Recognition of Peroxisomal Targeting Signal Type 1 by the Import Receptor Pex5p*

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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.

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 (16–20) and the two peroxisomal membrane-associated proteins Pex14p (21–25) and Pex17p (26). 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 (27, 28). 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) (29). 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, trpl, ura3-251, pbi-1122, pep4-3, gal2), BJ1991pex5Δ (MATα, pex5::LEU2, leu2, trpl, ura3-251, pbi-1122, pep4-3, gal2), HP7c (MATα, ara352, his3-200, ade2-101, lys2-801, trpl-901, leu2-3, 112, gal4-542, gal80-538, lys2::GAL1-UAS-GAl1-TATA-HIS3, URA3::GAL4-UAS-LacZ, and PCY2 [MATα, pga1, sga80, URA3::GAL1-lacZ, lys2-801, his3-200, trpl-901, 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 (30). 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 (31). 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, SphI, SsrI, and HindIII at the 3′-end of PEX5. Two additional restriction sites were introduced in PEX5 by silent mutations: a Xhol site at position 1140 by mutating the codon for leucine 381 from CTG to CTA and a SacI 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...
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PEX5 from pAN1 EcoRI-SpeI in the two-hybrid vector pCP86 (32). 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- SpeI in pAN1, resulting in pAN21. Subsequently, the two complementary 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 pCP97 (32). The acidic amino acid sequence (the extreme carboxyl terminus residues GMDELYQLQGSSKL) Gal4 DNA-binding domain fusions with the Pex13p SH3 domain (pGB17) and Pex14p (pGB47) have been described before (19). The oligonucleotides used were p325 (AAGGATCCATGGTCAAAGTCGCAATTCTTG), p326 (CGGATTCTGCGATGTCGATGAAATAGCTGTCG), p327 (AAAAAGCTTTAGAGGCATGAGAACCTT), p328 (CACAAGTCTGATGTCCGATTGCTGCGGATGAAATAGCTGTCG), p329 (TTCAGGGTTGGTGCATGAAATAGCTGTCG), p330 (ATGATGAGATGGAATGCTGCGGATGAAATAGCTGTCG), p331 (CCCAGGCTTTAGAGGCATGAGAACCTT), p332 (GGGGCTTCATTGGCCGCTTCCAATAGATCAGAGG), and p333 (ATGATGAGATGGAATGCTGCGGATGAAATAGCTGTCG).

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 lacking the 3′ → 5′ proofreading activity (35). pAN1 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, and 35). pAN1 was used as a template under standard reaction conditions by digestion of pEL102 with 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 Mdh3p was analyzed by Western blotting to determine whether 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 H76 expressing pDBMDH3-SEL was obtained by transformation of 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 the capacity to interact with Mdh3p, a Pex5p SKL-containing peroxisomal matrix protein (see “Experimental Procedures”). Each of the selected mutants was analyzed by Western blotting to determine whether full-length Pex5p was still produced at normal levels.

From the C-terminal pex5 library, 14 pex5 mutants were selected, whereas from the N-terminal pex5 library, only one muta-
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Fig. 1. Construction of the pex5 mutant libraries. The XhoI restriction site was used to swap wild-type PEX5 sequences for randomly mutagenized pex5 sequences.

Table I

| pex5 mutant | Substitution Location |
|-------------|-----------------------|
| pex5.14     | L531P                 | TPR7 |
| pex5.30     | L465P                 | TPR5 |
| pex5.38     | N393D                 | TPR3 |
| pex5.45     | N393D                 | TPR3 |
| pex5.46     | N393D                 | TPR3 |
| pex5.48     | N504P                 | TPR5 |
| pex5.70     | N539S                 | TPR3 |
| pex5.74     | G498E                 | TPR3 |
| pex5.79     | R485K                 | TPR3 |
| pex5.92     | R485K                 | TPR3 |
| pex5.97     | N504F, R526G          | TPR6, 7 |
| pex5.98     | S25L, G354C           | TPR6, 7 |

Pex5p and Mdh3p is direct and is dependent on 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 Pex5p-N393D 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.

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 (10, 12, 28). To determine whether 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

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pex5 suppressor mutants restore the interaction with a PTS1 mutant

Two-hybrid interaction was measured in the 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 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.

| Mutation/location | Two-hybrid interaction | Mdh3-SEL | Mdh3ΔSKL | Mdh3-ΔSKL |
|-------------------|------------------------|-----------|-----------|-----------|
| PEX5 (wild-type)  |                        |           |           |           |
| pex5.sup1         | S534L/TPR7             | –         | –         | +         |
| pex5.sup2         | E361K/TPR2             | +         | +         | *         |
| pex5.sup3         | N503D/TPR6             | –         | +         | *         |
| pex5.sup21        | N503D/TPR6             | +         | +         | +         |

suppressed the PTS1 mutation because it did not interact with Mdh3p from which 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 wild-type PTS1 (Mdh3-SEL), indicating that the mutations had no gross structural effects on the protein.

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 (29). 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 positions 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 positions 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 (42), leading to an antiparallel arrangement of α-helix A relative to 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 acids found at 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 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 positions found in the suppressor screen with Mdh3-SEL.

Fig. 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.

Table II

| Mutation/location | Two-hybrid interaction | Mdh3-SEL | Mdh3ΔSKL | Mdh3-ΔSKL |
|-------------------|------------------------|-----------|-----------|-----------|
| PEX5 (wild-type)  |                        |           |           |           |
| pex5.sup1         | S534L/TPR7             | –         | –         | +         |
| pex5.sup2         | E361K/TPR2             | +         | +         | *         |
| pex5.sup3         | N503D/TPR6             | –         | +         | *         |
| pex5.sup21        | N503D/TPR6             | +         | +         | +         |

Fig. 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). Stars 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 positions found in the suppressor screen with Mdh3-SEL.
FIG. 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 in blue are the side chains of the amino acids that are involved in PTS1 recognition. Glutamic acids and isoleucine are shown in dark blue, and asparagines are shown in light blue. Ile366 and Asn393 are located on one side of the small TPR groove, and Glu351 and Glu353 are on the other side. The general TPR groove is indicated. C, ribbon model of TPR3–7 with the side chain of Arg326 indicated. D, space-filling model of TPR5, 6 and 7. Indicated in blue is Arg326, sticking out into the general TPR groove. Also indicated in blue are Asn393 and Ser394, where suppressor mutations were found.

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 mutated 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 acids 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 (GFP-SK). 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-SK (Table III). The mutations N360A and E394A in the loops of TPR2 and TPR3, respectively, resulted in complete loss of GFP-SK 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-SK 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 asparagine 393
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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, o-nitrophenyl β-D-galactopyranoside.

| Location of mutation | Interaction with Mdh3p | Interaction with GFP-SKL |
|----------------------|------------------------|--------------------------|
| Wild-type Pex5p      | 544 (531–558)          | 40 (37–44)               |
| Pex5p-N325A          | 640 (569–711)          | 72 (71–72)               |
| Pex5p-N360A          | 574 (551–598)          | <1                       |
| Pex5p-E361K          | 277 (275–278)          | <1                       |
| Pex5p-E363A          | 127 (106–148)          | <1                       |
| Pex5p-I389D          | <1                     | <1                       |
| Pex5p-N393Y          | <1                     | <1                       |
| Pex5p-N393A          | <1                     | <1                       |
| Pex5p-N393D          | <1                     | <1                       |
| Pex5p-E394A          | 487 (465–508)          | <1                       |
| Pex5p-I503A          | 886 (819–953)          | 99 (96–104)              |
| Pex5p-S504A          | 832 (825–838)          | 77 (76–77)               |
| Pex5p-N505A          | 551 (550–553)          | 42 (41–43)               |
| Pex5p-R526A          | <1                     | <1                       |
| Pex5p-N557A          | 661 (651–671)          | 87 (67–68)               |

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 (29). 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 (43, 44). 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 mutations 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

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.

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 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 (29) is present in Pex5p TPR1–3 (Fig. 4B). For PP5, Das et al. (29) 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. 4, A and B). 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 mutant with Mdh3p and 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, o-nitrophenyl β-D-galactopyranoside.

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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 PTS1 that might contribute to receptor recognition has been suggested before (34, 41, 45, 46).

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 (29), two complex structures have been recently published (47, 48). The complexes between the adaptor protein Hsp 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. (29) based on the isolated PP5 structure. Interestingly, the second complex structure of the small GTPase Rac bound to the TPR domain of p67\textsuperscript{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 Pex5p 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 PTS1 (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 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. 4, A and B). These residues might be involved in binding the positively charged patch in TPR1–3 formed by strictly conserved glutamic acid residues in intra-repeat loops 2 and 3 (Fig. 4, C 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 (Mdh3\DeltaSKL), the other suppressors still displayed (a weak) binding to Mdh3\DeltaSKL. This phenotype might be related to the possible role of asparagine 503 (and serine 534) in peptide backbone recognition (51). Finally, the model of Gatto et al. (51) 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, G. J., Jr., Geisbrecht, B. V., Gould, S. J., and Berg, J. M. (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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