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 NH$_2$ terminus in the matrix and its COOH terminus in the cytosol. It contains a 25–amino acid peroxisome membrane-targeting signal at its NH$_2$ 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)$^\dagger$. 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 NH$_2$ terminus and has a consensus sequence of R/K-L/V/I-X$_5$-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-
somewhere, suggesting that the receptors shuttle from the cytoplasm to the peroxisomes, where they bind to the tightly associated peroxisomal membrane protein Pex13p (Girzalsky et al., 1999) and Pex14p (A Ibertini et al., 1997; Brooks et al., 1997; Fransen et al., 1998). Pex5p and Pex7p, as well as Pex14p, are in a complex with another peripheral peroxisomal membrane protein, Pex17p (H u s e et al., 1998). The binding of Pex5p and Pex7p to Pex17p, however, was dependent on the presence of Pex14p (H u s e 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 (E l g e r s m a et al., 1996; E r d m a n n and B l o b e l, 1996; G o u l d et al., 1996; A I bertini 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 n antibody against cytosolic HS P 70 inhibits the import of SK L-containing proteins into peroxisomes (W al t o n et al., 1994; Fransen et al., 1998). Deleting the gene encoding D j p 1p, a cytosolic D n a j -like protein, had a drastic effect on peroxisomal import of certain PTS-containing proteins (H e t t e m a et al., 1998). These data suggest that after being translated, PT S1- and PT S2-containing proteins are recognized by their respective receptors. This interaction could be facilitated by the action of chaperones and their cofactors. The complex of PT S-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. D ifferent consensus sequences for peroxisomal membrane targeting have been proposed (D yer et al., 1996; E l g e r s m a et al., 1997). D eleting the gene encoding D j p 1p, a cytosolic D n a j -like protein, had a drastic effect on peroxisomal import of certain PTS-containing proteins (H et t e m a et al., 1998). These data suggest that after being translated, PT S1- and PT S2-containing proteins are recognized by their respective receptors. This interaction could be facilitated by the action of chaperones and their cofactors. The complex of PT S-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.

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Materials and Methods

Cloning Procedures

Standard cloning procedures were used (Sambrook et al., 1989). D N A sequencing was performed according to the Sanger method (Sanger et al., 1977). R estriction site ends were made blunt with K l e n o w polymerase from Boehringer Mannheim. P C R was performed using Vent D N A polymerase from New England Biolabs. The resulting P C R products were cloned into p C R 2.1 by adding a 3' restriction sites from the p C R vectors were used. P C R fragments were cut within the primers to facilitate further cloning of the products, otherwise restriction sites from the p C R vectors were used. P C R fragments were cut out with specified restriction enzymes, purified with Q i a e x (Qiagen) and cloned into the specified vectors according to standard protocols.

Isolation of pex Mutants and Cloning of P E X 2 2

The isolation of pex mutants was performed according to Elgersma et al. (1998). A genomic library was transformed into the p e x 22.1 strain (S t a n d a r d p r o c e d u r e s were used (Sambrook et al., 1989). D N A sequencing was performed according to the Sanger method (Sanger et al., 1977). R estriction site ends were made blunt with K l e n o w polymerase from Boehringer Mannheim. P C R was performed using Vent D N A polymerase from New England Biolabs. The resulting P C R products were cloned into p C R 2.1 by adding a 3' restriction sites from the p C R vectors were used. P C R fragments were cut out with specified restriction enzymes, purified with Q i a e x (Qiagen) and cloned into the specified vectors according to standard protocols.

Construction of Disruptions

To disrupt P E X 2 2, the 5' and 3' regions of the gene were amplified with P C R (T K 45 and T K 46 for the 5' region and T K 47 and T K 48 for the 3' region). The 5' fragment was cloned as a B a m H I-S mal fragment into p b l u e s c r i pt SK I (Stratagene). The 3' fragment was then ligated as an E c o R I-S mal fragment into this vector. The resulting plasmid was cut with S m a l and a blunt-ended H a e l -B a m H I-Z e o c i n fragment (cut out from plasmid p P I C Z A:invitrogen) was inserted. The resulting plasmid, p T K 29, was cut with B a m H I and E c o R I and transformed into P P Y 12 and S M D 1163. The disruptions were confirmed by P C R .

The 5' and 3' regions of the P E X 4 gene were amplified with P C R...
(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 BamHI-Smal fragment into pBluescriptII. The 3’ fragment was then ligated as an HindIII-Smal fragment into this vector (cut with HindIII-Smal). The resulting fragment was then cut with Smal and a blunt-ended HaelI-BamHI I zeocin fragment was inserted. The resulting plasmid, pTK 35, was cut with BamHI and HindIII and transferred into pPYW 12 and SM 1663. The disruptions were confirmed by PCR.

The ScY A5 F plasmid 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 A5 F, followed by kanMX2, followed by the 3’ region of ScY A5 F. This construct was transferred into the S. cerevisiae strain BJ191 and G418-resistant colonies were checked for correct disruption of the ScY A5 F gene by PCR.

Construction of Plasmids

Plasmids used are in Table I and DNA primers are in Table II. Plasmid pBK20 contains the 1.1 kb BamHI fragment of pBS 13 in vector pSG560 (Gould et al., 1992). pBK21 contains the 0.7 kb BglII-BamHI fragment, pBK22 the 0.8 kb BamHI-HindIII fragment, pBK23 the 0.3 kb HindIII fragment, and pBK25 the 0.9 kb EcorI-V BamHI fragment. All these fragments were cloned into pSG560 either as a blunt-ended fragment or as a blunt-ended BamHI fragment containing one blunt end and one BamHI end.

Plasmid pTK 10, which expresses the PEX22 gene from the alcohol oxidase (AOX) promoter, was cloned as follows: the gene was amplified by PCR using TK31 and TK 40, thereby introducing a BamHI site immediately upstream of the A TG. The PEX 22 gene was excised with BamHI and EcoRI and cloned into pPIC3K (Invitrogen) with BamHI and EcoRI. This plasmid was cut with EcoRI-HindIII, blunt ended using Klenow enzyme.

Yeast two-hybrid plasmids were made by fusing appropriate gene fragments downstream of the DNA binding (DB) domain of LexA or the activation domain (D) of VP16. PEX 22 fusions were generated by cloning, in-frame, parts of or the full-length PEX 22 fused to either domains in plasmids pKN S55 (pBTM116 based) or pKN S52 (pVP16 based) (Faber et al., 1998). Plasmid pTK12 and pTK 13 were constructed by fusing a BamHI-EcoRI PCR fragment of PEX22 (primer TK34 and TK 40) with BamHI-EcoRI cut pKN S55 or pKN S52, respectively. Plasmid pTK 14, expressing a Pex22p lacking the first 25 NH2-terminal amino acids, was constructed by cloning the BamHI-EcoRI PCR fragment obtained with primers TK 35 and TK 40 into BamHI-EcoRI cut pKN S55. Plasmid pTK 16, expressing the COOH-terminal part of Pex22p, was constructed by cloning the BamHI-EcoRI PCR fragment obtained with primers TK 36 and TK 40 into BamHI-EcoRI cut pKN S55. Plasmid pTK 18, expressing the NH2-terminal part of Pex22p, was constructed by cloning the BamHI-EcoRI PCR fragment obtained with primers TK 34 and TK 37 into BamHI-EcoRI cut pKN S55.

Plasmids containing PEX4 (as a BamHI-EcoRI fragment made by PCR with primers K N F13 and K NF14) in pKN S55 or pKN S52 (Faber et al., 1998) for two-hybrid analysis were named pKNF119 and pKNF118, respectively. Plasmid pTK 21 was constructed as follows: a BamHI-EcoRI fragment of PEX4 (cut out of pKNF118) was cloned into pKN S55, which had been cut with BamHI and EcoRI (blunt ended). Plasmid pTK 23 contains a BamHI-SapI fragment of PEX 4 cloned into pKN S55. Plasmid pTK 25 contains an EcoRI-V-EcoRI fragment of PEX 4 cloned into pKN S55, cut with BamHI (blunt ended) and EcoRI. Plasmid pTK 27 contains an Sphi-EcoRI fragment of PEX4 in pKN S53, cut with BamHI (blunt ended) and EcoRI. Plasmid pTK 29, expressing a 6HIS-tagged Pex4p from the GAPDH promoter was made as follows: PEX4 was amplified with primers TK 51 and KNF14 (Faber et al., 1998) and cloned as a BamHI-I HindIII fragment into the BamHI-HindIII cut pQE30 (Qiagen).

PEX22 was then cut with SmaI and a blunt-ended HaeII-BamHI Zeocin fragment into this vector (cut with HindIII-SmaI). The resulting fragment was cloned as a BamHI-SmaI fragment into pKNSD55.

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**Table I. Primers Used in This Study**

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**Table II. Primers Used in This Study**

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Koller et al. *Role of PpPex22p in Peroxisome Biogenesis*
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 BamH1-BglII 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-BglII cut plasmid pTW103, which contains a full-length GFP fragment missing the ATG in pCR2.1 (Invitrogen). The resulting plasmid was cut with BamH1 and EcoR1 to excise the fragment containing Pex22(1-25)-GFP and cloned into BamH1-EcoR1 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 BamH1-EcoR1 cut PCR fragment with primer TK96 and TW6 was ligated into BamH1-EcoR1 cut pTW71 resulting in plasmid pTK44. Plasmid pTK44, expressing a GFP, fused to the amino acids 8-25 of Pex22p (Pex22(8-25)-GFP), was cloned as follows: PCR was performed with primer TK95 and TUSH primer 3′A OX (Invitrogen) with plasmid pTK32 as template. The resulting fragment was cut with BamH1 and EcoR1 and cloned into plasmid pTW71, cut with BglII and EcoR1.

To a fine powder using a pestle. This powder was incubated with undiluted sera at a concentration of 1% (wt/vol) at 4°C. After an overnight incubation the tube was centrifuged for 10 min, the supernatant collected and frozen. 1 ml of TCA was added to 5 ml of the supernatant to precipitate proteins. After an overnight incubation the sample was centrifuged for 10 min. The pellets were resuspended in sample buffer and glass beads added. The tube was vortexed for 1 min and heated at 100°C for 5 min. The suspension was centrifuged and the pellet washed five times with buffer containing 20 mM imidazole. The pellets were resuspended in sample buffer and part of it loaded onto an SDS 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; Cohu Inc.) and a CG-7 Frame Grabber (Scion Corp.). Samples for immunofluorescence were induced in methanol, spheroplasted, fixed, and prepared as described by Babst et al., 1998. α-Pex3p and α-A OX were used at a dilution of 1:1,000. Microscopy for immunofluorescence was described (Dorizzi et al., 1998).

Miscellaneous

TCA lysates were made as follows: 2 OD of cells were collected by centrifugation, resuspended in 0.1 N HCl and incubated for 1 h. 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. The procedure was repeated four times. The sample was separated from the glass beads and loaded on gels.

Dighton permeabilization was done according to Elgersma et al. (1998). Western blotting was performed according to standard procedures. A nitrocellulose filter was used at the following dilutions: α-Scalactase, 1:10,000; α-Thiolase, 1:10,000; α-ScG6PDH (glucose-6-phosphate dehydrogenase), 1:5,000 to 10,000; α-ScF-1 subunit of mitochondrial ATPase, 1:10,000; α-PpPex3p, 1:10,000; α-PpPex4p, 1:1,000; α-PpPex5p, 10,000; α-PpPex7p, 1:1,000; α-PpPex22p, 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 (BLE) was fused to 51 basepairs, encoding the NH₂-terminal 17 amino acids (containing the PTS2 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 (PpPex7.1 and Ppfox3.1) 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 pex22.1. This mutant was backcrossed twice against wild-type and the resulting strain (STK10) was used for further experiments.

Cloning of PEX22

The pex22.1 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 rechecked 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 acids. The deduced amino acid sequence is shown in 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).
peroxisomes were clearly present in both methanol (Fig. 4 A) and oleate (Fig. 4 B) grown cells. In Δpex22 cells, no normal peroxisomes could be observed (Figs. 4, C and D). However, in both growth media, small single-membrane organelles could be observed, suggesting that Δpex22 cells contain peroxisome remnants.

Differential centrifugation experiments confirmed the results obtained with the GFP fusions. Wild-type cells, SMD 1163 (for control), and the Δpex22 strain (STK 12) were grown in oleate to induce peroxisomes. Post-nuclear supernatants (PNS) from these strains were centrifuged at 27,000 g (27 k). The supernatant was spun further at 100,000 g (100 k). Equal portions of these fractions (PNS, 27-k pellet, 100-k pellet, and 100-k supernatant) were analyzed by immunoblotting. Both catalase and thiolase, which are PTS1- and PTS2-containing proteins, respectively, in yeasts and mammals, were localized in the 27-k pellet in the wild-type strain, whereas in the Δpex22 strain these proteins were cytosolic (100-k supernatant) (Fig. 5 A). Pex3p, however, was localized in the 27-k pellet in both strains. To check if the pelletable Pex3p is membrane bound, the 27-k pellet was resuspended in 65% sucrose and overlaid with layers of 50% and 30% sucrose, respectively. A first centrifugation, fractions were collected from the top and analyzed. Immunoblots showed that in both strains, Pex3p floated to the middle or top of the gradient, as did a mitochondrial marker (F1β-ATPase), suggesting that Pex3p is membrane-bound in the Δpex22 strain (Fig. 5 B). Together, these data suggest that both PTS1- and PTS2-containing proteins are not properly targeted in a Δpex22 strain, whereas peroxisomal membrane proteins (Pex3p) are targeted to membrane structures, most likely the peroxisome remnants seen by immunofluorescence and electron microscopy.

**Pex22p Is Localized to Peroxisomes**

Antibodies raised against Pex22p (see Materials and Methods) specifically detected a protein of ~23 kDa 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). In 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 organellar 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 migrated, 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 immunoelectron 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).

**Pex22p Is a Peroxisomal Membrane Protein with Its COOH Terminus Facing the Cytosol**

The topology of Pex22p within the peroxisomal membrane was analyzed by organelle subfractionation. The wild-type strain, SMD 1163, was grown in oleate and the 27-k pellet was fractionated into soluble and insoluble fractions after treatment with 0.1 M Na2CO3, pH 11.5, 10 mM Tris, pH 8.5 (no salt), 1 M NaCl in 10 mM Tris, pH 8.5 (high salt), and 0.1% Triton X-100. Pex22p behaved like Pex3p, a peroxisomal membrane protein (Wiemer et al., 1996), in all the experiments, whereas catalase, a soluble matrix protein, was found in the supernatant under all the conditions tested (Fig. 5 D). The 27-k pellet was further incubated with increasing amounts of trypsin in the presence or absence of Triton X-100 to assess the availability of Pex22p for the protease. Fig 5 E shows that Pex22p, as well as Pex3p, were degraded even in the absence of detergent. Thiolsate was well protected upon protease treatment in the absence of Triton X-100, but degraded in the presence of detergent. The immunocytochemistry experiment showed that several gold particles are actually localized on the cytosolic side of the peroxisomes (Fig. 6, B and D). Sequence analysis of Pex22p showed that it contains one putative membrane span near the NH2 terminus. The facts that the bulk of the protein is protease accessible even in the absence of Triton X-100 and that the antibody that detects Pex22p was raised against a protein lacking the first 25 amino acids, suggest that the NH2 terminus faces the peroxisomal lumen whereas the COOH terminus is cytosolic.

**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, 6PDH, 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.
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 Pex22p protein (construct Pex22.1) was needed for interaction with Pex4p, whereas the COOH-terminal 39% of Pex4p (construct Pex4.2) interacted with Pex22p (Fig. 8A). 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 dithiobioc(succinimidylpropionate) (DSP) was added to the lysates to cross-link neighboring proteins. 6HIS-Pex4p and associated proteins were precipitated with Ni<sup>2+</sup>-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. 8B), 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**

PpPex4p was previously characterized as a ubiquitin-conjugating enzyme, similar to ScPex4p (Crane et al., 1994). A Δpex4 strain (STK14) behaved similarly in differential

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Figure 5. Subcellular localization, floatation gradient, Nycodenz gradient, membrane extraction and protease protection assays for Pex22p. (A) Postnuclear supernatant (PNS) was produced from wild-type (SMD1163) and Δpex22 (STK12) cells grown in oleate and subfractionated into a 27,000-g pellet (27 k p), a 100,000-g pellet (100 k p) and a 100,000-g supernatant (100 k s). Equivalent volumes were loaded on gels, transferred to nitrocellulose and blotted for the specified proteins. (B) The 27-k pellet of wild-type and Δpex22 strains grown in oleate were overlaid with sucrose and centrifuged. Fractions were taken from the top and checked for the localization of Pex3p and the β-subunit of the mitochondrial F1-ATPase (F1). (C) PNS from wild-type cells (SMD1163) grown on oleate was loaded on top of Nycodenz gradients. Equal volumes of fractions from the gradient were analyzed by immunoblotting. (D) The 27-k pellet of oleate-grown wild-type cells was subfractionated into an insoluble pellet fraction (p) and a soluble fraction (s) after treatment with 0.1 M carbonate (pH 11.5), 10 mM Tris (pH 8), 10 mM Tris (pH 8), 1 M NaCl, and 0.1% Triton X-100. The distributions of the specified proteins between supernatant and membranous pellet fractions were examined by immunoblotting. (E) A 27-k pellet of oleate-grown wild-type cells was treated with the specified amount of trypsin in the presence (+) or absence (−) of 0.1% Triton X-100. The disappearance of the specified proteins was examined by immunoblotting.
centrifugation, as did a Δpex22 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. As shown in Fig. 9 A, all the strains showed similar amounts of Pex3p, whereas strains deleted for Δpex4 and Δpex22 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 Δpex22 strain.

Pex22p Anchors Pex4p at the Peroxisomal Membrane

NH-Pex4p expressed from its own promoter (strain STK15) complemented a Δpex4 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 Δpex22 strain. We overexpressed the NH-tagged Pex4p from the ACO promoter in wild-type (PPY12) and Δpex22 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 Δpex22 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. ΔScyaf5 strains grew on glucose like wild-type cells, whereas they did not grow on oleate. A ΔScyaf5 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,
whereas in the ΔScyaf5 strain this construct was localized in the cytosol (Fig. 10B). 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. As seen in Fig. 10C, 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
study 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 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.

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**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), by fluorescence microscopy showing colocalization of the fusion with a peroxisomal marker (Fig. 7 A), and by the release of the fusion protein from cells only with high concentrations of digitonin or by Triton X-100 (Fig. 7 B). Other experiments designed to show that the GFP portion of the fusion protein faces the cytosol failed because GFP is highly resistant to proteases (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 et al., 1995).
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 (Elgersma et al., 1997) in addition to the lumenal 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 nother 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 mPTSs are underway. Comparison of the different mPTSs found so far shows that there is a predominance of positively charged amino acids. Pex22p-GFP fusions 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 5A), 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* show that it only interacts with Pex4p, a UBC enzyme that is localized to the cytosolic face of peroxisomal membranes (Fig. 8A). The COOH-terminal cytosolic domain (amino acids 26–187) of Pex22p interacts with the COOH terminus (amino acids 125–204) of Pex4p. Although this domain of Pex4p includes the active site Cys (C133), Pex4p constructs containing Ala (C133A) or Ser (C133S) substitutions (Crane et al., 1994) at this location interacted normally with Pex22p in the yeast two-hybrid system (data not shown). This result demonstrates that the interaction of Pex22p and Pex4p is not dependent on the UBC activity of Pex4p. Likewise, the binding of Pex22p to Pex4p is not dependent on the stretch designated INS2 (insertion element 2) that is unique to Pex4p in comparison with several UBC enzymes (Fig. 8A). This segment has been postulated to be important for peroxisomal localization (Crane et al., 1994). Pex22p and Pex4p also physically interact because 6HIS-Pex4p expressed in *P. pastoris* was able to bind Pex22p specifically (Fig. 8B).

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 9A). 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. 9A). Fourth, NH-Pex4p is mislocalized to the cytosol in the Δpex22 strain (Fig. 9B). Many of these points are reminiscent of the relationship between Ubc7p and Cue1p in *S. cerevisiae*. Cue1p, an integral membrane protein of the E.R., is essential for the localization of Ubc7p, a UBC enzyme, to the cytosolic face of the E.R., and both these proteins are required for the degradation of aberrant proteins in the E.R. membrane and for the retrograde transport of luminal substrates out of the E.R. (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. 9B). 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. 9B).

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 (Kalisz, 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
Δpex22 and Δpex4 strains were directly attributable to the absence of Pex5p, PEX5 was overexpressed in the Δpex4 and Δpex22 strains expressing GFP-SKL. The introduction of the PEX5 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 Δpex22 strain overexpressing Pex4p (data not shown). Therefore, the phenotypes seen in the Δpex4 and Δpex22 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 Δpex4 and Δpex22 strains (Fig. 3, see also Wiebel and Kunau, 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 Pex22p/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 (Baerends et al., 1997; Komori et al., 1997). The function of Pex4p at the membrane might be to ubiquitinate and therefore target unfolded membrane proteins or nonstoichiometric 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 Iberitini et al., 1997; Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996), Pex14p (A Iberitini et al., 1997; Brocard et al., 1997; Fransen et al., 1998), or Pex17p (Huuse et al., 1998). Pex13p is stable in Δpex4 or Δpex22 strains (data not shown) and Pex5p is stable in a P. pastoris Δpex13 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, Scyaf5p (open reading frame YAL055w), appears to be the homologue of PpPex22p. Like PpPex22, 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, Scyaf5p 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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References


Elgersma, Y., H.M. E Igersma, T. Wenzel, J.M. McAffery, M.G. Farquhar, and...