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

Antonius Koller,* William B. Snyder,* Klaas Nico Faber,* Thibaut J. Wenzel,* Linda Rangell,† Gilbert A. Keller,‡ and Suresh Subramani*

*Department of Biology, University of California San Diego, La Jolla, California 92093-0322; and †Pharmacological Science, Genentech, South San Francisco, California 94080

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-
some, 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 Iberlini et al., 1997; Brocard 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 (Huhse et al., 1998). The binding of Pex5p and Pex7p to Pex17p, however, was dependent on the presence of Pex14p (Huhse et al., 1998). Deletion of the 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; Emdmann and Blobel, 1996; Goud et al., 1996; A Iberlini 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 nodule against cytosolic HSP70 inhibits the import of SKL-containing proteins into peroxisomes (Walt et al., 1994; Fransen et al., 1998). Deletion of the gene encoding Djp1p, a cytosolic DnaJ-like protein, had a drastic effect on peroxisomal import of certain PTS-containing proteins (Hettema 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). Deletion of the gene encoding Djp1p, a cytosolic DnaJ-like protein, had a drastic effect on peroxisomal import of certain PTS-containing proteins (Hettema 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.
(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 pBluescriptSK II. 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-BamH I Zeocin fragment was inserted. The resulting plasmid, pTK35, was cut with BamH I and HindIII and transferred into PPy 12 and SM D163. The disruptions were confirmed by PCR.

The ScYAF5 plasmid was disrupted according to Wach et al. (1994). Primers TK53, TK62, TK63, and TK 64 were used to isolate a fragment using PCR that contains the 5' region of ScYAF5, followed by KanMX2, followed by the 3' region of ScYAF5. This construct was transformed into the S. cerevisiae strain BJ2191 and G 418-resistant colonies were checked for correct disruption of the ScYAF5 gene by PCR.

### Construction of Plasmids

Plasmids used are in Table I and DNA primers are in Table II. Plasmid pTK10, which expresses the NH-PEX4 region of S. cerevisiae ScYAF5, was constructed by cloning a BamH I-Smal fragment into pBTM116 (Faber et al., 1998). Plasmids pTK12 and pTK13 were constructed by fusing appropriate gene fragments, containing one blunt end and one BamHI end, and EcoRI. Plasmid pTK23 contains a BamH I-SspI fragment of PEX4 cloned into pKNSD55, which had been cut with BamH I and EcoR I (blunt ended). Plasmid pTK25 contains an EcoR V-EcoR I fragment of PEX4 cloned into pKNSD53, cut with BamH I (blunt ended) and EcoR I. Plasmid pTK27 contains an SspI-EcoR I fragment of PEX4 in pKNSD53, cut with BamH I (blunt ended) and EcoR I. Plasmid pTK21 was constructed as follows: a BamH I-EcoRV fragment (cut out of pKNF118) was cloned into pKNSD55, containing one blunt-ended or the full-length PEX4 region. The 5' 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-BamH I Zeocin fragment was inserted. The resulting plasmid, pTK35, was cut with BamH I and HindIII and transferred into PPy 12 and SM D163. The disruptions were confirmed by PCR.

### Table I. Plasmids Used in This Study

| Plasmid | Relevant features | Source |
|---------|------------------|--------|
| pTW5 | GFP-SKL | Wiemer et al., 1996 |
| pTW66 | PTS2-GFP | Wiemer et al., 1996 |
| pTK10 | pTIC3K PEX22 | This study |
| pTK12 | pKNSD55 PEX22 | This study |
| pTK13 | pKNSD52 PEX22 | This study |
| pTK14 | pKNSD55 PEX22 (26-187) | This study |
| pTK16 | pKNSD55 PEX22 (88-187) | This study |
| pTK18 | pKNSD55 PEX22 (1-89) | This study |
| pTK20 | pQE30 PEX22 (26-187) | This study |
| pTK21 | pKNSD52 PEX4 (1-87) | This study |
| pTK23 | pKNSD52 PEX4 (1-124) | This study |
| pTK25 | pKNSD53 PEX4 (88-204) | This study |
| pTK27 | pKNSD53 PEX4 (125-204) | This study |
| pTK29 | pBluescript 5’ PEX22-Zeocon-3’ | This study |
| pTK30 | pTIC3K PEX22-GFP | This study |
| pTK32 | pTW71 PEX22(1-25)-GFP | This study |
| pTK34 | pTW71 PEX22(1-7)-GFP | This study |
| pTK35 | pBluescript 5’ PEX4-Zeocon-3’P EX4 | This study |
| pTK36 | pTW71 6HIS-P EX4 | This study |
| pTK37 | pQE30 PEX4 (1-124) | This study |
| pTK45 | RS3036 ScYAF5 | This study |
| pTK46 | pKNSD52 ScYAF5 | This study |
| pTK47 | pKNSD55 ScYAF5 | This study |
| pTK48 | pENS26 ScYAF5 | This study |
| pTK49 | pKNSD55 ScYAF4 | This study |
| pTK50 | pTW71 NH-PEX4 | This study |
| pTK51 | pBluescript 5’ PEX4-NH-PEX4-Zeocon-3’P EX4 | This study |
| pKNSD119 | pKNSD55 PEX4 | This study |
| pKNSD118 | pKNSD52 PEX4 | This study |

### Table II. Primers Used in This Study

| Primer | 5’-Sequence-3’ |
|--------|----------------|
| TK31 | 5’-GGATCCATGAGAATTCGAGGAGAAC |
| TK34 | 5’-GGATCCATACAACTCTAAGAGAAGAAC |
| TK35 | 5’-GGATCCAGTTTATTATATACCCGAG |
| TK36 | 5’-GGATCCCGGAGAGGAGAGTATC |
| TK37 | 5’-CTATCGAGCAGAGACACAG |
| TK40 | 5’-GGCAAAAGAAGAAGATCCGAG |
| TK41 | 5’-GGATCCTGGAGAATTGCTTTGAGG |
| TK42 | 5’-CCCGGTTTTTCTAGGATGAGG |
| TK43 | 5’-CCCGGTTAATTGCTTTGAGG |
| TK44 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK45 | 5’-CCCGGGGTTTCTTCTAGGAGG |
| TK46 | 5’-CCCGGGGTTGAGAATTCGAG |
| TK47 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK48 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK51 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK52 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK53 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK54 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK55 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK56 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK57 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK58 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK59 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK60 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK61 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK62 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK63 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK64 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK65 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK66 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK67 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK68 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK69 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TK70 | 5’-CCCGGGGTTGCTACTTATGAGG |
| TW6 | 5’-CCCGGGGTTGCTACTTATGAGG |
| KNF13 | 5’-CCCGGGGTTGCTACTTATGAGG |
| KNF14 | 5’-CCCGGGGTTGCTACTTATGAGG |

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 BglII-BamHI cut plasmid pTW313, 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 pCRII (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 TK96 and TW6 was ligated into plasmid pTK34. 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 Pichia primer 3′A OX (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 pTK51 expressing a NH2-tagged Pex4p from the acyl-CoA oxidase (ACO) promoter was cloned as follows: A BamHI-EcoRI fragment containing the full-length PEX4 was cloned into plasmid pM22 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 SmaI site of the pBuescriptSKI (containing the 5′ end of PEX4 and the 3′ end of PEX4 (see above). A blunt-ended HaelI-BamHI Zocin fragment was then cloned into the blunt ended EcoRI site in the 3′ end of PEX4. This whole fragment (5′-PEX4-NH-PEX4-Zocin-3′-PEX4) was cut out of the plasmid with Xbal-HindIII and transformed into a Δpex4 strain (PPY12, Δpex4-A RGC). Aarginine minus and Zocin resistant colonies were checked for their expression of NH-Pex4p.

A fragment of ScyA5 containing the full-length gene was amplified with primers TK52 and TK62 on genomic S. cerevisiae DNA. The resulting EcoRI fragment was cloned into pSR306, cut with EcoRI to yield plasmid pTK45. The two-hybrid vectors with ScyA5 were made as follows: ScyA5 was amplified with PCR with primers TK52 and TK53. The resulting BamHI-EcoRI fragment was cloned into either pKN55 (to yield plasmid pTK46) or pKN56 (to yield pTK47). Plasmids for the two-hybrid experiment expressing ScPEX4 were made by amplifying ScPEX4 with primers TK67 and TK68. The resulting fragment was cloned as a BamHI-EcoRI fragment into pKN55 (to yield pTK48) and pKN56 (to yield pTK49).

Production of Antibodies

For the construction of a 6HIS-Pex4p, a BamHI-EcoRI fragment of PEX4 produced by PCR with primers TK35 and TK40 was cloned into 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 Ehr, 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 lter 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 SDG gel.

Fluorescence and Electron Microscopy

Fluorescence microscopy for the detection of GFP-tagged proteins was done as described by Monosov et al. (1998). Fluorescence images were acquired using a CCD camera (model 4995; Cohn Inc.) and a CE-7 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 1:10,000. Microscopy for immunofluorescence was as described (O’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.

Dignition permeabilization was done according to Elgersma et al. (1998). Western blotting was performed according to standard procedures. A nitrobiotides were used at the following dilutions: α-Scatalase, 1:10,000; α-Schlorase, 1:10,000; α-ScGPDH (glucose-6-phosphate dehydrogenase), 1:3,000; α-F β subunit of mitochondrial ATPase, 1:10,000; α-PpPex3p, 1:10,000; α-PpPex4p, 1:1,000; α-PpPex5p, 10,000; α-PPex7p, 1:10,000; α-PpPex22p, 1,200; 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 plehymycin, 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 P52 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 plehymycin. 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 plehymycin. Two plehymycin
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 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 1. Map of PEX22, PEX22 disruption construct and complementation assay of PEX22 constructs. (A) Plasmid p82.09 and derivatives (p82.20–p82.25 and pTK10) containing the DNA indicated by a line were tested for ability to complement the pex22.1 mutant (STK20). Plus (+) and minus (−) signs indicate ability and inability, respectively, to complement the mutant. For the Δpex22 disruption, the whole open reading frame of PEX22 was replaced by the Zeocin-resistance gene (see Materials and Methods). (B) Δpex22::Zeocin (STK11) was transformed with an empty plasmid (pPIC3K) or plasmid pTK10 and streaked on minimal methanol medium (SMethanol). Wt is PPy12.

Figure 2. Nucleotide sequence of PEX22 and deduced amino acid sequence. Complete nucleotide sequence of the PEX22 open reading frame and flanking sequences are shown. The deduced amino acid sequence is denoted by the one-letter code. The putative transmembrane region is underlined. These sequence data are available from GenBank under accession number AF133103.
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, SM D1163 (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). 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, SM D 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. Thiolase 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, 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.
These plasmids were then transformed in combination into the \textit{S. cerevisiae} strain L40 and interaction of these proteins was assessed by the production of \( \beta \)-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. 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 \( \Delta \text{pex4} \) 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 dithiobis(succinimidylpropionate) (DSP) was added to the lysates to cross-link neighboring proteins. 6HIS-Pex4p and associated proteins were precipitated with \( \text{Ni}^{2+} \)-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 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.

\( \Delta \text{pex4} \) and \( \Delta \text{pex22} \) Strains Share Similar Phenotypes

PpPex4p was previously characterized as a ubiquitin-conjugating enzyme, similar to ScPex4p (Crane et al., 1994). A \( \Delta \text{pex4} \) strain (STK14) behaved similarly in differential

---

**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 \( \Delta \text{pex22} \) (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 \( \Delta \text{pex22} \) 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 \( \beta \)-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 (STK 12 and STK 14) 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 STK 15) 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 (PPY 12) 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 PPY 12, 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 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), 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
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). 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. 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 (K.alish, J.E., and S.J. Gould, 6th International Congress on Cell Biology, 1996, A bstract 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 X5 was overexpressed in the $\Delta$Pex4 and $\Delta$Pex22 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 a $\Delta$pe22 strain overexpressing Pex4p (data not shown). Therefore, the phenotypes seen in the $\Delta$pe4 and $\Delta$pe22 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 $\Delta$pe4 and $\Delta$pe22 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 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–UbCp7 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 (Baeerends 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 Ibberini et al., 1997; Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996), Pex14p (A Ibberini et al., 1997; Brocard et al., 1997; Fransen et al., 1998), or Pex17p (Hulse et al., 1998). Pex13p is stable in $\Delta$pe4 or $\Delta$pe22 strains (data not shown) and Pex5p is stable in a P. pastoris $\Delta$pe13 strain (Gould et al., 1996). Pex14p and Pex17p remain as reasonable targets for investigation because their deletion causes PT S1 and PT S2 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 Pex22p, we did find a protein of similar predicted size and topology in S. cerevisiae. The hypothetical protein, ScyAf5p (open reading frame YAL1055w), appears to be the homologue of PpPex22p. Like PpPex22, the ScY Af5 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.

We thank the members of the lab for support and helpful discussions and S. Emr for use of the fluorescence microscope.

A. Kollar was supported by a fellowship of the Swiss National Science Foundation (no. 5203-046677), W.B. Snyder from a fellowship from the American Cancer Society, K.N. Faber by a fellowship from the Human Frontier Science Program Organization, and T.J. Wenzel by a fellowship from the European Molecular Biology Organization. This work was supported by the National Institutes of Health (Grant DK 41737) to S. Subramani. Submitted: 4 March 1999

Revised: 17 May 1999

A accepted: 3 June 1999

References

A. Ibberini, M. P. Rehling, R. Erdmann, W. Girzalsky, J. A. Kiel, M. Veenhuis, and W. H. Kunau. 1997. Pex4p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. Cell. 89:83–92.

Babst, M., W. Endlind, E. J. Estepa, and S. D. Emr. 1998. The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. EMBO (Eur. Mol. Biol. Organ.) 17:2982–2993.

Baeerends, R. J., S. W. Rasmussen, R. E. Hilbrands, M. van der Heide, K. N. Faber, P. T.W. Ruëvekamp, J. A. W. K. Kiel, J. M. Cregg, I. J. van der Klei, and M. Veenhuis. 1996. The Hansenula polymorpha PER9 gene encodes a peroxisomal membrane protein essential for peroxisomal assembly and integrity. J. Biol. Chem. 271:8887–8894.

Baeerends, R. J., F. A. Salomons, K. N. Faber, J. A. Kiel, I. J. van der Klei, and M. Veenhuis. 1997. Deviant Pex3p levels affect normal peroxisome formation in H. polymorpha: high steady-state levels of the protein fully abolish matrix protein import. Yeast. 13:1437–1448.

Biedler, J. E., C. Völklein, and T. Sommer. 1997. Role of Cue1p in ubiquitination and degradation at the ER surface. Science. 278:1806–1810.

Bodnar, A. G., and R. A. Rachubinski. 1996. Characterization of the integral membrane polypeptides of rat liver peroxisomes isolated from untreated and clofibrate-treated rats. Biochim. Cell Biol. 69:499–508.

Brocard, C., G. L. Ametschandwerter, R. Koudelka, and A. Hartig. 1997. Pex14p is a member of the protein linkage map of Pex5p. EMBO (Eur. Mol. Biol. Organ.) 16:5491–5500.

Crane, D. J., J. E. Kahlil, and S. J. Gould. 1994. The Ficha pastoris PA54 gene encodes a ubiquitin-conjugating enzyme required for peroxisome assembly. J. Biol. Chem. 269:2183–2184.

Diestelkötter, P., and W. W. Just. 1993. Role of Cue1p in ubiquitination and degradation at the ER surface. Science. 278:1806–1810.

Dietzel, T., C. Volkwein, and T. Sommer. 1997. Role of Cue1p in ubiquitination and degradation at the ER surface. Science. 278:1806–1810.

Dietzel, T., and W. W. Just. 1993. In vitro insertion of the 22-kD peroxisomal membrane protein into isolated rat liver peroxisomes. J. Cell Biol. 123:1717–1725.

Dodd, G., and S. J. Gould. 1996. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. J. Cell Biol. 135:1769–1774.

Dyer, J. M., J. A. M. C. New, and J. M. Goodman. 1996. The sorting sequence of the peroxisomal integral membrane protein PM47 is contained within a short hydrophobic loop. J. Cell Biol. 135:269–280.

Elgersma, Y., M. van den Berg, H. F. Tabak, and B. Distel. 1993. An efficient positive selection procedure for the isolation of peroxisomal import and peroxisome assembly mutants of Saccharomyces cerevisiae. Genetics. 135:731–740.

Elgersma, Y., J. J. Kwaast, A. Klein, T. Voorn-Brouwer, M. van den Berg, B. Metzig, T. A. W. H. Tabak, and B. Distel. 1996. The SH3 domain of the peroxisomal membrane protein Pex11p functions as a docking site for Pex5p, a mobile receptor for peroxisomal proteins. J. Cell Biol. 135:97–109.

Elgersma, Y., J. J. Kwaast, M. van den Berg, W. B. Snyder, B. Distel, S. Subramani, and H. F. Tabak. 1997. Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in S. cerevisiae, causes proliferation of the endoplasmic reticulum membrane. EMBO (Eur. Mol. Biol. Organ.) 16:7326–7341.
S. Subramani. 1998. A mobile PTSS receptor for peroxisomal protein import in Pichia pastoris. J. Cell Biol. 140:807-820.

Erdmann, R., and G. Blobel. 1996. Identification of Pex13p a peroxisomal membrane receptor for the PTSS recognition factor. J. Cell Biol. 135:111-121.

Faber, K.N., J.A. Heyman, and S. Subramani. 1998. Two A A A family peroxins, PpPex1p and PpPex6p, interact with each other in an A T P-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes. Mol. Cell. Biol. 18:936-943.

Fransen, M., S.R. Terlecky, and S. Subramani. 1998. Identification of a human PTSS receptor docking protein directly required for peroxisomal protein import. Proc. Natl. Acad. Sci. USA. 95:8087-8092.

Fujiki, Y., R.A. Rachubinski, and P.B. Lazarow. 1984. Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. Proc. Natl. Acad. Sci. USA. 81:7127-7131.

Girzalsky, W., P. Rehling, K. Stein, J. Kipper, L. Blank, W.-H. Kunau, and R. Erdmann. 1999. Involvement of Pex13p in Pex14p localization and peroxisomal targeting signal 2-dependent protein import into peroxisomes. J. Cell Biol. 144:1151-1162.

Gould, S.J., D. McCollum, A.P. Spong, J.A. Heyman, and S. Subramani. 1992. Development of the yeast Pichia pastoris as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast. 8:613-628.

Gould, S.J., J.E. Kalish, J.C. Morrell, J. Bjorkman, A.J. Urquhart, and D.I. Crane. 1996. Pex13p is an SH3 protein of the peroxisome membrane and a docking factor for the predominantly cytoplasmic PTSS receptor. J. Cell Biol. 135:85-95.

Harlow, E., and D. Lane. 1988. A nontidies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 313 pp.

Hettema, E.H., C.C.M. Ruigrok, M.G. Kerkamp, M. van den Berg, H.F. Tabak, B. Distel, and I. Braakman. 1998. The cytosolic DnaJ-like protein Djpl3p is involved specifically in peroxisomal protein import. J. Cell Biol. 142:421-434.

Höflund, J., M. Veenhuis, and W.H. Kunau. 1991. PAS3, a Saccharomyces cerevisiae gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. J. Biol. Chem. 266:157-197.

Huuse, B., P. Rehling, M. A Ibertini, L. Blank, K. Meller, and W.H. Kunau. 1998. Pex17p of Saccharomyces cerevisiae is a novel peroxin and component of the peroxisomal protein translocation machinery. J. Cell Biol. 140:49-60.

Iclomori, M., S.W. Rasmussen, J.A.K.W. Kiel, R.J. S. Aarends, J.M. Cregg, I.J. van der Klei, and M. Veenhuis. 1997. The H. anomala polymorpha PE 14 gene encodes a novel peroxisomal membrane essential for peroxisome biogenesis. EMBO (Eur. Mol. Biol. Organ.) J. 16:44-53.

Monse, E.Z., T.J. Wenzel, G.H. Liers, J.A. Heyman, and S. Subramani. 1996. Labeling of peroxisomes with green fluorescent protein in living peroxisomes. J. Histochem. Cytochem. 44:381-389.

Odorizzi, G., M. Babst, and S.D. Emr. 1998. Fdlp1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell. 95:847-858.

Purde, P.E., X. Yang, and P.B. Lazarow. 1998. Pex1Bp and Pex21p, a novel pair of related peroxins essential for peroxisomal targeting by the PTSS pathway. J. Cell Biol. 143:859-869.

Rieder, S.E., and S.D. Emr. 1997. A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. Mol. Biol. Cell. 8:2307-2327.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463-5467.

Smith, T.F., and M.S. Waterman. 1981. Identification of common molecular subsequences. J. M. Biol. 147:195-197.

Subramani, S. 1998. Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement. Physiol. Rev. 78:171-188.

van den Bosch, H., R.B.H. Schutgens, R.J.A. Wanders, and J.M. Tager. 1992. Annu. Rev. Biochem. 61:157-197.

van der Klei, I.J., R.E. Hilbrands, J.A. Kiel, S.W. Rasmussen, J.M. Cregg, and M. Veenhuis. 1998. The ubiquitin-conjugating enzyme Pex6p of Hansenula polymorpha is required for efficient functioning of the PTSS import machinery. EMBO (Eur. Mol. Biol. Organ.) J. 17:3608-3618.

Wach, A., A. Brachat, R. Pohlmann, and P. Philipsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast. 10:1793-1808.

Walton, P.A., M. Wendland, S. Subramani, R.A. Rachubinski, and W.J. Welch. 1994. Involvement of 70-kd heat-shock proteins in peroxisomal import. J. Cell Biol. 125:1037-1046.

Wiebel, F.F., and W.H. Kunau. 1992. The Pas2 protein essential for peroxisomal biogenesis is related to ubiquitin-conjugating enzymes. Nature. 359:73-76.

Wiener, E.A., G. Liers, K.N. Faber, T. Wenzel, M. Veenhuis, and S. Subramani. 1996. Isolation and characterization of Pas2p, a peroxisomal membrane protein essential for peroxisome biogenesis in the methylotrophic yeast Pichia pastoris. J. Biol. Chem. 271:18973-18980.