Brief Report

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

William B. Snyder, Antonius Koller, A. Jobu Choy, and Suresh Subramani

Department of Biology, University of California, San Diego, La Jolla, California 92093-0322

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

W.B. Snyder and A. Koller contributed equally to this paper.

Address correspondence to Dr. Suresh Subramani, Department of Biology, University of California, San Diego, 3230 Bonner Hall, 9500 Gilman Drive, La Jolla, CA 92093-0322. Tel.: (858) 534-2327. Fax: (858) 534-0053. E-mail: ssubramani@ucsd.edu

[1]*Abbreviations 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).

Hettema et al., 1999, 2000). Partial defects in iPMP localization have been noted for *Pppex17Δ* mutants (Snyder et al., 1999b). However, no peroxin has been shown to bind mPTS regions to directly mediate iPMP targeting to the peroxisome. *pex19Δ* mutants show a severe biogenesis defect with no detectable remnants in *S. cerevisiae* (Hettema et al., 2000), and small vesicular remnants in *P. pastoris* (Snyder et al., 1999a). Despite these differences in phenotypes for *pex19Δ* mutants, Pex19p, a predominantly cytosolic protein (Götte et al., 1998; Snyder et al., 1999a), must be important for the proper assembly of peroxisomes.

Previously, it was shown that Pex19p and Pex3p from *S. cerevisiae* and *P. pastoris* interact (Götte et al., 1998; Snyder et al., 1999a), and that PpPex19p also interacted with PpPex10p (Snyder et al., 1999a) and PpPex17p (Snyder et al., 1999b). However, the nature, site, and relevance of these interactions remain unclear. We have continued our analysis of interactions among *P. pastoris* peroxins and discovered that Pex19p interacts with most of the iPMPs. Analyzing the sites to which Pex19p binds in the iPMPs, the identification of mPTSs, the subcellular site of interaction, and whether the interactions occur in the absence of new protein synthesis, has greatly extended our knowledge of the function of an important player in peroxisome biogenesis.

Materials and Methods

Molecular Biological Techniques

DNA procedures, yeast transformations, and cell growth was 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). All 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 2H2d; 2.1 with primers 2H2u and 2H2.6d; 2.3 with 5′2.3 and 2H2d; 3.1 with 5′3.1 and 2h3.1d; 3.2 with 5′3.2 and 2h3.2d; 3.3 described elsewhere (Snyder et al., 1999a); 10.1 with 5′10.1 and 3′10.1; 10.2 with 5′10.2 and 2h10d; 10.3 with 5′10.3 and 3′10.1; *PEX13*(FL) with 2h13u and 2h13d; 13.1 with 2h13u and P13TMD; 13.2 with 5′13.2 and 2h13.2d; 13.3 with P13SH3u and 2H13d; 17.1 with 2h17u and 2hMPTSd; 17.2 and 17.3 described elsewhere (Snyder et al., 1999b). All PCR products were cloned into pCRblunt (Invitrogen) excised with the following enzymes and ligated into compatibly cut pKNSD55: *PEX2* clones with BamHI and EcoRI; *PEX3* with BglII and PstI; *PEX10* with BamHI and EcoRI; *PEX13* with BglII and SpeI; *PEX17* with BglII and EcoRI.

Green Fluorescent Protein Fusions

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 and cloned into PstI-HindIII cut pIB2 (Sears et al., 1998). All fragments of the iPMPs were amplified with the indicated primers, ligated into pCRblunt, and cut with either BamHI or BglII (depending on the site in the 5′ primer) and NotI and cloned into a BamHI-NotI cut pIB2-GFP. Fragment 2.3 was amplified with primers 5′2.3 and 3′2.3; 2.1 with 5′2.1 and 3′2.1; 3.1 with 5′3.1 and 3′3.1; 3.2 with 5′3.2 and 3′3.2; 3.3 with 5′3.3 and 3′3.2; 10.1 with 5′10.1 and 3′10.1; 10.2 with 5′10.2 and 3′10.2; 10.3 with 5′10.3 and 3′10.1; 13.2 with 5′13.2 and 3′13.1; 13.3 with 5′13.3 and 3′13.3; 17.1 with 5′17.1 and 3′17.1; 17.2 with 5′17.2 and 3′17.2; 22.1 with TK31 (Koller et al., 1999) and 3′22.1; 22.2 with TK38 and 3′22.2.

Table I. Primers

Name	DNA sequence (5′→3′)
2h2u	GCGGATCCATGCCCAATAGGCTCATACC
2h2d	GATCCTGCAGCTATAACGCACCAACGAAAAAAC
5′2.1	AGGATCCATGCCCAATAGGCTCATACC
2h2.5d	AGAATTCCTAATCAGTCTCTAGATCATAATCTTC
5′2.5	AGGATCCATGGGAGAAGACTTGAGCACC
2H2.6d	AGAATTCCTAACTTCCAGATACCAGAAATGTAAC
5′3.1	AAGATCTATGTTGGAGTACACGGCAGG
2h3.1d	GCGCCTGCAGCTATGGTTCACAAAGTACTGGCAGTG
5′3.2	AAGATCTATGATTATGGACGATTTGCCAGTAG
5′3.3	AAGATCTATGGTAATCCACTGTGCTTAGTGACGATTTC
2h3.2d	GCGCCTGCAGCTAAGGATCAAAATTAGAGTATAC
5′10.1	AGGATCCATGCCCCCATCTGAAGAGATC
3′10.2hy	ACTAGGTCAAAAAATAAGGCAAGATGGCG
5′10.2	AGGATCCATGAGGTTATTTCGGCGATAAAATCC
2h10d	GAGAGAATTCTCAAAATACAACCAAGAAGTAC
5′10.3	AGGATCCGGGGAAGAATACGTTGACCTAATC
2h13u	GTCCAGATCTATGAGTGACTCATCAGCTCC
2h13d	CGCGACTAGTTTATGTCTTCATCTTCTGAAATTC
P13TMD	CGCGACTAGTTAAAAGATACGGAAATCCCAC
5′13.2	AGGATCCATGATCAACTGGTTGAAACGAATC
P13SH3u	GTCCAGATCTAAGAAATTAATTGCTCATCTTGC
2h17u	GTCCAGATCTATGTCGTCAAGGCGCAACG
2hMPTSd	GGAATTCCTAACGAGTCAAAAGAGCAG
17cytU	GCGCAGATCTCGACCTATGTTGAAGCTTC
2H17NB	GAATTCTTAAAACTTGATCGTCTGTCTTCC
3′3.1	AGCGGCCGCTGGTTCACAAAGTACTGGCAGTG
3′3.2	AGCGGCCGCAGGATCAAAATTAGAGTATACAC
3′10.1	AGCGGCCGCGGTCAAAAAATAAGGCAAGATGGCG
3′10.2	AGCGGCCGCAAATACAACCAAGAAGTACCCTAG
5′13.1	AAGATCTATGAGTGACTCATCAGCTCC
3′13.1	AGCGGCCGCAGCAATTAATTTCTTTAAAAGATACGG
5′13.3	AGGATCCATGCATCTTGCTGAGACCAGTC
3′13.3	AGCGGCCGCTGTCTTCATCTTCTGAAATTCTG
5′17.1	AGGATCCATGTCGTCAAGGCGCAACGTG
3′17.1	AGCGGCCGCAGGTCGAATCAGAAAGAGGGCC
5′17.2	AAGATCTATGTTGAAGCTTCAATACGAAAG
3′17.2	AGCGGCCGCGGTACTAGACCTATTTCTTTTC
TK38	GGATCCATGAAGAGTTTTATAACGTCCGAC
3′22.1	AGCGGCCGCGTAAACAGAGTACCCCAGTCC
3′22.2	AGCGGCCGCATTGTATATATATTGATTCACTG
5′GFPNotI	ACTGCAGCGGCCGCGAGTAAAGGAGAAGAAC
3′GFPHindIII	AAAGCTTTATTTGTATAGTTCATCCATGCC

Biochemical Techniques

Crude cell-free extracts, SDS-PAGE, and Western blot analyses were performed as described previously (Snyder et al., 1999a). Primary antibodies were as follows: α-Pex19p (1:2,000), α-Pex3p (1:10,000), α-Pex2p (1:2,000), α-Pex10p (1:2,000), α-Pex22p (1:2,000), α-GFP (1:2,000), α-Scglucose-6-phosphate dehydrogenase (G6PDH) (1:2,000), and rat 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 five A_{600} equivalents of oleate-induced cells spheroplasted as described previously (Faber et al., 1998). For each cross-linking reaction, spheroplasts (5 A_{600} units) were pelleted and resuspended in 1 ml lysis buffer (20 mM potassium phosphate pH 7.5, 1 mM EDTA, and protease inhibitors). DSP (dithiobis[succinimidyl propionate]) (Pierce Chemical Co.) was added to a final concentration of 200 μg/ml and incubated for 30 min at room temperature. DSP was dissolved in DMSO just before use at a concentration of 20 mg/ml. The cross-linker was quenched by adding

1 M hydroxylamine to a final concentration of 20 mM. The reaction was then adjusted to 5% TCA and incubated on ice for at least 20 min. The TCA precipitates were washed twice with 1 ml cold acetone and dried. For each whole cell lysate control, the TCA pellet was resuspended in 0.5 ml of urea sample buffer (6 M urea, 10% β-mercaptoethanol, 6% SDS, 125 mM Tris, pH 6.8, and 0.01% Bromophenol blue), heated to 65°C for 10 min, and further diluted 1:5 in urea sample buffer before loading on the gel. For immunoprecipitations, the TCA pellets were resuspended in 100 μl urea cracking buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS, and 6 M Urea) and heated to 65°C. For minus (−) cross-linker reactions, the TCA pellet was resuspended in 100 μl of urea cracking buffer with 10 mM β-mercaptoethanol to cleave the cross-linker before immunoprecipitation. This was done so that all samples would be treated with cross-linker since the migration of many proteins in SDS-PAGE is altered by treatment with DSP and it was desirable that the proteins in + and − cross-linker samples have the same mobility. 1 ml of IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, and 0.5% Tween-20) was then added to the samples and the insoluble material pelleted for 20 min in a microfuge. 1 ml of this cleared lysate was added to a clean tube. Immunoprecipitation was performed using crude Pex19p antisera and protein A–Sepharose or affinity-purified anti-Pex19p coupled to Affi-Gel Hz beads as described by the manufacturer (Bio Rad). The immunoprecipitates were washed two times with urea-IP buffer (100 mM Tris, pH 7.5, 200 mM NaCl, 2 M urea, and 0.5% Tween-20) and two times with IP buffer. The beads were then resuspended in 90 μl of 1.11× urea sample buffer lacking β-mercaptoethanol and heated to 37°C to dissociate the antigen complexes from the antibodies. 90 μl of these samples were removed from the beads and placed in a clean tube. 10 μl β-mercaptoethanol was added and the samples were heated to 65°C before SDS-PAGE to cleave the cross-linker and allow the resolution of individual proteins. Cycloheximide was added to cells for 45 min before the standard cross-linking procedure where indicated.

Subcellular Fractionations

Differential centrifugation was performed as described (Faber et al., 1998) following treatment with DSP. In brief, 750 ml of oleate-grown cells (~1,000 A_{600} total) where spheroplasted, washed with spheroplasting buffer, and resuspended in 50 ml spheroplasting buffer. DSP was added to 200 μg/ml and incubated for 30 min at room temperature. Spheroplasts were then pelleted and processed for differential centrifugation. Nycodenz gradient analysis of cellular fractions is described elsewhere (Faber et al., 1998).

Results

Pex19p Interacts with Six Different iPMPs in P. pastoris

We examined the protein interactions between Pex19p and all *P. pastoris* peroxins using the yeast two-hybrid system. Previously, we reported an interaction between Pex19p transcriptional activation-domain fusions and DNA-binding domain fusions of Pex3p, Pex10p, and Pex17p that leads to activation of transcription of the *HIS3* reporter-gene and a His^+ phenotype for the two-hybrid tester strain (Snyder et al., 1999a,b; Fig. 1). In this study, we confirmed these associations and, in addition, we observed interactions between Pex19p and Pex2p, Pex13p, and Pex22p (Fig. 1). We did not observe an interaction between Pex19p and Pex1p, Pex5p, Pex6p, Pex12p, or Pex14p. We could not test the interaction of Pex19p with Pex4p, Pex5p, Pex7p, and Pex8p since these proteins autoactivated transcription as DNA-binding domain fusion proteins. These results are significant because they demonstrate that Pex19p interacts with six of the seven iPMPs known in *P. pastoris.* The only known iPMP from *P. pastoris* that did not interact with Pex19p in the two-hybrid assay was Pex12p. However, as described below it may interact indirectly with Pex19p.

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 that 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

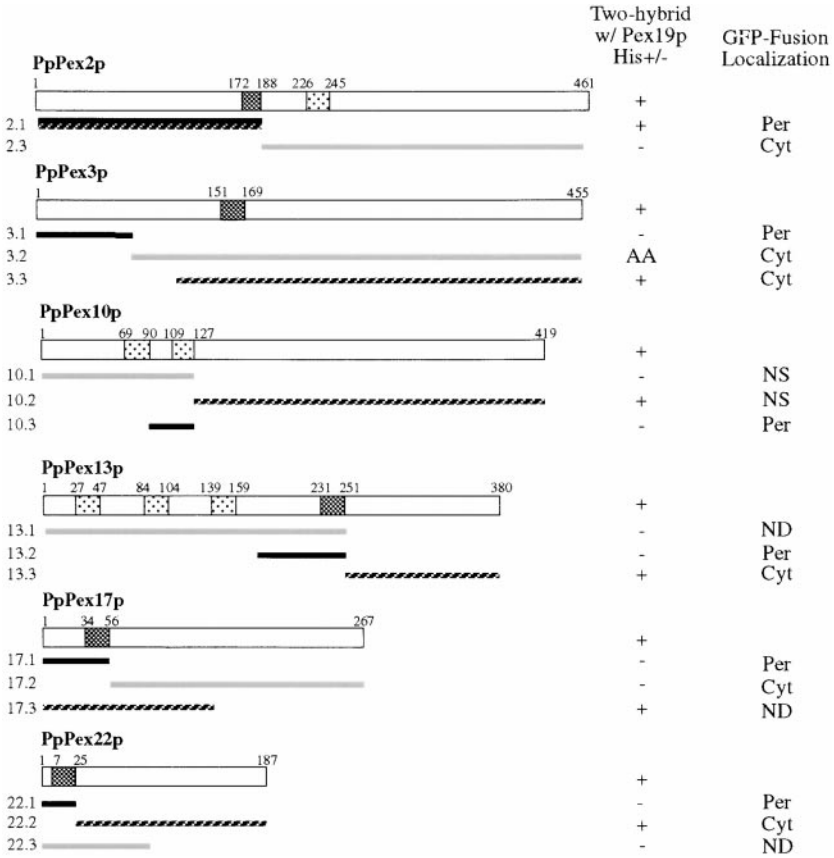

Figure 1. Summary of Pex19p interactions and localization of PMP-GFP fusions. Relative positions of iPMP domains tested for two-hybrid interaction with Pex19p and mPTS function are shown. Black bars indicate regions that functioned as a mPTS. Hatched bars indicate regions binding to Pex19p. Note that 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. AA 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.

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. These data revealed the mPTS regions of the examined iPMPs and suggest that the proteins probably do not contain multiple mPTSs.

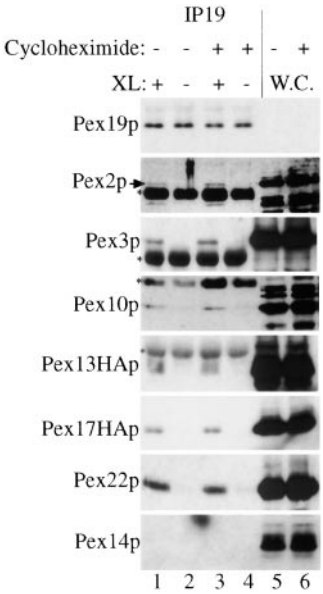

Figure 2. Coimmunoprecipitation of six iPMPs with Pex19p. Pex19p was immunoprecipitated from oleate-grown cells expressing Pex17HAp (S17HA) or Pex13HAp (S13HA). Cells treated with cycloheximide for 45 min, where indicated, before spheroplasting. Whole cell lysates (w.c.) were loaded as a control (0.02 A_{600} equivalent). The amount of immunoprecipitation loaded was 25-fold higher than that in the lane marked w.c. (0.5 A_{600} equivalent). Samples were immunoblotted to reveal the iPMPs. An asterisk indicates the IgG heavy chain. XL indicates the samples were cross-linked during immunoprecipitation.

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

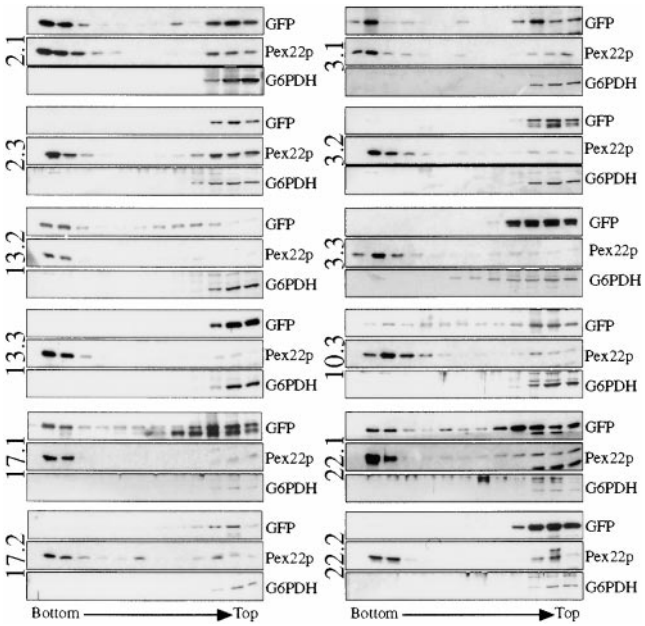

Figure 3. Analysis of mPTS function. Domains of iPMPs (see Fig. 1) were tested for mPTS function by colocalization of PMP-GFP (GFP) with either Pex22p (peroxisome) or G6PDH (cytosol) in the Nycodenz gradient.

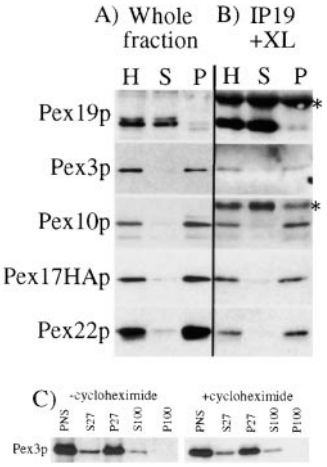

Figure 4. Coimmunoprecipitation of iPMPs from subcellular fractions with Pex19p. Homogenate (H), supernatant (S), and pellet (P) fractions were created as described in Materials and Methods. Whole fractions (A) or anti-Pex19p immunoprecipitates (B) were resolved by SDS-PAGE and immunoblotted. A and B do not show equivalent amounts or exposures. As asterisk indicates the IgG heavy chain. (C) Sequential, differential fractionation of oleate-grown, wild-type cells incubated in the presence (+) or absence (−) of cycloheximide for 45 min before lysis. PNS (post nuclear supernatant), S27 (27,000-*g* supernatant), P27 (27,000-*g* pellet), S100 (100,000-*g* supernatant), and P100 (100,000-*g* pellet).

function. This subcellular interaction site was determined by a cross-linking and subcellular fractionation experiment. Spheroplasted cells were incubated with DSP before the separation of subcellular fractions by differential centrifugation. The fractionation yielded equivalent proportions of the supernatant (S) and pellet (P) fractions from a 27,000-*g* centrifugation of the homogenate (H) fraction. The majority of iPMPs such as Pex3p, Pex10p, Pex17HAp, and Pex22p were in the pellet fraction (Fig. 4 A). We did observe a minor amount of iPMPs in the supernatant fractions and we believe that these proteins are released from the peroxisomes during the fractionation procedure (see below). These experiments show that cytosolic proteins were found in the supernatant fractions (not shown) as was the majority of Pex19p (Fig. 4 A). Immunoprecipitates of these fractions with anti-Pex19p contained >95% of the cross-linked Pex3p, Pex10p, Pex17HAp, and Pex22p in the pellet fractions (Fig. 4 B). It is important to note that these interactions occur with the small, peroxisome-associated pool of Pex19p, and not with the larger, cytosolic pool. This suggests that the peroxisome, and not the cytosol, is the steady state site of interaction between Pex19p and the iPMPs examined.

We suspected that the soluble iPMPs found in the fractionation procedure were the result of peroxisome rupture and not the newly synthesized pool. These procedures do result in significant breakage of peroxisomes resulting in the inability to pellet all peroxisomal proteins as described previously for *P. pastoris* (Snyder et al., 1999a, and references therein) and as reported recently in *S. cerevisiae* (Hettema et al., 2000). To differentiate between soluble iPMPs that result from organelle rupture and the newly synthesized pool of iPMPs that would also be soluble, we depleted the newly synthesized pools by treating the cells with cycloheximide before the fractionation. As shown in Fig. 4 C, we did not see a difference between the amount of soluble Pex3p (S27 and S100) in cells treated with cycloheximide for 45 min as compared with untreated cells. This result leads us to believe that the minor amounts of iPMPs in the supernatant fractions represent proteins released from the organelle and not the newly synthesized proteins.

Discussion

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 *S. cerevisiae* using *P. pastoris* proteins. Although we can not rule out bridging by an endogenous protein that binds to both the DNA-binding domain fusion proteins and the transcriptional activation-domain fusions in our heterologous system, 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. 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 can not 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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