Pex22p of *Pichia pastoris*, Essential for Peroxisomal Matrix Protein Import, Anchors the Ubiquitin-conjugating Enzyme, Pex4p, on the Peroxisomal Membrane

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Abstract. We isolated a *Pichia pastoris* mutant that was unable to grow on the peroxisome-requiring media, methanol and oleate. Cloning the gene by complementation revealed that the encoded protein, Pex22p, is a new peroxin. A Δpex22 strain does not grow on methanol or oleate and is unable to import peroxisomal matrix proteins. However, this strain targets peroxisomal membrane proteins to membranes, most likely peroxisomal remnants, detectable by fluorescence and electron microscopy. Pex22p, composed of 187 amino acids, is an integral peroxisomal membrane protein with its NH₂ terminus in the matrix and its COOH terminus in the cytosol. It contains a 25–amino acid peroxisome membrane-targeting signal at its NH₂ terminus. Pex22p interacts with the ubiquitin-conjugating enzyme Pex4p, a peripheral peroxisomal membrane protein, in vivo, and in a yeast two-hybrid experiment. Pex22p is required for the peroxisomal localization of Pex4p and in strains lacking Pex22p, the Pex4p is cytosolic and unstable. Therefore, Pex22p anchors Pex4p at the peroxisomal membrane. Strains that do not express Pex4p or Pex22p have similar phenotypes and lack Pex5p, suggesting that Pex4p and Pex22p act at the same step in peroxisome biogenesis. The *Saccharomyces cerevisiae* hypothetical protein, Yaf5p, is the functional homologue of *P. pastoris* Pex22p.

Key words: organelle • peroxin • peroxisome • protein transport • yeast

**Peroxisomes** are single-membrane–bound organelles present in all eukaryotic cells. They contain enzymes that are responsible for such metabolic pathways as hydrogen peroxide metabolism, β-oxidation of long-chain fatty acids, synthesis of plasmalogens, cholesterol, and bile acids, and degradation of purines and amino acids (for review see Van den Bosch et al., 1992). To ensure that all enzymes for these metabolic pathways are properly targeted to the peroxisomes, cells have evolved several mechanisms to direct these enzymes to their correct locations after they have been translated.

Matrix-localized enzymes contain either one of two peroxisome-targeting signals (PTSs). PTS1 is located at the extreme COOH terminus of peroxisomal proteins. It consists of three amino acids and has the sequence SKL or some variants of it. PTS2 is present at the NH₂ terminus and has a consensus sequence of R/K-L/V/I-X₅-H/Q-L/A (for review see Subramani, 1998). Each of these PTSs is recognized by a specific receptor, peroxin (Pex)5p or Pex7p, respectively. Mutants lacking functional Pex5p are still able to import PTS2-containing proteins, whereas cells lacking Pex7p are only able to import PTS1-containing proteins (for review see Subramani, 1998). These results suggested the existence of two distinct import pathways for peroxisomal matrix proteins. The localization of these two receptors is still controversial. It seems, however, that both receptors are localized to the cytosol and peroxi-

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1. Abbreviations used in this paper: AD, activation domain; AOX, alcohol oxidase; DB, DNA-binding domain; DSP, dithiobis(succinimidylpropionate); G6PDH, glucose-6-phosphate dehydrogenase; GFP, green fluorescent protein; IPTG, isopropyl β-D-thiogalactopyranoside; mPTS, membrane peroxisome targeting signal; Pex, peroxin; PNS, post nuclear supernatant; PTS, peroxisome-targeting signal; UBC, ubiquitin-conjugating enzyme.
osomes, suggesting that the receptors shuttles from the cytoplasm to the peroxisomes, where they bind to the tightly associated peroxisomal membrane protein Pex13p (Girzalsky et al., 1999) and Pex14p (Albertini et al., 1997; Bodnar and Rachubinski, 1991; Diserted from the cytoplasm into the membrane.

Pex13p is also targeted to the ER, at least when they are homologous to ubiquitin-conjugating enzymes (UBCs) (van der Klei et al., 1997). The binding of Pex5p and Pex7p to Pex17p, however, was dependent on the presence of Pex14p (Huhse et al., 1998). Deleting the genes encoding Pex14p or Pex17p inhibited both the PTS1- and PTS2-dependent import pathways, suggesting that these proteins function at a point of convergence for the two import pathways. Pex5p, Pex7p, and Pex14p were also shown to interact with the SH3 domain-containing, peroxisomal integral membrane protein Pex13p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1992; A Ibertini et al., 1997; Girzalsky et al., 1999). Pex7p is only targeted to the peroxisomes with the help of the interacting proteins Pex18p and Pex21p (Purdue et al., 1998). Several other proteins have been implicated in the import of peroxisomal matrix proteins. A ntidodies against cytosolic HSP70 inhibit the import of SKL-containing proteins into peroxisomes (Walten et al., 1994; Fransen et al., 1998). Deleting the gene encoding Djp1p, a cytosolic DnaJ-like protein, had a drastic effect on peroxisomal import of certain PTS-containing proteins (Hertema et al., 1998). These data suggest that after being translated, PTS1- and PTS2-containing proteins are recognized by their respective receptors. This interaction could be facilitated by the action of chaperones and their cofactors. The complex of PTS-containing protein and receptor is then transferred to the peroxisomes where the receptor is recognized by the complex comprised of Pex13p, Pex14p, and Pex17p. Then the receptor releases the cargo which is then transported into the peroxisome.

There is little known of the mechanism for targeting peroxisomal membrane proteins. Different consensus sequences for peroxisomal membrane targeting have been proposed (Dyer et al., 1996; Elgersma et al., 1997). D deflecting the gene encoding Djp1p, a cytosolic DnaJ-like protein, had a drastic effect on peroxisomal import of certain PTS-containing proteins (Hertema et al., 1998). These data suggest that after being translated, PTS1- and PTS2-containing proteins are recognized by their respective receptors. This interaction could be facilitated by the action of chaperones and their cofactors. The complex of PTS-containing protein and receptor is then transferred to the peroxisomes where the receptor is recognized by the complex comprised of Pex13p, Pex14p, and Pex17p. Then the receptor releases the cargo which is then transported into the peroxisome.

Another interesting protein involved in peroxisomal protein transport is Pex4p. Pex4p was shown to be highly homologous to ubiquitin-conjugating enzymes (UBCs) (Wiebel and Kunau, 1992; Crane et al., 1994; van der Klei et al., 1998) and is located on the peroxisomal membrane as a peripheral protein facing the cytosol. The protein binds ubiquitin and the active-site cysteine residue is important for Pex4p function (Cane et al., 1994). In S. cerevisiae and P. pastoris, deletion of PEX4 disrupts targeting via the PTS1 and PTS2 pathway (Wiebel and Kunau, 1992; Gould et al., 1992; our unpublished observation). In H. anomala polymorpha, only the PTS1 pathway was impaired and this defect could be rescued by overexpression of the PTS1 receptor, Pex5p (van der Klei et al., 1998), implying an important role for Pex4p in the import of PTS1-containing proteins in this yeast.

In this study we have characterized a novel peroxin, Pex22p, from P. pastoris. We analyzed the subcellular localization, targeting signal, topology, interacting partners, and the functions of this protein. Our data shed light on the roles of Pex4p and Pex22p in peroxisomal matrix protein import and show that these proteins are also conserved in S. cerevisiae.

Materials and Methods

Strains and Media

P. pastoris strains used in this study were as follows: parental wild-type strains PPY1, PPY12 (PPY1 arq4, his4), and SM D1163 (his4, pep4, prib1); STK 10 (PPY12, pex22.1, ARG4::PTW84 (PTS2-GFP [green fluorescent protein] (656G)A GADPH, PTS2-LEU2/GAPDH); STK 11 (PPY12, amp22:: Zeocin); STK 12 (SM D1163, pts22::Zeocin); STK 13 (PPY12, amp44:: Zeocin); STK 14 (SM D1163, pts24::Zeocin); STK 15 (PPY12, pTKS1 [PEX4:p::NH-PEX4, Zeocin]) S. cerevisiae strains used: BJ1991 (MAT a, leu2, trp1, ura3-52, prb1-1122, pep4-3); STK 16 (BJ1991, aja5); L40 (MAT a, his3a200, trp1-901, leu2-3,112, ade2, LYS2::(lexA op1);HIS3, URA3::(lexA op1),LacZ). The Escherichia coli strain used for cloning procedures was JM109 and for protein expression, SG13009. Yeast media were as described in Faber et al. (1998).

Cloning Procedures

Standard cloning procedures were used (Sambrook et al., 1989). DNA sequencing was performed according to the Sanger method (Sanger et al., 1977). Restriction site ends were made blunt with Klenow polymerase from Boehringer Mannheim. PCR was performed using Vent DNA polymerase from New England Biolabs. The resulting PCR products were cloned into pCR 2.1 by adding a 3' A-overhang with T4 ligase or into pCR-Blunt (Invitrogen). Usually, a restriction site was introduced within the primers to facilitate further cloning of the products, otherwise restriction sites from the pCR vectors were used. PCR fragments were cut out with specified restriction enzymes, purified with Qiaex (Qiagen) and cloned into the specified vectors according to standard protocols.

Isolation of pex Mutants and Cloning of PEX22

The isolation of pex mutants was performed according to Elgersma et al. (1998). A genomic library was transformed into the pex22.1 strain (STK 10). Five different plasmids (pB2.3, pB2.9, pB2.13, and pB2.15) restored growth on methanol and oleate medium. Restriction analysis of the inserts revealed that the five inserts contained an overlapping fragment of 1.1 kb. This fragment was excised from plasmid pB2.13 as a BamHI fragment, sequenced on both strands, and shown to include the PEX22 gene.

Construction of Disruptions

To disrupt PEX22, the 5' and 3' regions of the gene were amplified with PCR (TK45 and TK46 for the 5' region and TK47 and TK48 for the 3' region). The 5' fragment was cloned as a BamHI fragment into pBluescriptSKII (Stratagene). The 3' fragment was then ligated as an EcoRI-Smal fragment into this vector. The resulting plasmid was cut with Smal and a blunt-ended Hael I-BamHI Zeocin fragment (cut out from plasmid pPICZ A::Invitrogen) was inserted. The resulting plasmid, pTK29, was cut with BamHI and EcoRI and transformed into PPY12 and SM D1163. The disruptions were confirmed by PCR.

The 5' and 3' regions of the PEX4 gene were amplified with PCR.
(primers TK 41 and TK 42 for the 5' region and TK 43 and TK 44 for the 3' region). The 5' fragment was cloned as a BamH-I-Smal fragment into pBluescriptSKII. The 3' fragment was then ligated as a HindIII-Smal fragment into this vector (cut with HindIII-Smal). The resulting fragment was then cut with Smal and a blunt-ended HaelI-BamH-I Zeoic fragment was inserted. The resulting plasmid, pTK 35, was cut with BamH-I and HindIII and transformed into PYP 12 and SM D116. The insertions were confirmed by PCR.

The ScY A F5 gene was disrupted according to Wach et al. (1994). Primers TK53, TK 62, TK 63, and TK 64 were used to isolate a fragment using PCR that contains the 5' region of ScY A F5, followed by kanMX F2, followed by the 3' region of ScY A F5. This construct was transformed into the S. cerevisiae strain B191 and G418-resistant colonies were checked for correct disruption of the ScY A F5 gene by PCR.

### Construction of Plasmids

Plasmids used are in Table I and DNA primers are in Table II. Plasmid pTK12, which expresses the PEX22 gene from the GAPDH promoter was made as follows: the gene was amplified by PCR using primer TK31 and TK40, thereby introducing a BamHI site immediately upstream of the ATG. The Pex4p from the AOX promoter, was cloned as follows: the gene was amplified by PCR using primer TK31 and TK40, thereby introducing a BamHI site immediately upstream of the ATG. The Pex4p gene was excised with BamH-I and EcoRI and cloned into pTW71, which was blunt ended using Klenow enzyme.

The Pex4p gene from the AOX promoter was made as follows: the gene was amplified by PCR using primer TK31 and TK40, thereby introducing a BamHI site immediately upstream of the ATG. The Pex4p gene was excised with BamH-I and EcoRI and cloned into pTW71, which was blunt ended using Klenow enzyme.

Plasmids containing PEX4 (as a BamH-I-EcoRI fragment made by PCR with primers KNSD13 and KNSD14) in pKNSD55 or pKNSD52 (Faber et al., 1998) for two-hybrid analysis were named pKNSD119 and pKNSD118, respectively.
PEX22 fused to GFP, was made by amplifying PEX22 with primers TK31 and TK59, cutting the fragment with BglII and BamHI and cloning it into the BamHI-BamHI cut plasmid pTW133, which contains a GFP gene without the ATG in plasmid pTW71. The plasmid containing the first 25 amino acids of Pex22p fused to GFP was made as follows: the fragment encoding the first 25 amino acids was amplified by PCR with primers TK31 and TK61. This HindIII-BglII fragment was cloned into the HindIII-BamHI cut plasmid pTW103, which contains a full-length GFP fragment missing the ATG in pCR2.1 (Invitrogen). The resulting plasmid was cut with BamHI and EcoRI to excise the fragment containing Pex22(1-25)-GFP and cloned into BamHI-EcoRI cut pTW71 resulting in plasmid pTK32. The plasmid expressing the first 7 amino acids of Pex22p fused to GFP (Pex22(1-7)-GFP) was made as follows: a BamHI-EcoRI cut PCR fragment with primer TK95 and TK40 was repeated into plasmid pTK34. Plasmid pTK44, expressing a GFP, was fused to the amino acids 8-25 of Pex22p (Pex22(8-25)-GFP) was cloned as follows: PCR was performed with primer TK95 and Pichia primer 3′AOX (Invitrogen) with plasmid pTK32 as template. The resulting fragment was cut with BamHI and EcoRI and cloned into plasmid pTW71 cut with BglII and EcoRI.

Plasmid pTK0 expressing a NH-tagged Pex4p from the acyl-CoA oxidase (ACO) promoter was cloned as follows: a BamHI-EcoRI fragment containing the full-length PEX4 was cloned in plasmid pCM22 cut with BamHI-EcoRI (Elgersma et al., 1998). Plasmid pTK51, expressing NH-Pex4p from its own promoter was cloned as follows: the fragment expressing NH-Pex4p was cut out of pTK51 with BglII-EcoRI, treated with Klenow enzyme and cloned into the Smal site of the pBluescript KS I contain- ing the 5′end of PEX4 and the 3′end of PEX4 (see above). A blunt-ended HaelI-BamHI Zeocin fragment was then cloned into the blunt ended EcoRI site in the 3′end of PEX4. This whole fragment (5′-PEX4-NH-PEX4-Zeocin-3′PEX4) was cut out of the plasmid with Xbal-HindIII and transformed into a Δpex4 strain (PPY12, Δpex4: A R G4). A riginine minus and Zeocin resistant colonies were checked for their expression of NH-Pex4p.

A fragment of ScyA55 containing the full-length gene was amplified with primers TK52 and TK62 on genomic S. cerevisiae DNA. The resulting EcoRI fragment was cloned into pRS306, cut with EcoRI to yield plasmid pTK45. The two-hybrid vectors with ScyA55 were made as follows: ScyA55 was amplified with PCR with primers TK52 and TK53. The resulting BamHI-EcoRI fragment was cloned into either pKNSD55 (to get a 6HIS-Pex4p). This strain and SMD1163 as a control were grown in methanol and spheroplasts were prepared. Cross-linking of cell extracts was performed as previously described (Rieder and Emr, 1997). 50 μl of a 50% slurry of Ni2+-NTA agarose (Qiagen) was added with 10 mM imidazole to the supernatant to precipitate protein complexes. This mixture was incubated at 4°C for 1 h. A flow this incubation period the beads were washed five times with buffer containing 20 mM imidazole. The pellets were resuspended in sample buffer and part of it loaded onto an SD 5 gel.

Production of Antibodies
The construction of a 6HIS-Pex22p, a BamHI-EcoRI fragment of PEX22 produced by PCR with primers TK35 and TK40 was cloned into a BamHI-EcoRI cut plasmid pQE30 (Qiagen) creating plasmid pTK20. This plasmid, expressing Pex22p missing the first 25 amino acids, was transformed into strain SCYAF5, thereby rendering the cells sensitive to phleomycin. In

Differential Centrifugation, Nycodenz Gradient, Floatation Gradient, Membrane Extraction, and Protease Protection
Differential centrifugation and Nycodenz gradients were done as described (Faber et al., 1998). Floatation gradient was done as described (Faber et al., 1998) with the difference that the 27,000-g pellets were taken. Membrane extraction and protease protection experiments were done as described (Wimer et al., 1996).

Binding of Pex22p to Pex4p
A Δpex4 strain (STK14) was transformed with plasmid pTK36, expressing a 6HIS-Pex4p. This strain and SM D1163 as a control were grown in methanol and spheroplasts were prepared. Cross-linking of cell extracts was performed as previously described (Rieder and Emr, 1997). 50 μl of a 50% slurry of Ni2+-NTA agarose (Qiagen) was added with 10 mM imidazole to the supernatant to precipitate protein complexes. This mixture was incubated at 4°C for 1 h. A flow this incubation period the beads were washed five times with buffer containing 20 mM imidazole. The pellets were resuspended in sample buffer and part of it loaded onto an SD 5 gel.

Fluorescence and Electron Microscopy
Fluorescence microscopy for the detection of GFP-tagged proteins was done as described by Monosov et al. (1996). Fluorescence images were acquired using a CCD camera (model 4995; Cuhu Inc.) and a CC-77 Frame Grabber (Scion Corp.). Samples for immunofluorescence were induced in methanol, spheroplasted, fixed, and prepared as described (Babst et al., 1998). α-Pex3p and α-AOX were used at a dilution of 1:1,000. Microscopy for immunofluorescence was as described (Dorizzi et al., 1998).

Miscellaneous
TCA lysates were made as follows: 2 OD of cells were collected by centrifugation, resuspended in 10% TCA and incubated on ice for >30 min. The suspension was centrifuged and the pellet washed three times with acetone. The pellet was resuspended in sample buffer and glass beads added. The tube was vortexed for 1 min and heated at 100°C for 1 min. This procedure was repeated four times. The sample was separated from the glass beads and loaded on gels.

Digitonin permeabilization was done according to Elgersma et al. (1998). Western blotting was performed according to standard procedures. All antibodies were used at the following dilutions: α-Scatalase, 1:10,000; α-Schiolase, 1:10,000; α-SCG-PDH (glucose-6-phosphate dehydrogenase), 13,000; α-F-β subunit of mitochondrial ATPase, 1:10,000; α-PPeX3p, 1:10,000; α-Pex5p, 1:1,000; α-PeX7p, 1:10,000; α-PeX22p, 1:2,000; α-GFP, 1:2,000.

Results
Isolation of Peroxisomal Protein Import Mutants
The screen employed for the isolation of import mutants was based on a positive screening procedure (Elgersma et al., 1993, 1998). It used the bleomycin-resistance protein, which binds the toxic drug phleomycin, thereby preventing the drug from intercalating into DNA. The bleomycin gene (BL E) was fused to 51 basepairs, encoding the NH2-terminal 17 amino acids (containing the PT25 signal), of S. cerevisiae thiolase (FOX3). The fusion protein was targeted to the peroxisomes in P. pastoris wild-type cells, thereby rendering the cells sensitive to phleomycin. In pex mutants, however, this fusion protein would not be targeted to peroxisomes, therefore rendering the cells resistant to the drug. A wild-type yeast strain (PPY12 + pTW84; Elgersma et al., 1998) was mutagenized, grown in oleate and treated with phleomycin. Two phleomycin-
resistant mutants (PpPex7 and Ppfox3) did not grow on oleate, but grew on methanol (Elgersma et al., 1998; Koller, A., and S. Subramani, unpublished results). One other mutant did not grow on methanol and oleate, although it grew on glucose and glycerol, and was named pex22. This mutant was backcrossed twice against wild-type and the resulting strain (STK10) was used for further experiments.

Cloning of PEX22

The pex22 mutant (STK10) was transformed with a wild-type genomic library and plasmids (p82.2, p82.3, p82.9, p82.13, and p82.15) from colonies that grew on methanol medium were isolated and checked for their ability to restore growth on methanol and oleate. The five inserts contained an overlapping fragment of 1.1 kb which was isolated from p82.13 as a BamHI fragment and subcloned into the pSG560 vector (Gould et al., 1992) to check for complementation (p82.20; Fig. 1 A). The smallest, complementing fragment was the 0.9-kb EcoRV-BamHI fragment (p82.25). The whole 1.1-kb fragment was sequenced to obtain the PEX22 gene which is 564 bp long, encoding a protein of 187 amino acid (calculated molecular mass of 20,984 D and pl of 5.76; Fig. 2). The protein contains a putative membrane-spanning region between amino acids 7 or 8 and 24 or 25. Otherwise the protein does not contain any known motifs. The whole PEX22 gene was replaced in wild-type cells with the Zeocin-resistance gene (see Materials and Methods). The resulting Δpex22 strain grew normally on glucose, but not on methanol and oleate, for which growth was complemented upon reintroduction of PEX22 (pTK10; Fig. 1 B).

The Δpex22 Strain Does Not Import PTS1- and PTS2-Containing Proteins

The Δpex22 (STK11) strain was transformed with GFP constructs to determine the ability of this strain to import peroxisomal matrix proteins. The GFP constructs used were shown to be properly localized to peroxisomes in wild-type cells (Wiemer et al., 1996; Fig. 3). A PTS1-GFP (pTW51) introduced into the Δpex22 strain was not targeted into peroxisomes when grown in methanol medium but was localized in the cytosol (Fig. 3). A PTS2-GFP (expressing the first 17 amino acids of S. cerevisiae thiolase fused to GFP; pTW61) was also not targeted to peroxisomes when grown in oleate but was localized in the cytosol (Fig. 3). However, immunofluorescence with Pex3p antibody showed that this peroxisomal membrane protein localized to punctate structures in the cytosol in the mutant strain, suggesting that the Δpex22 strain retains the ability to target peroxisomal membrane proteins to some peroxisome-like structures, so called remnants (Fig. 3). Electron microscopy revealed that in wild-type cells, the
Figure 3. Detection of GFP-PTS1, PTS2-GFP, and Pex3p in wild-type and Δpex22 cells. Wild-type (PPY12) and Δpex22 (STK11) were transformed with constructs expressing GFP-PTS1 and PTS2-GFP. Cells expressing GFP-PTS1 were induced in methanol medium for 16 h, and those producing PTS2-GFP on oleate medium for 16 h. Pex3p was detected by immunofluorescence (IF) in methanol-induced cells. Pictures in the left column show the subcellular localization of the GFP constructs and Pex3p examined by fluorescence microscopy. Pictures in the right column were obtained by using Nomarski optics.

Pex22p Is Localized to Peroxisomes

Antibodies raised against Pex22p (see Materials and Methods) specifically detected a protein of ~23 kD in cells grown on oleate and methanol (Fig. 5 A). Cells grown in glucose only showed a faint band corresponding to Pex22p (data not shown). No band was apparent in Δpex22 strains as expected (Fig. 5 A). The same fractions as above (PNS, 27-k pellet, 100-k pellet, and 100-k supernatant) taken from the wild-type strain were checked for the presence of Pex22p by immunoblotting. Pex22p was localized to the 27-k pellet, suggesting an organelar localization for this protein (Fig. 5 A). The PNS of the wild-type strain was fractionated on a linear Nycodenz gradient and analyzed by immunoblotting. Catalase and thiolase, although with some trailing most likely due to rupture of some peroxisomes, near the bottom of the gradient, as did Pex3p (Fig. 5 C). Pex22p colocalized with the peroxisomal markers catalase, thiolase, and Pex3p. Further evidence that Pex22p is a peroxisomal protein was obtained by immunolectron microscopy. Sections of methanol- and oleate-grown cells were decorated with Pex22p antibodies followed by incubation with gold-conjugated protein A. The gold particles almost exclusively decorated the peroxisomal membrane in the wild-type (Fig. 6, B and D), but not the Δpex22 strain (Fig. 6 A). Sometimes, Pex22p was localized to patches on peroxisomes (Fig. 6 C).

The First 25 Amino Acids of Pex22p Contain an mPTS

Sequence analysis of Pex22p did not reveal an obvious
more, this fusion was organelle associated since the fusion protein (Pex22(1–25)-GFP) only leaked from cells at digitonin concentrations that released membrane proteins (Fig. 7 B). The cytosolic protein, G6PDH, was released into the supernatant at low concentrations (25 μg/ml), whereas the peroxisomal matrix protein GFP-SKL started to leak at digitonin concentrations of 50–100 μg/ml, and release was not complete until the concentration of digitonin was 500 μg/ml. Pex3p, a peroxisomal membrane protein, was only fully released into the supernatant at digitonin concentrations exceeding 1,000 μg/ml. The Pex22(1–25)-GFP fusion protein was released into the medium at very high concentrations (1,000–1,500 μg/ml), or when the cells were treated with 0.2% Triton X-100 (Fig. 7 B). These results show that the Pex22(1–25)-GFP construct is targeted to peroxisomal membranes.

**Pex22p Interacts with Pex4p**

To determine interactions of Pex22p with other Pex proteins, the yeast two-hybrid system was employed. **PEX22** was fused to the DB domain of LexA, or the AD of VP16. All published P. pastoris PEX genes (PEX1, PEX2, PEX3, PEX4, PEX5, PEX6, PEX7, PEX8, PEX10, PEX12, and PEX13) were also fused to these domains. mPTS. There is, however, a stretch of positively charged amino acids near the extreme NH2 terminus of Pex22p which does not completely fit the consensus sequence for an mPTS (Elgersma et al., 1997). This stretch is at the same location as the putative mPTS of Pex3p (Hp, Sc, Pp). Therefore, we constructed GFP fusions with full-length Pex22 (Pex22-GFP, pTK30, this construct is able to complement a Δpex22 mutant for growth on oleate and methanol), a second fusion with the first 25 amino acids of Pex22 (Pex22(1–25)-GFP; pTK32), containing the transmembrane domain, a third fusion with only the transmembrane domain (Pex22(8–25)-GFP; pTK44), and a fourth fusion with the first seven amino acids of Pex22 (Pex22(1–7)-GFP; pTK34), not containing the transmembrane domain. These constructs were transformed into PPY12 and the resulting strains induced on methanol. The constructs expressing full-length Pex22-GFP and Pex22(1–25)-GFP showed colocalization with alcohol oxidase, a bona fide peroxisomal matrix protein (Fig. 7 A), proving that these constructs get targeted to peroxisomes, whereas the other two constructs (Pex22(1–7)-GFP, Pex22(8–25)-GFP) were localized in the cytosol (data not shown). The Pex22(1–25)-GFP fusion protein could also be shown to colocalize with peroxisomes when an organelle fraction was separated on Nycodenz gradients (data not shown). Furthermore, this fusion was organelle associated since the fusion protein (Pex22(1–25)-GFP) only leaked from cells at digitonin concentrations that released membrane proteins (Fig. 7 B). The cytosolic protein, G6PDH, was released into the supernatant at low concentrations (25 μg/ml), whereas the peroxisomal matrix protein GFP-SKL started to leak at digitonin concentrations of 50–100 μg/ml, and release was not complete until the concentration of digitonin was 500 μg/ml. Pex3p, a peroxisomal membrane protein, was only fully released into the supernatant at digitonin concentrations exceeding 1,000 μg/ml. The Pex22(1–25)-GFP fusion protein was released into the medium at very high concentrations (1,000–1,500 μg/ml), or when the cells were treated with 0.2% Triton X-100 (Fig. 7 B). These results show that the Pex22(1–25)-GFP construct is targeted to peroxisomal membranes.

**Figure 4.** Electron microscopy of wild-type and Δpex22 cells. Morphological analysis of wild-type cells (PPY12) (A and B) and Δpex22 (STK11) (C and D). Cells were grown in methanol (A and C) or oleate medium (B and D), and prepared for electron microscopy. Arrows point to the peroxisome remnants. N, nucleus; P, peroxisome. Bars, 0.5 μm.
These plasmids were then transformed in combination into the S. cerevisiae strain L40 and interaction of these proteins was assessed by the production of β-galactosidase activity. Only the combination of Pex22p with Pex4p, a ubiquitin-conjugating enzyme, produced any detectable enzyme activity. Almost the whole Pex22 protein (construct Pex22.1) was needed for interaction with Pex4p, whereas the COOH-terminal 39% of Pex4p (construct Pex4.2) interacted with Pex22p (Fig. 8 A). Control experiments performed by exchanging the backbone vectors confirmed our findings (data not shown). We were also able to show that these two fragments of Pex22p (Pex22.1) and Pex4p (Pex4.2) interacted with each other (data not shown).

To show that Pex22p and Pex4p interact in vivo, 6HIS-Pex4p was expressed from the GAPDH promoter (plasmid pTK36). This plasmid was then transformed into the Δpex4 strain (STK14). The 6HIS-Pex4p complemented the disrupted strain as assessed by growth on methanol and oleate (data not shown). This strain was grown in methanol, and spheroplasts were prepared. The cross-linker dithiobiocin(succinimidylpropionate) (DSP) was added to the lysates to cross-link neighboring proteins. 6HIS-Pex4p and associated proteins were precipitated with Ni²⁺-NTA beads. Bound proteins were run on an SDS gel, blotted onto nitrocellulose and checked for the presence of Pex4p, Pex22p, and Pex3p. The 6HIS-Pex4p specifically bound Pex22p in the presence of the cross-linker DSP (Fig. 8 B), whereas no Pex22p could be detected in the sample without DSP. Pex3p, another peroxisomal membrane protein, did not bind to the beads or to 6HIS-Pex4p. Pex22p and Pex4p did also not bind to the beads, as seen in the wild-type strain, not expressing any 6HIS-tagged protein. These experiments confirm the specific interaction between Pex4p and Pex22p by two different methods.

Δpex4 and Δpex22 Strains Share Similar Phenotypes
PPex4p was previously characterized as a ubiquitin-conjugating enzyme, similar to ScPex4p (Crane et al., 1994). A Δpex4 strain (STK14) behaved similarly in differential...
centrifugation, as did a ∆pex22 strain (data not shown). TCA lysates were made from strains (STK12 and STK14) grown in methanol and oleate. Equal amounts of cells were loaded on a gel and blotted for the presence of Pex3p, Pex4p, Pex5p, Pex7p, and Pex22p. A s shown in Fig. 9 A, all the strains showed similar amounts of Pex3p, whereas strains deleted for ∆pex4 and ∆pex22 did not contain any detectable Pex5p. However, Pex7p was present in wild-type amounts in all the strains and was induced by oleate relative to methanol growth. Interestingly, we were unable to detect any Pex4p in a ∆pex22 strain.

**Pex22p Anchors Pex4p at the Peroxisomal Membrane**

NH-Pex4p expressed from its own promoter (strain STK15) complemented a ∆pex4 strain and was localized in the 27-k pellet during differential centrifugation (Fig. 9 B). The controls, Pex3p and G6PDH, were exclusively in the 27-k pellet and 100-k supernatant, respectively (Fig. 9 B). We were interested in seeing whether the localization of Pex4p is disturbed in a ∆pex22 strain. We overexpressed the NH-tagged Pex4p from the ACO promoter in wild-type (PPY12) and ∆pex22 strains and performed a differential centrifugation with oleate-induced cells. Interestingly, the wild-type Pex4p was undetectable in these strains (data not shown). In PPY12, the overexpressed NH-Pex4p was localized to the 27-k pellet and 100-k supernatant, whereas in a ∆pex22 strain, all of the NH-tagged Pex4p was in the cytosol (Fig. 9 B). This experiment suggests that Pex22p anchors Pex4p at the peroxisomal membrane.

**ScYaf5p Is a Homologue of PpPex22p**

PpPex22p was run against protein databases (SwissProt, SGD) with Blast and Fasta searches. No high-scoring homologue could be found. Only several low-scoring proteins could be found in the Saccharomyces Genome Database (SGD) database. Out of these, only ScYaf5p (open reading frame YAL055w) is of about similar size and exhibits a transmembrane region at the NH2 terminus similar to Pex22p, although it starts at amino acid 14-32 (Fig. 10 A). To determine if ScYaf5p is the real Pex22p homologue, the entire open reading frame of SCYAF5 was replaced by a PCR-generated kanMX2 cassette (Wach et al., 1994). Strains deleted for SCYAF5 were streaked on oleate and glucose plates. ∆Scyaf5 strains grew on glucose like wild-type cells, whereas they did not grow on oleate. A ∆Scyaf5 strain transformed with a plasmid expressing SCYAF5 from a catalase promoter complemented the growth defect on oleate (data not shown).

GFP-SKL is targeted to peroxisomes in wild-type cells, as shown in Fig. 6. Immuneelectron microscopy of wild-type and ∆pex22 cells. Cells (∆pex22 [A] and wild-type [PPY12] [B-D]) were grown in methanol (A and D) or oleate (B and C) and labeled with Pex22p antibody. Arrows in C show the patches of Pex22p on peroxisomes. Bars, 0.5 μm.
whereas in the ΔScyaf5 strain this construct was localized in the cytosol (Fig. 10 B). To test the interaction between ScYaf5p and ScPex4p, the genes encoding these proteins were cloned into the two-hybrid vectors and transformed into strain L40. A s seen in Fig. 10 C, only strains containing both constructs showed β-galactosidase activity. These results indicate that ScYaf5p is the functional homologue of Pex22p. However, overexpression of ScYAF5 from an alcohol oxidase promoter could not complement the growth phenotype of a P. pastoris Δpex22 strain on methanol. This could be explained by the fact that ScYaf5p does not interact in a two-hybrid experiment with PpPex4p (data not shown).

Discussion

Pex22p Is a Peroxisomal Integral Membrane Protein

The newly discovered peroxin, Pex22p, described in this
Koller et al. Role of PpPex22p in Peroxisome Biogenesis

The role of PpPex22p in peroxisome biogenesis is studied. The protein behaves like a peroxisomal integral membrane protein by several criteria. It is pelletable in differential centrifugations (Fig. 5 A) and colocalizes with peroxisomal markers in Nycodenz gradients (Fig. 5 C). In immunoelectron microscopy experiments, the protein was associated with the peroxisomal membrane (Fig. 6, B–D). The protein was not extracted from the membrane by buffers of low ionic strength, high salt or by alkaline sodium carbonate, indicating that it is an integral membrane protein (Fig. 5 D). Finally, most of the Pex22p is degraded upon addition of proteases, even in the absence of detergent, under conditions where thiolase, a matrix marker, is resistant (Fig. 5 E). These results, when combined with the prediction of a single transmembrane domain near the NH2 terminus of Pex22p, are consistent with a topology in which the NH2 terminus of Pex22p is in the peroxisomal matrix and the COOH terminus is in the cytosol. This topology makes it possible for the COOH terminus of Pex22p to be involved in protein interactions with the peroxisomal peripheral membrane protein, Pex4p, as discussed later.

We do not understand why Pex22p is localized in some immunoelectron microscopy pictures to patches at the peroxisomes. This is not seen in all the sections. It is possible that Pex22p clusters are required for its normal functions which are discussed later. The same behavior has also been observed for Pex14p in Hansenula polymorpha (Komori et al., 1997).

The mPTS of Pex22p Resides Within the NH2-terminal 25 Amino Acids

Pex22p contains a signal at the NH2 terminus that is sufficient for peroxisome targeting (Fig. 7 A). Fusing GFP to the first 25 amino acids of Pex22p targets the resulting fusion protein to peroxisomes. This conclusion is supported by the colocalization of this fusion protein with peroxisomal markers in a Nycodenz gradient (data not shown; Wiemer et al., 1996). GFP fusion proteins that contain the first 7 amino acids (lacking the transmembrane region) or amino acids 8–25 (containing only the transmembrane region) are not transported to the peroxisome but remain in the cytosol. The inability of the first 7 amino acids to function as an mPTS is noteworthy since in previous experiments with Pex3p and Pmp47 (Höhfeld et al., 1992; Baerends and...
al., 1996; Dyer et al., 1996; Wiemer et al., 1996) the mPTS did not require a transmembrane domain. The mPTS of ScPex15p, however, requires a transmembrane domain for targeting to the peroxisomal membrane (Egersma et al., 1997) in addition to the luminal portion of the protein.

At present, we are unable to decipher why some mPTSs require transmembrane domains to function while others do not. In the case of Pex22p, the seven amino acids fused to GFP could be buried and inaccessible to the putative receptor. That would explain why this fusion protein is seen in the cytosol. A nether possibility is that the targeting signal requires some amino acids that are located in the transmembrane domain of Pex22p. Experiments to determine the important amino acids of the mPTS are underway. Comparison of the different mPTSs found so far shows that there is a predominance of positively charged amino acids. Pex22p construct with alanine substitutions in two of the three positively charged amino acids of the seven-amino acid luminal stretch (K(2)→A and R(6)→A) do not properly localize to the peroxisome (data not shown). This result suggests that at least these two positive charges are important for proper targeting of the fusion protein.

**Requirement of Pex22p for Import of Peroxisomal Matrix, but Not Membrane Proteins**

Pex22p is important for peroxisome biogenesis and for growth of P. pastoris on methanol and oleate (Fig. 1). Functional peroxisomes are not formed in a Δpex22 strain (Fig. 4, C and D). Both exogenously expressed and endogenous PTS1- and PTS2-containing proteins accumulate in the cytosol (Figs. 3 and 5 A), whereas the membrane protein, Pex3p, is targeted to pelletable membranous structures that float in sucrose gradients (Fig. 5, A and B) and likely correspond to the peroxisomal remnants observed using fluorescence (Fig. 3) and electron microscopy (Fig. 4, C and D).

**Pex22p Interacts with Pex4p and Anchors It at the Peroxisomal Membrane**

Yeast two-hybrid experiments performed with Pex22p and all published peroxins of *P. pastoris* on methanol and oleate (Fig. 1). Functional peroxisomes are not formed in a Δpex22 strain (Fig. 4, C and D). Both exogenously expressed and endogenous PTS1- and PTS2-containing proteins accumulate in the cytosol (Figs. 3 and 5 A), whereas the membrane protein, Pex3p, is targeted to pelletable membranous structures that float in sucrose gradients (Fig. 5, A and B) and likely correspond to the peroxisomal remnants observed using fluorescence (Fig. 3) and electron microscopy (Fig. 4, C and D).

The interaction between Pex22p and Pex4p sheds light on the function of Pex22p. One possibility is that Pex22p is the elusive substrate for ubiquitination by Pex4p. However, this seems unlikely as Pex22p migrates in SDS gels at the predicted molecular mass (23 kD) and not as a protein with mono- or poly-ubiquitin modifications (Figs. 5 and 9 A). The molecular mass of Pex22p is also unchanged throughout oleate induction (data not shown).

An alternative possibility suggested by several experiments is that Pex22p anchors Pex4p on the peroxisomal membrane. First, Pex4p is a peripheral peroxisomal membrane protein facing the cytosol and is tightly associated with the peroxisomal membrane even though it has no transmembrane segment of its own (Wiebel and Kunau, 1992; Crane et al., 1994). Second, Pex22p and Pex4p interact (Fig. 8, A and B). It is noteworthy that the COOH-terminal domain of Pex22p which faces the cytosol interacts with Pex4p. Third, Pex4p is unstable in a Δpex22 strain (Fig. 9 A). Fourth, NH-Pex4p is mislocalized to the cytosol in the Δpex22 strain (Fig. 9 B). M any of these points are reminiscent of the relationship between Ubc7p and Cue1p in *S. cerevisiae*. Cue1p, an integral membrane protein of the ER, is essential for the localization of Ubc7p, a UBC enzyme, to the cytosolic face of the ER, and both these proteins are required for the degradation of aberrant proteins in the ER membrane and for the retrograde transport of luminal substrates out of the ER (Biederer et al., 1997). In a Δcue1 strain, Ubc7p could not be found and a myc-tagged Ubc7p, when overexpressed in this strain, was found in the cytosol. Pex4p is unstable in a Δpex22 strain and NH-Pex4p, when overexpressed from the acyl-CoA oxidase promoter, is localized to the cytosol in this strain. NH-Pex4p, in a wild-type strain, is localized equally in the 27-k pellet and 100-k supernatant, whereas wild-type levels of NH-Pex4p are localized solely to the 27-k pellet (Fig. 9 B). This shows that there is a saturable binding site for Pex4p on membranes. These results are consistent with the idea that Pex22p provides the binding site for Pex4p. Based on these data, we propose that Pex22p is the anchor protein at the peroxisomal membrane that recruits and holds Pex4p at this location. We are not able to explain why in the strains overexpressing NH-Pex4p, Pex3p is not only present in the 27-k pellet but also in the 100-k pellet and 100-k supernatant (Fig. 9 B).

This model would predict that Pex4p and Pex22p act together for import of peroxisomal matrix proteins. This hypothesis is supported by the observation that both the Δpex22 and Δpex4 strains do not contain wild-type levels of Pex5p, have similar phenotypes such as inability to grow on methanol and oleate, and are impaired in the import of peroxisomal matrix proteins, but not membrane proteins (Wiebel and Kunau, 1992; Crane et al., 1994). The instability of Pex5p in the P. pastoris Δpex4 strain has been observed by another group (Kalish, J.E., and S.J. Gould, 6th International Congress on Cell Biology, 1996, Abstract 2873) but this was not observed with *H. polymorpha* (van der Klei et al., 1998). Pex5p was also shown to be unstable in some mammalian, peroxisome-deficient complementation groups (CG1, CG4, and CG8), suggesting that more than one protein affects its stability (Dodt and Gould, 1996). To examine if some phenotypes (such as growth on methanol and import of GFP-SKL) observed in the


$\Delta pex22$ and $\Delta pex4$ strains were directly attributable to the absence of Pex5p, PE X 5 was overexpressed in the $\Delta pex4$ and $\Delta pex22$ strains expressing GFP-SKL. The introduction of the PE X 5 plasmid enhanced the level of Pex5p protein to wild-type levels as assessed by immunoblotting, but these strains remained unable to grow on methanol or import GFP-SKL into peroxisomes (data not shown). It is unlikely that Pex4p is solely responsible for the stability of Pex5p as we were unable to restore wild-type levels of Pex5p in a $\Delta pex22$ strain overexpressing Pex4p (data not shown). Therefore, the phenotypes seen in the $\Delta pex4$ and $\Delta pex22$ strains are not simply a consequence of Pex5p instability. This is supported by the fact that not only PTS1-mediated import, but also the import of PTS2-containing proteins is compromised in $\Delta pex4$ and $\Delta pex22$ strains (Fig. 3, see also Wiebel and K unau, 1992; Crane et al., 1994; our unpublished observation), despite the expression of stable Pex7p in these strains (Fig. 9 A ).

Models for the Role of PpPex22p/Pex4p in Peroxisomal Matrix Protein Import

Our data clearly support a role for Pex22p in the anchoring of Pex4p to the peroxisomal membrane. However, further experiments will be required to determine the role of this protein complex in peroxisome biogenesis. One possibility is that the Pex4p–Pex22p complex functions similar to the Cue1p–Ubc7p complex, regulating the proper assembly and/or correct stoichiometry of protein import complexes at the peroxisomal membrane. It is known that altered stoichiometry of peroxisomal integral or peripheral membrane proteins, Pex3p and Pex14p, can yield an import-deficient phenotype (B aerends et al., 1997; K omori et al., 1997). The function of Pex4p at the membrane might be to ubiquitinate and therefore target palmitoylated membrane proteins or nonpalmitoylated subunits of the complex, leading to their degradation by the 26S proteasome in the cytosol. If Pex4p and/or Pex22p were missing, the import complex might lose its ability to function, due to incorrect stoichiometry, leading to a block of matrix protein import, and this could in turn lead to an instability of Pex5p. Several proteins of the import complex could be affected by Pex22p and Pex4p, including Pex13p (A lbertini et al., 1997; E lgersma et al., 1996; E rdmann and B lobel, 1996; G ould et al., 1996), Pex14p (A lbertini et al., 1997; B roc ard et al., 1997; F ran sen et al., 1998), or Pex17p (H ushe et al., 1998). Pex13p is stable in $\Delta pex4$ or $\Delta pex22$ strains (data not shown) and Pex5p is stable in a P. pastoris $\Delta pex13$ strain (Gould et al., 1996). Pex14p and Pex17p remain as reasonable targets for investigation because their deletion causes PTS1 and PTS2 import defects, but are not yet available for testing in P. pastoris.

A variation of this model, equally compatible with the available data, is that Pex4p, instead of directly acting on these peroxisomal membrane proteins, negatively regulates (by ubiquitination and degradation) a protease, which in turn degrades peroxisomal membrane complexes. It is hoped that these testable models may lead, in the near future, to the function of Pex4p.

Conservation of PpPex22p in Other Yeasts

Although database searches did not reveal any proteins highly homologous to PpPex22p, we did find a protein of similar predicted size and topology in S. cerevisiae. The hypothetical protein, Scy af5p (open reading frame Y A L-055w), appears to be the homologue of PpPex22p. Like Pp Pex X 22, the Scy A F5 gene is essential for growth on oleate, and for the import of GFP-SKL, a fusion protein that is readily imported into peroxisomes in wild-type yeast. Furthermore, Scy af5p interacts with ScPex4p in a two-hybrid experiment. The conservation of Pex22p and its interacting partner, Pex4p, in other yeasts suggests that the functions of these proteins are likely to be conserved in all organisms.

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