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

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Chapter 2

Recognition of the peroxisomal targeting signal type 1 by the import receptor Pex5p

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Abstract

We have studied how Pex5p recognizes peroxisomal targeting signal type 1 (PTS1) containing proteins. A randomly mutagenized pex5 library was screened in a two-hybrid setup for mutations that disrupted the interaction with the PTS1 protein Mdh3p or for suppressor mutations that could restore the interaction with Mdh3p containing a mutation in its PTS1. All mutations localized in the tetratricopeptide repeat (TPR) domain of Pex5p. The Pex5p TPR domain was modeled based on the crystal structure of a related TPR protein. Mapping of the mutations on this structural model revealed that some of the loss-of-interaction mutations consisted of substitutions in α-helices of TPRs with bulky amino acids, probably resulting in local misfolding and thereby indirectly preventing binding of PTS1 proteins. The other loss-of-interaction mutations and most suppressor mutations localized in short, exposed, intra-repeat loops of TPR2, TPR3, and TPR6, which are predicted to mediate direct interaction with PTS1 amino acids. Additional site-directed mutants at conserved positions in intra-repeat loops underscored the importance of the loops of TPR2 and TPR3 for PTS1 interaction. Based on the mutational analysis and the structural model, we put forward a model as to how PTS1 proteins are selected by Pex5p.
Introduction

After synthesis in the cytosol, many proteins are sorted to the various organelles characteristic of a eukaryotic cell. For the import of matrix proteins into the peroxisome, two different pathways have been identified (for reviews, see Kunau, 1998; Hettema et al., 1999; Subramani et al., 2000). Most peroxisomal matrix proteins contain a peroxisomal targeting signal type 1 (PTS1) consisting of three amino acids at the extreme carboxyl terminus. A consensus sequence has been defined as (S/C/A)(K/R/H)(L/M) (Gould et al., 1989; Swinkels et al., 1992). Only a few peroxisomal proteins contain a peroxisomal targeting signal type 2 (PTS2). The consensus sequence for PTS2 is (R/K)(L/V/I)X₅(H/Q)(L/A), and it is located in the N-terminal part of a protein (Swinkels et al., 1991; Gietl et al., 1994; Glover et al., 1994).

The first step in the import process is the recognition of the targeting signal by the receptor protein. PTS1 is recognized by Pex5p (peroxins-5 protein) (McCollum et al., 1993; Dodt et al., 1995; Fransen et al., 1995; Tabak et al., 1995; Wiemer et al., 1995), and PTS2 by Pex7p (Marziocch et al., 1994; Zhang and Lazarow, 1995). After binding PTS1-containing proteins in the cytosol, the Pex5p-cargo complex docks at the peroxisomal membrane. Several proteins are thought to be part of the docking complex, e.g. the integral peroxisomal membrane protein Pex13p (Elgersma et al., 1996a; Erdmann and Blobel, 1996; Gould et al., 1996; Barnett et al., 2000; Bottger et al., 2000) and the two peroxisomal membrane-associated proteins Pex14p (Albertini et al., 1997; Brocard et al., 1997; Fransen et al., 1998; Girzalsky et al., 1999; Schliebs et al., 1999) and Pex17p (Huhse et al., 1998). Not much is known about the actual translocation step across the peroxisomal membrane.

Deletion studies have shown that the seven (or eight, depending on the organism) tetratricopeptide repeats (TPR) in the C-terminal part of Pex5p are important and also sufficient for the binding of PTS1 proteins (Brocard et al., 1994; Terlecky et al., 1995). To investigate in more detail how Pex5p binds PTS1-containing proteins, we have now used a different approach. A library of pex5 mutants was created by random mutagenesis of Saccharomyces cerevisiae PEX5. The yeast two-hybrid system was used to select pex5 mutants that had lost the capacity to bind the PTS1-containing protein Mdh3p (malate dehydrogenase-3 protein) from S. cerevisiae. In a separate screen, pex5 mutants were selected that had gained interaction with a mutant PTS1 protein (Mdh3-SEL). We also derived a structural model for the TPR motifs of Pex5p based on the crystal structure of the three TPR motifs in protein phosphatase-5 (PP5) (Das et al., 1998). By relating the mutations found in Pex5p to this structural model, we were able to explain why certain mutations affected the interaction with Mdh3p.
On the basis of orthologous sequence alignments, additional mutations were made in strongly conserved amino acids in Pex5p by site-directed mutagenesis. The selected mutants, together with the structural model, allowed us to put forward a proposal as to how PTS1-containing proteins are selected by Pex5p.

**Experimental Procedures**

**Strains and culture conditions.**

The yeast strains used in this study were *S. cerevisiae* BJ1991 (MATα, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2), BJ1991pex5Δ (MATα, pex5::LEU2, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2), HF7c (MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, gal4-542, gal80-538, LYS2::GAL1 UAS-GAL1 TATA-HIS3, URA3::GAL17mers(x3)-CTATGAll-TATA-lacZ), PCY2 (MATα, Agal4, Agal80, URA3::GAL1-lacZ, lys2-801, his3-Δ200, trp1-Δ63, 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.

**Cloning procedures.**

Standard techniques for DNA manipulations were used (Sambrook *et al.*, 1989). The construct pAN1 was made by cloning the complete open reading frame of PEX5 between EcoRI and HindIII in the multiple cloning site of pUC19. Some restriction sites in the multiple cloning site were deleted, and some additional sites were introduced: EcoRI and BamHI at the 5'-end of PEX5 and PstI, SpeI, SpeI and HindIII at the 3'-end of PEX5. Two additional restriction sites were introduced in PEX5 by silent mutations: a XbaI site at position 1140 by mutating the codon for leucine 381 from CTG to CTA, and a SalI site at position 1356 by mutating the codon for leucine 452 from TTA to TTG and the codon for serine 453 from AGC to TCG. The plasmid encoding the Gal4 activation domain fusion with PEX5 (pAN4) was constructed by cloning PEX5 from pAN1 EcoRI-SpeI in the two-hybrid vector pPC86 (Chevray and Nathans, 1992). The plasmid encoding the Gal4 DNA-binding domain fusion with GFP-SKL (pAN25) was constructed by PCR on GFP containing the S65T mutation with primers p330 and p331. The PCR product was cloned into EcoRI-PstI in pAN1, resulting in pAN21. Subsequently, the two complementary oligonucleotides
p332 and p333 were ligated between the PstI and SpeI sites of pAN21, resulting in pAN22. The insert from pAN22 was cloned into SalI-SpeI in the multiple cloning site of the two-hybrid vector pPC97 (Chevray and Nathans, 1992). The amino acid sequence of the extreme carboxyl terminus reads GMDELYLQGGGSKL. Gal4 DNA-binding domain fusions with the Pex13p SH3 domain (pGB17) and Pex14p (pGB47) have been described before (Bottger et al., 2000). pPR6/56 encodes a fusion protein of the Gal4 DNA-binding domain with Pex8p (amino acids 19-589) (Rehling et al., 2000). pDBMDH3 was made by PCR on pEL102 (Elgersma et al., 1996b). The PCR product was cloned into SalI-SpeI in the multiple cloning site of pPC97 (Chevray and Nathans, 1992). pDBMDH3-SEL was made by PCR on pMDH3-SEL (Elgersma et al., 1996b). The PCR product was cloned into SalI-SpeI in the multiple cloning site of pPC97. The glutathione S-transferase (GST)-Pex5p fusion protein (pGST-Pex5p) has been described before (Bottger et al., 2000). A GST fusion with the Pex5p-N393A mutant was made by site-directed mutagenesis (see below). A maltose-binding protein (MBP) fusion with Mdh3p (pAN60) was made by digestion of pEL102 with BamHI and cloning the fragment into pMal-c2 (New England Biolabs Inc.). An MBP fusion with MDH3ΔSKL (pAN56) was made by PCR on pEL102 with primers p325 and p326. The PCR product was cloned into BamHI-HindIII in pMal-c2. The oligonucleotides used were p325 (AAGGATCCATGGTCAAAGTCGCAATTCTTG) p326 (AAAAAGCTTCAAGAGTCTAGGATGAAACTCTTG), p330 (CGGAATTCTGTCGACTGGATCCATGAGTAAAGGAGAAGAACTTTTC), p331 CCCAAGCTTGGCATTGCCTGCAGGTATAGTTCATCCATGTG), p332 (GGGTGGTGTTCCAAGCTATGA) and p333 (CTAGTCATAGCCTGGAAACCACCCACCCCTGCA).

Construction of the pex5 mutant libraries
The PEX5 gene spanning 1839 base pairs was randomly mutagenized by error-prone PCR. We used Taq DNA polymerase that lacks the 3' → 5' proofreading activity (Eckert and Kunkel, 1991). pAN 1 was used as a template under standard reaction conditions (10 mM Tris-HCl (pH 7), 50 mM KCl; 1.5 mM MgCl2, 0.8 mM dNTPs, 0.03 units/μl Taq polymerase) (Zhou et al., 1991) using the M13/pUC primers (6 ng/μl) 1224 and 1233 (New England Biolabs Inc.).

The mutation frequency was determined by sequencing 23 randomly picked pex5 clones at 1.8 x 10⁻³ per nucleotide synthesized, resulting in 3.3 mutations per pex5 clone synthesized. The mutagenized pex5 was split into two parts using the XbaI site at position 1140. In this way, two sublibraries of pex5 mutants were made (see Fig. 1). The C-mutant pex5 library was made by replacing the 3'-XbaI-PstI fragment of the wild-type PEX5 sequence (nucleotides 1141-1839) in plasmid pAN1 with the
corresponding mutagenized \textit{pex5} sequence. This mutagenized region of \textit{PEX5} includes the motifs TPR3-7 (amino acids 381-612). The N-mutant \textit{pex5} library was made from the same PCR product as the C-mutant \textit{pex5} library by replacing the 5'-BamHI-XbaI fragment of the wild-type \textit{PEX5} region (nucleotides 1-1140) in plasmid pAN1 with the corresponding mutagenized \textit{pex5} sequence. This includes the motifs TPR1 and TPR2. For screening in the two-hybrid system (Fields and Song, 1989), both libraries were subcloned in plasmid pPC86 (using EcoRI-SpeI), leading to a fusion of the \textit{pex5} mutants with the Gal4 transcription activation domain. For \textit{in vivo} complementation studies, both libraries were subcloned (BamHI-PstI) behind the \textit{PEX5} promoter in plasmid pEL91.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{construction_of_pex5_mutant_libraries.png}
\caption{Construction of the \textit{pex5} mutant libraries.}
\end{figure}

The XbaI restriction site was used to swap wild-type \textit{PEX5} sequences for randomly mutagenized \textit{pex5} sequences.
Two-hybrid screen with the C-mutant and N-mutant pex5 libraries

Yeast strain HF7c expressing pDBMDH3 was transformed with the C-mutant pex5 library fused to the Gal4 transcription activation domain; 20,000 double transformants were selected on 2% glucose/leu- trp- plates. These transformants were replica-plated onto 2% glucose/leu- trp- his+ plates containing 25 mM 3-amino-1,2,4-triazole (3-AT) to test for the interaction between the PTS1 protein Mdh3p and the mutant pex5 library. About 300 transformants (1.5%) were selected that were not able to grow on plates without histidine. Total protein was isolated from 72 of the 300 transformants for Western blotting, using anti-Pex5p antibodies; 28 of the 72 transformants expressed a full-length Gal4 transcription activation domain-Pex5p fusion. The 28 plasmids coding for the full-length Pex5p were rescued and retransformed to HF7c for two-hybrid analysis. Nine of the 28 mutants displayed interaction with Mdh3p, indicating a first-round false-negative mutant. The remaining 19 clones were sequenced to determine the sites of the mutations. Five mutants contained more than two amino acid substitutions and were not further analyzed. The N-mutant pex5 library was screened for loss of interaction with Mdh3p in the same way as described for the C-mutant pex5 library. The percentage of mutants that could not grow was much larger for the N-mutant pex5 library than for the C-mutant pex5 library. Of the 3000 transformants, 100 were not able to grow. However, only 8 of the 100 selected mutants produced the full-length fusion protein, and from these mutants, the plasmid was rescued. After retransformation, only one of these mutants (pex5.42) still showed no interaction with Mdh3p.

Suppressor analysis

Yeast strain HF7c expressing pDBMDH3-SEL was transformed with either the C-mutant pex5 library or the N-mutant pex5 library and in each case, 100,000 transformants were made and plated onto 2% glucose/leu- trp- his+ plates containing 25 mM 3-amino-1,2,4-triazole. Transformants that were able to grow on these plates and thus contained pex5 mutants that had gained an interaction with Mdh3-SEL were selected. One pex5 suppressor mutant (pex5.sup2) was isolated from the N-mutant pex5 library, and three pex5 suppressor mutants (pex5.sup1, pex5.sup3 and pex5.sup21) were isolated from the C-mutant pex5 library. These mutants were sequenced to determine the sites of the mutations.

In vitro binding assay

The GST and MBP fusion proteins were expressed and isolated as described elsewhere (Barnett et al., 2000; Bottger et al., 2000). For the in vitro binding experiments, 250 μl of cleared lysate containing the MBP fusion protein was first

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bound to an amylose resin column; and subsequently, the purified GST fusion protein (100 µg) was passed over the column. After washing, bound proteins were eluted with maltose and analyzed by SDS-polyacrylamide gel electrophoresis. The fusion proteins used in these experiments were MBP-Mdh3p, MBP-Mdh3ASKL, GST-Pex5p and GST-Pex5p-N393A.

**Modeling of the Pex5p TPR domain**
The small amino acids at positions 8, 10, and 27 of the TPR motifs of Pex5p were aligned with the small amino acids of the PP5 TPR motifs. By using this optimized alignment, it was possible to model the Pex5p TPR domain based on the PP5 TPR crystal structure (Das et al., 1998). For the modeling procedure, the Pex5p TPR domain region was split into two parts, TPR motifs 1-3 and TPR motifs 5-7, thus excluding TPR4. Alignments and initial optimized template PP5 and model Pex5p overlays were generated using the Swiss-PdbViewer /SWISS-MODEL interface (Guex et al., 1999). Subsequent models were checked, refined, and energy-minimized using WHAT IF (Vriend, 1990) and the Biotech Validation Suite in combination with the Swiss-PdbViewer. Models show backbone root mean square deviations from the PP5 template of 0.4-0.8 Å. The final total energies were -4600 KJ/mol for TPR1-3 and -5900KJ/mol for TPR 5-7.

**Site-directed mutagenesis**
Site-directed mutations were introduced by using the Quickchange site-directed mutagenesis Kit (Stratagene) using pAN4 as a template. The oligonucleotides used were N325A (GCTGCCTACTGATGGAAGCCGGAGCCAAATTGAGCG), N360A (GGTCTAGTACAAAAACCCAGGAGCTGAAAGAGTTGAGCGGC), E363A (CCCCGAATGAAAAACGGTTGAAACGCTAAGC), I389D (GAGGCAATGAAAACTTTAGCCGAGCATATATACGCGGCC), N393A (CTTTAGCGATAAGTTATAGCCGGAGGTATTATGAGCGGCC), N393G (CTTTAGCGAGGTATTATGAGCGGCC), E394A (GCGATAAGTTATATAGCCGGAGGTATTATGAGCGGCC), N503A (GGGGCTTCATTGGCCGCTTCATAGATCAGAGG), S504A (GCTTCATTGGCATTCCCATAGATCAGAGG), N505A (GGCTTCATTGGCATTCCCATAGATCAGAGG), L531A (GTTAGAGCTCGCTATAATGCGGCGGTATCATCCATGAATATAGGC), N537A (GGCGGTATCATCCATGGGCTATAGGCTGTATTTTCAAAGGAGG). The mutations that were introduced using these primers are underlined. For each primer listed, also the complementary primer was used.

β-Galactosidase activity was determined as described (Miller, 1972; Lametschwandtner et al., 1998).
Results

Isolation of pex5 mutants disturbed in PTS1 recognition
To investigate which parts of Pex5p are responsible for contacting PTS1-containing proteins, mutations were introduced randomly into the S. cerevisiae PEX5 gene by PCR amplification (see “Experimental Procedures”). For technical reasons, the mutant library was divided into two halves (Fig. 1). The N-terminal part of the mutant library was linked to the wild-type C-terminal part, and the C-terminal part of the mutant library was linked to the wild-type N-terminal part. Both libraries were used for a yeast two-hybrid interaction screen to select mutants that had lost the capacity to interact with yeast Mdh3p, a PTS1 (SKL)-containing peroxisomal matrix protein (see “Experimental Procedures”). Each of the selected mutants was analysed by Western blotting to determine whether full-length Pex5p was still produced at normal levels.

From the C-mutant pex5 library, 14 pex5 mutants were selected, whereas from the N-mutant pex5 library, only one mutant (pex5.42) was selected that expressed full-length Pex5p (Table I). These numbers confirm previous results that the C-terminal TPR domain is responsible for PTS1 recognition (Brocard et al., 1994; Dodt et al., 1995; Terlecky et al., 1995).

None of the mutations we found gave rise to a gross structural alteration or instability of Pex5p since the two-hybrid interactions of these mutants with known partner peroxins such as Pex8p (Rehling et al., 2000), Pex13p (Elgersma et al., 1996a; Erdmann and Blobel, 1996; Gould et al., 1996; Barnett et al., 2000; Bottger et al., 2000), and Pex14p (Albertini et al., 1997; Brocard et al., 1997; Schliebs et al., 1999) were unaffected, and proteins were expressed at wild-type levels (data not shown). The pex5 mutants, when expressed in vivo under the control of the PEX5 promoter, were unable to complement the oleate non-utilizer (oun) phenotype of a pex5Δ strain, indicating that they had also lost their function in vivo (data not shown). The sites of the mutations in these pex5 mutants were determined, and we found that for every mutant, there was at least one mutation located in a TPR motif (Table I). This indicated that these motifs are important for the interaction with PTS1 proteins, i.e. in TPR2, TPR3, and TPR5-7. No mutations were found in TPR1 and TPR4, suggesting that these motifs do not contribute to PTS1 interaction. One residue, asparagine 393 in TPR3, was found to be mutated in five different clones (pex5.38, pex5.45, pex5.46, pex5.70, and pex5.97) suggesting an important role for this residue in PTS1 recognition.
Table 1. *pex5* mutants that have lost the interaction with Mdh3p.

Randomly mutagenized *pex5* libraries were screened for *pex5* mutants that had lost the interaction with the PTS1 protein Mdh3p in the two-hybrid system. The sites of the mutations were determined by sequencing. For the underlined substitutions, it is indicated in which TPR motif the mutations are located.

<table>
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<th>Substitution</th>
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*In vitro* binding studies

Previous studies of Pex5 proteins in different species have shown that the interaction between Pex5p and PTS1-containing proteins is direct (Fransen et al., 1995; Terlecky et al., 1995; Wiemer et al., 1995). To determine if the interaction between *S. cerevisiae* Pex5p and Mdh3p is direct and dependent on PTS1, an *in vitro* binding assay was carried out. The genes encoding Pex5p and Mdh3p were fused in frame to DNA sequences encoding GST and MBP, respectively, and the chimeric genes were expressed in *E.coli*. As a control, mutant Mdh3p lacking its PTS1 (Mdh3ΔSKL) was fused to MBP and expressed in *E.coli*. GST-Pex5p was purified on a glutathione-Sepharose column, and the purified fusion protein was tested for its ability to bind to a column with immobilized MBP-Mdh3p and MBP-Mdh3ΔSKL, respectively. Fig. 2 shows that GST-Pex5p interacted with MBP-Mdh3p, but not with MBP-Mdh3ΔSKL.
Furthermore, GST alone was not retained on the MBP-Mdh3p column (data not shown). These data indicate that the interaction between Pex5p and Mdh3p is direct and is dependent on the PTS1 of the latter protein. To determine whether asparagine 393, found to be mutated in five different clones, is also important for direct interaction with Mdh3p, an in vitro binding experiment was performed with the Pex5pN393D mutant. GST-Pex5p-N393D did not interact with Mdh3p, because it was not retained on a column with immobilized MBP-Mdh3p. These data underscore the important role of asparagine 393 in TPR3 of Pex5p for PTS1 interaction.

**Figure 2. In vitro binding experiments of Pex5p and Mdh3p.** Purified GST-Pex5p (100 μg) or GST-Pex5p-N393D (100 μg) was passed over an amylose column loaded with 250 μl of cleared lysate containing either MBP-Mdh3p or MBP-Mdh3ΔSKL. After washing, the columns were eluted with 20 mM maltose, and the proteins in the elution fractions were separated by SDS-polyacrylamide gel electrophoresis and revealed by staining with Coomassie blue WT, wild-type.

### Isolation of pex5 suppressor mutants
To identify additional residues in Pex5p involved in PTS1 recognition, we carried out a positive two-hybrid screen. Instead of screening for *pex5* mutants that had lost the interaction with a PTS1 protein, we screened for *pex5* mutants that gained interaction with the PTS1 mutant Mdh3-SEL. Four suppressor mutants were selected in this positive two-hybrid screen (Table II). Remarkably, one mutant (*pex5.sup2*) contained a glutamic acid-to-lysine substitution at position 361 in TPR2, a mutation that is exactly the opposite of the mutation introduced in the PTS1, i.e. lysine to glutamic acid. This suppressor mutant specifically suppressed the PTS1 mutation because it did not interact with Mdh3p from which the PTS1 had been deleted (Mdh3ΔSKL). The other suppressor mutants still showed a weak, but detectable interaction with Mdh3ΔSKL (see "Discussion"). It is noteworthy that all suppressors were still able to bind to Mdh3p with a wild type PTS1 (Mdh3-SKL) indicating that the mutations had no gross structural effects on the protein.
Table II. *pex5* suppressor mutants restore the interaction with a PTS1 mutant

Two-hybrid interaction was measured in two-hybrid reporter strain HF7c by growth on glucose plates lacking histidine and containing 25 mM 3-AT. (+, growth was observed after 4 days; -, no growth was observed after 4 days; +*, when double-transformed cells were first grown on glucose plates containing histidine and later were transferred to glucose plates lacking histidine (and containing 25 mM 3-AT), growth was observed on these his- plates. When cells were directly plated after the transformation onto glucose plates without histidine (and containing 25 mM 3-AT), no growth was detectable.

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**Modeling of the Pex5p TPR domain**

The mutations that altered PTS1 binding were not clustered in a small region of Pex5p as expected on the basis of the small PTS1 ligand comprising only three amino acids. Instead, the mutations were distributed over the entire TPR domain and were present in most TPR motifs, except TPR1 and TPR4 (Fig. 3 and Table I). To understand why the mutations led to a loss of Mdh3p interaction, we used the known three-dimensional structure of another TPR protein, PP5 (Das *et al.*, 1998). The crystal structure of PP5 shows that individual TPR motifs consist of two α-helices, α-helix A and α-helix B, which are antiparallel. The small hydrophobic amino acids at position 8, 20, and 27 are important for packing these α-helices close together. Most of the TPR motifs of Pex5p also contain these small hydrophobic amino acids such as glycine and alanine at position 8, 20, and 27 (Fig. 3). A common feature in many TPR motifs is a proline at position 32. This proline at the end of α-helix B probably supports a turn in the structure (Goebl and Yanagida, 1991) leading to an antiparallel arrangement of α-helix A relative to the α-helix B of the previous TPR. Analyzing the primary amino acid sequence of the seven TPR motifs of Pex5p for these features indicated that TPR4 differed from the other six TPR motifs. The amino acid sequence that should form the fourth TPR motif should be 34 amino acids, instead of the 42 amino acids found in between TPR3 and TPR5. In addition, there are no small amino
Mapping of the PTS1-binding site on Pex5p

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**Figure 3. Sequence alignment of Pex5p TPR motifs.**

Aligned are TPR1-3 and TPR5-7 from *S. cerevisiae* (Sc), *H. polymorpha* (Hp), *P. pastoris* (Pp), and *H. sapiens* (Hs). The part of the TPR motif that forms an α-helix or a loop (based on PP5) is indicated by arrows. The stars mark the small amino acids at positions 8, 20, and 27 of a TPR motif. These amino acids were aligned with the small amino acids of the TPR motifs of PP5. The hatched arrows indicate the positions where mutations were found that affect the packing of the α-helices such that PTS1 recognition is affected as a secondary effect. The black arrows indicate the positions involved in PTS1 binding. The white arrows indicate the mutant position found in the suppressor screen with Mdh3-SEL.
acids found at the positions 8, 20, and 27, and also proline 32 is not present. We suggest therefore that TPR4 is not a true TPR motif, but may rather function as a flexible hinge that connects two clusters of three TPR motifs. This may explain how two TPR subdomains can interact with the small ligand. However, to avoid possible confusion, we continued the numbering from TPR1 to TPR7.

It was possible to model the Pex5p TPR domain based on the PP5 TPR crystal structure by using an optimized alignment (see “Experimental Procedures”). Structural models were made of Pex5p TPR1-3 (Fig. 4, A and B) and Pex5p TPR5-7 (Fig. 4 C and D). Relating the loss-of-interaction mutations to the derived structural model of Pex5p allowed us to separate them into two groups as follows.

The first group of mutants contains an amino acid substitution that probably results in a small local change in the structure of a TPR domain. These mutations are located in the α-helix A or B, where they may interfere with correct packing of the α-helix in the structure. This can arise by a small amino acid changing into a more bulky one. An example is the G354C mutation in pex5.42; this glycine is present at position 8 of the second TPR motif. Another example is the G498E mutation in pex5.74; here the small amino acid at position 8 of TPR6 is mutated. The same effect is probably achieved when certain amino acids are replaced by a proline. These mutations are also localized in the α-helices of the TPRs. Mutants belonging to this group are pex5.14 (containing the L531P mutation in the α-helix A of TPR7), pex5.48 (containing the L518P mutation in the α-helix B of TPR6), pex5.30 (containing the L465P mutation in the α-helix A of TPR5), and pex5.79 (containing the L404P mutation in the α-helix B of TPR3). The introduction of a proline will probably disturb the continuity of an α-helix, and this may lead to (local) misfolding of a TPR motif. To test this hypothesis, we changed, by site-directed mutagenesis, the prolines in pex5.14, pex5.30, and pex5.79 to alanines, a residue that is accepted in an α-helix. Two-hybrid analysis revealed that in all cases, the alanine substitution restored PTS1 interaction (data not shown). This indicated that the prolines at these positions give a disturbance of the TPR structure and that the mutated residues (leucines in the α-helices of TPR3, TPR5 and TPR7) are not directly involved in binding of PTS1-containing proteins.

The second group consists of mutants that contain an amino acid substitution located in the small intra-repeat loop that connects α-helix A with α-helix B within a TPR motif (Fig. 4A). Here, several mutants contain a substitution of the same residue in the small loop of TPR3. Asparagine 393 was found to be mutated to aspartic acid (N393D) in pex5.38 and pex5.45, to tyrosine (N393Y) in pex5.46 and pex5.97, and to serine (N393S) in pex5.70. Unlike the α-helices these loops are somewhat projecting outwards from the folded TPR structure (Fig. 4A). The loop of TPR3 might therefore
be a position for direct contact with PTS1. This is in line with the substitution of asparagine 393 with alanine, which resulted in a loss of Mdh3p interaction in the two-hybrid system (Table III). Additional evidence that the intra-repeat loops are directly involved in PTS1 binding came from the screen for pex5 mutants that gained interaction with the PTS1 mutant Mdh3-SEL (Table II). This screen identified glutamic acid 361 in loop 2 as being directly involved in contacting the PTS1.

Table III. Quantification of two-hybrid interactions for Pex5p mutants made by site-directed mutagenesis

Two-hybrid interaction between the Pex5p mutants and either Mdh3p or GFP-SKL was quantitated in the two-hybrid reporter strain PCY2 by measuring β-galactosidase activity. Indicated is the average of two independent measurements with the range in parentheses. ONPG, α-nitrophenyl β-D-galactopyranoside.

<table>
<thead>
<tr>
<th>Location of mutation</th>
<th>β-galactosidase activity</th>
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<tr>
<td></td>
<td>Interaction with Mdh3p</td>
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<tr>
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<tr>
<td>Pex5p (N325A)</td>
<td>TPR1 (loop)</td>
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<tr>
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<td>TPR2 (loop)</td>
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<td>TPR3 (loop)</td>
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<td>TPR7 (α-helix A)</td>
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<td>Pex5p (N537A)</td>
<td>TPR7 (loop)</td>
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Site-directed mutagenesis of the Pex5p TPR motifs

The results from the two different screens for *pex5* mutants described above indicated that the intra-repeat loops of TPR2, TPR3, and TPR6, which connect the α-helices A and B within a TPR motif, are important for the interaction with PTS1 proteins. A sequence alignment of the Pex5p TPR motifs from *S. cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, and *Homo sapiens* showed that besides residues in the α-helices, also certain residues in the loops are well conserved (Fig. 3). The sequence conservation of amino acids in the intra-repeat loops is not a general feature of TPRs in other proteins. However, in the intra-repeat loops of Pex5p TPRs, with the exception of the loop of TPR5, some very well conserved asparagines are present, next to other conserved residues.

To further investigate the importance of the loops of the different TPR motifs in Pex5p, we mutared the conserved residues and studied the effect on PTS1 protein binding. In every TPR loop, except that in TPR5, at least one amino acid was mutated (Fig. 3 and Table III). The conserved asparagine 360 (loop 2), asparagine 393 (loop 3), asparagine 503 (loop 6), asparagine 505 (loop 6), and asparagine 537 (loop 7) residues were all mutated to alanines. Glutamic acid 361 and 363 (both in loop 2) and glutamic acid 394 (loop 3) were also mutated, as were the nonconserved asparagine 325 (loop 1) and serine 504 (loop 6) residues. These *pex5* mutants were still able to interact with Pex13p and Pex14p, indicating that the Pex5 protein is still at least partially functional (data not shown).

We tested the *pex5* mutants in the two-hybrid system for interaction with Mdh3p and an artificial PTS1 protein, green fluorescent protein (GFP) extended with the PTS1 SKL sequence at its carboxyl terminus (GFP-SKL). Mutations in the loops of TPR1 and TPR7 did not influence the binding of either protein (Table III). This indicated that amino acids in these loops do not directly participate in the binding of PTS1 proteins or that loss of a weak interaction is insufficient to evoke a phenotype. Mutations in the loops of TPR2 and TPR3 did have an effect on PTS1 protein binding and some of the mutants showed differences in interactions with Mdh3p and GFP-SKL (Table III). The mutations N360A and E394A in the loops of TPR2 and TPR3, respectively, resulted in complete loss of GFP-SKL binding, but interaction with Mdh3p was still present. Similarly, the suppressor mutant E361K and the site-directed mutant E363A in the loop of TPR2 showed complete loss of GFP-SKL binding, whereas interaction with Mdh3p was only 2-3 fold reduced.

Mutations in the loop of TPR6 (N503A, S504A, and N505A) did not disturb binding of either PTS1 protein. However, it should be noted that in our *pex5* suppressor screen, asparagine 503 was found to be mutated twice, suggesting that the loop of TPR6 contributes to the interaction with PTS1.
A much stronger phenotype was found when the asparagine 393 in the loop of TPR3 was mutated either to alanine or to glycine. In both cases, we found a complete loss of interaction with GFP-SKL and Mdh3p. This position was also found to be mutated several times in the screen for pex5 mutants with no Mdh3p interaction.

The structural model of the Pex5p TPR1-3 (Fig. 4A) suggests that the intra-repeat loops of TPR2 and TPR3 are localized close together and that the intra-repeat loop of TPR1 is farther away. A groove similar to that found in the structure of the TPR of PP5 (Das et al., 1998) is present in Pex5p TPR1-3 (Fig. 4B). For PP5, Das et al. postulated that this is the binding groove for target proteins. In our model, besides the general TPR groove, there is a smaller second groove in the area where the intra-repeat loops of TPR2 and TPR3 come together (Fig. 4B). There is a high sequence conservation in this area, and close to the residues of the loops of TPR2 and TPR3 is isoleucine 389, located in α-helix A of TPR3 (Fig. 4A and 4B). Because of its conservation among species and its close position to the residues of the loops of TPR2 and TPR3, we decided to mutate this hydrophobic residue to aspartic acid. The interaction of this Pex5p-I389D with Mdh3p and GFP-SKL in the two-hybrid system was completely lost (Table III).

The structural model for TPR5-7 (Fig. 4D) shows that the strictly conserved arginine 526, located in α-helix A of TPR7, projects outwards from the TPR groove. This might indicate that this amino acid is important for the interaction with target proteins. In line with this suggestion, arginine 526 was found mutated to glycine in the screen for pex5 mutants that had lost the interaction with Mdh3p (Table I). This mutant (pex5.98), however, contained a second mutation (S504P) in the loop of TPR6. To investigate the contribution of each residue to PTS1 interaction, single alanine mutants were generated by site-directed mutagenesis. The R526A mutation resulted in a complete loss of interaction with both Mdh3p and GFP-SKL, whereas the S504A mutation had no effect on the interaction with either PTS1 protein (Table III). These results indicate that arginine 526 might also be involved in the interaction with PTS1 proteins. Such an interaction might be possible due to the flexible hinge region discussed before, which could allow the TPR subdomains to come together.

Discussion

We carried out a structure-function analysis of the PTS1 receptor Pex5p to obtain insight into how recognition of PTS1 proteins destined for import into peroxisomes is accomplished. To this end, mutations in Pex5p were isolated that affected the binding of the peroxisomal matrix protein Mdh3p in a yeast two-hybrid trap. Two types of
mutants were isolated: loss-of-interaction mutants and suppressor mutants, *i.e.* mutants that gained interaction with Mdh3p containing a mutation in its PTS1. The *pex5* mutants were all located in the C-terminal half of Pex5p containing six TPRs. Rather surprisingly, they did not cluster in a particular region within the TPR domain. To be able to interpret the location of the *pex5* mutations in relation to its structure, we derived a homology model of the TPR domain of Pex5p based on the crystal structure of the three TPRs from PP5 (Das *et al.*, 1998).

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**Figure 4. Structural model of the TPR motifs of Pex5p.**

(A) Ribbon model of TPR1-3. Each TPR motif consists of two α-helices connected by a short intra-repeat loop. Side chains of amino acids involved in PTS1 interaction are indicated. (B) Space-filling model of TPR1-3. Indicated are the amino acids that are involved in PTS1 recognition. Ile^{380} and Asn^{393} are located on one side of the small TPR groove, and Glu^{61} and Glu^{363} are on the other side. The general TPR groove is indicated. (C) Ribbon model of TPR5-7 with the side chain of Arg^{526} indicated. (D) Space-filling model of TPR5-7. Indicated is Arg^{526}, sticking out into the general TPR groove. Also indicated are Asn^{503} and Ser^{534}, where suppressor mutations were found.
Mapping of the PTS1-binding site on Pex5p

Mapping of the mutations onto this structural model showed that some of the loss-of-interaction mutations consisted of amino acid substitutions with prolines or bulky amino acids in the α-helices of TPRs. These mutations are predicted to disrupt the regular packing of the TPR helices such that PTS1 protein recognition is affected as a secondary effect. Indeed, we showed that changing a mutational proline in an α-helix to alanine rescued Mdh3p recognition. Several inactivating mutations in Pex5 proteins of different species have been reported in the literature (Otera et al., 1998; Szilard and Rachubinski, 2000). These mutations were found to involve substitutions of glutamic acid residues (a bulky amino acid) for glycine residues located at position 8 of helix A in TPRs. Our modeling studies suggest that the stacking of the TPR helices might be compromised in these mutant Pex5 proteins.

The other loss-of-interaction mutations and most suppressor mutations were located in the short hairpin loops of TPR2, TPR3, and TPR6 that connect helices A and B (Fig. 4). These loops are somewhat exposed from the folded TPR structure and probably form the direct contact site for PTS1 proteins. In support of this, we found that changing a disabling mutation in the loop of TPR3 to alanine did not restore PTS1 protein recognition. Apparently, a much more critical property is involved here, related to the side chain of the original amino acid, which would be in line with direct interaction with Mdh3p. Additional site-directed mutagenesis of conserved residues in intra-repeat loops underscored the essential role of the loops of TPR2 and TPR3 in PTS1 interaction. Interestingly, some of these mutants showed a differential effect when tested in the two-hybrid trap against Mdh3p and GFP-SKL: interaction with Mdh3p remained or was slightly reduced, but interaction with GFP-SKL was completely lost. One possible explanation for this differential effect is that Mdh3p, an authentic peroxisomal matrix protein of yeast, contains, in addition to its PTS1, other sequences (so-called accessory sequences) that contribute to Pex5p binding. Most likely, a heterologous, non-peroxisomal protein like GFP does not contain such additional sites that can interact with Pex5p. Therefore, it is completely dependent on the added PTS1 for the interaction with Pex5p. The presence of amino acid sequences outside the PTS1 that might contribute to receptor recognition has been suggested before (Elgersma et al., 1995; Elgersma et al., 1996b; Purdue and Lazarow, 1996; Lametschwandtner et al., 1998).

A number of TPR structures have now been described that have contributed significantly to our understanding of how TPR domains interact with their targets. In addition to the x-ray structure of the isolated TPR domain of PP5 (Das et al., 1998), two complex structures have been recently published (Lapouge et al., 2000; Scheufler et al., 2000). The complexes between the adaptor protein Hop and peptides derived from either Hsp70 or Hsp90 showed that the peptides bind to a groove formed on the
helix A face of the TPR domain (the general binding groove; see also Fig. 4). This general binding groove for peptides in TPR domains had been predicted by Das et al. (1998) based on the isolated PP5 structure. Interestingly, the second complex structure of the small GTPase Rac bound to the TPR domain of p67^{phox} revealed a novel mode of interaction involving only the loop regions connecting TPR motifs. Our data now show the importance of the intra-repeat loops of TPRs in target recognition, suggesting yet another structural variation of TPR motif-mediated protein-protein interaction.

Our Pex5 modeling studies suggest that the TPR domain does not form a tandem array of seven TPRs, but rather two distinct clusters of three TPR motifs (TPR1-3 and TPR5-7) that are connected by a (flexible) linker of 42 amino acids (TPR4). Given the relative small size of the PTS1 signal (three amino acids) and the distribution of mutations affecting PTS1 binding over both TPR clusters, it is tempting to speculate that the two clusters of TPRs are localized close together in space forming a single binding site for the PTS1. The absence of a crystal structure of Pex5p prevents the description of the interaction of the TPRs with the PTS1 amino acids at the molecular level. However, based on our mutational analysis and the homology model, some predictions can be made. Very striking is the negatively charged patch in TPR1-3 formed by strictly conserved glutamic acid residues in intra-repeat loops 2 and 3 (Fig. 4A and 4B). These residues might be involved in binding the positively charged amino acid at position -2 of the PTS1 via electrostatic interactions. In Mdh3p and GFP-SKL, the -2 residue is lysine, but other positively charged amino acids like arginine and histidine can also be found at this position (Gould et al., 1989; Swinkels et al., 1992). This notion is supported by the charge-shift suppressor mutation E361K in the loop of TPR2, which was isolated in a screen with Mdh3p containing a negatively charged residue (glutamic acid) at position -2. Close to the negatively charged residues in the loops of TPR2 and TPR3, two conserved residues are located that are essential for PTS1 binding: asparagine 393, found to be mutated in several independent clones in the loss-of-interaction screen, and isoleucine 389 (valine in human Pex5p). An asparagine residue can be involved in different types of interactions because its side chain is able to both donate and accept hydrogen bonds. In particular, interactions of asparagine side chains with the backbone of short peptides have been well documented (Stern et al., 1994; Conti et al., 1998). Isoleucine 389 might be important for contacting the hydrophobic side chain of leucine at position -1. Other residues that can be found at this position in PTS1 signals are either large hydrophobic (methionine) or aromatic (phenylalanine) amino acids.

The exact contribution of TPR5-7 to PTS1 binding cannot be easily extracted from our data. However, while our work was in progress a speculative model for the
interaction of only TPR5-7 of human PEX5 with the PTS1 was published (Gatto et al., 2000). This model, which is based solely on homology modeling and orthologous sequence information, highlights the importance of four strictly conserved asparagine residues in the A helices of TPR 6 and TPR7. These asparagine residues are predicted to recognize the backbone of the PTS1. One of these asparagine residues (Asn\textsuperscript{503}) (Fig. 4C and D) was found to be mutated in two independent clones (pex5.sup3 and pex5.sup21) in our suppressor screen. Remarkably, the phenotype of these suppressors (and the S534L suppressor) was different from that of the previously mentioned charge-shift suppressor E361K. Whereas, the E361K mutant showed no interaction with Mdh3p without its PTS1 (Mdh3ASKL), the other suppressors still displayed (a weak) binding to Mdh3ΔSKL. This phenotype might be related to the possible role of asparagine 503 (and serine 534) in peptide backbone recognition (Gatto et al., 2000). Finally, the model of Gatto et al. (2000) predicts a role for an absolutely conserved arginine residue in helix A of TPR7 (Arg\textsuperscript{526} in yeast Pex5p) in binding the carboxylate oxygens of the PTS1 C-terminus. Our experimental data support this prediction since substitution of arginine 526 with alanine or glycine completely abrogated PTS1 interaction (Tables I and III).

The work described above demonstrates that by combining homology modeling and mutational analysis, we were able to put forward a possible model as to how PTS1 is recognized by the TPR domain of Pex5p. Further refinement of this model requires the crystal structure of the Pex5p TPR domain in complex with a PTS1 protein.

Addendum:
While our work was under review the crystal structure of the human Pex5p TPR domain complexed with a PTS1 peptide was published (Gatto et al. (2000) Nat. Struct. Biol. 7, 1091-1095). Superimposition of the modeled yeast Pex5p TPR domain presented in our work onto the human Pex5p TPR crystal structure revealed that our predicted structure closely matches the crystal structure (overall backbone root mean square deviation = 0.9-1.2 Å). Also, our prediction of the PTS1-binding site is completely in line with the results of Gatto et al.

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References


Mapping of the PTS1-binding site on Pex3p


