Brief Report

The Peroxin Pex19p Interacts with Multiple, Integral Membrane Proteins at the Peroxisomal Membrane

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Abstract. Pex19p is a protein required for the early stages of peroxisome biogenesis, but its precise function and site of action are unknown. We tested the interaction between Pex19p and all known Pichia pastoris Pex proteins by the yeast two-hybrid assay. Pex19p interacted with six of seven known integral peroxisomal membrane proteins (iPMPs), and these interactions were confirmed by coimmunoprecipitation. The interactions were not reduced upon inhibition of new protein synthesis, suggesting that they occur with preexisting, and not newly synthesized, pools of iPMPs. By mapping the domains in six iPMPs that interact with Pex19p and the iPMP sequences responsible for targeting to the peroxisome membrane (mPTSs), we found the majority of these sites do not overlap. Coimmunoprecipitation of Pex19p from fractions that contain peroxisomes or cytosol revealed that the interactions between predominantly cytosolic Pex19p and the iPMPs occur in the organelle pellet that contains peroxisomes. These data, taken together, suggest that Pex19p may have a chaperone-like role at the peroxisome membrane and that it is not the receptor for targeting of iPMPs to the peroxisome.

Key words: organelle biogenesis • protein localization • peroxin • chaperone • mPTS receptor

Introduction

The machinery for protein import into peroxisomes has been characterized from many organisms (reviewed in Hettema et al., 1999). Genetic studies in several different yeast species identified at least 23 genes (PEX) that encode proteins (peroxins) necessary for peroxisomal protein import and biogenesis (Hettema et al., 1999; Koller et al., 1999; Brown et al., 2000). Orthologs of many of these proteins are known in human cells and are often defective in patients suffering from specific human genetic diseases. Despite this rapid growth in the identification of components required for peroxisome protein localization, functional characterization of these proteins has lagged.

The early stages of peroxisome matrix protein import have been characterized mainly by studies of protein–protein interactions (reviewed in Hettema et al., 1999). Peroxisome matrix proteins are synthesized in the cytosol and usually contain one of two peroxisome-targeting signals (PTSs),1 PTS1 or PTS2. These PTSs interact specifically with predominantly cytosolic targeting receptor proteins. The targeting receptors for PTS1 and PTS2 proteins are encoded by PEX5 and PEX7, respectively, and defects in these lead to impaired import of either PTS1- or PTS2-containing proteins. Both PTS receptors interact with a complex of proteins at the peroxisome membrane by binding to Pex13p and/or Pex14p, and this docking precedes membrane translocation by an unknown mechanism.

Whereas all pex mutants have defects in the localization of peroxisome matrix proteins, only a subset show defects in the biogenesis of the peroxisome membrane (reviewed in Hettema et al., 1999; Snyder et al., 1999b; Hettema et al., 2000). Most pex mutants contain organelle remnants that proliferate under peroxisome-inducing conditions, and these remnants contain iPMPs. In contrast, a few pex mutants are distinguished by the fact that they do not contain the typical iPMP-containing remnants. For example, yeast pex3Δ mutants and the mammalian PEX16 mutant contain no detectable peroxisome remnants (reviewed in Hettema et al., 1999).

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1Abbreviations used in this paper: DSP, dithiobis(succinimidyl propionate); GFP, green fluorescent protein; iPMP(s), integral peroxisome membrane protein(s); mPTS(s), integral peroxisome membrane protein targeting signal(s); PTS(s), peroxisome targeting signal(s).
Materials and Methods

Molecular Biological Techniques

DNA procedures, yeast transformations, and cell growth were performed by standard methods (Snyder et al., 1999a). All DNA-oligonucleotide primers are listed in Table I.

Two-Hybrid Analysis

Cloning vectors, tester strains, and screening by two-hybrid analysis have been described (Faber et al., 1998). A II two-hybrid clones were tested for autoactivation. Two-hybrid clones containing PEX3, PEX10, PEX17, PEX19, PEX22, and PEX22 sub-domains were described previously (Koller et al., 1999; Snyder et al., 1999a; Snyder et al., 1999b). Two-hybrid clones containing full-length (FL) PEX genes and subdomains were amplified by PCR using primers as follows: PEX2 (FL) with 2H2u and 2H2.6d; 2.3 with 5

Green fluorescent protein (GFP) hybrids were constructed as follows: GFP was amplified with primers 5′GFPNotI and 3′GFPHindIII. The resulting fragment was cut with PstI and HindIII cloned into PstI-HindIII cut pBluescript SK+ (Sears et al., 1998). All fragments of the pMPs were amplified with the indicated primers, ligated into pCRblunt, and cut with either BamHI and EcoRI (depending on the site in the 5′ primer) and NotI and cloned into a BamHI-NotI cut pBluescript SK+ (Rieder and Emr, 1997) with minor modifications. Cross-linking was performed using DSP. DSP was dissolved in DMSO just before use at 600 µg/ml and incubated for 30 min at room temperature. DSP was dissolved in DMSO just before use at 100 µg/ml and incubated for 30 min at room temperature. DSP was dissolved in DMSO just before use at 100 µg/ml and incubated for 30 min at room temperature.

Biological Techniques

Crude cell-free extracts, SDS-PAGE, and Western blot analyses were performed as described previously (Snyder et al., 1999a). Primary antibodies were used as follows: α-Pex19p (1:2,000), α-Pex3p (1:1,000), α-Pex10p (1:2,000), α-Pex22p (1:2,000), α-GFP (1:2,000), α-Sgcglu6-phosphate dehydrogenase (G6PDH) (1:2,000), and α-HA (1:1,000). Secondary antibodies and detection methods have been described (Snyder et al., 1999b).

The cross-linking was a standard procedure described previously (Rieder and Emr, 1997) with minor modifications. Cross-linking was performed from April 9, 2017.
We confirmed the interactions observed in the two-hybrid analysis by coimmunoprecipitation experiments. For this analysis, we used strains expressing HA epitope-tagged, fully functional versions of Pex13p (Johnson, M.A., W.B. Snyder, M. Veenhuis, S. Subramani, and J.M. Cregg, manuscript submitted for publication) and Pex17p (Snyder et al., 1999b) to facilitate the detection of the coprecipitated proteins in anti-Pex19p immunoprecipitations. Protein complexes were cross-linked with DSP to allow immunoprecipitation under denaturing conditions. In these Pex19p immunoprecipitations, we observed that Pex2p, Pex3p, Pex10p, Pex13HAp, Pex17HAp, and Pex22p coimmunoprecipitated with Pex19p in a cross-linker-dependent manner (Fig. 2, lanes 1 and 2). Previously, we reported that this technique does not cross-link all peroxisomal proteins into one complex (Koller et al., 1999; Snyder et al., 1999b) and we did not observe Pex14p, a PMP (Johnson, M.A., W.B. Snyder, M. Veenhuis, S. Subramani, and J.M. Cregg, manuscript submitted for publication), in the Pex19p immunoprecipitations (Fig. 2). This indicates that Pex19p specifically forms complexes with these six iPMPs under physiological conditions. Since 25-fold more cell extract is shown from the immunoprecipitations than the whole cell lysates, cross-linking between Pex19p and a given iPMP is very inefficient, with <1% of the total cellular pool of each iPMP found in the coimmunoprecipitates with Pex19p. The remaining iPMP, which did not show an interaction with Pex19p in the two-hybrid assay, Pex12p, also formed a complex with Pex19p (not shown), but this interaction might be indirect since it was not observed in the two-hybrid test (see Discussion) and was not examined further.

Pex19p Interacts with Preexisting, and Not with Newly Synthesized, Pools of iPMPs

To determine whether the interactions occurred between Pex19p and the newly synthesized iPMPs, we disrupted all new protein synthesis and performed the coimmunoprecipitation assay. Treatment of P. pastoris with 1 mg/ml of cycloheximide inhibits ~90% of protein synthesis (Tuttle and Dunn, 1995, and our unpublished data). We treated cells with cycloheximide for 45 min and then performed the cross-linking and coimmunoprecipitation as usual. We did not see any difference in the quantity of coimmunoprecipitated proteins in the presence or absence of cycloheximide (Fig. 2). This suggests that complex formation between Pex19p and the six iPMPs examined occurs in the absence of new protein synthesis with the preexisting pools which are at the peroxisome membrane.

Pex19p-binding Sites on the iPMPs Generally Do Not Overlap with mPTSs

To determine if Pex19p, a predominantly cytosolic protein, functions like a targeting receptor by binding the mPTSs, we first needed to identify the mPTS regions in the iPMPs. For this purpose the mPTS regions must be sufficient to bring iPMPs to the peroxisome, but are not necessarily required for insertion into the membrane. GFP fusions were constructed which contained fragments of an iPMP at the amino terminus of GFP (Fig. 1). The localization of these PMP-GFP fusion proteins was assayed.
by density gradient centrifugation and Western blotting, to determine the positions of marker proteins and the PMP-GFP fusions in the gradient. As shown in Fig. 3 and summarized in Fig. 1, a region of the iPMPs that is able to function as a mPTS was defined for all of the iPMPs (Figs. 1 and 3, constructs 2.1, 3.1, 10.3, 13.2, 17.1, and 22.1). For Pex2p, Pex3p, Pex17p, and Pex22p the remaining portions of the proteins did not contain targeting information, at least in the context of the GFP fusions we created, thereby demonstrating the necessity of the mPTS. For Pex10p the remaining regions, 10.1 and 10.2, were not expressed, or were unstable in yeast. For Pex13p only the large cytosolic, SH3 containing, domain (13.3) was additionally tested and did not function as a mPTS. Several sub-domains of the 17.3 region were tested but did not interact (not shown). It is unclear where Pex19p binds in Pex17p, but Pex19p did not show an interaction with the mPTS (17.1). Only for Pex2p did we observe Pex19p interacting with segments containing the mPTS (2.1). This clearly demonstrates that the Pex19p-binding sites are distinct from the mPTS regions in at least four of the six iPMPs examined.

The hypothesis that Pex19p is the cytosolic receptor for the targeting of iPMPs to the peroxisome was further tested by determining whether the site of Pex19p binding in the iPMPs coincided with the mPTSs. Fig. 1 summarizes the data of the two-hybrid analysis between Pex19p and the various sub-domains of the iPMPs examined. We narrowed the subdomain of Pex19p interaction for all of the iPMPs except Pex17p. For Pex3p, Pex10p, Pex13p, and Pex22p the interaction domain did not function as a mPTS. Several sub-domains of the 17.3 region were tested but did not interact (not shown). It is unclear where Pex19p binds in Pex17p, but Pex19p did not show an interaction with the mPTS (17.1). Only for Pex2p did we observe Pex19p interacting with segments containing the mPTS (2.1). This clearly demonstrates that the Pex19p-binding sites are distinct from the mPTS regions in at least four of the six iPMPs examined.

**Site of Interaction of Pex19p with iPMPs**

Identification of the subcellular site of interaction between Pex19p and the iPMPs is critical for understanding Pex19p

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**Figure 1. Summary of Pex19p interactions and localization of PMP-GFP fusions.** Relative positions of iPMP domains tested for two-hybrid interaction with Pex19p and mPTS function are shown. Black bars indicate regions that functioned as a mPTS. Hatched bars indicate regions binding to Pex19p. Note that the mPTSs and Pex19p-binding sites are clearly nonoverlapping for Pex3p, Pex13p, and Pex22p. The symbol + indicates growth of the strain on media lacking histidine. A A indicates autoactivation and growth on media lacking histidine in the absence of a Pex19p activation domain fusion. Data for PMP-GFP localization are in Fig. 3. Cyt indicates that the PMP-GFP fusion was at the top of the gradient and did not colocalize with peroxisomes. Per indicates that the PMP-GFP fusion showed significant colocalization with the peroxisome peak. The approximate positions of transmembrane domains from published reports are shown by the dark boxes. The light boxes represent putative transmembrane regions predicted from computer programs. NS indicates that the fusion was not stable or not synthesized.
Pex19p Interacts with Preexisting Peroxisomal Pools of Many Different iPMPs

We have shown, using the yeast two-hybrid system, that Pex19p interacts with 6 (Pex2p, Pex3p, Pex10p, Pex13p, Pex17p, and Pex22p) of seven iPMPs in P. pastoris. We did not observe an interaction between Pex19p and Pex12p, except in coimmunoprecipitation experiments. It is likely that the observed interactions in the two-hybrid system are direct because the test is performed in situ, such bridging was not observed in the same test pairings of P. pastoris proteins (Snyder et al., 1999b) as it was in S. cerevisiae (Huhse et al., 1998). Furthermore, such bridging between the endogenous S. cerevisiae proteins and the P. pastoris two-hybrid fusion proteins seems unlikely, since S. cerevisiae peroxins fail to complement the corresponding P. pastoris deletion mutants (Koller et al., 1999; Snyder et al., 1999b).

The two-hybrid interactions observed were confirmed by coimmunoprecipitation and occurred in the absence of new protein synthesis. Using a standard cross-linking procedure we observed Pex2p, Pex3p, Pex10p, Pex13HAp, Pex17HAp, and Pex22p in Pex19p immunoprecipitations. This procedure does not cross-link all peroxisomal proteins (Koller et al., 1999; Snyder et al., 1999b). In fact, we can only cross-link a very small percentage (<1%) of iPMPs.
to Pex19p (Fig. 2) and a control protein, Pex14p, was not complexed to Pex19p (Fig. 2).

The prevailing model of peroxisome biogenesis posits that iPMPs are synthesized in the cytosol and targeted directly to the peroxisome (Lazarow and Fujiki, 1985). Working within this model, we would predict that if Pex19p bound to the newly synthesized pool of iPMPs either as a chaperone, a targeting receptor, or membrane insertion factor, we should have observed a decrease in the amount of iPMPs that cross-linked to Pex19p. The coimmunoprecipitation of the iPMPs with Pex19p was not disrupted or reduced by pretreatment of the cells with cycloheximide, a treatment known to disrupt new protein synthesis (Tuttle and Dunn, 1995). This strongly suggests that Pex19p does not form complexes with the newly synthesized iPMPs, and points to the peroxisome as the site of interaction because that is where the preexisting iPMPs are located.

Multiple Lines of Evidence Show that Pex19p Is Unlikely to Function as the mPTS Receptor

The mPTSs and Pex19p-binding Sites Are Distinct in Multiple iPMPs. A very attractive model for Pex19p function is one in which Pex19p binds to multiple iPMPs at the mPTS and consequently brings them from the cytosol to the peroxisome. However, our analysis of the Pex19p-binding sites on the iPMPs and our identification of the regions that are responsible for targeting them to the peroxisome, the mPTSs, suggests that Pex19p is not the cytosolic receptor for the mPTS. We have shown for Pex3p, Pex13p, and Pex22p that a Pex19-binding site is separate from the mPTS regions in these iPMPs. These data show that the mPTSs and Pex19p-binding domains are clearly distinct in three of the iPMPs tested.

Experimental limitations with the other iPMPs tested pose a few potential caveats. Pex2p interacts with Pex19p in a domain that also functions as a mPTS. However, our analysis can not currently differentiate between the amino acids responsible for Pex19p binding and those which are critical solely for mPTS function in Pex2p. In a second case, we were unfortunately unable to find a distinct Pex19p interaction domain in Pex17p, but two-hybrid experiments with S. cerevisiae proteins show that ScPex19p binds to amino acids 52-88 of ScPex17 (W.H. Kunau, unpublished results), which is separate from the corresponding region in PpPex17p that functions as a mPTS. Unfortunately, we were also unable to test if the Pex19p interaction domain in Pex10p functions as a mPTS due to the instability of the GFP fusion protein. None of these caveats, however, necessarily support the hypothesis that Pex19p is indeed the mPTS receptor in light of the additional evidence presented in this paper. Our conclusions are therefore based on correlative evidence, that in the majority of the iPMPs tested (Pex3p, Pex13p, Pex17p, and Pex22p), the available data show that the mPTS and Pex19p-binding sites are indeed nonoverlapping.

The Peroxisomal Interaction of Pex19p with Preexisting iPMPs Is Inconsistent with a Role for Pex19p as the Cytosolic mPTS Receptor. A analyzing the subcellular site of Pex19p and iPMP interactions further defined their significance. Treatment of cells with cross-linker before lysis did not alter the fractionation pattern of the proteins examined when compared with previous reports. The fact that the majority of Pex19p is in the supernatant fraction (Fig. 4 A, lane S), whereas the iPMPs are found in the organelle pellet (Fig. 4 A, lane P), may seem paradoxical with the fact that the proteins interact. Indeed, immunofluorescence microscopy has also revealed that the majority of Pex19p is cytosolic (not shown) and is not simply released from the organelle during fractionation procedures. In the anti-Pex19p immunoprecipitations, iPMPs were in the pellet fractions (Fig. 4 B, lane P), with very little (few percent of total) in the supernatant fractions (Fig. 4 B, lane S). Although most of the Pex19p was cytosolic, only the Pex19p in the organelle pellet was significantly complexed with iPMPs. The minor amounts of iPMPs that are in the supernatant fractions are likely to be the result of peroxisome rupture, an often observed problem with these fractionation procedures (Snyder et al., 1999a, and references therein; Hettema et al., 2000). This minor pool of iPMPs that might be released from the membrane could also be complexed with Pex19p, thus resulting in a very low percentage of iPMPs in the supernatant of the Pex19p immunoprecipitations. Indeed, our control experiments suggested that the nonpelletable pool of Pex3p does not represent the newly synthesized pool since treatment with cycloheximide did not diminish cytosolic Pex3p in fractionation experiments (Fig. 4 C). Taken together, our results point to the fact that the organelle-associated pool of Pex19p interacts with the iPMPs at the peroxisome. This conclusion explains the apparent paradox stated above regarding the Pex19p–iPMP interactions. We must point out that if Pex19p initially bound to iPMPs in the cytosol and then rapidly targeted to the peroxisome membrane, we might miss this cytosolic interaction in our steady state analysis. This seems unlikely because the cytosolic iPMPs would represent the newly synthesized pool that has not yet been targeted to the peroxisome, but our experiment with cycloheximide treatment does not reveal a reduction in the Pex19p–iPMP interactions (Fig. 2). Therefore, our conclusion that the interactions occur at the peroxisome membrane is supported by multiple pieces of evidence (Figs. 2 and 4).

The Peroxisomal Interaction of Pex19p with iPMPs Is Consistent with the Topology of Most iPMPs. Pex19p may interact on the cytosolic side of the peroxisome membrane as evidenced by the fact that the domains of Pex2p, Pex13p, and Pex22p that interact with Pex19p are known or predicted to be cytosolic. There are problems with incorporating the published topology of Pex10p into the Pex19p interaction model as previously described (Snyder et al., 1999a). For Pex3p and Pex17p, the tested domains that interact with Pex19p span the membrane and the precise interaction domain still needs to be identified. Nonetheless, the identification of cytosolic Pex19p-binding sites on the three of the iPMPs further supports our conclusion based on evidence from the cycloheximide treatments and fractionation data that the interactions occur at the cytosolic face of the peroxisome membrane.

Models for Pex19p Action

All the data presented clearly rule out the attractive model in which the interactions between Pex19p and the iPMPs
mediate targeting of newly synthesized iPMPs from the cytosol to the peroxisome, i.e., the mPTS-receptor model. This model makes several predictions. First, Pex19p would be expected to interact with the iPMPs in the cytosol, but we show that the interactions occur at the peroxisome. However, this analysis might not detect cytosolic interactions if the iPMP-Pex19p complexes are recruited rapidly to the membrane, but the evidence outlined in the other points below suggests that this is not the case. Second, as the newly synthesized pools of iPMPs are depleted during cycloheximide treatment, cross-linking between Pex19p and the iPMPs should diminish, but the cross-linking remains the same. Third, Pex19p should bind to the mPTS domain, but most of the Pex19p-binding domains on the iPMPs do not function as a mPTS. Finally, pex19Δ mutants should accumulate iPMPs in the cytosol, but Pex3p (Snyder et al., 1999a), Pex17p, and Pex22p (our unpublished results) localized to membranous remnants. In S. cerevisiae no iPMP-containing remnants were observed in pex19Δ mutants (Hettema et al., 2000), but this could be the result of defects in peroxisome biogenesis, not iPMP targeting, or the inability to detect the remnants. For the proteins examined here, Pex19p does not seem to function as a mPTS receptor, but we cannot rule out that possibility for other iPMPs.

While this manuscript was being revised, work was published showing that in human cells multiple iPMPs interact with Pex19p (Sacksteder et al., 2000). Our work complements that of Sacksteder et al. (2000) and reaffirms the evolutionary conservation of the interaction of Pex19p with multiple iPMPs. In the human system, Sacksteder et al. (2000) conclude that Pex19p interacts with the newly synthesized pool of iPMPs by binding to domains that contain the mPTS. Several models for Pex19p function were suggested, but the data could not distinguish between them. The favored hypothesis was that Pex19p functions as the cytosolic receptor mediating the localization of iPMPs or as a chaperone in the cytosol for newly synthesized iPMPs. Our data do not support these two hypotheses. In fact, when we began this study our working model was that Pex19p might be the mPTS receptor, but our findings based on multiple, complementary experiments (see above) have led us away from this idea.

Our data point to a role for Pex19p interacting with the preexisting iPMPs at the peroxisome membrane. None of the data are inconsistent with this conclusion, despite a few potential caveats described above. The pex19Δ mutants contain small, vesicular remnants suggesting that Pex19p functions at an early step in peroxisome biogenesis (Snyder et al., 1999a). The interaction of Pex19p with multiple iPMPs somehow allows maturation of the small vesicles to mature peroxisomes. For iPMPs to function properly they may need to assemble and disassemble dynamically into multiple, heteroligomeric complexes that carry out essential functions. Evidence for iPMPs forming mutually exclusive complexes with either Pex19p or the PTS receptors has been provided (Snyder et al., 1999b). It is unlikely that Pex19p is required for the targeting or insertion of iPMPs into the peroxisomal membrane since it interacts mainly with iPMPs at the peroxisome, and because it does not interact with the newly synthesized iPMPs, at least three of which are sorted to the membranes of remnants in a Pex19p-independent manner. Rather, we envision that Pex19p functions as an assembly or disassembly factor, or as a chaperone, to regulate the complexes comprising the iPMPs already in the peroxisomal membrane. Exactly how Pex19p does this remains a subject for future work.

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