The Di-aromatic Pentapeptide Repeats of the Human Peroxisome Import Receptor PEX5 Are Separate High Affinity Binding Sites for the Peroxisomal Membrane Protein PEX14*

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PEX5 functions as a mobile import receptor for peroxisomal matrix proteins with a peroxisomal targeting signal 1 (PTS1). A critical step within the PTS1-import pathway is the interaction between PEX5 and the peroxisome membrane-associated protein PEX14. Based on two-hybrid analyses in mammalian cells and complementary in vitro binding assays, we demonstrate that the evolutionarily conserved pentapeptide repeat motifs, WX(E/D/Q/A/S)(E/D/Q)(F/Y), in PEX5 bind to PEX14 with high affinity. The results obtained indicate that each of the seven di-aromatic pentapeptides of human PEX5 interacts separately at the same binding site in the N terminus of PEX14 with equilibrium dissociation constants in the low nanomolar range. Mutational analysis of the PEX14-binding motifs reveals that the conserved aromatic amino acids at position 1 or 5 are essential for high affinity binding. We propose that the side chains of the aromatic amino acids are in close proximity as part of an amphipathic α-helix and together form hydrophobic anchors for binding PEX5 to individual PEX14 molecules.

Import of peroxisomal matrix proteins is a posttranslational multistep process which involves recognition of the peroxisomal proteins in the cytosol by specific soluble receptors, docking of the receptor-cargo complex at the surface of the organelle, and translocation of the matrix proteins with or without their receptors across the membrane (for recent reviews, see Refs. 1–4). The current model proposes that the receptors shuttle back to the cytoplasm after release of their substrate (5, 6).

PEX5 and PEX7 were identified as receptors for peroxisomal matrix proteins in numerous species (for review see Ref. 7). Both proteins are capable of interacting with peroxisomal proteins via their peroxisomal targeting signals PTS1 or PTS2. A minor class of peroxisomal proteins, recognized by PEX7, contains the PTS2 signal which consists of the sequence (R/K)(L/I/V/IX5(H/Q/K/L/A) near the N terminus. The majority of matrix proteins that interact with PEX5 do so via their PTS1 which consists of the tripeptide SKL (or a conserved variant thereof) at the extreme carboxyl terminus of the protein.

The hypothesis of mobile receptors is in line with the cellular distribution of both PEX5 and PEX7. In most cases only small portions of the otherwise cytosolic receptors are associated with the peroxisome (for review see Ref. 7). Two likely candidates for the cargo-receptor docking site at the peroxisomal membrane are the proteins PEX13 and PEX14, which both interact with PEX5 and with each other via the cytoplasmic SH3-domain of PEX13 (for review see Ref. 1).

Despite the fact that the key components of the cytosolic steps of protein import, namely PEX5, PEX7, PEX13 and PEX14, are conserved in lower and higher eukaryotes, the mode of interactions appears to differ between mammalian and yeast cells in several aspects. First, the point of convergence of the PTS1 and PTS2 targeting pathways in yeast is the docking complex at the peroxisomal membrane (PEX13/PEX14) (8–10), whereas in mammalian cells the PTS receptors seem to interact in the cytoplasm directly (11, 12). Second, in mammalian cells PEX14 provides the initial docking site for both receptors. This was suggested because PEX5 was localized mostly in the cytosol in cell lines deficient in PEX14, whereas it accumulated at peroxisomal membranes in cells overexpressing PEX14 (12). This seems to be in disagreement with the observation that in the methylotrrophic yeast Hansenula polymorpha PEX5 can associate with the peroxisomal membrane in absence of PEX14 (13). Third, it was shown for several yeast species that PEX13 interacts directly with the PTS1 receptor (14–16), whereas a physical interaction between mammalian PEX13 and PEX5 remains unclear (12, 17).

Structural analysis of several mammalian and yeast PEX5 orthologs revealed that the binding regions for peroxisomal PTS1 proteins and for docking events are clearly separated. Binding of the PTS1 targeting signal requires the entire C-terminal half of PEX5 containing six tetratricopeptide repeat (TPR) domains (18), whereas the putative docking proteins PEX14 and PEX13 bind to the N-terminal half of the molecule (10, 19, 20). One of the most striking structural features of the N-terminal region is the presence of multiple WXXX(F/Y) pentapeptide repeats. Di-aromatic pentapeptide motifs are found in the N-terminal halves of all PEX5 orthologs but in different numbers and spacings. The human PEX5 contains seven of these motifs.

In a previous study we have proposed that recombinant human PEX5 possesses multiple binding sites for human PEX14 within its N-terminal half and that the conserved seven WXXX(F/Y) motifs may represent the binding sites on PEX5 (19). Here we demonstrate by in vitro and in vivo analyses that

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1 The abbreviations used are: PTS, peroxisomal targeting signal; TPR, tetratricopeptide repeat; GST, glutathione-S-transferase; CAT, chloramphenicol acetyltransferase.
WXXX(F/Y) Pentapeptide Repeats of Human PEX5

EXPERIMENTAL PROCEDURES
Preparation of Recombinant Proteins and Synthetic Peptides—Expression and purification of His6-tagged PEX5, PEX14-(1–78), and PEX14-(1–78) fused to the C terminus of glutathione-S transferase (GST) was carried out as described previously (19). Purity of proteins was checked by standard SDS-polyacrylamide gel electrophoresis or Tricine-SDS-polyacrylamide gel electrophoresis (21). Protein concentrations were determined by Coomassie or BCA-based colorimetric assay (Pierce) using bovine serum albumin as a standard. Alternatively the absorbance at 280 nm or 214 nm was measured.

16mer peptides were synthesized by the Fmoc (N-(9-fluorenyl) methoxycarbonyl) method of solid phase peptide chemistry on a Biosynthesis-2900 PEP-synthesizer (Milligen). Cleavage of the peptide from the resin and removal of the protecting groups was achieved by treating the peptide with 90% trifluoroacetic acid, 2.5% phenol, 2.5% 1,2-ethanedithiol, 1% triisopropylsilane (all from Sigma), and 5% water (22). The peptides used for competition experiments were high pressure liquid chromatography-purified. Cellulose-bound peptides were generated by the spots technique according to the manufacturer’s protocol (Anticog-Kit-Recombinant Arthrographis). PEX5 fusion protein was expressed and purified on a Biacore 2000 instrument (Amersham Pharmacia Biotech) after incubation with horseradish peroxidase coupled secondary antibody (Sigma).

Surface Plasmon Resonance—For competition studies we used surface plasmon resonance spectroscopy with a Biacore 2000 instrument (Biacore AB). Anti-GST antibodies were covalently linked to the sensor surfaces of a CM5 sensor chip as described previously (19). Specific surfaces were created by capturing GST-PEX14-(1–78), or, as a control, GST without fusion part to an immobilization level of 850 and 600 resonance units, respectively.

Mixtures of 50 nm PEX5 with varying amounts of W1 peptide were passed over these surfaces for 5 min at a flow rate of 20 ,/min. The concentrations of peptide in these mixtures ranged from 68 ,/min to 6.8 ,/min in 10-fold steps. At the end of each association phase the response was recorded and the data obtained from the control surface were subtracted from the specific surface. These specific responses were plotted against the peptide concentration on a logarithmic scale. The response obtained without peptide was set to 0.68 ,/min because concentration less than 68 nm produced no further effect. From these data an IC50 value was extracted by using the “one site competition” model of the Prism Graph Pad Software, version 3.02 (H. J. Motulsky, Graph Pad Software Inc.). If the KD value for the competing reaction is known, the inhibitory constant, Kc, can be calculated according to the equation of Cheng and Prusoff (24).

Steady State Fluorescence Spectroscopy—Binding of tryptophan-containing peptides to PEX14-(1–78) was monitored using the intrinsic fluorescence of tryptophan. The excitation wavelength was 295 nm. Emission spectra of peptides with and without PEX14-(1–78), each at a concentration of 600 nm, were recorded from λ = 300 nm to λ = 500 nm in 150 nm NaCl and 20 nm Tris-HCl, pH 8, at 20 °C. Titration curves were recorded at 340 nm by adding 20 nm (1 ml of a 16 ,/stock stock) PEX14-(1–78) in a stepwise fashion to 200–800 μl solution of peptide at 200 nm (20 °C). Excitation and emission slit widths were both set to 5 nm. The spectra were recorded with a PerkinElmer Life Sciences LS-50B spectrophotometer.

A plot of the relative fluorescence intensity versus PEX14-(1–78) concentration was used to extract Kc values. Equations 1–4 were used to fit the data with the Software Graph Pad Prism 3.02.

\[
K_D = \frac{C_{\text{peptide}} \cdot C_{\text{Complex}}}{C_{\text{Peptide}} - C_{\text{Complex}}}
\]

\[
C_{\text{Peptide}} = C_{\text{Peptide,0}} - C_{\text{Complex}}
\]

\[
F = F_{\text{peptide}} \cdot (C_{\text{peptide}} - C_{\text{complex}}) + F_{\text{complex}} \cdot C_{\text{Complex}}
\]

RESULTS

WXXX(F/Y) Motifs Are Required for PEX5-PEX14 Interaction in Mammalian Two-hybrid Assays—We previously demonstrated that human PEX5 possesses multiple binding sites for PEX14 and proposed that the various WXXX(F/Y) pentapeptide repeats might provide the structural basis for these interactions (19). To map the PEX14-binding regions of PEX5 we expressed the long form of PEX5 and various truncated forms fused to the Gal4 DNA-binding domain in human fibroblast cells (Fig. 1). The truncated versions of PEX5 contained either none or different numbers of WXXX(F/Y) motifs as indicated in Fig. 1. The interaction between these PEX5 fragments with PEX14 fused to the Gal4 DNA-activating domain was monitored by the expression level of the reporter protein CAT. Deletion of the complete C-terminal half of PEX5 containing the whole TPR-domain had no significant effect on the interaction with PEX14. This result is in line with two recent observations. We found that recombinant PEX5 fragments PEX5-(335–639) fused to GST and maltose binding protein did not bind recombinant PEX14 (data not shown), and Chang et al. reported that no interaction between the C-terminal half of PEX5 and PEX14 could be detected using a yeast two-hybrid assay (26).

However, our mammalian two-hybrid results confirm our previous in vitro findings that at least two different binding sites for PEX14 exist within the N-terminal half of PEX5. This
The loci containing the di-aromatic pentapeptide repeats are indicated in the schematic illustration of the long form of human PEX5 as white bars. The full-length protein (639 amino acids) and fragments of PEX5 were fused to the Gal4 DNA-binding domain, and together with PEX14 fused to the Gal4 DNA-activating domain expressed in human skin fibroblasts. The interaction was quantified by measuring the Gal4p-dependent expression rate of chloramphenicol acetyltransferase (CAT). Data from one typical experiment are shown after correction for CAT expression obtained after cotransfection of the corresponding PEX5 plasmids together with pVP16.

All PEX5 fragments containing WXXX(F/Y) motifs interacted in the two-hybrid assay. In contrast, two fragments without the WXXX(F/Y) motif, PEX5-(1–113) and PEX5-(191–222), did not interact with PEX14 in the assay used. Although there is not a strong correlation between the number of WXXX(F/Y) motifs and the expression level of CAT, these results indicate that the conserved pentapeptide repeats are essential for complex formation between PEX5 and PEX14.

All Seven Di-aromatic Pentapeptide Repeats Form High Affinity Binding Sites for PEX14-(1–78) in Vitro—To further substantiate that the multiple di-aromatic pentapeptides determine PEX5 binding to PEX14 we measured their individual binding affinities. For this purpose, we performed fluorescence measurements with eight synthetic 16-mer peptides corresponding to different tryptophan-containing regions of PEX5 (Fig. 2B). In addition to the seven peptides containing the consensus sequence WX(A/S/D/E/Q)(D/E/Q)(F/Y) we included an eighth one, which contained the pentapeptide sequence WDKLQ. The latter peptide was chosen because of its sequence similarity to the WXXX(F/Y) motifs and because it is a conserved pattern in the known PEX5 proteins of different organisms. The purified PEX5-binding domain of PEX14 consisting of the N-terminal 78 amino acids that does not contain tryptophan was used as a binding partner (19). The emission spectra of the intrinsic tryptophan in the peptides before and after incubation with PEX14-(1–78) are shown in Fig. 3. The formation of complexes between each of the peptides W1, W2, W3, W5, W6, and W7 and PEX14-(1–78) were accompanied by a 2- to 3-fold increase in the fluorescence intensity and a significant change of the maximum emission wavelength (Fig. 3). The blue shift of ~20 nm in fluorescence after binding of each of the peptides to PEX14-(1–78) indicated a less polar environment in the vicinity of tryptophan. Significant changes in fluorescence properties of tryptophan were not observed for peptides W4 and WQ indicating that the affinities of these peptides for PEX14-(1–78) were substantially lower.

The affinity for each peptide (at 200 nM) was quantified by PEX14-(1–78) titration. The emission was recorded at a wavelength of 340 nm (Fig. 4A). The shapes of the titration curves obtained with W1-W7 imply that saturation of complex formation occurs for each at a concentration of ~200 nM PEX14-(1–78), indicating a stoichiometry of 1:1 between the peptide and the PEX14 fragment.

Equilibrium binding constants calculated from the titration curves exhibit high affinity binding in the nanomolar range for all peptides containing a WXXX(F/Y) motif (Fig. 4B). The highest affinities were observed for the peptides W1, W2, W3, and W5 with K_D values below 20 nM, whereas peptide W4 displayed lower affinity with a K_D of about 100 nM. Thus, the binding affinities found by fluorescence spectroscopy for each of the peptides containing one WXXX(F/Y) motif are in the same range as that determined for full-length PEX5 toward PEX14-(1–78) using surface plasmon resonance spectroscopy (19).

The Two Aromatic Amino Acid Side Chains within the Repeats Are Crucial for High Affinity Interaction with PEX14-(1–78)—To analyze the structural requirements for the binding of the di-aromatic peptides to PEX14 PEX5-derived 15-mer oligopeptides were synthesized on a cellulose membrane support and probed with GST-PEX14-(1–78). Specific anti-GST antibodies were used to visualize the complex formation. No staining was observed when GST without fusion protein was used (data not shown). With the exception of the WQ peptide, all seven peptides containing WXXX(F/Y) motifs (W1-W7) interacted with PEX14-(1–78) (Fig. 5).

Several mutated versions of the di-aromatic motif within the W1 peptide were used to determine which of its five amino acids are strictly required. Replacing all five residues of the motif with alanine abolished binding to PEX14-(1–78) completely (Fig. 5).

To identify the critical residues within this pentapeptide sequence further alanine substitutions were carried out at the conserved positions 1, 5, and 3/4 of the motif. Removing each of the aromatic side chains at the positions 1 and 5 resulted in a
complete loss of binding. In contrast, mutation of the conserved charged residues at position 3 and 4 did not affect the interaction with PEX14-(1–78). Inversion of the pentapeptide sequence or exchange of the aromatic residues revealed that not only the presence of both side chains but also their positions within the motif sequence is essential for interaction with PEX14. As expected from the degeneracy of the pentapeptide motif a phenylalanine at position 5 could be replaced with a tyrosine without significant loss of binding.

The results shown demonstrate that tryptophan at position 1 of the first motif is one of the two residues crucial for the high affinity interaction of the W1 peptide with PEX14-(1–78). To test if all other pentapeptide sequences (W2-W7) interact in a similar way with PEX14-(1–78) we substituted the first amino acid position of all seven di-aromatic motifs with alanine. In all cases the interaction is not detectable or significantly reduced (Fig. 5).

Fig. 3. Fluorescence emission spectra of synthetic peptides derived from tryptophan-containing regions of the N-terminal half of PEX5. The spectra of peptides W1-W7 and WQ (each at 600 nM) were recorded in the absence (dashed line) or presence (solid line) of 600 nM PEX14-(1–78). The spectrum obtained solely by PEX14-(1–78) is shown in the W1 panel (upper left, thin line). The maximum emission wavelengths at 360 nm and 340 nm are indicated by vertical dashed lines.

**DISCUSSION**

Two essential functions of the PTS1 receptor PEX5 are associated with different regions of this protein. It binds peroxisomal matrix proteins in a PTS1-dependent manner in the cytosol with the six TPR motifs in its C-terminal half (18). In contrast, the docking event to the peroxisomal membrane proteins PEX13 and/or PEX14 requires the N-terminal half of PEX5. We reported previously that N-terminal fragments of human PEX5 form stable multimeric complexes with an N-
terminal fragment of the peroxisomal membrane protein PEX14 (19). In the present study we demonstrate that multiple pentapeptide repeats in the N-terminal half of human PEX5 provide the molecular basis for this interaction. These pentapeptide repeats appear in different numbers in all known PEX5 orthologs and have been recognized previously (27), but no function could be clearly assigned to this motif. We show herein that each of the seven motifs of human PEX5 is capable of forming a single, high affinity binding site for the N-terminal PEX5-binding domain of PEX14. The consensus sequence of the PEX14-binding motif derived from human PEX5 is WXXX(F/Y). Our data indicate that both aromatic amino acids tryptophan and phenylalanine/tyrosine at position 1 and 5, respectively, are indispensable for the high affinity interaction with PEX14. Based on secondary structure prediction we previously suggested that each WXXX(F/Y) motif forms a part of an amphipathic α-helix (19). We propose from our recent data that the aromatic groups of tryptophan and phenylalanine/tyrosine, which are positioned on the same face of the α-helix, bind directly to a hydrophobic region of PEX14. The proposed binding mechanism is supported by the observed changes in the fluorescence spectra of tryptophan that were recorded before and after complex formation with PEX14 (Fig. 3). The drastic blue shift indicates a much less polar environment. A similar binding mechanism was also described for the extended A-helix of the catalytic subunit of cAMP-dependent protein kinase. In this case, phenylalanine 26 and tryptophan 30 as a part of the amphipathic α-helix mediate a tight intramolecular interaction by filling a deep hydrophobic pocket between the two lobes of the catalytic core (28).

**Fig. 4.** Fluorescence titration analysis of the interaction of PEX14-(1–78) with synthetic peptides W1-W7 and WQ. A, the increase in intrinsic tryptophan fluorescence of peptides (200 nM) upon addition of increasing amounts of PEX14-(1–78) was followed at 340 nm. Symbols used are ■, W1; ○, W2; △, W3; □, W4; ▲, W5; ○, W6; ●, W7; X, WQ. B, equilibrium binding constants $K_D$ for peptides W1-W7 were estimated from the binding curves as described under “Experimental Procedures.” Error bars represent the standard errors as derived from the fitting procedure.

**Fig. 5.** Mutational analysis of PEX14-binding regions of PEX5. 15-mer peptides of the indicated sequences were synthesized directly on a cellulose membrane. Bold letters indicate the pentapeptide motifs and their mutated variants. Dashes represent identical amino acids. After blocking of the membrane and incubation with GST-PEX14-(1–78) bound fusion protein was visualized via anti-GST antibodies as described under “Experimental Procedures.”

**Fig. 6.** Competition of PEX5 and peptide W1 for binding to PEX14-(1–78). The influence of peptide W1 on binding of PEX5 to GST-PEX14-(1–78) was studied by surface plasmon resonance spectroscopy. Mixtures of 50 nM full-length His₆-PEX5 and varying concentrations of peptide were passed over surfaces with immobilized GST-PEX14-(1–78). The response in resonance units, RU, at the end of a 5-min association phase corrected for the signal obtained for a control surface (GST alone) is shown.
cated by mutational analysis (Fig. 5). The strong preference for glutamine, glutamate, and aspartate at these positions at the opposite side of the α-helix might indicate that these residues could be involved in other intra- or intermolecular interactions.

Several lines of evidence suggest that the tryptophan-dependent binding mechanism is identical for all seven PEX14-binding sites of human PEX5. First, tryptophan is an essential part of all WXXX(F/Y) peptides for the formation of stable complexes with PEX14(1–78) as indicated by peptide spot overlay assays (Fig. 5). Second, the change of fluorescence properties during complex formation with PEX14(1–78) for each tryptophan of six of the seven WXXX(F/Y) peptides is essentially the same (Fig. 3). Third, all seven WXXX(F/Y) peptides bind with similar high affinities with $K_D$ values in the low nanomolar range (Fig. 4). Finally, competition analysis indicates that all WXXX(F/Y) peptides can occupy the same site on the PEX14 fragment (amino acids 1–78) (Fig. 6).

Binding of WXXX(F/Y) repeats to PEX14 strongly depends on their molecular environment, i.e., the accessibility of the aromatic side chains. We demonstrated previously that recombinant PEX5 formed a stable multimeric complex with the N-terminal PEX14(1–78) fragment with a stoichiometry of 1:6–7 (19). Therefore, we suggest that at least six WXXX(F/Y) motifs of human PEX5 provide authentic binding sites for PEX14. This must not be necessarily true for all motifs existing in other PEX5 orthologs. Two recent reports about PEX5 proteins from the yeasts *Saccharomyces cerevisiae* are relevant here. In both cases no interaction between PEX5 fragments containing one or more WXXX(F/Y) motifs and PEX14 could be detected using overlay and pull-down assays (20, 29). Instead, the WXXX(F/Y) motifs are essential parts of much larger PEX13 binding sites. In *S. cerevisiae* the interaction between PEX5 and PEX13 requires in addition to both aromatic residues of the pentapeptide motif at least two negatively charged residues to the C terminus (on the same side of a proposed α-helix). These are not conserved in the context of the human PEX5 WXXX(F/Y) motifs. In *P. pastoris* the minimal fragment length required for binding to PEX13 comprises 114 amino acid residues including three WXXX(F/Y) motifs, and a peptide containing one single WXXX(F/Y) motif is not sufficient to compete this interaction. The data from both groups suggest that the PEX13 interacting region of these two yeast PEX5 orthologs is more complex than a single PEX14-binding site defined in human PEX5.

It seems important in this context that in mammalian cells probably only PEX14 provides the initial binding protein for PEX5 at the peroxisomal membrane (12), whereas in yeast both PEX13 and PEX14 can fulfill their function as docking proteins (10). Thus, one feature of WXXX(F/Y) motifs in both yeasts and mammals is that they mediate contact between the PTS1 receptor and the organism-specific preferred interacting partner at the peroxisomal membrane.

Multiple high affinity binding sites for PEX14 on PEX5 were not expected when the former was proposed as docking protein for both cytosolic import receptors (8). In principle, a single binding site on PEX5 could provide the structural basis for such a docking event. Therefore, it is an important question whether all binding sites on human PEX5 are occupied simultaneously or sequentially in *vivo*. Gouveia et al. recently isolated PEX5 from membranes of rat liver peroxisomes as a transmembrane complex with PEX14 and showed that the complex contained PEX5 and PEX14 with a 1:5 stoichiometry (30). Our *in vitro* data provide a rationale for the formation of such a membrane complex. It is tempting to speculate that a function of the WXXX(F/Y) pentapeptide repeats of PEX5 is first the docking of the cargo-receptor complex to the integral membrane protein PEX14 and subsequently the formation of the transmembrane multimeric complex.

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