Membrane Association of the Cycling Peroxisome Import Receptor Pex5p*

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Peroxisomal proteins carrying a peroxisome targeting signal type 1 (PTS1) are recognized in the cytosol by the cycling import receptor Pex5p. The receptor-cargo complex docks at the peroxisomal membrane where it associates with multimeric protein complexes, referred to as the docking and RING finger complexes. Here we have identified regions within the Saccharomyces cerevisiae Pex5p sequence that interconnect the receptor-cargo complex with the docking complex. Site-directed mutagenesis of the conserved tryptophan residue within receptor-cargo complex with the docking complex. Site-directed mutagenesis of the conserved tryptophan residue within

suggest that a translocation-competent state of the PTS1 receptor enters the membrane via protein-lipid interactions before it tightly associates with other peroxisomes.

Peroxisomes post-translationally import folded and oligomeric proteins of very different sizes from the cytosol across the single membrane into their matrix (1–4). This is in contrast to most other translocation systems that transport unfolded polypeptide chains (5). Although the identities of many proteins, collectively called peroxins, that are required for this process are known, the mechanism of protein translocation across the peroxisomal membrane is poorly understood.

Current evidence favors a cycling receptor model for matrix protein import (6–8). Two soluble import receptors, Pex5p and Pex7p, bind their cognate peroxisomal targeting signals (PTS)3 in the cytosol and then shuttle to peroxisomes, where the PTS proteins are imported. After releasing their cargo, the receptors recycle to the cytosol for additional rounds of import. Most of the peroxisomal matrix proteins possess one of two evolutionarily conserved PTS, the C-terminal PTS1 or the N-terminal PTS2, which are specifically recognized by Pex5p and Pex7p, respectively (2, 3). A few matrix proteins are known that contain a completely different targeting signal that is also recognized by Pex5p (9–12). This import receptor was shown to consist of two functionally distinct domains. Although binding of the PTS1 proteins is mediated by six tetratricopeptide repeats within its C-terminal half (13), essential transport steps of the receptor cycle seem to be performed by its N-terminal half (9, 14).

Numerous reports demonstrate that mammalian and yeast Pex5p tightly associate with peroxisomal membranes (15–20). Membrane peroxins that bind directly to both receptors, Pex5p and Pex7p, are Pex13p (21–24) and Pex14p (25–27). These two proteins together with Pex17p have been established as members of a membrane-bound docking subcomplex (28, 29). An even larger complex, termed the importomer, which in addition to the docking subcomplex contains Pex8p and the three RING finger peroxins, Pex2p, Pex10p, and Pex12p, has been shown to exist in the peroxisomal membrane (29). The ATP-dependent dislocation of the PTS1 receptor from the peroxisomal membrane into the cytosol is mediated by the AAA peroxins Pex1p and Pex6p (30). The interaction of Pex5p and Pex14p, which seems to be the initial binding partner of the receptors at the peroxisomal membrane, has been studied intensively (31–34). It was found that the N terminus (amino acid residues 1–78) of human Pex14p directly interacts with Pex5p with a binding affinity in the nanomolar range (32). Moreover, the seven conserved WXXXXF motifs within the N-terminal half of human Pex5p form individual high affinity sites for Pex14p (35). These motifs are present in variable number and spacing in all known Pex5 proteins. Recently it was shown that the PTS1 and the PTS2 receptors not only associate with the outer surface of the membrane (cycling receptor model) but actually traverse the membrane. This has led to the extended shuttle

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We dedicate this manuscript to Helmut Kindl in honor of his 70th birthday and his contribution to the field of peroxisome biogenesis.

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The abbreviations used are: PTS, peroxisomal targeting signal; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; PMSF, phenylmethylsulfonyl fluoride; MES, 4-morpholineethanesulfonic acid; aa, amino acid; ProtA, Protein A.
receptor model (6). Despite these accumulating data about the translocation machinery, a translocation channel has not yet been identified.

In this study, we investigated the interaction of Saccharomyces cerevisiae Pex5p (ScPex5p) at the peroxisomal membrane in greater detail. The identification of a novel Pex14p-binding site in the Pex5p sequence enabled us to generate a mutant Pex5p that has lost the ability to associate stably with the docking and the RING finger complex. Most unexpectedly, this Pex5p variant associated together with cargo protein with the peroxisomal membrane and was still able to mediate matrix protein import. From these findings we speculated that protein-lipid interaction rather than protein-protein interactions could anchor the receptor-cargo complex at the membrane. In a first step to verify this assumption, we show in vitro that Pex5p has the ability to spontaneously insert into phospholipid monolayers and bilayers.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—Yeast strains used in this study are derivatives of S. cerevisiae UTI-7A if not stated otherwise (Table 1). Strains expressing proteins fused to tobacco etch virus (tobacco etch virus-protease cleavage site)-protein A instead of wild-type Pex5p were generated by genomic integration into the PEX5 locus. This was achieved by transforming haploid yeast cells with the PCR products according to Knop et al. (36). The yeast strain pex3Δpex19Δ was generated according to Güldener et al. (37) using primer pairs Ku365/Ku700 (Table 2) for PEX19 deletion and Ku575/Ku862 for PEX3 deletion. The construction of the pex13Δ pex14Δ pex5Δ strain required the disruption of PEX5 with the removable loxP-kanMX4-loxP marker using the oligonucleotides Ku301 and Ku976b. The deletion of PEX13 was generated using the primer pair Ku274/RE534, whereas PEX14 was deleted with Ku301 and Ku976b. The deletion of PEX13 was generated using loxP-kanMX4-loxP (Belgium). The corresponding coding DNA regions were amplified by PCR using genomic DNA of the wild-type strain as template. The oligonucleotides used in this study are listed in Table 2 and were obtained from Eurogentec (Belgium).

The PEX5 full-length fragment was amplified by using the primers Ku875 and Ku888. The amplification product was cloned using primer-generated endonuclease recognition sites into Sall/BgIII-digested pWK-PEX5-(aa 1–313) (14) and into Sall/BgIII-digested pPC86 (39) resulting in pWK-PEX5 and pPC86-PEX5. The PEX5-(aa 1–313) fragment was obtained by Sall/BgIII digestion from the vector pWK-PEX5-(aa 1–313) (14) and ligated into a Sall/BgIII-digested two-hybrid vector pPC86 resulting in pPC86-PEX5-(aa 1–313). The same procedure was used to obtain the PEX5-(aa 313–612) fragment from the vector pDK-PEX5-(aa 313–612) (14) resulting in pPC86-PEX5-(aa 313–612). The PEX5-(aa 1–245) fragment was amplified using the primer pair Ku875/Ku876. The amplification product was ligated after MluI/NotI restriction into an MluI/NotI-digested pWK-PEX5 resulting in pWK-PEX5 and into pWK-PEX5-(aa 1–313) (14) and ligated into a Sall/BgIII-digested two-hybrid vector pPC86 resulting in pPC86-PEX5-(aa 1–245). The PEX5 fragment encoding amino acids 246–267 was amplified by two PCRs. In a first step a fragment corresponding to amino acids 246–258 fused to the GAL-4-activating domain was amplified using the two-hybrid vector pPC86 as template and the primer pair Ku1436/Ku1433 in which the latter one codes for the PEX5 fragment. The PCR product serves as template for the final fragment coding for amino acids 246–267, which was amplified by the primer pair Ku1436/Ku1433 in which the latter one codes for the PEX5 fragment (aa 252–267) and contains a Nots restriction site. The amplification product was ligated after MluI/Nots restriction into an MluI/Nots-digested pPC86 vector resulting in pPC86-PEX5-(aa 246–267).

Point mutations in PEX5 were introduced using overlap extension PCR (40). All base pair changes were verified by sequencing. For the mutation Pex5p(W120A), PCR products were amplified by primer pairs Ku875/Ku856 and Ku855/Ku888 using genomic DNA of wild-type strain as template. Ku856 and Ku855 contain a substituted base pair triplet coding for alanine at amino acid position 120. The exterior primers Ku875 and Ku888 together with both PCR products were used for the overlap extension PCR. The amplification product was digested with Sall and BgIII and ligated into Sall/BgIII-digested pPC86 resulting in pPC86-PEX5(W120A). To introduce the double mutation W120A/W204A into the PEX5 sequence, the PCR product PEX5(W120A) was used as a template. Overlapping PCR was carried out as described above using primers Ku857 and Ku858 for the substitution W204A and the exterior primers Ku875 and Ku888. The PCR product was digested with Sall and BgIII and ligated into Sall/BgIII-digested pPC86 resulting in pPC86-PEX5(W120A;W204A). To introduce the single mutation Pex5p(Trp-204) the same cloning procedure was carried out as described above but using genomic DNA of wild-type strain as template resulting in pPC86-PEX5(W204A).

TABLE 1

| Strain          | Description               | Ref. |
|-----------------|---------------------------|------|
| UTL-7A (wild-type) | MATa, ura 3-2, trp1, leu2-3/11 | 38   |
| Wild-type Pex5p-ProtA | MATa, ura 3-2, trp1, leu2-3/112, pex5-LEU2 | 14   |
| pexΔ            | MATa, ura 3-2, trp1, leu2-3/112, pex5-LEU2 | 14   |
| pex5Δpex19Δ     | MATa, ura 3-2, trp1, leu2-3/112, pex5-LEU2, pex5::loxPkan | 30   |
| pex19Δ          | MATa, ura 3-2, trp1, leu2-3/112, pex5::loxPkan, pex19::loxP | This study |
| pexΔpex13Δpex14Δ | MATa, ura 3-2, trp1, leu2-3/112, pex5::loxPkan, pex13::loxP, pex14::loxPkan | This study |
| PCY2            | Mata, Dgalk4, Dgatl80, URA::GAL1-LacZ, lyz2-801::his3-D200, trp1-D63, leu2 ade2-101pol+ | 39   |
Additionally, the PCR fragment was ligated into Sall/BglIII-digested pWK-PEX5-(aa 1–313) resulting in pDK-PEX5(W204A). To obtain Pex5p(W261A), PCR products were amplified by primer pairs Ku875/Ku1458 and Ku888/Ku1457 using genomic DNA of wild-type strain as template. Ku1458 and Ku1457 contain a substituted base pair triplet coding for alanine at amino acid position 261. The exterior primers Ku875 and Ku1457 were used for the overlap extension PCR. The amplification product was digested with Sall and BglIII and ligated into Sall/BglIII-digested pWK-PEX5-(aa 1–313) (14) and into Sall/BglIII-digested pC86 (39), resulting in pDK-PEX5(W261A) and pPC86-PEX5(W261A). To introduce the double mutation W204A/W261A into the PEX5 sequence, PEX5(W204A) was used as a template. Overlapping PCR was carried out as described above using primers Ku1558 and Ku1457 for the substitution W204A and the exterior primers Ku875 and Ku1584 (containing a "salvaged" SalI restriction site) by using genomic DNA isolated from yeast strain PCY2 (Clontech) was performed according to the method of Fields and Song (44), open reading frames of selected PEX genes were fused to the DNA-binding domain or transcription-activating domain of GAL4 in the vectors pC86 and pPC97 (39). Cotransformation of two-hybrid vectors pPC86 and pWK-PEX5p(W204A), pWK-PEX5p(W204;261A), and pDK-PEX5p(W204;261A) resulted in pDK-PEX5p(W204A)ProtA, pDK-PEX5p(W204;261A)ProtA, and pDK-PEX5p(W204;261A)ProtA.

For bacterial expression of yeast Pex5p, the vector pET9d-His-ScPex5p was kindly provided by K. Niederhoff (Bochum, Germany). For expression of human Pex5p, the vector pET9d-His-HsPex5pLp was used (32).

Two-hybrid Assay—For two-hybrid assays based on the method of Fields and Song (44), open reading frames of selected PEX genes were fused to the DNA-binding domain or transcription-activating domain of GAL4 in the vectors pC86 and pPC97 (39). Cotransformation of two-hybrid vectors into yeast strain PCY2 (Clontech) was performed according to the protocols of the manufacturer. Transformed yeast cells were plated on SD synthetic medium without tryptophan and leucine. β-Galactosidase filter tests were performed as described previously (45).

Western Blotting and Densitometry—Western blots were incubated with polyclonal rabbit antibodies raised against human Pex5p and the S. cerevisiae proteins thiolase, Fox1p, Pex5p, Pex10p, Pex12p, Pex13p, Pex14p, Pex17p, Pex3p, cat- alase A, porin, fructose-1,6-bisphosphatase (all raised in our laboratory), aconitase (a kind gift of R. Lill, University of Marburg, Germany), Mdh3p (a kind gift of L. McAlister-Henn, University of California), and Sec72p (a kind gift of E. Hartmann, University of Lübeck, Germany). Horseradish peroxidase coupled with anti-rabbit IgG in combination with the ECL system (Amersham Biosciences) was used to detect immunoreactive complexes.
Lipid Binding Activity of Pex5p

For semi-quantitative analyses of Western blot signals the band density on film was measured with a scanner using Scion image software. The relative density of signals was calculated in the area encompassing the immunoreactive protein band and subtracting the background of an adjacent nonreactive area in the same lane of the protein of interest.

**Yeast Cell Fractionation**—Spheroplasting of yeast cells, homogenization, and differential centrifugation at 25,000 × g of post-nuclear supernatants were performed as described previously (38). Cell fractionation by means of density gradient centrifugation was carried out as described previously (14), but instead of postnuclear supernatant 3–4 mg of protein of 25,000 × g organellar pellet were loaded onto the gradient. For membrane extraction of organelar pellets, aliquots were adjusted to a final concentration of 0.1 M Na₂CO₃, pH 11.5, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Samples were incubated for 30 min on ice and subjected to centrifugation at 100,000 × g for 1 h. Protease protection experiments were carried out with organellar pellets containing 100 μg of protein and increasing amounts of proteinase K (Sigma) in buffer (0.6 M sorbitol, 5 mM MES, 0.5 mM EDTA, 50 mM KCl, pH 6.0) with or without 1% Triton X-100. Digestion was carried out on ice for 10 min and stopped by adding PMSF (240 μg/ml) and SDS-PAGE sample buffer. All samples were analyzed by Western blotting.

For flotation analyses of cell lysates, olate grown yeast cells were lysed according to Lamb et al. (46) using glass beads and lysis buffer (20 mM HEPES; 100 mM KOAc; 5 mM MgOAc; pH 7.5) containing protease inhibitors (240 μg/ml PMSF, 2 μg/ml aprotinin, 0.35 μg/ml bestatin, 1 μg/ml pepstatin, 2.5 μg/ml leupeptin, 0.16 mg/ml benzamidine, 5 μg/ml antipain, 0.21 mg/ml NaF, 6 μg/ml chymostatin). 1.5 mg of protein from the lystate were adjusted to a concentration of 45% (w/v) sucrose. The samples were laid onto sucrose cushions (220 μl of lysis buffer with 50% (w/v) sucrose) and overlaid with 500 μl of buffer II (lysis buffer; 40% (w/v) sucrose), 1900 μl of buffer I (lysis buffer; 25% (w/v) sucrose), and 1000 μl of lysis buffer. After ultracentrifugation for 3 h at 170,000 × g in a swing-out rotor, the flotation gradient was collected as 10 fractions from top (fraction 10) to bottom (fraction 1). The fractions were analyzed by SDS-PAGE and Western blot detection.

The immunopurification of membrane-associated Pex5p-ProtA and its variants with IgG-coupled Sepharose was performed as described previously (14, 29). For densitometric analyses of Western blot signals, at least two independent experiments and various exposure times were used. During the course of all fractionation experiments, SDS-PAGE samples of homogenates and lysates were taken immediately after cell breakage.

**Electron Microscopy**—For electron microscopy, olate-induced cells were fixed with 1.5% KMnO₄ and prepared as described by Erdmann et al. (38).

**Expression, Purification, and Analysis of Recombinant Proteins**—Expression and purification of His₆-tagged human and yeast Pex5p were carried out as described previously (32) with slight modifications. After binding to the nickel-nitriolotriacetic acid matrix (Qiagen, Germany), yeast proteins were eluted with 100 mM imidazole, and dithiothreitol was added to the eluate to a final concentration of 10 mM.

**In Vitro Assays**—The membrane insertion experiments into lipid monolayers and liposomes were carried out with mixtures of 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), purchased from Avanti Polar Lipids, Inc., and stock solutions of lipids were prepared in chloroform. The phospholipid phosphorus concentration was determined using the method of Rouser et al. (47). The final lipid mixtures were made at a concentration of 10 mM.

Lipid monolayers were spread on subphase buffer of 20 mM Tris-HCl, pH 7.4, 50 mM NaCl (continuously stirred with a magnetic bar) to give an initial surface pressure of 20 mN/m. Proteins were injected through a hole at the side of the monolayer trough (volume 5 ml); the injection volume was less than 1% of the volume of the subphase. The surface pressure of the monolayer was measured by the Wilhelmy plate method, using a Cahn 200 electrobalance (48). Experiments were performed in a thermostatically controlled cabinet at room temperature.

For liposome preparation DOPC and DOPE were mixed together at a molar ratio of 7:3 in chloroform. The organic solvent was removed by placing the flask on a rotary evaporator (Buchi Rotavapor, Switzerland) operating at 80 rpm under a low vacuum at 40 °C, until a thin lipid film was produced on the bottom. The dried lipid film was hydrated in 20 mM Tris, 50 mM NaCl, pH 8, to a final concentration of 10 mM. Small unilamellar vesicles were obtained by sonication of the multilamellar vesicle suspension using a Branson sonifier (Branson Ultrasonics Corp.). The procedure was continued at room temperature until the solution became clear. The vesicles were stored at 4 °C and used within 24 h.

Purified human and *S. cerevisiae* Pex5p (0.75 nmol) were incubated for 1 h at room temperature in the absence or presence of liposomes (ratio of 1:750) in a final volume of 80 μl and mixed with 170 μl of buffer III (20 mM Tris-HCl; 50 mM NaCl; 65% (w/v) sucrose, pH 7.4). The samples were transferred to 4-ml ultracentrifuge tubes onto a sucrose cushion (220 μl of buffer III) and overlaid with 500 μl of buffer II (20 mM Tris-HCl; 50 mM NaCl; 40% (w/v) sucrose, pH 7.4), 1900 μl of buffer I (20 mM Tris-HCl; 50 mM NaCl; 25% (w/v) sucrose, pH 7.4), and 1 ml of buffer 0 (20 mM Tris-HCl; 50 mM NaCl, pH 7.4). After ultracentrifugation for 3 h at 170,000 × g in a swing-out rotor, the gradient was collected as 10 fractions from top to bottom. The fractions were analyzed by SDS-PAGE and Western blot detection.

For protease protection assays, floated fractions from gradients containing Pex5p were mixed with trypsin (Sigma) in a 60:1 ratio (according to Western blot signals). As a control, the corresponding load fractions were used. Protease treatment was carried out on ice. Trypsin was inactivated by incubation with SDS sample buffer for 5 min at 95 °C. Samples were taken after 0, 0.5, 1, 3, 5, and 10 min and analyzed by SDS-PAGE and immunoblotting.

**In Vitro Export**—*In vitro* import/export studies were performed as described previously (30).
Identification of the Pex5p-binding Site for the Conserved N-terminal Domain of Pex14p in S. cerevisiae—Although accumulated evidence demonstrates that the PTS1 receptor Pex5p binds to several peroxins organized in multisubunit complexes at the peroxisomal membrane (2), studies in a PEX8 deletion strain of S. cerevisiae strongly suggested that the initial association occurs with the docking subcomplex consisting of Pex13p, Pex14p, and Pex17p (29). To investigate the association of the receptor-cargo complex with the peroxisomal membrane in more detail, we mapped the Pex14p-binding site on Pex5p. Previous studies in higher eukaryotes demonstrated that the conserved N-terminal region of the membrane-associated Pex14p, referred to as Pex14p-N, interacts with the PTS1 receptor Pex5p via di-aromatic pentapeptide repeats (31, 33–35). ScPex5p possesses two of these WXXXF motifs, one of them is part of its Pex13p-binding site (21). We substituted the conserved tryptophans in both motifs of ScPex5p with alanine and tested the two-hybrid interaction with Pex13p and Pex14p-N. In line with previous reports, the W204A mutation abolished the interaction between Pex5p and Pex13p. However, neither of the two WXXXF motifs was required for binding of Pex14p-N (Fig. 1A).

To map the Pex14p-N-binding sites within the sequence of ScPex5p, we generated a number of truncated Pex5p versions and examined the fragments for their two-hybrid interaction with Pex14p-N (Fig. 1A). The smallest fragment in our analysis which retained interaction with Pex14p-N was located between amino acid positions 246 and 267. One striking feature within this stretch of amino acids is a di-aromatic pentapeptide that represents an inverted WXXXF motif. The sequence FQEVW is highly conserved among the Pex5p sequences of fungi and C. elegans. Sequences were aligned using ClustalX. Black boxes denote 100% sequence conservation, and gray boxes indicate more than 50% conserved amino acid residues. The asterisk marks the position of the conserved tryptophan, Trp-261, in S. cerevisiae Pex5p sequence. FXXXW indicates the inverted WXXXF motif. C, a single point mutation, W261A, within the Pex5p sequence abolishes binding to Pex14p-(aa 1–58). The indicated mutations W204A and W261A were introduced into the two-hybrid plasmid pPC86-PEX5 and transformed together with any of the following plasmids: pPC97-PEX8, pPC97-PEX13(SH3), pPC97-PEX14, pPC97-PEX14-(aa 1–58), pPC97-PEX14-(aa 225–341), and pPC97-PTS1. Selected transformants were analyzed for β-galactosidase activity with a filter assay.
with the peroxisomal membrane and, in particular, with constituents of the docking and the RING finger complexes. As shown above, these point mutations specifically disrupt the interactions of the PTS1 receptor with Pex13p and the N-terminal domain of Pex14p, respectively. The mutations were introduced in Pex5p-Protein A (ProtA) expression plasmids, and extracts of digitonin-solubilized membranes of cells expressing the mutant Pex5p-ProtA fusion proteins were applied to affinity purification using IgG-Sepharose columns. We have demonstrated previously that the C-terminal ProtA tag does not affect the functionality of the PTS1 receptor (14). Fig. 2A (2nd lane) shows in accordance with previous results that affinity-purified fractions using Pex5p-ProtA as a bait contain Pex13p, Pex14p, Pex17p, as well as the RING finger peroxins Pex10p and Pex12p. As a negative control, Pex3p was not found associated with Pex5p. It is important to note that in addition to known binding partners of Pex5p at the membrane, malate dehydrogenase 3 (Mdh3p) could be detected in all eluates of the IgG-Sepharose column. This soluble peroxisomal enzyme had been reported to be one of the most abundant PTS1-containing matrix proteins in cells of *S. cerevisiae* after oleate induction (50, 51). It is very likely that membrane-associated Mdh3p represents a cargo on its way into the peroxisome. Thus, the presence of Mdh3p in all eluates indicated the ability of the tested Pex5p variants to bind PTS1 proteins and to target their cargo to the peroxisomal membrane (Fig. 2A, 2nd to 5th lanes). Specific disruption of the Pex5p-Pex14p-N interaction by the single W261A mutation only slightly reduces the amounts of Pex5p-associated Pex13p, Pex14p, Pex17p, Pex10p, and Pex12p (Fig. 2A, 4th lane). The Pex5p variant harboring only W204A, in agreement with the two-hybrid results, led to affinity-purified eluates that selectively lacked Pex13p (Fig. 2A, 3rd lane) but still contain Pex14p, Pex17p, Pex10p, and Pex12p. In contrast, in eluates obtained from cells expressing the Protein A-tagged Pex5p with both mutations not any component of the docking complex was detected. The lack of an interaction with Pex17p is probably due to the fact that Pex5p interacts with Pex17p indirectly through Pex14p. Moreover, the components of the RING finger subcomplex were not found. To test the absence of binding partners of Pex5p(W204A;W261A) more rigorously, Western blots obtained from two independent affinity purifications were subjected to prolonged exposure

In contrast, this mutation did not impair the interaction of the PTS1 receptor with Pex8p, Pex13p, and PTS1 peptide indicating that the Pex5p variant is folded correctly (Fig. 1C). Therefore, we conclude that the ScPex5p fragment consisting of amino acids 246–267 and containing the inverted WXXXF motif FQEVW is sufficient and necessary for the interaction between Pex5p and the conserved N-terminal domain of Pex14p. However, Pex5p(W261A) still interacts with full-length Pex14p and with a C-terminal fragment of Pex14p consisting of amino acids 235–341 in the two-hybrid assay (Fig. 1C). It has been shown recently that Pex14p contains an additional binding site for the PTS1 receptor within the C-terminal domain of Pex14p (41, 49). Nonetheless, our data clearly prove that the Pex14p-C-binding site of Pex5p, which has not been mapped so far, is not identical with the inverted WXXXF motif.

**Disruption of the Interaction of Cargo-laden Pex5p with the Docking and the RING Finger Complex**—As a next step, we studied the effect of the two point mutations, W204A and W261A, in ScPex5p on the association of the PTS1 receptor and, consequently, on the functionality of the PTS1 receptor with the docking and the RING finger peroxins. It has been shown recently that Pex14p contains an additional binding site for the PTS1 receptor within the C-terminal domain of Pex14p (41, 49). Nonetheless, our data clearly prove that the Pex14p-C-binding site of Pex5p, which has not been mapped so far, is not identical with the inverted WXXXF motif.

**FIGURE 2. Membrane-associated Pex5p(W204A;W261A) does not form stable complexes together with the docking and the RING finger peroxins.** A, the point mutations W204A and W261A alone or in combination affect the association of Pex5p with the docking and the RING finger complex. *S. cerevisiae* wild-type cells, in which the genomic PEX5 open reading frame was replaced by a DNA region coding for Pex5p-ProtA, pex5Δ cells expressing Pex5p(W204A)-ProtA, Pex5p(W261A)-ProtA, and Pex5p(W204A;W261A)-ProtA from plasmids and as a control for wild-type cells (Pex5p), were grown on oleate medium. Protein complexes were isolated from equal amounts of 1% (w/v) digitonin-solubilized membranes subjected to IgG affinity chromatography. Bound proteins were eluted with SDS gel electrophoresis sample buffer and subjected to immunoblot analysis with antisera directed against Pex5p, Mdh3p, Pex13p, Pex14p, Pex17p, Pex10p, Pex12p, and Pex3p (*left panel*). Sample volumes correspond to equal amounts of the indicated bait proteins. The *right panel* displays semiquantitative densitometric analysis of Western blot signals obtained for Pex5p-associated Pex13p and Pex14p given in % of maximum (amount of protein bound to Pex5p-ProtA). The results presented are representative of two independent IgG-Sepharose purifications and various exposition times. Standard errors are indicated by bars. B, schematic representation of interactions between the ScPex5p-cargo complex and membrane peroxins as deduced from the experiment shown above.
times. Therefore, traces of Pex13p and Pex14p but still no RING finger peroxins were found (Fig. 2A, right panel). Taking into account the known interaction between Pex13p and Pex14p (21, 42, 52), we can deduce from our results a complex network of interactions between Pex5p-cargo and constituents of the docking and RING finger complexes as shown schematically in Fig. 2B. Our data strongly suggest that either Pex13p or Pex14p-N is required to associate Pex5p stably to peroxisoms acting downstream of the docking event.

Pex5p(W204A;W261A) Mediates PTS1 Protein Import into Peroxisomes—In order to study the effect the uncoupling of Pex5p from the docking complex has on peroxosome protein import, the mutant Pex5 proteins, Pex5p(W204A), Pex5p(W261A), Pex5p(W204A;W261A), and the corresponding Protein A-tagged variants were expressed in a PEX5 deletion strain. Surprisingly, all tested variants of Pex5p are able to partially complement the oleate growth defect of pex5Δ cells as indicated by the oleate plate drop dilution assay (Fig. 3A, left panel) or growth in liquid oleate medium (Fig. 3A, right panel). The unexpected ability of these mutants to facilitate import of peroxisomal matrix proteins is further substantiated by electron microscopy and subcellular fractionation. Electron micrographs of cells expressing these mutated Pex5 proteins revealed the presence of membrane-bound organelles that resemble in size and number wild-type peroxisomes (Fig. 3B). Subcellular fractionation of cells expressing Pex5p(W204A;W261A) by means of 25,000 × g centrifugation (Fig. 3C) clearly showed that the import of the PTS1 proteins catalase and Mdh3p as that of the PTS2 protein thiolase into peroxisomes is not drastically affected. In contrast, acyl-CoA oxidase (Fox1p) is found mostly in the 25,000 × g supernatant suggesting a cytosolic mislocalization. This import defect is consistent with recent observations of Klein et al. (10). The authors demonstrated that the W261A mutation drastically weakens binding efficiency of Pex5p with acyl-CoA oxidase and peroxisomal carnitine acetyltransferase, both of which utilize unusual non-PTS1 non-PTS2 targeting signals. Therefore, it seems likely that the reduced growth rates of the W261A single and double mutants on oleate compared with wild-type Pex5p (Fig. 3A) are due to lower binding efficiency of this specific subset of cargo proteins rather than to a general failure of this mutant to facilitate import of peroxisomal proteins.

25,000 × g sediments obtained from pex5Δ cells complemented with Pex5p(W204A;W261A) were further subjected to density gradient centrifugation (Fig. 3D). Peroxisomal matrix and membrane proteins cofractionated at a density of 1.16 g/cm³, whereas wild-type peroxisomes were found at a density of 1.17 g/cm³. This difference could be explained by the partial import defect caused by the W261A mutation. In line with the results of the functional complementation analysis, Pex5p(W204A;W261A) is clearly associated with the peroxisomal fraction. However, scanning densitometric analysis of Pex5p Western blot signals in both gradients revealed that the amount of Pex5p(W204A;W261A) associated with mature, intact peroxisomes (17.8%) is significantly less than wild-type Pex5p (31.8%). A major portion of Pex5p(W204A;W261A) seems to be associated also with other cellular organelles, probably mitochondria. Therefore, disruption of the binding sites for Pex14p and Pex13p results in more unspecific binding of the PTS1 receptor to other membranes.

In summary, the wild-type like morphology of peroxisomes (Fig. 3B) and the relative amounts of PTS1 matrix enzymes associated with these organelles (Fig. 3, C and D) in the double mutant cells clearly support the notion that the Pex5p(W204A;W261A) variant is able to target PTS1 proteins to peroxisomes and to facilitate their translocation across the peroxisomal membrane.

Membrane Binding of Pex5p Does Not Require a Stable Association with the Docking and the RING Finger Complex—Next we asked whether disruption of interacting sites affects the membrane binding properties of the PTS1 receptor. To quantify the amounts of Pex5p associated with membranes, oleate-induced cells were disrupted with glass beads, and the resulting homogenates were subjected to flotation analyses. Both Pex5p(W204A;W261A) and its Protein A-tagged variant cofractionate like wild-type Pex5p in light density regions of the gradients together with typical membrane marker proteins for peroxisomes, mitochondria, and endoplasmic reticulum (Fig. 4A). Remarkably, the amount of membrane-associated Pex5p(W204A;W261A), which barely binds to other membrane proteins, is comparable with that of wild-type Pex5p. In light of these findings, we conclude that the interactions of Pex5p either with Pex13p or with Pex14p-N, which are required to connect the PTS1 receptor with the docking and the RING finger complex, are not necessary for membrane attachment of cargo-laden Pex5p.

In order to test whether mutant Pex5p binds exclusively to peroxisomal membranes, we used the double deletion strain pex3Δpex19Δ, which should not contain peroxisomal membranes at all (53). Unexpectedly, a small fraction of the PTS1 receptor and higher amounts of both mutant forms were found in the floated membrane fraction indicating unspecific binding to other cellular vesicles (Fig. 4A) in the absence of peroxisomal membranes.

In order to study the influence of Pex14p and Pex13p binding on the membrane topology of Pex5p, we applied carbonate extraction and proteinase K treatment to 25,000 × g organellar pellets obtained from wild-type cells and from pex5Δ cells expressing Pex5p(W204A;W261A). In contrast to integral membrane proteins like Pex3p, both Pex5p proteins, wild-type and double mutant, seem to exist in various membrane-bound states. Although most of the organellar Pex5 proteins were sensitive to carbonate extraction (Fig. 4B) and proteinase K digestion (Fig. 4C), small fractions of both proteins were completely resistant against these treatments. A possible interpretation of these results is that a large fraction of organellar Pex5p is more loosely associated with the membrane, most likely the cytosolic side, whereas a minor portion is intrinsically embedded in the membrane. Apparently, the mutant Pex5p(W204A;W261A) that is not stably associated with other peroxins exhibits the same membrane binding properties as wild-type Pex5p.

Lipid Binding Activity of Pex5p

Membrane-associated Pex5p(W204A;W261A) Can Shuttle Back to the Cytosol in a Pex1p- and ATP-dependent Manner—In order to test whether the impairment to associate stably with the importomer affects recycling of the PTS1 receptor, we performed in vitro import/export studies with the Pex5p
FIGURE 3. The Pex5p variants Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) can mediate the import of PTS1 proteins into peroxisomes. A, expression of the Pex5p variants Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) under control of the endogenous promoter partially rescue the PTS1 import defect in pex5Δ cells. For the plate growth assay (left panel), pex5Δ cells expressing Pex5p(W204A)-ProtA, Pex5p(W261A)-ProtA, and Pex5p(W204A;W261A)-ProtA were grown in 0.3% glucose medium for 16 h. Cells were washed and diluted to 3 × 10⁷ cells/ml in distilled water. Aliquots were applied as a series of 10-fold dilutions onto an oleic acid plate, whereas the first spots on the left side correspond to 2 × 10⁴ cells. The growth plates were incubated at 30 °C for 5 days. For the growth curves (right panel) cells expressing Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) were grown to mid-log phase in 0.3% glucose medium and inoculated at A₆00 of 0.1 in liquid oleate medium. Growth was followed with time by measuring the absorbance at 600 nm for 60 h.

B, peroxisomes of cells expressing the mutated Pex5p variants exhibit wild-type like morphology. Electron microscopy analysis of wild-type cells, pex5Δ cells, and pex5Δ cells expressing Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) were carried out with cells grown for 12 h in oleic acid medium. L, lipid drops; M, mitochondria; N, nucleus; V, vacuole; P, peroxisomes; Bars, 1 μm. C, wild-type cells, pex5Δ cells, and pex5Δ cells expressing Pex5p(W204A;W261A) were grown for 14 h on oleate and subjected to subcellular fractionation. Equivalent volumes of the 600 × g postnuclear supernatant (H), 25,000 × g sediment (P), and 25,000 × g supernatant (S) were analyzed by Western blotting. Antibodies used were directed against Pex5p, Mdh3p, catalase, Fox1p, thiolase, Pex14p, cytosolic fructose-1,6-bisphosphatase, and mitochondrial aconitase.

D, oleate-grown pex5Δ cells expressing Pex5p(W261A;W204A) and wild-type cells were further analyzed by density gradient centrifugation. 25,000 × g sediments were loaded on top of linear Optiprep gradients (15.5 to 36% (w/v) Iodixanol) containing 18% (w/v) sucrose and subjected to centrifugation. Fractions were collected from the bottom (fraction 1) of the gradient. Equal quantities of each second gradient fraction were analyzed by Western blotting using antibodies directed against Pex5p, Mdh3p, catalase, Fox1p, thiolase, Pex14p, fructose-1,6-bisphosphatase, and aconitase. The right panel displays the corresponding densitometric analyses of anti-Pex5p Western blot signals. Results are given in percent of the sum of all signals in each gradient.

FIGURE 3. The Pex5p variants Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) can mediate the import of PTS1 proteins into peroxisomes.
mutants as described previously (30). To achieve insertion of Pex5p into membranes first, cytosolic fractions from Pex1p-deficient cells containing wild-type or mutant Pex5 proteins were incubated with whole cell membranes obtained from pex1/H9004 pex5/H9004 cells. Like wild type, significant amounts of the single and double mutants associate with the membranes in vitro (Fig. 5 A, import pellet fractions). Further sample analyses revealed that all membrane-associated Pex5 proteins can be released again (Fig. 5 A, export supernatant fractions).

The export of wild-type and mutant proteins depends on the presence of ATP and Pex1p, demonstrating that all tested variants are substrates for AAA peroxins and ATP-dependent export and thus are capable of cycling between the peroxisomal and cytosolic compartment. The fact that only peroxisomal membranes contain functional Pex5p export machinery further proves that a considerable amount of Pex5p(W204A;W261A) enters the right compartment. However, the relative amounts of Pex5p mutants in the release fractions are lower when compared with wild type. In particular, Pex5p(W204A) and Pex5p(W204A;W261A) are drastically reduced. One possible explanation could be that the interactions between the mutated PTS1 receptors and the docking complex, in particular Pex13p, are critical for an efficient transport of Pex5p along and from the peroxisomal membrane. This notion is further supported by using Pex5p-associated membrane preparations from pex13/pex14Δ cells in the in vitro export assay (Fig. 5B).

In the absence of both Pex13p and Pex14p, no specific release of membrane-bound Pex5p could be detected.

**Human and Yeast Pex5p Can Insert Spontaneously into Phospholipid Membranes in Vitro**—The results described above indicate that yeast Pex5p might not require stable interactions with any of the tested membrane-bound peroxins for its functional association with the peroxisomal membrane. In order to study the nature of the ability of Pex5p to bind to peroxisomal membranes, we investigated the potential of Pex5p from human and yeast to interact directly with lipids. As a first approach, we tested the insertion of recombinant purified PTS1 receptors into phospholipid monolayers. All experiments were carried out with a phospholipids mixture consisting of 3 volumes DOPE and 7 volumes DOPC. This composition was based on previous reports demonstrating that these lipids are the principal constituents, 28% phosphoethanolamine and 57% phosphocholine, respectively, of the membranes of rat liver peroxisomes (54). A mixture of both was spread on an aqueous subphase to form monolayers at the air/water interphase with an initial surface pressure of 20 mN/m. The insertion of ScPex5p and HsPex5p purified from *Escherichia coli* extracts was monitored after their injection below the monolayer surface. In both experiments, monolayer surface pressure

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**FIGURE 4.** Pex5p(W261A;W204A) and wild-type Pex5p exhibit the same membrane properties. A, membrane association of Pex5p is not affected by the point mutations W204A and W261A. Oleate grown wild-type cells, pex3/pex19Δ cells, and pex5Δ cells expressing Pex5p-ProtA, Pex5p(W204A)-ProtA, Pex5p(W261A)-ProtA, or Pex5p(W204A;W261A)-ProtA under control of the endogenous promotor were disrupted with glass beads. 1.5 mg of protein from the lysate was applied to a flotation gradient. After ultracentrifugation at 170,000 × g the gradient was collected as 10 fractions from top (fraction 10) to bottom (fraction 1). Equal portions of each fraction were subjected to immunoblot analysis with antisera directed against Pex5p, Pex3p, thiolase, Sec72p, and porin. B and C, organellar fractions were obtained from lysates of wild-type and pex5Δ cells expressing Pex5p(W204A;W261A) by a 25,000 × g centrifugation step and applied to alkaline and proteinase K treatment. The organelles were incubated with 0.1 M sodium carbonate, pH 11.5, for 30 min (B). Extracted proteins (S, supernatant) were separated from the membrane fraction (P, pellet) by centrifugation. Equal amounts of proteins were analyzed by Western blotting for the presence of Pex5p, the integral membrane protein Pex3p, and the matrix protein thiolase. The membrane topology of wild-type Pex5p and mutant Pex5p(W204A;W261A) was tested by treating organellar pellets for 10 min with varying amounts of proteinase K and analyzed by Western blotting using anti Pex5p antibodies (C).
increased as a function of the amount of Pex5p injected resulting in maximal increases of 5 and 7 mN/m, respectively (Fig. 6A). In the absence of a lipid film, the proteins themselves showed a surface pressure increase less than 1 mN/m (data not shown). Thus, we conclude that the increase in surface pressure was not due to the surface properties of the proteins but to the insertion of Pex5 proteins into the lipid monolayer. The specificity of this lipid binding assay is demonstrated by the usage of two other purified soluble proteins, both of which play major roles in bacterial protein translocation processes. SecA, one of the two control proteins, has been reported to possess lipid binding activity as shown previously by monolayer experiments (55). Using our assay conditions, SecA affects the surface pressure in a similar range as the PTS1 receptors, whereas the cytoplasmic receptor SecB remained in the water phase without a significant disturbance of the surface tension (Fig. 6B) (56).

To extend the studies concerning the lipid binding activity of the recombinant PTS1 receptors for bilayer membranes, we incubated human and yeast Pex5p with artificial liposomes generated by a phospholipid mixture DOPE:DOPC (3:7). Subsequently, the vesicles were floated by sucrose density gradient centrifugation, and the fractions were analyzed by Western blotting. Fig. 5B shows that a significant portion of both HsPex5p and ScPex5p comigrate with the floated liposomes. Although in both preparations many degradation products of Pex5p could be detected, only the full-length and in the case of ScPex5p one additional degradation product are found in the lighter fractions of the gradients. The fact that some major degradation products do not float with liposomes demonstrates that the liposome association of the intact PTS1 receptors is not due to unspecific binding. To address the question if the full-length Pex5 proteins associate peripherally with the liposomes or insert into the bilayer phase, we further applied protease protection assays to Pex5p fractions (Fig. 5C). A significant fraction of both human and yeast Pex5p became resistant to trypsin treatment when associated with liposomes. This result is in line with previous studies demonstrating that human membrane-bound Pex5p remains uncleaved when intact organelles were incubated with trypsin (15). Taken together, our in vitro studies suggest that both PTS1 receptors can insert spontaneously into bilayer membranes.
DISCUSSION

During each receptor cycle the cytosolic PTS1 receptor Pex5p binds tightly to the peroxisomal membrane. It is generally believed that cargo-laden receptor associates with the membrane via protein-protein interactions. This view is primarily based on the lack of predictable membrane spans in the sequence of Pex5p and its direct binding to several membrane-bound peroxins, including the docking complex constituents Pex14p and Pex13p as well as the RING finger peroxin Pex12p (2). Here we demonstrate that Pex5p has the ability to insert in an import-competent state into the peroxisomal membrane without a stable association with these peroxins. This conclusion is based on the following lines of in vivo and in vitro evidence. First, lipid binding activity of both human and yeast Pex5p could be demonstrated directly by spontaneous insertion of the purified proteins into artificial phospholipid mono- and bilayers. Second, in a PEX5-deleted yeast strain we have expressed a mutant, Pex5p(W204A;W261A), defective in the interaction with Pex13p and Pex14p. These cells contain cargo-laden PTS1 proteins and can be exported from the membrane in a Pex10p- and ATP-dependent manner. However, the active mutant has lost the ability to form a stable complex with any of the components of the docking complex and in addition with Pex10p and Pex12p suggesting that it is held by protein-lipid interactions rather than protein-protein interactions in the peroxisomal membrane.
Lipid Binding Activity of Pex5p

For disconnecting the PTS1 receptor from the docking and RING finger complexes, we have identified the binding site of yeast Pex5p for the conserved N-terminal domain of Pex14p. This region was shown to interact directly with WXXXF motifs of mammalian and plant Pex5p. Although yeast Pex5p contains two of these motifs, these are not directly involved in Pex14p binding. Remarkably, the mapped Pex14p binding region contains a single reverse WXXXF motif that is conserved among fungi and C. elegans. Site-directed mutagenesis of the conserved tryptophan into an alanine (W261A) within this novel motif confirmed the importance of this residue for the Pex14p-N binding and thereby emphasized the similarity between WXXXF motifs (in which tryptophan is a key residue) and the FXXXW motif in the mode of binding to the N-terminal domain of Pex14p.

As Azevedo and co-workers (57) reported that the insertion of mammalian Pex5p into the peroxisomal membrane requires Pex14p, we expected that this W261A mutation in yeast Pex5p would lead to an exclusively soluble PTS1 receptor. However, our data show that the cargo-laden PTS1 receptor still associates with the docking and the RING finger complex most likely via its interaction with Pex13p (see Fig. 2C). Therefore, we combined the mutation within the reverse WXXXF motif, W261A, with a single point mutation within the Pex13p-binding site W204A. In fact, introduction of both mutations into Pex5p abolished the formation of stable Pex5p-docking-RING finger complexes.

Surprisingly, the mutations did not prevent the membrane association of the PTS1 receptor (Fig. 2A and Fig. 4A), whereas subcellular fractionation analyses indicate that the double mutant Pex5p is localized not only to peroxisomal but also to other cellular membranes (Fig. 3D). The partial mislocalization of the double mutant to other non-peroxisomal organelles/membranes suggests that the formation of a stable Pex5p-docking complex contributes to the specific association of Pex5p with the peroxisomal membrane. Accordingly, the amount of peroxisome-associated Pex5p seems to be negligible in pex13Δpex14Δ cells (Fig. 5B). With respect to the partial peroxisomal association of Pex5p(W204A,W261A), it is relevant to note that ScPex14p possesses a second binding site for Pex5p (41, 49). Although the first Pex5p-binding site of ScPex14p is within the membrane-protected N-terminal domain (58), the second one is located at the cytosolic C-terminal end of Pex14p and is reported to provide the docking function (41). It is important to note that the presented results also indicate that Pex5p possesses two independent binding sites for Pex14p as follows: the inverted WXXXF motif for the Pex14p-N representing the conserved binding mode, and another one for Pex14p-C that has not yet been mapped and that so far has been found only in S. cerevisiae. It may well be possible that this second binding site transiently interacts with Pex5p. This could also explain the residual less than 2% of Pex14p-bound intermediate of the receptor-cargo complex might exist under physiological conditions. The complex network of interactions with its initial binding partners suggests a novel sequence of events for the docking step. The most simple interpretation of all of these findings regarding the properties of Pex5p and Pex14p would be that the C-terminal binding site of Pex14p tethers the Pex5p-cargo complexes to the membrane and thereby triggers its insertion into the membrane. Subsequently, the N-terminal binding site of Pex14p together with Pex13p stably connects the membrane-embedded Pex5p with the docking and the RING finger complex. In this model the N-terminal domain of Pex14p is acting like an adaptor domain, which could help to increase the efficiency of matrix protein translocation and/or receptor recycling.

There is additional experimental evidence that supports this view. Salomons et al. (59) reported that in the yeast Hansenula polymorpha a massive overproduction of Pex5p can partially rescue the import defect of Pex14p-depleted cells. This could mean that a high concentration of Pex5p partially compensates for the lacking tethering step, and under these circumstances Pex5p inserts without any help into the membrane.

The proposed but still not yet proven model of the initial events at the peroxisomal membrane can be experimentally tested, e.g. by identification of the lipid-binding sites of Pex5p and generation of mutants that fail to associate with liposomes in vitro and peroxisomes in vivo. Such experiments to evaluate the physiological relevance of a lipid-bound state of Pex5p are underway.
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