14p is not only required to maintain the peroxisomal localization but also influences the stability of Pex17p. The dependence on Pex14p led to the question of how Pex17p is targeted to its destination at the peroxisomal membrane. Pex17p obviously does not contain a PTS1 or PTS2, and targeting of the protein to peroxisomes is independent of the corresponding import receptors Pex5p and Pex7p (42). On the other hand, Pex17p contains a putative binding site for Pex19p, the chaperone and/or import receptor required for the topogenesis of integral peroxisomal membrane proteins (28, 53). In line with a possible role for Pex19p in Pex17p targeting, we found a small but significant amount of Pex19p associated with the Pex17p complex (Fig. 2B). As Pex19p was shown to be indispensable for proper targeting of integral peroxisomal membrane proteins (11, 54), the presence of Pex19p in the Pex17p complex might be due to its association with other docking components such as the integral Pex13p. However, in line with previous findings for *P. pastoris* (9), we have shown by use of the yeast two-hybrid system that Pex17p interacts with Pex19p (Fig. 4). This interaction is independent of Pex14p, and *in vitro* binding studies with synthetic peptides (28) or recombinant proteins (this study) provide evidence for a direct interaction between the proteins. To gain deeper insight into the function of the Pex17p/Pex19p interaction, we limited the Pex19p-binding site of Pex17p to amino acid residues 52–88, which in conjunction with the Pex14p-binding site, proved to be sufficient for the peroxisomal targeting of an otherwise cytosolic reporter protein (Fig. 6). The identified Pex19p-binding fragment contains a sequence motif that is also present in a number of integral peroxisomal membrane proteins (28). To date, this motif was shown to be essential for Pex19p binding and peroxisomal targeting of membrane proteins in yeast and human (28, 53, 55). In line with these findings, substitution of crucial amino acid residues of this motif within Pex17p prevented the Pex17p/Pex19p interaction (Fig. 5). Hence, mutated Pex17p was mostly mistargeted to a cytosolic fraction (Fig. 6). However, we cannot exclude that a very weak interaction below detection level exists, which could explain the small fraction of mutated Pex17p associated with peroxisomes.

Further evidence for a functional role of Pex19p in Pex17p targeting is provided by the fact that cells lacking Pex19p displayed significantly reduced levels of Pex17p (Fig. 2A), an observation that was also made for *P. pastoris* cells lacking Pex14p (46) (the second Pex17p-binding partner) and that is also true for *S. cerevisiae* pex14A cells (data not shown). However, Pex14p remained unaffected in pex19A cells (Fig. 2A). Thus, deficiency in Pex14p cannot be the reason for the observed instability of Pex17p, but in view of the accumulated evidence, the most likely explanation is that Pex19p itself is required for Pex17p peroxisomal targeting and stability.

The function of Pex19p is still a matter of debate (57). Stabilization of membrane proteins by Pex19p via a chaperone-like
function is proposed, as is the function of Pex19p as an import receptor that recognizes peroxisomal membrane proteins in the cytosol and directs them to the peroxisomal membrane (26, 57). Recently, the dynamic-like protein Vps1p has been demonstrated to associate with peroxisomes in a Pex19p-dependent manner (53). However, the authors propose that Pex19p acts as a chaperone but not as a receptor for the peroxisomal targeting of Vps1p. The rapid degradation of membrane proteins, including Pex17p, in the absence of Pex19p supports the idea of a chaperone function of the protein. Here we have provided evidence for an additional targeting function of Pex19p. We have shown that the 68-amino-acid fusion construct, consisting only of the Pex19p- and Pex14p-binding sites of Pex17p, is targeted to the peroxisomal membrane in a Pex19p-dependent manner (Fig. 6). Neither the Pex19p- nor the Pex14p-binding site, which both lack putative transmembrane spans or expanded hydrophobic regions, alone is sufficient for peroxisomal targeting. Consequently, the most plausible explanation is that Pex19p recognizes and binds Pex17p in the cytosol, which would in a stabilization of the protein. Pex19p is then supposed to target Pex17p to the peroxisomal membrane where it is anchored to the docking complex by binding to Pex14p. These data strongly support the idea that Pex19p does not only function as a chaperone but is also directly involved in the peroxisomal targeting of peroxisomal membrane proteins. Furthermore, our data show that this function of Pex19p is not restricted to integral membrane proteins but that peripheral membrane proteins such as Pex17p may rely on interaction with Pex19p to reach their final destination at the peroxisomal membrane. Moreover, previously, the targeting signal for PMPs (mPTS) was defined as a region comprising a Pex19p-binding motif in conjunction with a transmembrane segment (28). As a consequence of our data on Pex17p-targeting, we propose to extend the definition for an mPTS to regions comprising a Pex19p-binding motif and a peroxisomal anchor sequence.

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