A Role for the Peroxin Pex8p in Pex20p-dependent Thiolase Import into Peroxisomes of the Yeast *Yarrowia lipolytica*

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Peroxisomes are organelles required for peroxisome assembly. The cytosolic peroxin Pex20p binds directly to the β-oxidation enzyme thiolase and is necessary for itsimerization and peroxisomal targeting. The intraperoxisomal peroxin Pex8p has a role in the import of peroxisomal matrix proteins, including thiolase. We report the results of yeast two-hybrid analyses with various peroxins of the yeast Yarrowia lipolytica and characterize more fully the interaction between Pex8p and Pex20p. Immunoprecipitation showed that Pex8p and Pex20p form a complex, while in vitro binding studies demonstrated that the interaction between Pex8p and Pex20p is specific, direct, and autonomous. Pex8p fractionates with peroxisomes in cells of a disruption strain, indicating that Pex20p is not necessary for the targeting of Pex8p to peroxisomes. In cells of a PEX8 disruption strain, thiolase is mostly cytosolic, while Pex20p and a small amount of thiolase associate with peroxisomes, suggesting the involvement of Pex8p in the import of thiolase after docking of the Pex20p-thiolase complex to the membrane. In the absence of Pex8p, peroxisomal thiolase and Pex20p are protected from the action of externally added protease. This finding, together with the fact that Pex8p is intraperoxisomal, suggests that Pex20p may accompany thiolase into peroxisomes during import.

Peroxisomes are members of the microbody family of organelles and are found in organelles from yeasts to humans and in most cell types. They are the site of many important biochemical processes that vary depending on the organism or cell type. Among their many metabolic activities, peroxisomes perform the β-oxidation of fatty acids, bile acid synthesis, plasmalogensynthesis, cholesterol metabolism, and methanol oxidation (1). Soluble enzymes and other proteins found in the peroxisomal matrix are synthesized on polysomes free in the cytosol (1) and are targeted to peroxisomes by cis-acting peroxisomal targeting signals (PTS). Matrix proteins are targeted most commonly by a PTS1, a tripeptide of the sequence SKL or a conserved variant thereof, located at the extreme carboxyl terminus of proteins. Less commonly, matrix proteins are targeted by a PTS2, a nonapeptide located near or at the amino terminus of proteins and having the consensus sequence (R/K)(L/V/I)X,(H/Q)(L/A) (reviewed in Refs. 2–4). Interestingly, unlike other organelles in which proteins traverse the membrane in an unfolded conformation, peroxisomes are capable of importing oligomeric protein complexes (5, 6).

Peroxisomes are organelles required for peroxisome assembly. A subset of peroxins is required for the targeting and import of peroxisomal matrix proteins. The PTS receptors Pex5p and Pex7p bind PTS1- and PTS2-containing proteins, respectively, and are necessary for targeting these proteins to peroxisomes (reviewed in Refs. 2–4). There are conflicting findings regarding the localization of these receptors in the cell. Both receptors have been found in the cytosol, associated with the peroxisomal membrane or in the peroxisomal matrix (reviewed in Refs. 3 and 4). Pex5p and Pex7p have therefore been proposed to be mobile receptors that bind cargo proteins in the cytosol, dock at the peroxisomal membrane, enter the peroxisome, release their cargo in the matrix, and recycle to the cytosol (2–4, 7, 8). Pex13p and Pex14p are integral and peripheral peroxisomal membrane peroxins, respectively, necessary for PTS receptor docking (reviewed in Refs. 2–4). Since Pex13p and Pex14p interact with each other and with both PTS receptors, it has been suggested that although PTS1- and PTS2-dependent targeting pathways are divergent, the import pathways are convergent (9, 10). Pex8p is a peroxin associated with the inside of the peroxisomal membrane, and, in its absence, PTS1- and PTS2-containing proteins are mislocalized to the cytosol (11–14). Recently, a physical interaction was detected between Pex8p and the PTS1 receptor Pex5p in the yeast Saccharomyces cerevisiae, and Pex8p was suggested to function in protein translocation across the peroxisomal membrane following the docking of Pex5p-cargo complexes (14).

Peroxisomal targeting signals and the peroxisomal import apparatus have been strongly conserved during evolution, but interestingly some divergence has been recognized. In certain mammalian systems, two isoforms of Pex5p have been identified, a short isoform necessary for PTS1 import and a long isoform necessary for the import of both PTS1- and PTS2-containing proteins (15, 16). In Yarrowia lipolytica, the PTS2 receptor Pex7p has not been identified. In this yeast, Pex20p, a peroxin with sequence similarity to Pex5p, is necessary for the dimerization and peroxisomal targeting of thiolase, a PTS2

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containing protein (17). Since Pex20p has been shown to be found in both the cytosol and associated with the peroxisomal membrane, it has been proposed to act as a cycling protein that picks up thiolase in the cytosol, docks at the peroxisomal membrane, and then repeats the circuit (17).

Here we report the identification and characterization of a direct interaction between Pex8p and Pex20p. Pex20p is apparently not required for the targeting of Pex8p to peroxisomes, because Pex8p is still peroxisomal in cells of a PEX20 disruption strain. Instead, we provide data that are consistent with Pex8p being directly involved in the import of thiolase into peroxisomes at a stage following docking of the Pex20p-thiolase complex at the peroxisomal membrane. Our data also suggest that, like Pex7p and Pex5p, Pex20p may accompany its cargo into peroxisomes during import.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Culture Conditions**—The yeast strains used in this study are listed in Table I. Media components were as follows: YEPD, 1% yeast extract, 2% peptone, 2% glucose; YPD, 1% yeast extract, 2% peptone, 2% sodium acetate; YPD, 0.3% yeast extract, 0.5% peptone, 0.5% K2HPO4, 0.5% KH2PO4, 1% Brij 35, 1% (w/v) Tween 40, 0.2% (w/v) oleic acid; YNA, 1.34% yeast nitrogen base without amino acids, 0.05% (w/v) Tween 40, 1.34% yeast nitrogen base without amino acids, 2% glucose; YNO, 0.2% (w/v) oleic acid; YND, 1.34% yeast nitrogen base without amino acids, 2% glucose; YNO agar plates were supplemented with leucine, uracil, and lysine, unless the appropriate amino acids or nucleotides) (Bio 101, Vista, CA). Yeast Strains and Culture Conditions—

**Two-hybrid Analyses**—Physical interactions between peroxisins were detected using the Matchmaker Two-Hybrid System (CLONTECH). Chimeric genes were generated by amplifying the open reading frames (ORFs) of PEX genes from Y. lipolytica genomic DNA by the polymerase chain reaction (PCR) and ligating them in-frame and downstream of the DNA encoding the transcription-activating domain (AD) and the DNA-binding domain (DB) of the GAL4 transcriptional activator in the plasmids pGAD424 and pGBT9, respectively (Table II). To generate pGAD-P8pANC, a plasmid encoding AD-Pex8p (12–644), a portion of the PEX8 gene with flanking EcoRI sites was amplified from genomic DNA with oligonucleotides 122-1 and 549 (Table III) and ligated into EcoRI-digested pGAD424. To generate pGAD-P8-(12–370), pGAD-P8pANC was digested with EcoRI and BamIII, and the resulting 1080-bp fragment was ligated into the corresponding sites of pGAD424. pGAD-P8-(164–644) encoding AD-Pex8p (164–644) was generated by digesting pGAD-P8pANC with Apol and EcoRI and ligating the 1442-bp fragment into EcoRI-digested pGAD424. pGAD-P20-(1–76) encoding AD-Pex20p (1–76) was generated by excising a BglII/SmaI fragment from pGBT-PEX20 (Table II).

**Construction of Chimeric Genes and Isolation of Recombinant Proteins**—Chimeric genes encoding maltose-binding protein (MBP) fusions and glutathione S-transferase (GST) fusions were generated as described below. The PEX8 ORF was excised from pGAD-PEX8 (Table II) with EcoRI and BglII and ligated into EcoRI/BamHI-digested pBlueScript SKIII (+) (Stratagene, La Jolla, CA) to generate the plasmid pBS-PEX. To generate pMAL-P8X encoding MBP-Pex8p, pEX8 was excised from pBS-PEX with EcoRI/XbaI and ligated into EcoRI/XbaI-digested pMAL-c2 (New England Biolabs, Beverly, MA). To generate pGEX-PEX20 encoding GST-Pex20p, pGBT-PEX20 (Table II) was digested with EcoRI, and the PEX20 ORF was ligated into EcoRI-digested pGEX-4T1 (Amersham Pharmacia Biotech).

The plasmids pGEX-4T1, pGEX-PEX20, pMAL-c2, and pMAL-PEX8 were introduced into the Escherichia coli strain BL21 (DE3) (Novagen, Madison, WI). Induction and purification of GST fusion proteins were performed according to the manufacturer’s (Amersham Pharmacia Biotech) specifications, except that expression of chimeric genes was induced at 30 °C with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Cells were lysed with B-PER reagent (Pierce). Induction and purification of MBP fusion proteins was performed according to the manufacturer’s (New England Biolabs) specifications. All purified proteins were dialyzed against buffer (20 mM HEPES-KOH, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 20% (w/v) glycercrol) and stored at −70 °C.

**In Vitro Binding Assay with Recombinant Proteins**—An in vitro binding assay was performed using recombinant GST, GST-Pex20p, MBP, and MBP-Pex8p purified as described above. Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) was washed with RW buffer (20 mM HEPES-KOH, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 0.1% (w/v) Tween 20, 0.1% casamino acids, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 2.5 μg/ml antipain, 0.21 mg/ml NaF, 0.1 mg/ml Pefabloc SC) and resuspended in 0.5 ml of RW buffer for 20 min at room temperature. Beads were collected by centrifugation and washed three times with 0.5 ml of RW buffer. Purified MBP or MBP-Pex8p (5 μg reaction) was added to 100 μl of RW buffer containing GST or GST-Pex20p linked to beads and tumbled end-over-end for 30 min at room temperature. Beads were collected by centrifugation, and supernatants were retained as the unbound fractions. The beads were resuspended in 0.5 ml of RW buffer and applied to spin filters (Cytosignal, Irvine, CA), washed three times with 0.5 ml of RW buffer, and eluted with 50 μl of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at room temperature. 50% of bound fractions and 25% of unbound fractions were run on 9% polyacrylamide gels, and proteins were detected by staining with Coomassie Brilliant Blue R250.

**In Vitro Binding Assay with Yeast Lysate**—Aliquots of glutathione-Sepharose 4B (10 μl of packed beads/reaction) were washed four times with 1 ml of RW buffer (20 mM HEPES-KOH, pH 6.8, 150 mM potassium acetate, 2 mM magnesium acetate, 0.5% (w/v) Triton X-100, 0.1% (w/v) casamino acids, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 2.5 μg/ml antipain, 0.21 mg/ml NaF, 0.1 mg/ml Pefabloc SC) and resuspended in 0.5 ml of RW buffer. 3 μg of GST or GST-Pex20p was added to the beads, and reactions were tumbled end-over-end at room temperature for 80 min. Beads were collected by centrifugation and washed three times with 0.5 ml of RW buffer. YPBO-grown cells of the PEX8-HA strain (Table I) were collected by centrifugation, washed three times with water, and washed once with RW buffer. Cells were disrupted in RW buffer by agitation with glass beads. The lysate was clarified by centrifugation at 20,000 × g in a microcentrifuge for 45 min at 4 °C. 120 μl of the cleared supernatant (10 mg of protein/ml) or of RW buffer alone was tumbled end-over-end in batch with glutathione-Sepharose-bound GST or GST-Pex20p for 2 h at 4 °C. Beads were washed twice with 0.8 ml of RW buffer, applied to spin filters, and washed four times with 0.5 ml of RW buffer. Bound proteins were eluted in 40 μ1 of SDS-PAGE sample buffer according to the manufacturer’s protocol. The cleaved proteins were run on 9% polyacrylamide gels and detected using the Matchmaker Two-Hybrid System (CLONTECH).
instructions. Proteins in equal fractions of each sample were separated on 10% polyacrylamide gels, transferred to nitrocellulose, and subjected to immunoblotting.

Epitope Tagging of Pex8p—A modified PEX8 gene encoding Pex8p tagged at its carboxyl terminus was made by inserting a DNA fragment encoding two copies of the influenza virus hemagglutinin (HA) epitope in frame and downstream of the PEX8 ORF. A fragment containing the PEX8 ORF flanked by 2.0 and 1.2 kilobase pairs of genomic DNA at its 5' and 3'-ends, respectively, was excised from plasmid p16TH (22) by digestion with EcoRI. A modified Gene flanking the proposed dIII site was introduced immediately upstream of the stop codon of PEX8 and downstream of the gene (A1924T), creating a premature stop codon2 and cannot grow on oleic acid medium (13). Transformants were selected for reestablished growth on oleic acid medium (YNO agar) and characterized by Southern blotting and electron microscopy. One strain, PEX8HA, having the correct genotype and wild-type morphology, was chosen for further study.

To make a PEX8HA expression plasmid, PEX8-HA was excised from pG7PEX8-HA with HindIII and integrated into the genome of the pex8–1 mutant strain (Table I) by homologous recombination to replace the pex8–1 allele of PEX8. The pex8–1 allele has a substitution mutation in the PEX8 gene (A1924T), creating a premature stop codon2 and cannot grow on oleic acid medium (13). Transformants were selected for reestablished growth on oleic acid medium (YNO agar) and characterized by Southern blotting and electron microscopy. One strain, PEX8-HA, having the correct genotype and wild-type morphology, was chosen for further study.

A fragment containing the modified PEX8 gene was excised from pG7PEX8-HA+ with HindIII and integrated into the genome of the pex8–1 mutant strain (Table I) by homologous recombination to replace the pex8–1 allele of PEX8. The pex8–1 allele has a substitution mutation in the PEX8 gene (A1924T), creating a premature stop codon2 and cannot grow on oleic acid medium (YNO agar) and characterized by Southern blotting and electron microscopy. One strain, PEX8-HA, having the correct genotype and wild-type morphology, was chosen for further study.

A modified PEX8 gene encoding Pex8p tagged at its carboxyl terminus was made by inserting a DNA fragment encoding two copies of the influenza virus hemagglutinin (HA) epitope in frame and downstream of the PEX8 ORF. A fragment containing the PEX8 ORF flanked by 2.0 and 1.2 kilobase pairs of genomic DNA at its 5' and 3'-ends, respectively, was excised from plasmid p16TH (22) by digestion with EcoRI. A modified Gene flanking the proposed dIII site was introduced immediately upstream of the stop codon of PEX8 and downstream of the gene (A1924T), creating a premature stop codon2 and cannot grow on oleic acid medium (YNO agar) and characterized by Southern blotting and electron microscopy. One strain, PEX8-HA, having the correct genotype and wild-type morphology, was chosen for further study.

Table II
Generation of plasmids for two-hybrid system analyses

| Name         | Function of Pex8p and Pex20p in Thiolase Import |
|--------------|--------------------------------------------------|
| pGBT-PEX1    | pEX1                                             |
| pGAD-PEX1    | pEX1                                             |
| pGBT-PEX2    | pEX2                                             |
| pGAD-PEX2    | pEX2                                             |
| pGBT-PEX5    | pEX5                                             |
| pGAD-PEX5    | pEX5                                             |
| pGBT-PEX6    | pEX6                                             |
| pGAD-PEX6    | pEX6                                             |
| pGBT-PEX8    | pEX8                                            122–2 and 336 |
| pGAD-PEX8    | pEX8                                            122–2 and 336 |
| pGBT-PEX9    | pEX9                                             |
| pGAD-PEX9    | pEX9                                             |
| pGBT-PEX16   | pEX16                                           NA* |
| pGAD-PEX16   | pEX16                                           NA* |
| pGBT-PEX20   | pEX20                                           398 and 399 |
| pGAD-PEX20   | pEX20                                           398 and 399 |

a Parent plasmids are pGAD424 and pGBT9 for constructs beginning with pGAD and pGBT, respectively.

b PEX2, PEX5, PEX6, PEX8, and PEX9 were formerly designated PAY5, PAY3, PAY4, PEX17, and PAY2, respectively.

NA, not applicable. Constructs containing PEX16 were not generated by PCR. Instead, PEX16 was excised from plasmid p16TH (22) by digestion with EcoRI.

d The complete ORFs of the PEX genes in these constructs were determined to be correct by sequencing. For all other constructs, the gene fusion junction was determined to be correct by sequencing.

A fragment containing the modified PEX8 gene was excised from pG7PEX8-HA+ with HindIII and integrated into the genome of the pex8–1 mutant strain (Table I) by homologous recombination to replace the pex8–1 allele of PEX8. The pex8–1 allele has a substitution mutation in the PEX8 gene (A1924T), creating a premature stop codon2 and cannot grow on oleic acid medium (YNO agar) and characterized by Southern blotting and electron microscopy. One strain, PEX8-HA, having the correct genotype and wild-type morphology, was chosen for further study.

To make a PEX8-HA expression plasmid, PEX8-HA was excised from pG7PEX8-HA+ with HindIII and ligated into the HindIII site of the E. coli Y. lipolytica shuttle vector pINA445 to make the plasmid pG7PEX8-HA encoding Pex8p-HA+. pINA445 contains the Y. lipolytica LEU2 gene for positive selection of yeast transformants and the Y. lipolytica ARS68 gene for autonomous plasmid replication in Y. lipolytica cells (19).

Coimmunoprecipitation—Coimmunoprecipitation was performed by immunoaffinity chromatography using protein A-Sepharose CL-4B (Sigma). All steps were performed at 4°C unless otherwise specified. YPO-brown PEX8-HA cells were collected by centrifugation, washed three times with water, and washed once with RX buffer. Cells were resuspended in RX buffer and lysed by disruption with glass beads. The total cell lysate was clarified by centrifugation at 100,000 g of protein of the cell lysate, were separated by SDS-PAGE, and the protein A-Sepharose suspension was added to each reaction, and reactions were incubated end-over-end for 40 min. Antibody complexes were pelleted by centrifugation and resuspended in 0.5 ml of RX buffer. For each eluate, along with the protein A-Sepharose suspension was added to each reaction, and reactions were incubated end-over-end for 40 min. Antibody complexes were pelleted by centrifugation and resuspended in 0.5 ml of RX buffer. Samples were then applied to spin filters and washed three times with RX buffer, and antibody complexes were eluted in SDS-PAGE sample buffer at room temperature. Proteins in 20% of each eluate, along with 10 μg of protein of the cell lysate, were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted.

Subcellular Fractionation—Subcellular fractionation of Y. lipolytica was performed as described (17, 20) using 5 mM MES, pH 5.5, 1 μM sorbitol containing 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 2.5 μM antipain, 0.21 mg/ml NaF, 0.1 mg/ml Pefabloc SC

2 J. Smith and R. A. Rachubinski, unpublished results.
subfractionation of peroxisomes and protease protection analysis. Two-hybrid interactions were used to confirm the physical interaction between Pex8p and Pex20p, as well as other peroxins. The results were confirmed by liquid β-galactosidase assays and the yeast two-hybrid system. The interactions were monitored by the detection of β-galactosidase activity in yeast colonies. The results showed that Pex20p interacted with multiple peroxins, including Pex1p, Pex6p, Pex8p, and Pex5p. These interactions were also detected by immunoblotting, demonstrating the presence of protein-protein interactions.

**RESULTS**

**Detection of Y. lipolytica Peroxin Interactions with the Yeast Two-hybrid System**

A limited yeast two-hybrid screen (30) was performed to identify physical interactions between *Y. lipolytica* peroxins Pex1p, Pex2p, Pex5p, Pex6p, Pex8p, Pex9p, Pex16p, and Pex20p. Others have used this methodology successfully to detect interactions between peroxins (for examples, see Refs. 10 and 31–33). Chimeric genes were generated by ligating *Y. lipolytica* PEX genes in frame and downstream of sequences encoding the designated fusion (x axes). β-Galactosidase activity is measured in arbitrary units (U) as defined by the manufacturer (CLONTECH). A, Pex1p and Pex6p physically interact. Each column is the measure of the average β-galactosidase activity of at least three individual transformants. B, Pex8p and Pex5p physically interact. Each column is the measure of the average β-galactosidase activity of at least six individual transformants. The levels of activity from strains synthesizing DB-Pex8p and AD-Pex5p and synthesizing DB-Pex5p and AD-Pex8p are significantly different from those of the corresponding control strains at confidence levels of 95 and 90%, respectively. Error bars represent S.D. values.
For the filter assay of b shown (last column) two representative independent transformants for each strain are 6 terminus of Pex20p in the yeast two-hybrid system.

Indicating that Pex8p and Pex5p interact physically. To characterize this interaction further, fusions to the amino terminus of Pex20p, and the 11 amino-terminal and 27 COOH-terminal amino acid residues at the amino and carboxyl termini of peroxins. The activity of β-galactosidase obtained from the liquid assay (third column) is the average of the activities of three independent transformants ± S.D.

For the filter assay of β-galactosidase activity, the color intensities of two representative independent transformants for each strain are shown (last column).

Lysates of strains transformed with constructs encoding DB-Pex8p or DB-Pex20p showed high levels of β-galactosidase activity, but so did the lysates of control strains synthesizing one or the other of the fusion proteins (Fig. 1B). Using the Smith-Satterthwaite test for statistical significance (34), it was determined that the levels of β-galactosidase activity from strains synthesizing both DB-Pex8p and AD-Pex5p or both DB-Pex5p and AD-Pex8p were significantly higher than those of the corresponding control strains at confidence levels of 95 and 90%, respectively, indicating that Pex8p and Pex5p interact physically.

Lysates of strains transformed with constructs encoding DB-Pex8p or DB-Pex20p showed high levels of β-galactosidase activity, making it difficult to identify an interaction between Pex20p and Pex8p. Therefore, a plasmid encoding DB-Pex20p-(1–76), a fusion between the DB domain and the amino-terminal 76 amino acid residues of Pex20p, was generated. DB-Pex20p does not have intrinsic transcription activation activity. Lysates of strains synthesizing both AD-Pex8p and DB-Pex20p-(1–76) showed significantly higher β-galactosidase activity than did lysates of control strains synthesizing one or the other of the fusion proteins (Fig. 2, compare row 1 with rows 5 and 6), demonstrating that Pex8p and Pex20p interact physically. To characterize this interaction further, fusions to smaller domains of Pex8p were assayed. DB-Pex20p-(1–76) still interacted with AD-Pex8p-(12–644), containing Pex8p truncated at both its amino and carboxyl termini, but did not interact with AD domain fusions to more extensive amino- or COOH-terminal truncations of Pex8p (Fig. 2, compare row 2 to rows 3 and 4). Therefore, Pex8p physically interacts with the amino terminus of Pex20p, and the 11 amino-terminal and 27 COOH-terminal amino acid residues of Pex8p are dispensable for this interaction.

The Interaction between Pex8p and Pex20p Is Direct and Autonomous—Some interactions between peroxins that have been detected using the yeast two-hybrid system have been shown to be indirect (10, 33). Therefore, it is possible that an endogenous S. cerevisiae protein may have bridged the observed interaction between Pex8p and Pex20p. To determine whether the interaction between Pex8p and Pex20p is direct and autonomous, an in vitro binding assay was performed. The

Table: DNA binding domain fusion | β-galactosidase activity Liquid assay | Filter assay
--- | --- | ---
1 | 671 | 1.76 | 5.15 ± 0.35
2 | 644 | 1.76 | 15.11 ± 1.03
3 | 370 | 1.76 | 0.12 ± 0.00
4 | 664 | 1.76 | 0.14 ± 0.01
5 | 664 | 1.76 | 0.09 ± 0.01
6 | 671 | 1.76 | 0.10 ± 0.00

Fig. 2. Analysis of the interaction of Pex8p with the amino terminus of Pex20p in the yeast two-hybrid system. SYP526 cells doubly transformed with chimeric genes encoding AD (first column) and DB (second column) fusion proteins were tested for β-galactosidase activity. The Pex8p and Pex20p portions of fusions are represented by shaded bars and open bars, respectively. Numbers indicate the amino acid residues at the amino and carboxyl termini of peroxins. The activity of β-galactosidase obtained from the liquid assay (third column) is the average of the activities of three independent transformants ± S.D.

The Pex8p-Pex20p Interaction Is Specific—An in vitro binding experiment was performed to determine whether GST-Pex20p could interact specifically with Pex8p tagged with the hemagglutinin epitope, Pex8p-HA, in a yeast lysate. GST and GST-Pex20p were immobilized on glutathione-Sepharose beads. A total cell lysate was prepared from PEX8-HA, a strain synthesizing Pex8p-HA, and incubated with immobilized GST or GST-Pex20p. The proteins bound to each column and the proteins of the total cell lysate were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with various antibodies (Fig. 4). Pex8p-HA interacted specifically with GST-Pex20p, while GST or MBP alone did not interact with MBP-Pex8p or GST-Pex20p, respectively. We conclude that the interaction between Pex8p and Pex20p is direct and autonomous.

Pex8p-HA and Pex20p Coimmunoprecipitate—To determine whether Pex20p was capable of interacting with Pex8p-HA in vivo, Pex20p was immunoprecipitated under native conditions from a total lysate of cells grown in YPBO medium, and coimmunoprecipitating proteins were analyzed by SDS-PAGE and immunoblotting with various antibodies (Fig. 5). All the proteins tested were present in the total cell lysate, Pex8p-HA coimmunoprecipitated specifically with Pex20p but not with peroxiosomal isocitrate lyase or the peroxin Pex6p. Therefore, Pex8p-HA interacts with Pex20p in vivo. As previously reported (17), the precursor form of thiolase also specifically coimmunoprecipitated with Pex20p.

Pex20p Is Not Required for Targeting Pex8p-HA to Peroxisomes—Since Pex20p binds thiolase in the cytosol and is nec-
necessary for its targeting to peroxisomes (17) and because Pex20p interacts with Pex8p-HA+, we investigated whether Pex20p might also bind Pex8p in the cytosol and act in targeting it to peroxisomes. To test this hypothesis, Pex8p-HA+ was localized by subcellular fractionation of cells of the wild-type strain E122, the PEX20 disruption strain pex20ko, and the original PEX20 mutant strain pex20–1 (Table I), each transformed with the expression plasmid pPEX8-HA+ encoding Pex8p-HA+. The postnuclear supernatant fraction of each strain was divided by centrifugation at 20,000 × g into a supernatant fraction (20KgS) enriched for cytosol and high-speed pelletable organelles and a pellet fraction (20KgP) enriched for peroxisomes and mitochondria. The 20KgS fraction of each strain was divided by centrifugation at 200,000 × g into supernatant fraction (200KgS) highly enriched for cytosol and pellet fraction (200KgP) enriched for high-speed pelletable peroxisomes, including high-speed pelletable peroxisomes.

For high-speed pelletable peroxisomes but not in the highly enriched cytosol fraction (200KgS) of cells of all strains. For all strains, Pex8p-HA+ cofractionated with peroxisomal marker proteins in a sucrose density gradient (data not presented). Together, these results suggest that Pex20p is not required for the targeting of Pex8p to peroxisomes. As expected, thiolase was preferentially localized to peroxisome-enriched fractions of cells of the wild-type strain and to cytosol-enriched fractions of cells of the PEX20 mutant strains.

Pex20p and a Small Amount of Thiolase Associate with Peroxisomes in Cells of a PEX8 Disruption Strain—Interaction between Pex20p and Pex8p may occur at the level of the peroxisomal membrane during the targeting or import of thiolase. Consistent with this scenario, only a small fraction of thiolase is peroxisomal in a PEX8 disruption strain (13). To further elucidate the role of Pex8p in the import of thiolase, the localization of Pex20p and the subperoxisomal localization of peroxisome-associated thiolase were determined in cells of the PEX8 disruption strain, pex8-ka (13) (Table I). Double labeling immunofluorescence microscopy of YPBO-grown cells demonstrated that as expected, Pex20p showed a diffuse localization characteristic of a cytosolic protein (17), while the peroxisomal membrane protein Pex2p and the peroxisomal matrix protein thiolase showed a punctate pattern of localization characteristic of peroxisomes in cells of the wild-type strain E122 (Fig. 7).

Interestingly, in cells of the pex8-ka strain, Pex20p colocalized with Pex2p to punctate structures characteristic of peroxisomes, while as expected, thiolase had a diffuse localization characteristic of a cytosolic location. These results suggest that while Pex20p is predominantly cytosolic in wild-type cells, as has been reported previously (17), a significant fraction of Pex20p associates with peroxisomes in cells lacking Pex8p.

Pex20p and thiolase were also localized by subcellular fractionation of cells of the wild-type E122 and pex8-ka strains. In E122 cells, Pex20p was found largely in the 20KgS fraction enriched for cytosol and high-speed pelletable organelles, while thiolase was primarily in the 20KgP fraction enriched for peroxisomes, as expected (Fig. 8A). In pex8-ka cells, by contrast, Pex20p was found primarily in the 20KgS fraction and thiolase was found primarily in the 20KgS fraction, although a small amount of thiolase fractionated to the 20KgP, since some thiolase is peroxisomal in pex8-ka cells (13). The results of subcel-
Pex20p is peroxisomal in the PEX8 disruption strain, pex8-KA. The PEX8 disruption strain pex8-KA (top panels) and the wild-type strain E122 (bottom panels) were grown overnight in YEPD medium and transferred to YPBO medium and grown for an additional 9 h. Cells of each strain were processed for immunofluorescence microscopy. Cells were double-labeled with guinea pig anti-Pex2p antibodies (left panels) and rabbit anti-Pex20p antibodies (middle panels) or labeled with guinea pig anti-thiolase antibodies (right panels). Guine pig primary antibodies were detected with rhodamine-conjugated donkey anti-guinea pig IgG secondary antibodies. Rabbit primary antibodies were detected with fluorescein-conjugated donkey anti-rabbit IgG secondary antibodies.

Fig. 8. Peroxisomal Pex20p from the PEX8 disruption strain is membrane-associated and protected from the action of externally added proteases. A, immunoblot analysis of subcellular fractions of the wild-type strain E122 and of the PEX8 disruption strain pex8-KA. Equal portions of each digest were separated by SDS-PAGE and analyzed by immunoblotting (Fig. 8B). Pex20p localized primarily to the T18S fraction from both wild-type and pex8-KA cells along with peroxisomal membrane protein Pex2p, whereas thiolase was primarily found in the T18S fraction from both strains. These data indicate that in both wild-type and pex8-KA cells, Pex20p associates with the peroxisomal membrane. In addition, since thiolase was present in the soluble T18S fraction derived from pex8-KA cells, either thiolase is in the matrix of peroxisomes in the absence of Pex8p or membrane-associated thiolase is released during the extraction incubation.

In wild-type cells, Pex20p is localized to the outer surface of peroxisomes, as demonstrated by its susceptibility in vitro to the action of external proteases (17). To determine whether in the absence of Pex8p, peroxisome-associated Pex20p and thiolase are protected from the action of proteases by the peroxisomal membrane, protease protection analysis was performed on a 20KgP fraction isolated from cells of the pex8-KA strain. Equal portions of the 20KgP fraction were incubated with increasing amounts of trypsin in the absence or presence of the nonionic detergent, Triton X-100. The proteins in equal portions of each digest were separated by SDS-PAGE and analyzed by immunoblotting (Fig. 8C). Both Pex20p and thiolase showed greater resistance to trypsin in the absence of detergent than in its presence, suggesting that they are afforded protection from the action of proteases by the peroxisomal membrane in pex8-KA cells.

DISCUSSION

Pex20p is a peroxin that binds thiolase in the cytosol and is necessary for its oligomerization and targeting to peroxisomes (17). Pex8p is an intraperoxisomal peroxin required for the import of a number of peroxisomal proteins, including thiolase (11–14). Using the yeast two-hybrid system, we identified an interaction between Pex8p and the amino terminus of Pex20p. The Pex8p-Pex20p interaction was confirmed by the isolation of a complex containing Pex8p and Pex20p from yeast lysates by coimmunoprecipitation and by in vitro binding studies with recombinant proteins showing that this interaction is specific, direct, and autonomous. Although Pex20p and Pex8p are in different cellular compartments, two readily possible scenarios for their interaction can be proposed. One scenario is that Pex20p binds Pex8p in the cytosol and is required for the oligomerization and/or peroxisomal targeting of Pex8p. The second scenario is that Pex8p interacts with Pex20p at the peroxisomal membrane at a step in the import of thiolase into peroxisomes. We localized Pex8p and Pex20p in cells of PEX20 and PEX8 disruption strains, respectively, to elucidate the mechanism of interaction of these two proteins. Pex20p-dependent Targeting and/or Oligomerization of Pex8p—The possibility of a role for Pex20p in the targeting and/or oligomerization of Pex8p is consistent with evidence that suggests that Pex8p of the yeast Hansenula polymorpha may be an oligomer (11). It is also compatible with the fact that Pex8p is intraperoxisomal and in most yeasts contains PTS1 (12) or both PTS1 and PTS2 motifs (11, 14). Since Pex8p shares PTS with peroxisomal matrix enzymes, it is conceivable that it may also be targeted to peroxisomes by the same peroxins involved in targeting these enzymes. Although such an explanation is plausible, two lines of evidence suggest that Pex20p is not necessary for the targeting and/or oligomerization of Pex8p. First, assuming that mislocalized or nonoligomerized Pex8p molecules are nonfunctional and that Pex20p is necessary for the oligomerization and/or the targeting of Pex8p, then a PEX20 disruption strain should present a phenotype that is as severe as or more severe than that of a PEX8 disruption strain. However, this is not the case, since several matrix proteins are

lular fractionation are consistent with those of immunofluorescence analysis.

The suborganelar locations of Pex20p and thiolase in wild-type cells and in pex8-KA cells were now compared. The 20KgP fraction of wild-type and pex8-KA cells was disrupted by incubation in dilute Tris buffer. The 20KgP-D was then separated by centrifugation into a pellet fraction (T18S) enriched for membranes and a supernatant fraction (T18S) enriched for soluble proteins. Proteins in equal portions of each fraction were separated by differential centrifugation. Proteins in equal portions of each digest were separated by SDS-PAGE and analyzed by immunoblotting (Fig. 8B)....
mislocalized in \textit{PEX8} disruption strains (11–14), while only thiolase is mislocalized in a \textit{PEX20} disruption strain (17). Second, an epitope-tagged version of Pex8p was localized to subcellular fractions from cells of \textit{pex20} mutant strains enriched for peroxisomes. Although these data strongly suggest that Pex20p is not required for the targeting and/or oligomerization of Pex8p, we cannot rule out the possibility that redundant systems exist for these functions, with only one requiring Pex20p.

A Role for Pex8p in Pex20p-dependent Import of Thiolase—The alternative scenario that Pex8p and Pex20p interact at the peroxisomal membrane in a step in the targeting or import of thiolase is consistent with previously published findings. Pex8p has been shown to be associated with the peroxisomal membrane in three different yeasts, including \textit{Y. lipolytica} (12–14). It has also been shown that in cells of a \textit{Y. lipolytica} \textit{PEX8} disruption strain, thiolase is primarily cytosolic, although a small amount of thiolase associates with peroxisomes (13). The localization of Pex20p and thiolase in cells of a \textit{PEX8} disruption strain also points to a direct role for Pex8p in the Pex20p-dependent import of thiolase. While a small amount of total Pex20p has previously been shown to be associated with peroxisomes in wild-type cells (17), we find that a large fraction of Pex20p associates with peroxisomal membranes in cells devoid of Pex8p relative to wild-type cells. These data point to a role for the interaction between Pex8p and Pex20p in the import of thiolase at a stage following the docking of Pex20p-thiolase complexes at the peroxisomal membrane. The exact role of Pex8p in thiolase import is unknown; however, since Pex20p has been shown to interact with Pex8p in the absence thiolase (or any other protein), it is tempting to speculate that Pex8p has a role either in dissociating thiolase from Pex20p or in recycling Pex20p after dissociation. Future experiments will be aimed at studying the interactions between Pex8p and Pex20p bound to thiolase.

Interestingly, we find that the peroxisome-associated Pex20p and thiolase in cells of the \textit{PEX8} disruption strain are protected from the action of external proteases. These data suggest that in the absence of Pex8p, both Pex20p and thiolase are protected by membranes. This, together with the fact that Pex20p directly interacts with Pex8p, an intraperoxisomal peroxin, suggests that Pex20p could enter peroxisomes with its cargo. This event may not be readily detectable in wild-type cells (17), either because the steady-state level of the population of intraperoxisomal Pex20p is very low or because Pex20p may exit the peroxisome through a putative translocation channel during fractionation of wild-type cells.

A Role for Pex8p in Pex5p-dependent Import of PTS1-containing Proteins—As mentioned previously, thiolase is mislocalized in cells lacking Pex20p, while several matrix proteins, including PTS1-containing proteins, are mislocalized in cells lacking Pex8p (13). One possible reason for this more extensive mislocalization of matrix proteins in \textit{pex8} mutant strains is that in these strains, Pex20p becomes trapped in the peroxisomal membrane, thereby clogging the translocation apparatus. Since the PTS1- and PTS2-dependent import pathways are apparently convergent (9, 10), this obstruction may prevent PTS1-containing proteins from being imported into the peroxisome. Alternatively, Pex8p may be directly involved in both Pex5p-dependent and Pex20p-dependent import. Our identification of an interaction between Pex8p and Pex5p in the yeast two-hybrid system is consistent with this possibility. In addition, Pex8p and Pex5p of \textit{S. cerevisiae} have recently been shown to interact, even in the absence of the PTS1 of Pex8p (14). Although Pex8p and Pex5p have not been shown to interact directly, Pex5p and Pex5p may interact at their amino termini, as we have shown that Pex5p and Pex20p interact at their amino termini, and Pex20p and Pex5p show sequence similarity at their amino termini (17).
A Role for the Peroxin Pex8p in Pex20p-dependent Thiolase Import into Peroxisomes of the Yeast *Yarrowia lipolytica*
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