Interaction of Pex5p, the Type 1 Peroxisome Targeting Signal Receptor, with the Peroxisomal Membrane Proteins Pex14p and Pex13p*

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Aaron J. Urquhart‡, Derek Kennedy§, Stephen J. Gould¶, and Denis I. Crane††

From the ‡School of Biomolecular and Biomedical Science, Griffith University, Nathan, Queensland 4111, the §Center for Molecular and Cellular Biology, University of Queensland, St. Lucia, Queensland 4072, Australia, and the ¶Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Pex5p, a receptor for peroxisomal matrix proteins with a type 1 peroxisome targeting signal (PTS1), has been proposed to cycle from the cytoplasm to the peroxisomal membrane where it docks with Pex14p and Pex13p, the latter an SH3 domain-containing protein. Using in vitro binding assays we have demonstrated that binding of Pex5p to Pex14p is enhanced when Pex5p is loaded with a PTS1-containing peptide. In contrast, Pex5p binding to Pex13p, which involves only the SH3 domain, occurs at 20–40-fold lower levels and is reduced when Pex5p is preloaded with a PTS1 peptide. Pex14p was also shown to bind weakly to the Pex13p SH3 domain. Site-directed mutagenesis of the Pex13p SH3 domain attenuated binding to Pex5p and Pex14p, consistent with both of these proteins being binding partners for this domain. The SH3 binding site in Pex5p was determined to lie within a 114-residue peptide (Trp100-Glu213) in the amino-terminal region of the protein. The interaction between this peptide and the SH3 domain was competitively inhibited by Pex14p. We interpret these data as suggesting that docking of the Pex5p-PTS1 protein complex at the peroxisomal membrane occurs at Pex14p and that the Pex13p SH3 domain functions as an associated component possibly involved in sequestering Pex5p after relinquishment of the PTS1 protein cargo to components of the translocation machinery.

The import of proteins into the peroxisomal matrix from their site of synthesis on cytosolic ribosomes requires at least two targeting signals. Peroxisomal targeting signal (PTS) type 1 consists of a tripeptide sequence (Ser-Lys-Leu, or conserved variant) at the extreme carboxyl terminus and is utilized by the majority of matrix proteins (1). PTS2 is an amino-terminal signal that is used by a small number of proteins (2, 3). The PTS1 receptor has been identified as Pex5p, a protein with TPR domains that bind the PTS1 signal (4, 5) and the PTS2 receptor. Initially reported as a peroxisomal integral membrane protein, Pex5p, has been demonstrated to bind Pex5p via a cytoplasmically oriented SH3 domain (10–12), and a reinvestigation of the subcellular localization of Pex5p in these studies concluded that Pex5p was predominantly cytoplasmic, with a small proportion associated with the peroxisomal membrane. Yeast pex13 mutant strains exhibit defective PTS1 protein import (10–12) and also a marked reduction in the level of membrane-associated Pex5p (10). These findings have been taken to suggest that Pex5p acts as a cycling PTS1 receptor that is docked at the peroxisomal membrane by Pex13p.

Interestingly, pex13 mutants also display defective PTS2 protein import (10–12). The recent finding that the amino-terminal region of Pex13p binds Pex7p (13) is one possible explanation for this effect. Pex14p, a peripheral membrane protein on the cytoplasmic surface of the peroxisome, has also been shown to bind Pex7p, a finding implicating it as a docking protein for this receptor (14). Interestingly, Pex14p has also been shown to bind Pex5p and also weakly to the Pex13p SH3 domain. Another peroxisomal peripheral membrane protein, Pex17p, is also involved in these import pathways and has been shown to associate with Pex14p (15). Thus, Pex13p and Pex14p represent putative convergence points for the two import pathways and provide initial direct evidence of a common translocation machinery for PTS1 and PTS2 proteins.

Despite these important findings, many elements of the import mechanisms remain unknown. In particular, the different roles of the proteins implicated in docking Pex5p at the peroxisome membrane have not been resolved. Thus, Pex13p and Pex14p may compete for binding of Pex5p or form part of a protein import cascade in which a receptor-cargo protein complex moves from one component to another (14). A clarification of the interactions among Pex5p, Pex13p, and Pex14p is therefore warranted, and in this paper we report on molecular analyses that address these questions. On the basis of the findings presented, we propose a revised model for the interactions of Pex5p, Pex13p, and Pex14p in the PTS1 import pathway.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Roche Molecular Biochemicals (Germany). Polymerase chain reaction (PCR) was performed using EXPAND DNA polymerase (Roche Molecular Biochemicals). Oligonucleotide primers were synthesized by Pacific Oligos (Australia) or Bresatec (Adelaide, Australia). [35S]Methionine was purchased from Amersham Pharmacia Biotech (Buckinghamshire, U. K.) or Bresatec. Peptides were synthesized by Chiron Technologies (Melbourne, Australia).

Construction, Expression, and Purification of GST Fusion Proteins—DNA fragments corresponding to Pex13p amino acids Pro927s, Pro934s, Pro936s-Thr939s, and His382s-Thr390s were generated via PCR using the following primer pairs

A: 5'-GGGGATCCACGACAGTTGAGTGT-3' and 5'-GCGGAAAAATTTCGTTGATG-3'

B: 5'-GGGGATCCACGACAGTTGAGTGT-3' and 5'-GCGGAAAAATTTCGTTGATG-3'

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The GST fusion plasmids were electroporated into electrocompetent DH10B E. coli cells (Life Technologies, Inc.) using a GenePulser (Bio-Rad) according to the manufacturer's protocol and then plated onto LB agar (Life Technologies, Inc.) containing 100 μg/ml ampicillin in order to select for successful transformants. Positive constructs were identified via restriction enzyme digest and confirmed by direct sequencing using dyeoxy terminator cycle sequencing on an Applied Biosystems 373 DNA sequencer.

GST fusion proteins were expressed by seedling 1-liter overnight cultures of LB containing 100 μg/ml ampicillin with DH10B cells harboring the relevant plasmid, to an absorbance at 600 nm (A600) of 0.95. Cells were grown to an A600 of 0.5, at which point expression of the GST fusion protein was induced by addition of 0.5 mM isopropyl-β-D-thiogalactoside (Promega, Madison, WI). The cells were gently shaken and sonicated, and the lysate was cleared by centrifugation (30 min at 12,000 × g) and mixed gently for 1 h at room temperature with 1 ml of GSH resin (Amersham Pharmacia Biotech). The lysate/resin mix was loaded onto a column and washed with approximately 40 ml of lyso buffer. The GST fusion proteins were eluted from the resin with 50 mM Tris-HCl and 10 mM glutathione and stored at −80 °C until required. The purity of fusion proteins was confirmed by SDS-polyacrylamide gel electrophoresis.

To quantify the level of binding between the various GST fusions and proteins, equivalent mol of protein were used as determined by methionine determination using the Bio-Rad Protein Assay (Promega). Briefly, in triplicate measurements, 2 μl of coupled transduction reaction product was added to 98 μl of 1 mM NaOH and 2% H2O2, vortexed and incubated at 37 °C for 10 min. 900 μl of ice-cold 25% trichloracetic acetic acid/protein mix was then vacuum filtered onto GF/C filter paper (Whatman, Maidstone, U. K.) pre-wet with 5% trichloroacetic acid. The filters were washed with 10% BLOTTO 3 times, dried and exposed to x-ray film (Eastman Kodak, Rochester, NY) and bound radioactivity determined using densitometry and the analysis software NIH Image, version 1.61. To quantify the level of binding between the various GST fusions and 35S-labeled protein, individual dots were cut from the dried nitrocellulose membrane (Bio-Rad). A negative control of no protein was used to monitor background binding. The nitrocellulose was then blocked for 2 h at room temperature using TBST (137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.4, 0.1% Tween 20) or TBS (137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.4) containing 10% w/v skimmed milk powder (BLOTTO). After blocking, immobilized proteins were probed (overnight at 4 °C) with anti-GST polyclonal antibody (Promega) and the blots were then washed in TBST or TBS (4 × 5 min) and dried at room temperature. At this stage, some of the dried membranes were exposed overnight to x-ray film (Eastman Kodak, Rochester, NY) and bound radioactivity determined using densitometry and the analysis software NIH Image, version 1.61.

When comparing the degree of binding of different 35S-labeled protein, equivalent mol of protein were used as determined by methionine determination using the Bio-Rad Protein Assay (Promega). Briefly, in triplicate measurements, 2 μl of coupled transduction reaction product was added to 98 μl of 1 mM NaOH and 2% H2O2, vortexed and incubated at 37 °C for 10 min. 900 μl of ice-cold 25% trichloracetic acid proton acid mixture was added and the mix incubated on ice for 30 min to precipitate the translation product. 250 μl of the trichloroacetic acid/protein mix was then vacuum filtered onto GF/C filter paper (Whatman, Maidstone, U. K.) pre-wet with 5% trichloroacetic acid, 2% dextrose, 20 g/liter bacteriological agar) to select for HIS+ transformants, which were subsequently tested for their ability to grow in minimal media containing methanol, SM (1.7 g/liter yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories), 5 g/liter ammonium sulfate, 0.5% methanol).
acid; the filter was rinsed three times with 1 ml of ice-cold 5% trichloracetic acid and once with 1 ml of acetone and then dried at room temperature. Dry filter paper was then placed in 10 ml of scintillation fluid and counted, as above, and the results used to determine mol of protein (based on methionine content) for use in dot blot assays as well as the relative molar binding levels.

Competitive binding between radiolabeled Pex5p and unlabeled Pex5p for membrane-immobilized GST-SH3 was tested by adding a constant volume (30 µl) of 35S-labeled Pex5p with various molar ratios (up to 90 µl) of unlabeled Pex5p, both created by in vitro transcription/translation, in 1 ml of TBST BLOTTO. Competitive binding between Pex5p and the Trp100-Glu213 peptide for membrane-immobilized GST-SH3 was tested by adding a constant volume (15 µl) of 35S-labeled Pex5p with various molar ratios (up to 50 µl) of unlabeled Trp100-Glu213 peptide, both created by in vitro transcription/translation, in 1 ml of TBST BLOTTO. Competitive binding between the Trp100-Glu213 peptide and Pex14p for membrane-immobilized GST-SH3 was tested by adding a constant volume (10 µl) of 35S-labeled Trp100-Glu213 peptide with various molar ratios (up to 50 µl) of unlabeled Pex14p, created by in vitro transcription/translation, in 1 ml of TBST BLOTTO. In all cases, membrane-bound radioactivity was determined by scintillation counting. For all competitive binding assays, the amount of unlabeled translation product was approximated from molar calculations of duplicate in vitro transcription/translation reactions in which products were labeled with [35S]methionine, as above.

The specificity of binding of a PTS1-containing peptide to Pex5p was tested by conjugating the peptide CRYHLKPLQSKL (which contains the SKL PTS1 signal) or CRYHLKQLSK (a control peptide lacking the signal) to SulfoLink Coupling Gel (Pierce, Rockford, IL), according to the manufacturer’s protocol. 25 µl of gel conjugate was then mixed with 35 µl of TBST buffer and 15 µl of either 35S-labeled Pex5p or 35S-labeled Pex14p, or 15 µg of purified GST-SH3 fusion protein, and incubated for 16 h at 4 °C. Following incubation, the mixtures were pelleted by centrifugation to yield a supernatant and gel pellet. The gel pellet was washed four times with 1 ml of TBST and resuspended in a final volume of 50 µl of TBST. Proportional volumes of supernatant and pellet were resolved by SDS-polyacrylamide gel electrophoresis. 35S-labeled proteins were visualized by autoradiography, and GST-SH3 was detected by Western analysis using a rabbit anti-SH3 antibody (10).

The effect of PTS1 peptide loading of Pex5p was determined using the same peptides. 50 µl of 35S-labeled Pex5p, created using coupled in vitro transcription/translation, was incubated at 4 °C for 1 h with a 60 µM concentration of either peptide (19) in 1 ml of TBS BLOTTO. These mixtures were then used to probe various membrane-immobilized GST fusion constructs, as described above.

**Three-dimensional Structural Modeling and Eisenberg Analysis**

The technique of protein modeling by homology (20) was used to assign three-dimensional structure to the unknown structure of the Pex13p SH3 domain based on structural information from Fyn, another member of the SH3 domain family. The underlying principle of protein modeling by homology is that within members of a protein family there are evolutionarily conserved regions that tend to be at the inner core of proteins where differences in peptide chain topology would have significant effects on the overall conformation of the protein (20). Several modeled proteins have been subsequently defined by x-ray crystallography, demonstrating the success of this method (21, 22). Coordinates were assigned from the crystal structure of the SH3 domain of Fyn (pdbP) (23) to the secondary structural regions of Pex13 protein according to the alignment shown in Fig. 6A. All suitable loop structures were obtained by searching the Brookhaven Protein Data bank for proteins containing loops of the same length joining similar secondary structural units. All splice junctions were relaxed to a bond length of 1.34–1.37 Å and a bond angle greater than 166°. The resultant crude model was then refined by energy minimization using Biosyn’s consistent valence force field to a convergence criterion of less than 1.0 kcal/mol Å (maximum derivative). The accuracy of three-dimensional model was tested using the Eisenberg method (24–26), which utilizes a comparison of the amino acid sequence of the protein with the model, using a three-dimensional profile which is calculated from the atomic coordinates of the structure. Each residue in the three-dimensional model is characterized by its environment, which is defined by the area of the residue side chain that is buried in the protein structure, the fraction of the side chain that is covered by polar residues, and the local secondary structure. Thus the probability of finding each amino acid in the assigned environment is calculated from a three-dimensional–one-dimensional scoring table derived from a statistical analysis of known protein structures (26). The amino acid sequence of the protein is then compared with the three-dimensional profile generated from the environment classifications and gives rise to an overall profile score and a graphical presentation of the assessment (see inset graphs for Fyn and Pex13 in Fig. 6D). The maximum expected score for a protein is given by: $S_{calc} = \exp\left(-0.833 + 1.008 \times length\right)$, where length is the number of residues. Based on studies of all coordinate entries in the Brookhaven Data Bank derived by x-ray, NMR, and computational methods, along with a number of deliberately misfolded proteins, Eisenberg et al. (24) suggest that a cutoff of $<0.65 \times S_{calc}$ is useful for identifying grossly misfolded structures. Models with profile scores between 0.45 × $S_{calc}$ and $S_{calc}$ are considered correct, although it is still possible that locally misfolded regions occur. It is suggested the examination of profile score in a moving window scan of 10 residues is sufficient to identify locally misfolded regions.

**RESULTS**

**Pex5p Interacts with Pex13p and Pex14p in Vitro**—The results of previous yeast studies have demonstrated that Pex5p interacts with both Pex13p (10–12) and Pex14p (14). The former interaction has been proposed to occur via the SH3 domain of Pex13p. To study these interactions in detail for the P. pastoris proteins, we developed in vitro overlay binding assays in which 35S-labeled “probe” proteins, generated by in vitro transcription/translation (Fig. 1A), were tested for binding to
purified GST fusion proteins (Fig. 1B) immobilized on nitrocellulose membrane. The validity of these binding assays was evaluated by immobilizing various amounts of purified GST-SH3 fusion protein and probing with a constant amount of Pex5p; complex formation, quantified as the amount of membrane-bound radioactivity, increased with immobilized protein in the range 0–16 μg (data not shown). In subsequent experimentation we employed approximately 8 μg (0.2 nmol) of GST fusion protein. Under these conditions, complex formation was proportional to added [35S]-labeled Pex5p up to the maximum volume tested of 50 μl (Fig. 2A) and was competitively inhibited by the addition of unlabeled Pex5p (Fig. 2B). The inability to observe “stoichiometric” competition in this latter experiment was because saturation binding of GST-SH3 was not able to be achieved due to the prohibitively large volumes of labeled Pex5p (i.e. translation product) required. This binding interaction was saturable, however, as demonstrated in separate experiments using 2 μg of GST fusion protein (data not shown). We nevertheless chose 8 μg of GST fusion protein for the routine binding assay to ensure levels of bound radioactivity which would allow accurate and reproducible comparisons among various protein interactions.

The possible involvement of protein regions upstream and downstream of the Pex13p SH3 domain in the interaction with Pex5p was first clarified using different Pex13p fusion constructs. As shown in Fig. 3, which also serves to exemplify the dot blot results obtained in these experiments, Pex5p bound all of the GST-SH3 fusion proteins tested in these experiments, but most effectively to that containing just the SH3 domain. These data confirm that the SH3 domain alone is responsible for binding to Pex5p. We also assessed Pex5p binding specificity by testing for potential binding to another, related SH3 domain, that of the human Pex13 protein (10). These assays showed that binding of [35S]-labeled Pex5p to the GST-human SH3 fusion protein was undetectable, being at the same level as that for the GST control (data not shown).

An important initial question for these studies was whether the preloading of Pex5p with a PTS1-containing protein, an expected prerequisite for the docking of this cytoplasmic receptor at the peroxisomal membrane, would influence the interaction of this receptor with either Pex13p or Pex14p. We therefore synthesized two peptides, one containing a consensus carboxyl-terminal PTS1 targeting signal, CRYHLKPLQSL, and a control peptide lacking this signal, CRYHLKPLQLKS. The specificity of binding of the PTS1-containing peptide to Pex5p was established by determining the binding of [35S]-labeled Pex5p to agarose gel conjugated to either the PTS1 peptide or the control peptide. These results are presented in Fig. 4A and show that Pex5p bound the PTS1 peptide but not the
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control peptide. In further experiments, we tested for possible interaction of these same gel-conjugated peptides with Pex14p and the Pex13p SH3 domain. In both cases, no binding was observed (Fig. 4A).

Pex5p overlay binding assays were carried out with equimolar amounts of immobilized GST-SH3 and GST-Pex14p in the presence of these peptides separately. As shown in Fig. 4B, Pex5p preloaded with the PTS1 peptide bound to both the GST-Pex13p-SH3 fusion protein and to GST-Pex14p and minimally to the GST control fusion protein. Importantly, the amount of Pex5p bound to Pex14p was 20–40 times greater on a molar basis than that to Pex13p-SH3. Binding of Pex5p to Pex13p almost doubled in the absence of the PTS1 peptide, whereas Pex5p binding to Pex14p was reduced in the absence of PTS1 peptide. The results presented here represent results of assays carried out in the absence of 0.1% Tween 20 (see “Experimental Procedures”). This modification to the binding assay was introduced to test whether Pex5p binds PTS1 peptides less efficiently in the presence of detergent; however, we obtained essentially equivalent results whether detergent was present or not (data not shown). Overall, these data suggest that Pex5p binds more effectively to Pex14p when associated with PTS1-containing cargo and to the Pex13p SH3 domain when dissociated from cargo.

Pex14p has also been shown to interact weakly with the Pex13p SH3 domain in Saccharomyces cerevisiae (13, 14, 27). We were able to demonstrate this interaction for the P. pastoris proteins using the in vitro binding assay (Fig. 5). This interaction was less on a molar basis than that seen between Pex5p and the Pex13p SH3 domain, but again it was more pronounced with the GST-SH3 fusion protein that lacked SH3 domain flanking regions.

Modeling of the Pex13p SH3 Domain—To provide a structure suitable for undertaking mutagenesis of the Pex13p SH3 domain to address the observed interactions between both Pex5p and Pex14p, the SH3 domain was modeled to the SH3 domain of Fyn according to the principles of protein modeling by homology. Recently, similar models have been used successfully to design point mutation experiments to determine structure and function of proteins and interactive residues (28). The crystal structure of Fyn (pdbFyn.ent) (23) was selected as a suitable template to model the Pex13p SH3 domain because compared with other solved structures it has high resolution at 2.3 Å and contains a synthetic ligand in its interactive region. In addition, there is significant sequence similarity (64%) between the two SH3 domains with 35% identity over the co-linear alignment used for the modeling (Fig. 6A). The proposed model is presented as a ribbon diagram in Fig. 6B. This model was assessed by Eisenberg analysis, which gives an overall profile score and a graphical presentation of the assessment (see inset graphs for Fyn and Pex13p in Fig. 6B). The profile score of 21.0 (cutoffs 12.5–27.8) obtained for Pex13p indicates that this SH3 domain can achieve a fold similar to that of the Fyn SH3 domain, specifically the antiparallel five stranded β-sandwich. One distinguishing feature of the Pex13p SH3 domain is the extended n-Src loop between the second and third β-strands.

Mutations in the SH3 Domain Affect Pex13p Function in Vivo and in Vitro—Studies on other SH3 domain-ligand interactions have identified SH3 domain residues believed to be involved in direct ligand binding or stabilization of the bound ligand complex. These studies, when applied to the model proposed in Fig. 6, implied that the Pex13p SH3 domain residues Glu291, Glu293, Glu313, Trp321, and Trp322 were potential candidate residues involved in the interaction of the Pex13p SH3 domain with Pex5p and Pex14p. We therefore modified these residues by site-directed mutagenesis and tested the functional activity of the mutated proteins both in vivo and in vitro. The in vivo effect of these mutations was assessed as the ability of the corresponding mutant Pex13 proteins to rescue the growth deficiency of a pex13Δ strain on methanol-containing media, a substrate that requires peroxisomal alcohol oxidase activity and hence functional peroxisomes (29). As shown in Fig. 7A, only expressed Pex13 proteins incorporating the E291K and E296K mutations were unable to rescue growth of the pex13Δ strain, implying loss of Pex13p function in vivo. The specific biological consequences of these mutations were tested in the in vitro assay, as the binding of radioactive Pex5p or Pex14p (Fig. 7B). For Pex5p binding, the E291K and E296K mutations again produced the most perturbation, reducing SH3 binding to 40% and 20%, respectively, of the wild-type level. E291Q and E296Q, which neutralize the negative glutamic acid charge in each case, reduced Pex5p binding only slightly. Mutation of a glutamic acid residue in the n-Src loop, E318K, and the double mutation targeting the conserved tryptophan residues, W321L/
The Pex5p mutants were also evaluated for possible perturbation of the SH3 domain fold as a cause of disrupted protein function. On the basis of energy calculations and profile scores, none of the introduced mutations changed the predicted SH3 fold to any significant degree; all of the Eisenberg scores, a measure of the validity and accuracy of the predicted three-dimensional models (24, 25), were within the acceptable range expected for the wild-type SH3 fold (data not shown). Although it should be noted that this computational analysis would not detect changes that could affect post-translational folding, another independent measure of correct structure and/or susceptibility to proteolysis is the level of soluble fusion protein expression in E. coli (30). In this regard, none of the GST-SH3 fusion proteins was noticeably affected by the introduced mutations (data not shown). These data imply that the observed changes in binding activity are not caused by structural changes in the SH3 domain per se but rather by perturbations of the interactions between the SH3 domain and its ligand(s).

SH3 domain mutations thus affect the biological activity of Pex13p, determined as the function of Pex13p in peroxisome biogenesis in vivo and the binding of Pex5p and Pex14p in vitro. Some of the observed changes in Pex13p function in vivo are consistent with disruption of the binding of the Pex13p SH3 domain to Pex5p, for example, effects of the E291K and E296K mutations. Other data, however, indicate a lack of correlation between effects noted in vitro and those in vivo. One explanation for these differences is that particular SH3 domain mutants may still retain residual Pex13p activity in vivo. Another possible explanation is that the effects of the SH3 modifications may be complicated by the interaction of this domain with binding partners, including Pex5p and Pex14p.

Mapping of the Pex13p Binding Site in Pex5p—The Pex5p dot blot overlay was utilized further to determine the SH3 binding region in Pex5p. In these experiments, Pex5p constructs generated by deletion mutagenesis and labeled using *in vitro* transcription/translation were used to probe nitrocellulose-immobilized GST-SH3 fusion proteins. To allow a more quantitative assessment of relative binding efficiencies, Pex5p constructs were added at equivalent molar ratios determined by the level of radioactive incorporation in each case. The results are shown in Fig. 8. The carboxyl-terminal half of Pex5p, which contains the PTS1-binding TPR domain (4), did not bind the Pex13p SH3 domain. The binding site was found to reside instead in the amino-terminal half of the protein. The minimal Pex5p sequence that produced quantitative binding under these conditions was that encoded by pAU211 and corresponding to the extreme amino-terminal region of Pex5p (4, 18). Deletion of sequence both amino-terminal and carboxyl-terminal to this peptide resulted in minimal binding activity; the extreme amino-terminal region of Pex5p per se had no binding activity (Fig. 8A). The Trp<sup>106</sup>-Glu<sup>213</sup> peptide sequence contains no proline-rich peptide. It does, however, include a number of pentapeptide loose repeats of the form WXXQ/F/WXQDF (downward pointing arrows in Fig. 8A) (4, 18) and a high content of glutamine residues (19.5%). In separate experiments, we have been unable to demonstrate that a synthetic
peptide, QTQWEDQFKDI, which contains the sequence of one of these motifs, displaces Trp\textsuperscript{100}-Glu\textsuperscript{213} peptide binding to the Pex13p SH3 domain (data not shown). We tested for the specificity of binding of the Trp\textsuperscript{100}-Glu\textsuperscript{213} peptide to GST-SH3 by substituting the GST-SH3 point mutants used previously. As with full-length Pex5p, the E291K and E296K substitutions reduced binding of the Trp100-Glu213 peptide to GST-SH3 by substituting the GST-SH3 domain. Further research will be required to determine the nature of the Pex14p binding site. Pex5p and Pex14p Compete for Binding to the Pex13p SH3 Domain—As noted above, both Pex5p and Pex14p are able to bind the Pex13p SH3 domain, albeit with different apparent affinities. The identification of the Pex5p Trp\textsuperscript{100}-Glu\textsuperscript{213} peptide provided a means of testing possible binding competition of Pex5p and Pex14p for the Pex13p SH3 domain; the Trp\textsuperscript{100}-Glu\textsuperscript{213} peptide does not bind to Pex14p, thereby obviating the complication of ternary interaction among the full-length Pex5p, Pex14p, and Pex13p SH3 domain. As shown in Fig. 9, Pex14p competed with Pex5p for binding to the SH3 domain. Together with the results on the effects of the different SH3 mutations, these results suggest that the Pex5p and Pex14p binding sites in the SH3 domain overlap. The region of Pex14p which interacts with the SH3 domain has not been identified. Interestingly the P. pastoris Pex14p contains a putative class II SH3 ligand, APPLPER, and it has been shown recently that mutation of the identical ScPex14p peptide abolished its binding to the ScPex13p SH3 domain, suggesting that this is the SH3 domain ligand (13). We would note that using our dot blot experiments, we have been unable to demonstrate displacement by this peptide of the binding of Pex14p to the SH3 domain (data not shown), but we concede that possible reasons for this may include the low affinity of this interaction and the need for flanking residues present in the full-length Pex14p to achieve a higher affinity interaction.

DISCUSSION

The data presented in this report shed new light on the possible roles of Pex14p and Pex13p in importing PTS1 proteins into the peroxisomal lumen. First, both of these proteins have been shown to interact with Pex5p, the PTS1 receptor, with the interaction with Pex14p predominating when based
on the molar ratio of bound Pex5p. Second, we have established that the loading of Pex5p with PTS1 peptide has provided a means of differentiating the different functions of Pex13p and Pex14p in the PTS1 import pathway. Specifically, loading of Pex5p with PTS1 peptide enhances its interaction with Pex14p but diminishes that with Pex13p. Taken together, these observations suggest a sequence of interaction of Pex5p with these two membrane components in which cargo-laden Pex5p is first docked at the peroxisome membrane via interaction with Pex14p. Subsequent to, or coincident with, release of this PTS1 protein cargo, Pex5p is shifted to Pex13p (Fig. 10). The reason for the shift of Pex5p from Pex14p to Pex13p is not yet clear but may provide a means by which the receptor is able to cycle back to the cytoplasm. The possible influence of other proteins in this proposed receptor transfer mechanism has not been addressed in this study.

The existence of this receptor docking complex raises some interesting questions about the nature of the individual component interactions. The data presented here confirm the contention by us and others that the SH3 domain of Pex13p is indeed the region of Pex13p required for the interaction with Pex5p. Implicit in this hypothesis is that the region of Pex5p involved in this SH3 interaction is separate from the region required for recognition of nascent PTS1 proteins. It is now well established that the TPR domain of Pex5p, located in the carboxyl-terminal half of the protein, is responsible for PTS1
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signal recognition and binding (4, 5). Not surprisingly then, our data here show that the SH3 binding region of Pex5p is distinct from the TPR domain and is situated instead in the amino-terminal half of the protein. Our preliminary data on the Pex14p binding site in Pex5p are consistent with this site also lying in the amino-terminal half of the protein, albeit separate from the SH3 binding site, a result that accords with recent findings on the interaction of human Pex14p with Pex5p (31). These distinct structural features of Pex5p are consistent with a tri-functional role of this receptor in terms of protein interaction: PTS1 protein recognition in the cytoplasm, peroxisomal membrane docking of this receptor-protein complex at Pex14p, and displacement of the unloaded Pex5p to Pex13p.

Our results indicate that Pex14p and Pex5p compete for binding to the SH3 domain of Pex13p. A number of questions arise as to the mechanisms by which this SH3 domain manages two different binding partners. SH3 ligands are assumed to comprise peptides that form left-handed helices and are therefore normally, though not always, proline-rich peptides (32, 33). Promiscuous behavior of SH3 domains in ligand binding is not new. Recently, for example, the Btk SH3 domain has been shown to bind two partners, although in this case both ligands were of the classical polyproline structure (34). The P. pastoris Pex14p contains a putative class II ligand, APPLPER, and the identical peptide is found in the S. cerevisiae Pex14p (14). Both of these proteins interact weakly with their cognate Pex13p SH3 domains (this study; 14). A recent report on the S. cerevisiae proteins has shown that mutations in this polyproline ligand disrupt the interaction of Pex14p with the Pex13p SH3 domain (13). The SH3 binding region of Pex5p, on the other hand, appears to represent a different type of SH3 ligand in that Pex5p does not contain proline-rich stretches and therefore does not apparently fit with the classical structure of such ligands. Whether elements of this peptide sequence do form a non-proline left-handed helix is unknown. One identifiable feature of the SH3 binding site of Pex5p is the presence of a pentapeptide repeat motif WXQXF/WXDQF, a feature also apparent in the S. cerevisiae and human Pex5 proteins (18). Our binding data here, however, indicate that a single motif alone is not effective in competitive inhibition of the binding between the Pex5p Trp100-Glu213 peptide and the SH3 domain in addition, the Trp100-Glu213 peptide, which contains all of the pentapeptide repeats, was the minimal effective binding sequence in our studies. Thus the SH3 domain binding site in Pex5p may be significantly different from classical ligands in both sequence and length. The paper by Schliebs and co-workers (31) is relevant here. The authors note that these pentapeptide repeats form amphipathic a-helices and speculate that they may be binding sites, not for the SH3 domain but instead for Pex14p. In view of this suggestion, it is unclear as to why the Pex5p Trp100-Glu213 peptide, which contains all of the pentapeptide repeats in P. pastoris Pex5p, did not bind Pex14p in our experiments; but again, this may have to do with the requirement in these interactions for flanking or other regions of Pex5p.

The other interesting feature of the Pex13p SH3 domain is the extended n-Src loop. Whether this loop confers unusual ligand specificity is not known. A specific involvement of acidic residues was tested in these studies in light of other studies (35) that have implicated salt bridge formation between SH3 glutamic acid residues and arginine residues in the binding ligand. Substitution of an n-Src loop glutamic acid residue (E318K) was the only mutation tested which abolished binding to both Pex14p and the Pex5p Trp100-Glu213 peptide, a result that suggests that this loop is required for the interactions with both Pex14p and Pex5p. In contrast, Pex5p, but not Pex14p, binding was also attenuated by similar substitutions of two RT-loop glutamic acid residues (Glu291 and Glu296), suggesting that Pex5p binding involves this loop as well. The n-Src loop and the RT loop define the spatial arrangement of the Pex13p SH3 domain putative ligand binding groove. If Pex14p and Pex5p do in fact behave like classical SH3 ligands and interact by binding to the SH3 ligand binding groove, the possible different positioning of these ligands in and around this groove is germane to an understanding of the mechanisms involved. Our data suggest that the Pex14p class II ligand may occupy a relatively small space of the SH3 groove close to the n-Src loop. In contrast, if, as our data suggest, the Pex5p ligand is larger and more structurally complex, it may occupy a greater space in the groove as well as exterior to the SH3 domain and thus involve both the n-Src and RT loops. In this model, competition for SH3 domain binding by Pex5p and Pex14p would be primarily regulated by the n-Src loop, the length of which in Pex13p is atypical in the SH3 protein family. It is of interest here that only the E291K and E296K mutations suppressed
Pex13p function in vivo. The reason for this is not clear but may reflect greater functional significance of the interaction of the SH3 domain with Pex5p rather than with Pex14p. For the S. cerevisiae proteins, Pex13p SH3 binding to Pex14p is not essential for membrane association of Pex14p (13). Binding of Pex5p to the Pex13p SH3 domain, on the other hand, may represent a biological imperative; in our model this would be the Pex13p-mediated shuttling of Pex5p back to the cytoplasm.

The mechanism by which Pex5p may be shuttled from Pex14p to Pex13p has not been directly addressed in these studies, but the speculation by Schliebs et al. (31) that the pentapeptide motifs of Pex5p, in representing potentially different affinity binding sites for Pex14p, is intriguing in that it may provide a means of transferring Pex5p along Pex14p for transfer to the Pex13p SH3 domain. The interaction observed between Pex14p and the Pex13p SH3 domain may also contribute to the mechanisms proposed here. This apparent low affinity interaction between these components may represent a transient interaction to allow Pex5p transfer. It is also tempting to speculate here that the pentapeptide repeats of Pex5p may function in a manner similar to that proposed above to enable Pex5p detachment from the Pex13p SH3 domain for cycling back to the cytoplasm.

The role of Pex7p, the PTS2 receptor, in this putative import complex has not been addressed in these studies. Pex14p was characterized initially as the membrane docking factor for Pex7p (14), but recently it has also been shown that Pex13p functionally interacts with Pex7p via a cytoplasmically oriented amino-terminal region, with this complex enabling the interaction between Pex5p and Pex7p (13). Further studies will be required to incorporate Pex7p into this emerging model and to provide links between PTS1 and PTS2 protein import.

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