Pex5p, a guide for import of proteins into peroxisomes
Klein, A.T.J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 4

Saccharomyces cerevisiae acyl-CoA oxidase follows a novel, non-PTS1, import pathway into peroxisomes that is dependent on Pex5p

André T. J. Klein, Marlene van den Berg, Gina Bottger, Henk F. Tabak and Ben Distel

Department of Biochemistry, Academic Medical Center, The Netherlands

Abstract

The peroxisomal protein acyl-CoA oxidase (Poxlp) of *Saccharomyces cerevisiae* lacks either of the two well-characterized peroxisomal targeting sequences known as PTS1 and PTS2. Here we demonstrate that peroxisomal import of Poxlp is nevertheless dependent on binding to Pex5p, the PTS1 import receptor. The interaction between Pex5p and Poxlp, however, involves novel contact sites in both proteins. The interaction region in Pex5p is located in a defined area of the amino-terminal part of the protein outside of the tetratricopeptide repeat domain involved in PTS1 recognition; the interaction site in Poxlp is located internally and not at the carboxyl terminus where a PTS1 is normally found. By making use of *pex5* mutants that are either specifically disturbed in binding of PTS1 proteins or in binding of Poxlp, we demonstrate the existence of two independent, Pex5p-mediated import pathways into peroxisomes in yeast as follows: a classical PTS1 pathway and a novel, non-PTS1 pathway for Poxlp.
Introduction

Proteins destined for import into the peroxisomal matrix are synthesized on free polyribosomes in the cytoplasm. For targeting to their proper destination, these proteins possess a peroxisomal targeting signal (PTS) that directs them to peroxisomes. Two different PTSs have been identified, PTS1 and PTS2. The majority of peroxisomal matrix proteins contain a PTS1 and only a few have a PTS2. The PTS1 is located at the extreme carboxyl terminus of a peroxisomal matrix protein and was first defined as three amino acids with the consensus sequence (S/C/A)(K/R/H)(L/M) (Gould et al., 1989; Swinkels et al., 1992). The PTS2 is positioned at the amino-terminal part of a protein and has the consensus sequence (R/K)(L/V/I)X5(H/Q)(L/A) (Swinkels et al., 1991; Gietl et al., 1994; Glover et al., 1994b; Tsukamoto et al., 1994). The PTS1 and PTS2 are recognized and bound in the cytosol by specific receptor proteins, Pex5p (peroxin-5 protein) (McCollum et al., 1993; Van der Leij et al., 1993; Brocard et al., 1994; Dodt et al., 1995; Fransen et al., 1995; Szilard et al., 1995; Terlecky et al., 1995; Wiemer et al., 1995; Dodt and Gould, 1996) and Pex7p (Marzioch et al., 1994; Zhang and Lazarow, 1995; Rehling et al., 1996; Zhang and Lazarow, 1996; Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997; Elgersma et al., 1998), respectively. For Pex5p it has been shown that an array of tetratricopeptide repeats (TPR) in the carboxyl-terminal part of the protein mediates the binding of PTS1 (Brocard et al., 1994; Dodt et al., 1995; Terlecky et al., 1995). The details of the interaction between Pex5p and PTS1 have been resolved by an extensive mutational analysis of Pex5p (Klein et al., 2001) and determination of the crystal structure of a Pex5p-PTS1 peptide complex (Gatto et al., 2000). Those studies revealed that the TPR domain of Pex5p forms two clusters of three TPR motifs that are close together in space and form a single binding site for the PTS1. Amino acids from both TPR clusters are interacting with the PTS1 peptide backbone and with the amino acid side chains. How binding of PTS2 by Pex7p, a WD-40 repeat protein, takes place is still unclear.

The receptor-cargo complex docks on the peroxisome via the interaction with a protein complex located in the peroxisomal membrane. Although some of the details vary between different species, it has been shown that Pex13p, Pex14p, and Pex17p are part of this docking complex (Elgersma et al., 1996a; Erdmann and Blobel, 1996; Gould et al., 1996; Albertini et al., 1997; Brocard et al., 1997; Fransen et al., 1998; Huhse et al., 1998; Girzalsky et al., 1999; Schliebs et al., 1999; Shimizu et al., 1999; Snyder et al., 1999; Will et al., 1999; Barnett et al., 2000; Bottger et al., 2000; Urquhart et al., 2000; Saidowsky et al., 2001). Proteins implicated in the translocation
over the peroxisomal membrane are Pex2p, Pex10p, and Pex12p (Dodt and Gould, 1996; Chang et al., 1999). However, it is still unclear how the actual translocation over the peroxisomal membrane takes place, except that protein unfolding is not a prerequisite for translocation (Glover et al., 1994a; McNew and Goodman, 1994; Walton et al., 1995; Elgersma et al., 1996b; Hausler et al., 1996; Lee et al., 1997; Yang et al., 2001). The first PTS1 identified was that of firefly luciferase and consists of the carboxyl-terminal tripeptide SKL (Gould et al., 1987; Gould et al., 1989). This tripeptide proved not only essential for the import of luciferase but was also shown to be sufficient to direct other proteins to peroxisomes (Gould et al., 1988; Gould et al., 1989; Gould et al., 1990; Swinkels et al., 1992). However, a number of observations (Gould et al., 1989; Sommer et al., 1992; Motley et al., 1995) suggest that the definition of a PTS1 as being both necessary and sufficient for the import of proteins into peroxisomes needs some adjustment. These studies have shown that whether or not a carboxyl-terminal tripeptide can function as a PTS1 depends on its context. For instance, targeting of alanine:glyoxylate aminotransferase I to peroxisomes in humans depends on the carboxyl-terminal tripeptide KKL (Motley et al., 1995). However, this carboxyl-terminal KKL was not sufficient to direct the reporter protein luciferase to peroxisomes in human fibroblasts (Motley et al., 1995) and in monkey kidney CV-1 cells (Gould et al., 1989) or to glycosomes in Trypanosoma brucei (Sommer et al., 1992). For peroxisomal malate dehydrogenase (Mdh3p), it was also shown that in the homologous context many variations that do not comply with the consensus sequence could still direct this protein to peroxisomes in Saccharomyces cerevisiae (Elgersma et al., 1996b). These results can be explained by the presence of accessory sequences in a peroxisomal matrix protein that, when this protein is presented in its homologous context, contribute to the binding of the PTS1-containing protein to Pex5p. These accessory sequences can sometimes be located close to the PTS1 and can influence the binding to Pex5p in a species-dependent manner, as was shown for hexadecapeptides containing a PTS1 (Lametschwandtner et al., 1998). In other cases a PTS1 is not essential at all. This is most evident for carnitine acetyltransferase (Cat2p); its targeting to peroxisomes in S. cerevisiae is Pex5p-dependent, but after deletion of the PTS1 most of the carnitine acetyltransferase is still directed to peroxisomes (Elgersma et al., 1995). Deletion of the PTS1 in Cat2p also does not affect its interaction with Pex5p in the two-hybrid system. These results suggest that in some cases accessory or alternative sequences can be used for binding to Pex5p and that these can function as a targeting signal.

Import of proteins in a PTS1- or PTS2-independent way can be explained in various ways. In genetically constructed S. cerevisiae strains import into peroxisomes can take
place by formation of homo-oligomers between subunits without a PTS and subunits with a PTS (Glover et al., 1994a; McNew and Goodman, 1994; Elgersma et al., 1996b; Lee et al., 1997). Similarly, it has been shown that S. cerevisiae $\Delta^3\Delta^2$-enoyl-CoA isomerase (Eci1p) can hetero-oligomerize with $\Delta^3\Delta^2$-dienoyl-CoA isomerase (Dci1p) resulting in the import of Eci1p from which the PTS1 had been deleted (Yang et al., 2001). In a natural context, there are several peroxisomal matrix proteins that are not equipped with a recognizable PTS1 or PTS2. Examples of such proteins are Hansenula polymorpha malate synthase (Bruinenberg et al., 1990) and acyl-CoA oxidases of the yeasts Candida tropicalis (Small et al., 1988), Candida maltosa (Hill et al., 1988) S. cerevisiae (Dmochowska et al., 1990) and Yarrowia lipolytica (Wang et al., 1998). How targeting of these proteins to peroxisomes takes place, via piggybacking or via alternative targeting sequences in these proteins, is not known (Small et al., 1988). Remarkably, in human (Fournier et al., 1994), rat (Miyazawa et al., 1989), mouse (Nohammer et al., 2000), and in the yeast Pichia pastoris (Koller et al., 1999) acyl-CoA oxidase is imported via its PTS1.

Here we show that S. cerevisiae acyl-CoA oxidase (Pox1p) binds directly to Pex5p and that binding is not dependent on the carboxyl-terminal 17 amino acids of Pox1p. By using a pex5 mutant that is specifically disturbed in the interaction with and the import of PTS1 proteins, we show that S. cerevisiae Pox1p is imported into peroxisomes in a PTS1-independent manner. The site of Pox1p interaction on Pex5p was identified and shown to be located in a region outside of the TPR domain. A pex5 mutant containing an Y253N substitution within the Pox1p-binding region is specifically disturbed in the interaction with and the import of Pox1p. These results demonstrate a novel, non-PTS1 mediated import route for Pox1p that is dependent on Pex5p.

**Experimental Procedures**

**Strains and culture conditions**

The yeast strains used in this study are as follows: S. cerevisiae BJ1991 (MATa, leu2, trpl, ura3-251, prbl-1122, pep4-3, gal2); BJ1991pex5$\Delta$ (MATa, pex5::LEU2, leu2, trpl, ura3-251, prbl-1122, pep4-3, gal2); BJ1991pex3$\Delta$ and BJ1991pex7$\Delta$ were described previously (Hettema et al., 2000); HF7c (MATa, ura3-52, his3-200, ade2-101, lys2-801, trpl-901, leu2-3,112, gal4-542, gal80-538, LYS2::GAL1_UAS-GAL1_TATA-HIS3, URA3::GAL4 17mers(x3r)-CyCl_TATA-lacZ); and PCY2 (MATa, $\Delta$gal4, $\Delta$gal80, URA3::GAL1-lacZ, lys2-801, his3-$\Delta$200, trpl-$\Delta$63, leu2, ade2-101). The Escherichia
coli strain DH5α (recA, hsdR, supE, endA, gyrA96, thi-1, relA1, lacZ) was used for all transformations and plasmid isolations. Yeast transformations were carried out as described (Gietz et al., 1992). Transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, and amino acids as needed. Cell culture conditions are as follows: cells were pre-grown overnight on minimal 0.3% glucose medium (0.3% glucose, 0.67% yeast nitrogen base (YNB; Difco) and amino acids (20-30 μg/ml) as required). These cultures were inoculated in fresh 0.3% glucose medium and further grown to log phase. For induction on oleate these cultures were inoculated 1:10 in fresh oleate medium (0.5% potassium phosphate buffer, pH 6.0, 0.5% peptone and 0.3% yeast extract, 0.1% oleate, 2% Tween 40) and grown overnight at 28°C.

Cloning procedures

Standard techniques for DNA manipulations were used (Sambrook et al., 1989). The following plasmids have been described previously: pGST-Pex5p, encoding a fusion of glutathione S-transferase (GST) with Pex5p (Bottger et al., 2000); pAN4, encoding a fusion of the Gal4 trans-activating domain (Gal4AD) with Pex5p (Klein et al., 2001); pDBMDH3, encoding a fusion of the Gal4 DNA-binding domain (Gal4BD) with Mdh3p (Klein et al., 2001); pEL128, encoding a fusion of Gal4BD with ΔN-Cat2-AC (Elgersma et al., 1995); pGB17, encoding a fusion of Gal4BD with the Pex13pSH3 domain (Bottger et al., 2000); pGB47, encoding a fusion of Gal4BD with Pex14p (Bottger et al., 2000). The plasmid for expression of Pex5p in yeast (pTI98) was created by subcloning the PEX5 insert of pAN1 (Klein et al., 2001) behind the PEX5 promoter in pEL91 (Bottger et al., 2000) using BamHI and PstI. pex5 mutants were subcloned in pEL91 in the same way. pGB37, encoding NH-tagged Mdh3p was generated by subcloning the SacI-HindIII fragment of pEL143 (Elgersma et al., 1996b) behind the CTAL promoter in pEW111 (Hettema et al., 1998). pAN81, encoding a fusion of Gal4BD with Pox1p, was constructed by a PCR on genomic DNA of S. cerevisiae with primers pr34 and pr35. The PCR product was cloned in pGEM-T (Promega) without A-tailing, generating pAN74, which was used as template in a second PCR with primers pr34 and pr52. This PCR product was cloned SalI-Spel in pPC97 (Chevray and Nathans, 1992). pAN82, encoding a fusion of Gal4BD with Pox1p from which the last 3 amino acids had been deleted, was made by a PCR on pAN74 with primers pr34 and pr53. The PCR product was cloned SalI-Spel in pPC97. pAN83, encoding a fusion of Gal4BD with Pox1p from which the last 17 amino acids had been deleted, was made by a PCR on pAN74 with primers pr34 and pr54. The PCR product was cloned SalI-Spel in pPC97. pAN88, encoding a fusion of maltose-
binding protein (MBP) with Pox1p, was generated by subcloning the XbaI-SpeI insert of pAN81 in the XbaI site of pMAL-c2 (New England Biolabs Inc.). For the construction of pAN87, encoding a MBP fusion with ΔN-Cat2-ΔC, the SacI-HindIII fragment of pEL99 (Elgersma et al., 1995) was subcloned in pUC19 (New England Biolabs Inc.) generating pAN85. The EcoRI-HindIII insert of pAN85 was subsequently subcloned in pMAL-c2. pMAL-c2 was used for expression of MBP.

pAN37, encoding a fusion of Gal4AD with amino acids 252-612 of Pex5p, was made by PCR on pTI98 with primers p184 and p403. The PCR product was cloned EcoRI-SpeI in pPC86 (Chevray and Nathans, 1992). pAN39, encoding a fusion of Gal4AD with amino acids 307-612 of Pex5p, was made by PCR on pTI98 with primers p184 and p405. The PCR product was cloned EcoRI-SpeI in pPC86. pHZ3, encoding a fusion of Gal4AD with amino acids 307-612 of Pex5p, was made by PCR on pTI98 with primers pex5-1 and pex5-427. The PCR product was cloned SalI-SpeI in pPC86. pAN92, encoding a Gal4AD fusion with amino acids 239-300 of Pex5p was generated by PCR on pAN4 with primers pr66 and pr68. The PCR product was cloned EcoRI-SpeI in pPC86. pAN94, encoding a GST fusion with amino acids 239-300 of Pex5p, was generated by PCR on pAN4 with primers pr66 and pr68. The PCR product was cloned EcoRI-SpeI in pRP265nb (Barnett et al., 2000). pRP265nb was used for expression of GST.

For introducing single amino acid substitutions, the QuickChange site-directed mutagenesis kit (Stratagene) was used. The oligonucleotides pr64 and pr65 were used for introducing the D262G substitution, and pr62 and pr63 were used for introducing the I264T substitution.

Subcellular fractionation and protease protection assays
Subcellular fractionation experiments were performed as described previously (Bottger et al., 2000). Protease protection was performed on oleate-grown cells (200 OD units) that were spheroplasted and lysed in hypotonic buffer similar as described for the preparation of homogenates for subcellular fractionation. 20 µg of proteinase K (Roche Molecular Biochemicals) was added to 50 µg of protein sample and incubated with or without Triton X-100 (final concentration 0.15%) at room temperature for 5, 10, 15, and 30 min. Protease activity was stopped by addition of an equal volume of 20% trichloroacetic acid, and proteins were precipitated on ice for a minimum of 1 h. Samples were centrifuged for 30 min at 20,000 x g, and pellets were washed with acetone and resuspended in Laemmli sample buffer (Sambrook et al., 1989).
### Table 1. Primer compositions

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>p184</td>
<td>CGGACTAGTAAGCTGCATGCCTGCAG</td>
</tr>
<tr>
<td>p403</td>
<td>CGGAATTCAATCAATCTGATTTC</td>
</tr>
<tr>
<td>p405</td>
<td>CGGAATTCACAAGGATATTTAATAATTCC</td>
</tr>
<tr>
<td>pex5-1</td>
<td>ACGCGTCGACCAGCAGTGAAGCTTGTC</td>
</tr>
<tr>
<td>pex5-427</td>
<td>ACGCGTCGACCAGCAGTGAAGCTTGTC</td>
</tr>
<tr>
<td>pr34</td>
<td>GGACTAGTTCATTTGCAGAAATTTGCTCTTGTTC</td>
</tr>
<tr>
<td>pr35</td>
<td>ACGCGTCGACCAGCAGTGAAGCTTGTC</td>
</tr>
<tr>
<td>pr52</td>
<td>ACGCGTCGACCAGCAGTGAAGCTTGTC</td>
</tr>
<tr>
<td>pr53</td>
<td>GGACTAGTTCATTTGCAGAAATTTGCTCTTGTTC</td>
</tr>
<tr>
<td>pr54</td>
<td>GGACTAGTTCATTTGCAGAAATTTGCTCTTGTTC</td>
</tr>
<tr>
<td>pr62</td>
<td>CCAAGAAGTGTGGGGATAGCACACAAGGAGCTCAAGAG</td>
</tr>
<tr>
<td>pr63</td>
<td>CTTCCTCAGGCTTGTGGCTACACTTCTTCTTG</td>
</tr>
<tr>
<td>pr64</td>
<td>CTGATTTCCAAGAAGTGTGGGGTGAGCACACAAAGGAGCTG</td>
</tr>
<tr>
<td>pr65</td>
<td>CAGCGTCCCTGGTGATGCTACACTCCTTCTTGG</td>
</tr>
<tr>
<td>pr66</td>
<td>CGGAATTCCTGGCAGTACACAGAACGACGCTCAG</td>
</tr>
<tr>
<td>pr68</td>
<td>GGACTAGTTCATTTGCAGAAATTTGCTCTTGTTC</td>
</tr>
</tbody>
</table>

### Miscellaneous

The GST and MBP fusion proteins were expressed and isolated as described previously (Barnett et al., 2000; Bottger et al., 2000). The in vitro binding assay has also been described before (Klein et al., 2001).

Catalase A enzyme activity was measured as described by Lucke (Lucke, 1963), and β-galactosidase enzyme activity was determined as described before (Miller, 1972; Lametschwandtner et al., 1998).

Western blots were incubated with rabbit polyclonal antibodies raised against catalase A, 3-ketoacyl-CoA thiolase, Pex5p (all raised in our own laboratory), Pox1p (a kind gift from Dr. J.M. Goodman, Dallas), NH (a kind gift from Dr. P. van der Sluijs, Utrecht, The Netherlands), GST (Sigma), and mouse monoclonal antibodies against MBP (Sigma). Secondary antibodies used were goat anti-rabbit Ig-conjugated alkaline phosphatase or goat anti-mouse Ig-conjugated alkaline phosphatase. The pex5 mutant library and the screening procedure for pex5 mutants have been described before (Klein et al., 2001). *Candida albicans* sequences homologous to *S. cerevisiae*
Pex5p and Pox1p were retrieved from the Stanford Genome Technology Center by performing a blast search with these proteins at sequence- www.stanford.edu/group/candida. Contig6-2210 and contig6-2346 contain the C. albicans sequences homologous to S. cerevisiae Pox1p and Pex5p, respectively.

Results

The import of Pox1p into peroxisomes is mediated by Pex5p but is independent of the PTS1-binding site in Pex5p

S. cerevisiae Pox1p does not contain any recognizable peroxisomal targeting sequence. It is therefore unclear how this protein is imported into the peroxisomal matrix and whether it uses one of the known import receptors, Pex5p or Pex7p. To investigate this we examined the targeting of Pox1p to peroxisomes in wild-type, pex5Δ, and pex7Δ cells. Cells were homogenized, and a post-nuclear supernatant was centrifuged at 17,500 x g. Equivalent volumes of the organellar pellet and the supernatant fractions were analyzed by Western blotting with antibodies specific for Pox1p, the NH-tag to detect NH-Mdh3p (a PTS1 protein expressed from a co-transformed plasmid), and 3-ketoacyl-CoA thiolase (a PTS2 protein) (Fig. 1A). The distribution of catalase A (a PTS1 protein) was determined by measuring the enzyme activity (Fig. 1B). In wild-type cells Pox1p, catalase A, NH-Mdh3p, and thiolase were located in the pellet fraction, indicating that each of these proteins was targeted to peroxisomes. In pex5Δ cells Pox1p, catalase A and NH-Mdh3p were mis localized to the supernatant fraction indicating that peroxisomal targeting of Pox1p, like the PTS1 proteins catalase A and NH-Mdh3p, is dependent on Pex5p. Although a significant fraction of NH-Mdh3p was recovered in the organellar pellet, this does not represent peroxisomal import (see below). The localization of the PTS2 protein thiolase was not affected in pex5Δ cells. In pex7Δ cells only thiolase was mislocalized to the supernatant fraction, and both Pox1p and catalase A (not shown) were recovered in the pellet fraction. To investigate further the role of Pex5p in the import of Pox1p, we made use of the Pex5p(N393D) mutant. The N393D mutation specifically affects the interaction of Pex5p with PTS1 proteins (Klein et al., 2001). Subcellular fractionation of pex5Δ cells expressing Pex5p(N393D) showed that the PTS1 proteins NH-Mdh3p and catalase A were mislocalized to the supernatant fraction (Fig. 1). However, this mutation in Pex5p did not affect the distribution of Pox1p; the protein was mainly located in the pellet fraction, like in pex5Δ cells expressing wild-type PEX5 from a plasmid.
Figure 1. Targeting of Pox1p to peroxisomes is dependent on Pex5p.
Wild-type cells, pex5Δ cells, pex5Δ cells expressing Pex5p(N393D), all (co)transformed with a plasmid expressing NH-tagged Mdh3p, and pex7Δ cells were grown on oleate and subjected to subcellular fractionation. Equivalent volumes of the 600 x g post-nuclear supernatant (H), 17,500 x g pellet (P) and 17,500 x g supernatant (S) were analyzed by Western blotting (A). The antibodies used were directed against Pox1p, the NH epitope to detect NH-tagged Mdh3p and thiolase. Note that the subcellular localization of NH-tagged Mdh3p in pex7Δ cells was not studied. Distribution of catalase A was determined by measuring enzyme activity (B).
To prove that Pox1p, recovered from the pellet fraction of \( pex5\Delta \) cells expressing Pex5p(N393D), was imported into peroxisomes, we carried out a protease protection experiment. Wild-type, \( pex5\Delta, pex7\Delta, pex3\Delta, \) and \( pex5\Delta \) cells expressing Pex5p(N393D) were spheroplasted and lysed in hypotonic buffer. Equal amounts of cleared homogenates were exposed to proteinase K in the absence or presence of detergent (Fig. 2). The PTS2 matrix protein thiolase was used as an internal control for peroxisomal membrane integrity in the wild-type and \( pex5\Delta \) strains. In wild-type cells Pox1p was protected from protease degradation in the absence of detergent but was completely degraded in the presence of detergent, indicating that Pox1p has been imported into peroxisomes (Fig. 2A). Similar results were found in \( pex7\Delta \) cells, showing that Pox1p does not use the PTS2 targeting pathway for its import into peroxisomes. However, in \( pex5\Delta \) cells Pox1p was rapidly degraded in the absence of detergent, whereas thiolase was not affected by proteinase K treatment. These results confirmed that the import of Pox1p into peroxisomes is dependent on Pex5p. The protease protection experiment in the \( pex3\Delta \) strain served as a control for protein degradation in the absence of detectable peroxisomal membrane remnants (Baerends et al., 1996; Wiemer et al., 1996; Hettema et al., 2000). In this case both Pox1p and thiolase were rapidly degraded. To confirm that Pex5p-mediated import of Pox1p into peroxisomes is not dependent on the PTS1-binding site in Pex5p, we performed a protease protection experiment on a homogenate of \( pex5\Delta \) cells expressing Pex5p(N393D). Fig. 2B shows that Pox1p was completely protected from the protease in the Pex5p(N393D) mutant, indicating that Pox1p had been translocated across the peroxisomal membrane. In contrast the PTS1 protein NH-Mdh3p was rapidly degraded in the absence of detergent and thus not protected by a membrane. This finding suggests that the presence of NH-Mdh3p in the organellar pellet of the Pex5p(N393D) mutant in the subcellular fractionation (Fig. 1) is the result of a specific association of the protein with membranes or aggregation. Taken together, the results from the subcellular fractionation and protease protection experiments show that peroxisomal import of Pox1p is mediated by Pex5p but is not dependent on the PTS1-binding site of Pex5p.

**Pex5p interacts directly with Pox1p**

The preceding data demonstrate that Pox1p is targeted to peroxisomes by Pex5p. To study the interaction between Pex5p and Pox1p in an *in vitro* reconstituted system, we made use of bacterially expressed fusion proteins; Pex5p was fused to GST, and Pox1p was fused to maltose-binding protein (MBP). GST-Pex5p was purified on a glutathione-Sepharose 4B column, and the purified protein was loaded onto an
amylose column with bound MBP-Pox1p. After extensive washing of the column, to remove specifically bound proteins, MBP-Pox1p was eluted with maltose, and the eluates were analyzed by Western blotting. As shown in Fig. 3 GST-Pex5p co-eluted

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>pex5Δ</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>- TX100</td>
<td>+ TX100</td>
<td>- TX100</td>
<td>+ TX100</td>
</tr>
<tr>
<td></td>
<td>0  5  15 30</td>
<td>0  5  15 30</td>
<td>0  5  15 30</td>
<td>0  5  15 30</td>
</tr>
</tbody>
</table>

**Figure 2.** Membrane translocation of Pox1p requires Pex5p but is independent of the PTS1-binding site in Pex5p.

Wild-type cells, pex5Δ cells, pex3Δ cells, and pex7Δ cells (A) and pex5Δ cells co-expressing NH-Mdh3p and Pex5p(N393D) (B) were grown on oleate and converted to spheroplasts. Cleared homogenates were exposed to proteinase K for the times indicated in either the absence or the presence of 0.15% Triton X-100 (TX100). Samples were analyzed by Western blotting with antibodies specific for Pox1p, thiolase and the NH epitope to detect NH-tagged Mdh3p.
with MBP-Poxlp indicating that GST-Pex5p is able to bind to MBP-Poxlp. No co-collution was observed when MBP was used together with GST-Pex5p or MBP-Poxlp together with GST. These results show that Pex5p and Poxlp can interact directly with each other without support of other proteins.

**Figure 3. Pex5p interacts directly with Poxlp and AN-Cat2-ΔC.**
Purified GST-Pex5p or GST alone (100 μg each) was passed over an amylose column loaded with 250 μl of cleared lysate containing either MBP alone, MBP-Poxlp, or MBP-AN-Cat2-ΔC. After extensive washing, the column was eluted with 20 mM maltose, and the proteins in the elution fractions were subjected to SDS-PAGE followed by Western blotting. Antibodies were directed against MBP (**top panel**) or GST (**lower panel**).

**The interaction of Poxlp with Pex5p is not dependent on its carboxyl-terminal three amino acids**
Most peroxisomal matrix proteins are imported in a Pex5p-dependent manner into peroxisomes by virtue of a PTS1. Although the sequence of the three amino acids at the extreme carboxyl terminus that forms the PTS1 is rather degenerate, a general consensus sequence has been defined as (S/C/A)(K/R/H)(L/M) (Gould *et al.*, 1989; Swinkels *et al.*, 1992). The last three amino acids of *S. cerevisiae* Poxlp are I-N-K (Dmochowska *et al.*, 1990) and, hence, do not comply with this consensus sequence. We therefore did not expect it to behave as a PTS1. To investigate this we deleted the last 3 or 17 amino acids of Poxlp and studied the effect of these deletions on the
interaction with Pex5p in the two-hybrid system. The strength of the Pex5p-Pox1p interaction was quantified by measuring the β-galactosidase activity in a two-hybrid assay. Deletion of the last 3 or 17 amino acids of Pox1p did not reduce the interaction with Pex5p compared with that of full-length Pox1p (Fig. 4). These results show that Pox1p does not contain a typical PTS1 and that the interaction is not dependent on the last 17 residues of the protein.

Figure 4. Carboxyl-terminal deletions of Pox1p do not affect the interaction with Pex5p.
The last 3 (Pox1Δ3) or 17 (Pox1Δ17) amino acids of Pox1p were deleted. The strength of the interaction between Pex5p (fused to Gal4AD) and wild-type Pox1p, Pox1Δ3, or Pox1Δ17 (all fused to Gal4BD) was quantified in a two-hybrid assay by measuring the β-galactosidase activity. As a control the strength of the interaction between Pex5p and the empty Gal4BD (BD) and between wild-type Pox1p and the empty Gal4AD (AD) was determined. The values given are the mean ± S.D. of three measurements on independent transformants. The Pex5p-Pox1p interaction was set to 100%.

The region of Pex5p responsible for Pox1p interaction is clearly distinct from the PTS1 interaction site
The results thus far implicate that Pox1p has a different binding site on Pex5p when compared to PTS1 proteins. The TPR motifs in the carboxyl terminus form the binding
site for PTS1 (Brocard et al., 1994; Dodt et al., 1995; Terlecky et al., 1995), and the amino acids that mediate this interaction have been identified (Gatto et al., 2000; Klein et al., 2001). To determine the responsible regions for Pox1p interaction, we made several deletions in PEX5 giving rise to truncated proteins. The effect of these truncations on the interactions with Pox1p and Mdh3p, respectively, was studied (Fig. 5). We also included carnitine acetyltransferase from which the mitochondrial targeting signal and the PTS1 had been deleted (ΔN-CAT2-ΔC). Previously, it has been shown that this protein can still be targeted to peroxisomes in a Pex5p-dependent manner, indicating that this protein has an additional, internal peroxisomal targeting signal (Elgersma et al., 1995). The interaction between ΔN-CAT2-ΔC and Pex5p is direct as shown in Fig. 3.

<table>
<thead>
<tr>
<th>Gal4AD fusion</th>
<th>Gal4BD fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pex5p</td>
<td>β-galactosidase activity (% ± SD)</td>
</tr>
<tr>
<td>Pox1p</td>
<td>Mdh3p</td>
</tr>
<tr>
<td>Pox5(252-612)</td>
<td>39.8 ± 0.70</td>
</tr>
<tr>
<td>Pox5(307-612)</td>
<td>13.5 ± 0.09</td>
</tr>
<tr>
<td>Pox5(1-427)</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Pex5(N393D)</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

Figure 5. The Pox1p binding region in Pex5p is clearly distinct from the PTS1 interaction site.
Truncated versions of Pex5p (fused to Gal4AD) were tested in a two-hybrid assay for their interaction with Mdh3p, Pox1p and ΔN-Cat2-ΔC (all fused to Gal4BD). The strength of the interaction was determined by measuring β-galactosidase activity. For each protein fused to Gal4BD, the interaction with wild-type Gal4AD-Pex5p was set to 100%. The values given are the mean ± S.D. of three measurements on independent transformants. < 1 means that no interaction could be detected. For every Gal4AD fusion protein indicated, the interaction with the empty Gal4BD was also tested. Likewise, for every Gal4BD fusion protein, the interaction was tested against the empty Gal4AD. In each of these cases no interaction could be detected (not shown).
The relative strength of the interaction of the deleted versions of Pex5p with the above-mentioned proteins was determined in the two-hybrid system by quantifying the \( \beta \)-galactosidase activity. Deletions of the amino terminus of Pex5p (Pex5p-(252-612) and Pex5p-(307-612)) had a much more severe effect on the interaction with Pox1p and \( \Delta N \)-CAT2-\( \Delta C \) when compared with the interaction with the PTS1 protein Mdh3p (Fig. 5). The Gal4AD fusion with the TPR motifs of Pex5p (Pex5p-(307-612)) could still interact with Mdh3p, although with reduced efficiency when compared with wild-type Pex5p. However, the same construct expressing only the TPR motifs of Pex5p did not interact with either Pox1p or \( \Delta N \)-CAT2-\( \Delta C \). Conversely, deletion of the last three TPR motifs (Pex5p-(1-427)) completely abolished the interaction with Mdh3p, whereas the interaction with both Pox1p and \( \Delta N \)-CAT2-\( \Delta C \) could still be detected.

The N393D mutation in Pex5p, which has previously been shown to abolish completely the interaction with PTS1 proteins (Klein et al., 2001), did not severely affect the interaction with either Pox1p or \( \Delta N \)-CAT2-\( \Delta C \). Together the results show that the interaction of Pex5p with PTS1 proteins is clearly distinct from that with Pox1p and \( \Delta N \)-CAT2-\( \Delta C \). The two-hybrid results for the Pex5p(N393D) mutant were also in line with the results obtained with this mutant in the subcellular fractionation and protease protection experiments. This mutation has a severe effect on the import of PTS1 proteins into peroxisomes and leads to their mislocalization in the cytosol, but import of Pox1p is not affected.

**Mapping of the binding site for Pox1p on Pex5p**

To further delineate the Pox1p-binding site on Pex5p, we used a randomly mutagenized \( pex5 \) library. This library has been described before (Klein et al., 2001) and was used to identify the binding sites for PTS1 proteins (Klein et al., 2001) and Pex13p (Barnett et al., 2000; Bottger et al., 2000) on Pex5p. The library of \( pex5 \) mutants, fused to Gal4AD, was screened for mutants that had lost the interaction with Pox1p, fused to Gal4BD, in a two-hybrid assay. Loss of interaction was scored by the inability of transformants to grow on media lacking histidine. These mutants were subsequently analyzed by Western blotting for their ability to synthesize full-length Pex5p. Of the 20,000 transformants screened, 9 synthesized full-length Pex5p. Table II shows the results of the sequence analysis of these \( pex5 \) mutants. Although almost every \( pex5 \) mutant contained multiple amino acid substitutions, a clustering of mutations was observed in a small region in the amino terminus of Pex5p, spanning amino acids 253-264 (Fig. 6). Moreover, two residues in this region, Tyr-253 and Trp-261, were each found to be mutated in three different isolated mutants. These results indicate that this region in Pex5p is important for the interaction with Pox1p and are in
line with the deletion studies of Pex5p, which showed that the binding site for Pox1p is located somewhere between amino acids 252 and 427 of Pex5p.

To investigate whether these mutations specifically disturbed the interaction with Pox1p or resulted in a general loss of interaction with partner proteins because of a change in the overall structure of the mutant Pex5p, we tested the interaction with two proteins that bind to the amino terminus of Pex5p: Pex13p (Barnett et al., 2000; Bottger et al., 2000; Urquhart et al., 2000) and Pex14p (Schliebs et al., 1999; Saidowsky et al., 2001). Mdh3p was used as an example of a protein that binds to the carboxyl-terminal TPR motifs. We also included ΔN-CAT2-ΔC because of its similar behavior as Pox1p in the two-hybrid assay with the deleted versions of Pex5p (Fig. 5). For some of the isolated pex5 mutants, we first created single amino acid substitutions by site-directed mutagenesis. The mutants included in this analysis were Pex5p(Y253N), Pex5p(Q258R,A369T), Pex5p(W261A), Pex5p(D262G), and Pex5p(I264T). The results are summarized in Fig. 7. Based on the interactions, the pex5 mutants could be divided into two different groups.

Table II. pex5 mutants that have lost the interaction with Pox1p

<table>
<thead>
<tr>
<th>mutant</th>
<th>substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pex5.4</td>
<td>N73S W261R</td>
</tr>
<tr>
<td>pex5.10</td>
<td>N163S W261G</td>
</tr>
<tr>
<td>pex5.31</td>
<td>K31R Y253H</td>
</tr>
<tr>
<td>pex5.43</td>
<td>F155L W261R</td>
</tr>
<tr>
<td>pex5.54</td>
<td>Y253N</td>
</tr>
<tr>
<td>pex5.55</td>
<td>I264T N312D</td>
</tr>
<tr>
<td>pex5.76</td>
<td>N11S S174R D262G</td>
</tr>
<tr>
<td>pex5.163</td>
<td>Q258R A369T</td>
</tr>
<tr>
<td>pex5.215</td>
<td>R70G Q125R P181L Y253N E342G</td>
</tr>
</tbody>
</table>
Figure 6. Multiple sequence alignment of the region in Pex5p important for Pox1p interaction.
Sequences were aligned using ClustalX. White text on a black background denotes a sequence residue identity, and black text on a gray background indicates a similarity. The positions where mutations were found that disturb the interaction with Pox1p are indicated by arrowheads.

The first group consisted of Pex5p(Y253N), Pex5p(D262G), and Pex5p(I264T) (Fig. 7A). These mutants were specifically affected in the interaction with Pox1p and in the case of Pex5p(I264T) also with ΔN-CAT2-ΔC. The interaction of these mutants with Pex13p and Pex14p was only slightly reduced to about 60-80% of the interaction strength of wild-type Pex5p. Remarkably, we observed an increase in the strength of the interaction with Mdh3p for each of these pex5 mutants. These results indicate that these amino acid substitutions specifically affect the binding of Pox1p (and of ΔN-CAT2-ΔC in the case of Pex5p(I264T)) but do not disturb the overall structure of Pex5p. In contrast, Pex5p(Q258R,A369T) and Pex5p(W261A) that form the second group were severely affected in every interaction that takes place in the amino terminus of Pex5p (Fig. 7B). Probably the amino acid substitutions in these mutants affect the correct folding of the amino terminus of Pex5p.

Residues 239-300 of Pex5p are sufficient to bind Pox1p directly
To test whether the identified region in Pex5p is sufficient to bind Pox1p, we fused residues 239-300 of Pex5p to the Gal4AD domain. In a two-hybrid assay we could clearly detect an interaction between this Pex5 peptide of 62 amino acids and Pox1p resulting in growth of yeast colonies on plates without histidine (data not shown). The strength of this two-hybrid interaction was quantified by measuring the β-galactosidase activity (Fig. 8A). Although the strength of the interaction of the Pex5 peptide with Pox1p was reduced to 10% when compared with that of full-length Pex5p, it was still 200-fold above the background value (empty Gal4AD and Pox1p). A similar result was found for the interaction between the Pex5 peptide and ΔN-CAT2-ΔC. Introduction of either the Y253N or the I264T mutation in the Pex5 peptide by site-directed mutagenesis completely abolished the interaction with Pox1p (data not shown). These two-hybrid results were confirmed by in vitro binding experiments with purified proteins. For this we fused the Pex5p-(239-300) peptide to GST. We also
created two other GST-Pex5 peptides containing either the Y253N or the I264T mutation. These fusion peptides were purified over a glutathione-Sepharose 4B column and loaded onto an amylose column to which MBP-Poxlp was bound. After extensive washing of the column, to remove aspecifically bound proteins, MBP-Poxlp was eluted with maltose, and the eluates were analyzed by Western blotting. As can be seen in Fig. 8B, GST-Pex5p-(239-300), like the full-length fusion of Pex5p (Fig. 3), co-eluted with MBP-Poxlp. Furthermore, in agreement with the two-hybrid results, the mutated forms of the Pex5p-(239-300) peptide, containing either the Y253N or the I264T mutation, were unable to associate with MBP-Poxlp. These results show a specific and direct interaction between the identified region of Pex5p and Poxlp.

**Pex5p(Y253N) is specifically disturbed in the targeting of Poxlp**

To investigate the *in vivo* effect of the Pex5p(Y253N) mutation on the targeting of proteins to peroxisomes, we cloned this *pex5* mutant in a plasmid under the control of the *PEX5* promoter. Pex5p(Y253N) was co-expressed in *pex5Δ* cells with green fluorescent protein (GFP) containing a PTS1, GFP-SKL. Similarly, either Pex5p or Pex5p(N393D) was co-expressed in *pex5Δ* cells with GFP-SKL. As a control we used *pex5Δ* cells expressing only GFP-SKL. In *pex5Δ* cells expressing wild-type Pex5p from a plasmid, GFP-SKL showed clear punctated fluorescence, indicative for the import of GFP-SKL into peroxisomes (Fig. 9A). In *pex5Δ* cells only cytosolic fluorescence could be detected, and the same was found in *pex5Δ* cells expressing Pex5p(N393D), which is disturbed in the binding of PTS1 proteins (Klein *et al.,* 2001). In contrast, the *pex5Δ* cells expressing Pex5p(Y253N) showed a punctated pattern of GFP-SKL fluorescence indicating that the Y253N mutations did not affect the import of this artificial PTS1 protein into peroxisomes.

The effect of the Y253N mutation on the localization of Poxlp was also studied by a subcellular fractionation. We used the same cells as follows; *pex5Δ, pex5Δ* expressing Pex5p, *pex5Δ* expressing Pex5p(N393D), and *pex5Δ* expressing Pex5p(Y253N). Equal amounts of homogenate, organellar pellet, and supernatant were analyzed by Western blotting (Fig. 9B). Poxlp was localized in the pellet fraction in *pex5Δ* cells expressing wild-type Pex5p and mislocalized to the supernatant in *pex5Δ* cells. The N393D mutation did not affect Poxlp localization (see also Fig. 1A). However, in *pex5Δ* cells expressing Pex5p(Y253N), we found that Poxlp was mislocalized to the supernatant. The Pex5p(Y253N) mutation did not disturb peroxisomal targeting of the PTS1 proteins NH-Mdh3p and catalase A. These data show that disruption of the Pex5p-Poxlp interaction, caused by the Y253N mutation in Pex5p, specifically affects the *in vivo* targeting of Poxlp to peroxisomes.
Figure 7. Effect of amino acid substitutions in Pex5p on the interaction with partner proteins.

The strength of the two-hybrid interaction between Pex5p (fused to Gal4AD) and a number of partner proteins (fused to Gal4BD) was quantified by measuring the β-galactosidase activity. For each partner protein used, the interaction with wild-type Pex5p was set to 100%. The values given are the mean ± S.D. of three measurements on independent transformants. Three pex5 mutants were specifically affected in the interaction with Pox1p (A) whereas two other mutants had lost most of the interactions that were tested (B).
A novel Pex5p-mediated import pathway into peroxisomes

Figure 8. Amino acids 239-300 of Pex5p are sufficient to bind Pox1p.
Amino acids 239-300 of Pex5p were fused to Gal4AD and tested in a two-hybrid assay for the interaction with Gal4BD fusions of Pox1p and ΔN-Cat2-ΔC (A). The strength of the interaction was determined by measuring β-galactosidase activity. For each protein fused to Gal4BD the interaction with wild-type Pex5p (fused to Gal4AD) was set to 100%. The values given are the mean ± S.D. of three measurements on independent transformants. As a control the interaction of Pox1p and ΔN-Cat2-ΔC with the empty Gal4AD (AD) was measured. Pex5p and Pex5p-(239-300) did not interact with the empty Gal4BD (not shown). Amino acids 239-300 of wild-type (WT) Pex5p (GSTpepWT), Pex5p(Y253N) (GSTpepY253N), and Pex5p(I264T) (GSTpepI264T) were fused to GST and tested in an in vitro binding assay for their interaction with MBP-Pox1p (B). Purified GST fusion proteins (100 μg each) were passed over an amylose column loaded with 250 μl of cleared lysate containing MBP-Pox1. After extensive washing, the column was eluted with 20 mM maltose, and the proteins in the elution fractions were subjected to SDS-PAGE followed by Western blotting. Antibodies were directed against Pox1p (top panel) or GST (lower panel).
Discussion

There are two well-characterized peroxisomal targeting sequences, PTS1 and PTS2, that direct proteins into peroxisomes. Soluble receptors have been identified, Pex5p for PTS1 and Pex7p for PTS2, that specifically interact with these PTSs and are absolutely required for their import into peroxisomes. Only a few peroxisomal matrix proteins have neither a PTS1 nor a PTS2 and the signals that target these proteins to peroxisomes remain to be characterized (Hill et al., 1988; Small et al., 1988; Bruinenberg et al., 1990; Dmochowska et al., 1990; Wang et al., 1998). In this study we have analyzed in detail how one of these proteins, S. cerevisiae acyl-CoA oxidase (Poxlp), reaches its subcellular destination.

Targeting of Poxlp is dependent on Pex5p, which functions as receptor for PTS1 proteins. This dependence is based on direct interaction because Poxlp and Pex5p bind to each other in a yeast two-hybrid trap and in in vitro reconstitution assays. The carboxyl-terminal part of Poxlp, where the PTS1 is normally found, is not required for binding since 3- or 17-amino acid terminally deleted versions of Poxlp bind equally well to Pex5p. Also the way in which Pex5p interacts with Poxlp is unorthodox. Previous studies demarcated the part of Pex5p involved in PTS1 recognition to the carboxyl-terminal half containing the TPR repeats (Brocard et al., 1994; Dodt et al., 1995; Terlecky et al., 1995; Gatto et al., 2000; Klein et al., 2001). Import of Poxlp, however, does not require this well-defined PTS1-binding site on Pex5p; the Pex5p(N393D) mutant, which is selectively disturbed in the interaction with PTS1 proteins (Klein et al., 2001), mislocalized PTS1 proteins to the cytosol, but Poxlp was efficiently imported into peroxisomes. In line with these findings, this pex5 mutant was still able to interact with Poxlp, whereas the interaction with the PTS1 protein Mdh3p was abolished. Recently, similar observations were reported by Yang et al. (2001). They showed that a point mutation within the TPR domain of S. cerevisiae Pex5p at position 495 (Asn to Lys) abolishes the import of catalase, a PTS1 protein, but not that of Poxlp. These data suggest, therefore, that Pex5p binds Poxlp in a way that is clearly distinct from the interaction with PTS1 proteins. We have located the Pex5p part interacting with Poxlp amino-terminally of the TPR containing half by a combination of deletion and mutational experiments and in vitro reconstitution assays. The part of Pex5p consisting of amino acids 239-300 is sufficient for binding Poxlp, and important residues are located in the area spanning amino acids 253-264. Multiple sequence alignment of this area using the Pex5p sequences from S. cerevisiae, C. albicans, P. pastoris, Y. lipolytica, and H. sapiens shows a high amino acid identity between S. cerevisiae and C. albicans. It is noteworthy that of the five residues that
AA novel Pex5p-mediated import pathway into peroxisomes

Figure 9. Effect of amino acid substitutions in Pex5p on the localization of peroxisomal matrix proteins.
Wild-type cells, pex5Δ cells, and pex5Δ cells expressing either Pex5p(N393D) or Pex5p(Y253N) were all (co) transformed with a plasmid expressing GFP-SKL. Subcellular distribution of GFP-SKL was visualized by fluorescence microscopy (A). Wild-type cells, pex5Δ cells, and pex5Δ cells expressing Pex5p(N393D) or Pex5p(Y253N) were all (co) transformed with a plasmid expressing NH-tagged Mdh3p and subjected to subcellular fractionation. Equivalent volumes of the 600 x g post-nuclear supernatant (H), 17,500 x g pellet (P), and 17,500 x g supernatant (S) were analyzed by Western blotting (B). The antibodies used were directed against Pox1p, catalase A (Cta1p), or the NH epitope to detect NH-tagged Mdh3p.

were found to be mutated in S. cerevisiae Pex5p, four are strictly conserved in C. albicans Pex5p. Considering this high sequence similarity between S. cerevisiae and C. albicans in this region, it is tempting to speculate that a similar mechanism exists for the targeting of acyl-CoA oxidase in both species. In line with this suggestion, the carboxyl-terminal three amino acids of C. albicans acyl-CoA oxidase (LSK) do not match the PTS1 consensus. Also in the closely related species C. tropicalis the carboxyl terminus of acyl-CoA oxidase does not resemble a PTS1 sequence. For C.
tropicalis acyl-CoA oxidase it has been suggested that it contains internal targeting sequences that direct the protein to peroxisomes (Small et al., 1988). Unfortunately, the C. tropicalis Pex5p sequence is not available. It remains to be determined, therefore, whether there is high sequence similarity in this region of Pex5p between S. cerevisiae and C. tropicalis. There is less conservation in this Pex5p region in the other three species, Y. lipolytica, P. pastoris, and H. sapiens. In the latter two cases this may be explained by the fact that acyl-CoA oxidase in these species is targeted to peroxisomes via the classical PTS1 pathway (Fournier et al., 1994; Koller et al., 1999).

The amino-terminal half of Pex5p contains a number of WXXXF motifs. Two of these motifs are present in S. cerevisiae and seven in H. sapiens. In humans it has been shown that these motifs form multiple binding sites for Pex14p (Schliebs et al., 1999; Saidowsky et al., 2001), whereas in S. cerevisiae one of these motifs is essential for the association with the SH3 domain of Pex13p (Barnett et al., 2000; Bottger et al., 2000). However, there is an additional inverted motif, FXXFW, in Pex5p that is conserved in yeasts but not in human. In human only the tryptophan is conserved in this region, and phenylalanine is not present. In S. cerevisiae the FXXXW motif is located in the core of the Pox1p-interacting region and therefore may play a pivotal role in this interaction. The conserved Trp-261 within this motif was found mutated in three independent clones in our mutant screen. However, substitution of this conserved residue affected several interactions that take place in the amino-terminal half of Pex5p, including the interaction with Pex14p. This may indicate that this highly conserved tryptophan residue is also important for the correct folding of the amino terminus of Pex5p.

Our study also indicates that the binding sites on Pex5p for Pox1p and ΔN-CAT2-AC are partially overlapping. Some pex5 mutants that have lost the interaction with Pox1p are also affected in the interaction with ΔN-CAT2-AC; Pex5p(I264T) shows a complete loss of interaction with ΔN-CAT2-AC, and Pex5p(D262G) shows a strongly reduced interaction with ΔN-CAT2-AC. The Y253N mutation, however, is specific for the Pox1p interaction because it completely abolishes the association with Pox1p but does not affect ΔN-CAT2-AC binding. Furthermore, in support of the partially overlapping binding sites for Pox1p and ΔN-CAT2-AC on Pex5p we found that the Pex5p-(239-300) peptide interacted with both proteins in a two-hybrid assay. The fact that Cat2p, from which the PTS1 has been deleted can still interact with Pex5p (Elgersma et al., 1995) indicates that besides the PTS1 there are other residues in this protein that contact Pex5p. As shown here this additional site of interaction for Cat2p
A novel Pex5p-mediated import pathway into peroxisomes

on Pex5p is located outside the TPR domain and partially overlaps with the Pox1p-binding site.

There have been other reports of peroxisomal matrix proteins that use accessory sequences to interact with Pex5p. For human catalase the lysine at the -4 position is essential for its import into peroxisomes (Purdue and Lazarow, 1996). Lametschwandtner et al. (1998) showed that in the hexadecapeptides they studied, residues upstream of the carboxyl-terminal tripeptide influenced the interaction strength with Pex5p. Although the appealing and simple concept of the original definition of a PTS1 may still hold for most proteins, we like to suggest on the basis of accumulating data in the literature and our own in depth analysis of Pox1p that there might be a whole spectrum of peroxisomal matrix proteins that differ in their dependence on a PTS1 for Pex5p-mediated targeting to peroxisomes. At one end there are proteins that use a consensus PTS1 to interact with Pex5p only at the PTS1-binding site. Then there are proteins, like Cat2p, that use two different ways to interact with Pex5p via their PTS1 and via accessory sequences. In the case of Cat2p these accessory sequences alone are sufficient to interact with Pex5p and direct the protein to peroxisomes (Elgersma et al., 1995, and our results). Another protein that might also use accessory sequences is S. cerevisiae Mdh3p. In the homologous context, i.e. Mdh3p expressed in S. cerevisiae, many alterations of the PTS1 of this protein are allowed without disrupting the interaction with Pex5p or targeting to peroxisomes (Elgersma et al., 1996b). One explanation for this finding could be that the interaction of accessory sequences in Mdh3p with Pex5p compensates for weaker binding of the PTS1 peptide to its binding site. However, when these non-consensus PTS1s are fused to a heterologous reporter protein, they fail to target to peroxisomes, presumably because these compensatory interactions cannot occur between the reporter protein and Pex5p. At the other end of the spectrum are proteins that do not have a recognizable PTS1. S. cerevisiae Pox1p is an example of such a protein, and the accessory sequences in this protein are sufficient to bind Pex5p and to target it to peroxisomes. This protein does not use the PTS1-binding site of Pex5p but the identified region just upstream of the Pex5p TPR domain. Thus in the case of Pox1p the accessory sequences function as an internal peroxisomal targeting signal of a new type, PTS3. Because protein folding probably precedes import into peroxisomes, it is conceivable that the internal PTS3 does not consist of a linear epitope, like the PTS1, but is composed of conformational epitopes within the folded protein. Future studies should reveal the structural details of this targeting signal.

Many studies have indicated a remarkable variation in the peroxisomal protein import pathway. The high degeneracy of the PTS1 and the use of accessory sequences in peroxisomal matrix proteins is one example. Also in the PTS2 import pathway a
number of variations exist, depending on the protein and the organism. For instance, glyoxysomal malate dehydrogenase of watermelon is different from other peroxisomal malate dehydrogenases because it contains a PTS2 (Gietl et al., 1994), whereas in the other organisms it possesses a PTS1. Even more remarkable is the absence of the entire PTS2 pathway in *Caenorhabditis elegans* (Motley et al., 2000). Proteins that contain a PTS2 in other organisms, like thiolase (Osumi et al., 1991; Swinkels et al., 1991; Erdmann, 1994; Glover et al., 1994b), alkylhydroxyacetonephosphate synthase (de Vet et al., 1997) and phytanoyl-CoA hydroxylase (Jansen et al., 1997) are equipped with a PTS1 in *C. elegans*. Whether the identified PTS3 pathway is only present in some yeast species or whether it is a more general peroxisomal import pathway, conserved among different proteins and different organisms, remains to be investigated.

**Acknowledgements**

We thank Will Stanley and members of our group for helpful discussions.

**References**


A novel Pex5p-mediated import pathway into peroxisomes


Lee, M.S., Mullen, R.T. and Trelease, R.N. (1997) Oilseed isocitrate lyases lacking their essential type 1 peroxisomal targeting signal are piggybacked to glyoxysomes. Plant Cell, 9, 185-197.


