MOLECULAR AND CELLULAR BIOLOGY, Oct. 2000, p. 7516–7526 Vol. 20, No. 20
0270-7306/00/$04.00+0

The Peroxisome Biogenesis Factors Pex4p, Pex22p, Pex1p, and Pex6p Act in the Terminal Steps of Peroxisomal Matrix Protein Import

CYNTHIA S. COLLINS,[1] JENNIFER E. KALISH,[1] JAMES C. MORRELL,[1]
J. MICHAEL MCCAFFERY,[2] AND STEPHEN J. GOULD[1]*

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205,[1] and Integrated Imaging Center, Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218[2]

Received 20 March 2000/Returned for modification 1 May 2000/Accepted 1 August 2000

Peroxisomes are independent organelles found in virtually all eukaryotic cells. Genetic studies have identified more than 20 *PEX* genes that are required for peroxisome biogenesis. The role of most *PEX* gene products, peroxins, remains to be determined, but a variety of studies have established that Pex5p binds the type 1 peroxisomal targeting signal and is the import receptor for most newly synthesized peroxisomal matrix proteins. The steady-state abundance of Pex5p is unaffected in most *pex* mutants of the yeast *Pichia pastoris* but is severely reduced in *pex4* and *pex22* mutants and moderately reduced in *pex1* and *pex6* mutants. We used these subphenotypes to determine the epistatic relationships among several groups of *pex* mutants. Our results demonstrate that Pex4p acts after the peroxisome membrane synthesis factor Pex3p, the Pex5p docking factors Pex13p and Pex14p, the matrix protein import factors Pex8p, Pex10p, and Pex12p, and two other peroxins, Pex2p and Pex17p. Pex22p and the interacting AAA ATPases Pex1p and Pex6p were also found to act after Pex10p. Furthermore, Pex1p and Pex6p were found to act upstream of Pex4p and Pex22p. These results suggest that Pex1p, Pex4p, Pex6p, and Pex22p act late in peroxisomal matrix protein import, after matrix protein translocation. This hypothesis is supported by the phenotypes of the corresponding mutant strains. As has been shown previously for *P. pastoris pex1*, *pex6*, and *pex22* mutant cells, we show here that *pex4*Δ mutant cells contain peroxisomal membrane protein-containing peroxisomes that import residual amounts of peroxisomal matrix proteins.

To form a functional peroxisome, peroxisome membranes must be generated and the subsequent import of both membrane and matrix proteins must occur. Various studies have established that both peroxisomal membrane proteins (PMPs) and peroxisomal matrix proteins are made in the cytosol and delivered posttranslationally to the peroxisome (26). However, the similarities between PMP import and peroxisomal matrix protein import appear to end there. Peroxisomal matrix proteins are targeted to the peroxisome lumen via the presence of either a PTS1 or a PTS2 targeting signal (40). The PTS1 signal, which consists of a -SKL sequence (or a conserved variant thereof) at the extreme carboxy terminus of the protein (16), is present on the vast majority of peroxisomal matrix proteins. The PTS2 signal consists of the sequence R/K-L/V/I-X_5-H/Q-L/A near the amino terminus (41) and has so far been identified in only a handful of proteins. Both types of signals are recognized by specific receptors: Pex5p for the PTS1 signal (6, 29) and Pex7p for the PTS2 signal (27, 31). After binding to the receptor, matrix proteins are taken to the peroxisome surface and inserted into the organelle lumen through an as yet unknown mechanism. For Pex5p, it has been proposed that the receptor is then recycled back to the cytosol and can undergo further rounds of import (7). In contrast, PMPs lack PTS1 and PTS2 motifs (40), are imported independently of Pex5p and Pex7p (2), and require a distinct PMP-binding protein, Pex19p, for their import (19, 28, 34).

In addition to Pex5p, Pex7p, and Pex19p, a variety of other proteins required for peroxisome biogenesis have been identified (19). These include peroxins necessary for the biogenesis of peroxisome membranes (Pex3p), docking factors for the PTS receptors (Pex13p and Pex14p), proteins that act downstream of receptor docking but are required for translocation of proteins across the peroxisome membrane (Pex8p, Pex10p, and Pex12p), and other proteins whose functions are less clear (Pex1p, Pex2p, Pex4p, Pex6p, Pex17p, and Pex22p).

Despite the identification of so many components of peroxisome biogenesis, there is still little direct evidence as to the order in which these peroxins act. In the course of investigating the effects that different peroxins have on Pex5p, the PTS1 receptor, we observed that the loss of Pex1p or Pex6p in either yeast or human cells leads to an accelerated turnover of Pex5p and a significant drop in steady-state levels of Pex5p (7, 49). Koller et al. (25) have recently reported a similar phenotype for *Pichia pastoris pex4* and *pex22* mutants. Here we show that the phenotype of reduced Pex5p abundance in *P. pastoris* is unique to selected *pex* mutants and can be used to determine the epistatic relationships among the different peroxins. The results of our epistasis analysis suggest that Pex4p, Pex22p, Pex1p, and Pex6p act in the terminal steps of peroxisomal matrix protein import. This hypothesis is consistent with the reported phenotypes of *P. pastoris pex1*, *pex6*, and *pex22* mutants, all of which contain peroxisomes and import detectable levels of peroxisomal matrix proteins. Similarly, we show here that the *pex4* mutant also contains peroxisomes that are capable of peroxisomal matrix protein import.

MATERIALS AND METHODS

Yeast media. The various media used were based on those described earlier for *P. pastoris* (17). Specifically, YPD (1% yeast extract, 2% peptone, 2% dextrose) was used as rich medium. YPM (1% yeast extract, 2% peptone, 0.5% methanol) was used as rich methanol medium. YPOLT (1% yeast extract, 2% peptone, 0.18% oleic acid, 0.02% Tween 40) was used as rich oleic acid medium. SD + histidine (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, 0.1% L-histidine, 2% dextrose) was used as minimal-dextrose medium. SM + histidine + arginine (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, 0.1% L-histidine, 0.1% L-arginine, 0.5% methanol) was used as minimal-methanol medium. Sporu-

* Corresponding author. Mailing address: Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 955-3424. Fax: (410) 955-0215. E-mail: sgould@jhmi.edu.

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
SGY22	*arg4-1 pex3-1 his4Δ::ARG4*	23
SGY23	*arg4-1 pex6-1 his4Δ::ARG4*	23
SGY24	*arg4-1 pex13Δ::ARG4 his4Δ::ARG4*	15
SGY26	*arg4-1 pex8-3 his4Δ::ARG4*	23
SGY27	*arg4-1 pex2-2 his4Δ::ARG4*	23
SGY29	*arg4-1 leu2::ARG4 pex5Δ::LEU2 his4Δ::ARG4*	Laboratory stock
SGY55[a]	*arg4-1 his4Δ::ARG4*	4
SGY132	*arg4-1 leu2::ARG4 pex10Δ::LEU2 his4Δ::ARG4*	23a
SGY311	*arg4-1 pex4Δ::ARG4 his4Δ::ARG4*	Laboratory stock
SGY443	*arg4-1 pex12Δ::ARG4 his4Δ::ARG4*	23a
SGY501	*arg4-1 pex1Δ::ARG4 his4Δ::ARG4*	Laboratory stock
STK11	*arg4 pex22Δ::Zeocin his4*	25
CYPP01	*arg4-1 pex4Δ::ARG4 pex10Δ::LEU2 his4Δ::ARG4*	This study
CYPP03	*arg4-1 pex1Δ::ARG4 pex10Δ::LEU2 his4Δ::ARG4*	This study
CYPP04	*arg4-1 pex6-1 pex10Δ::LEU2 his4Δ::ARG4*	This study
CYPP05	*arg4-1 pex1Δ::ARG4 pex4Δ::ARG4 his4-1*	This study
CYPP06	*arg4-1 pex4Δ::ARG4 pex8-3 his4Δ::ARG4*	This study
CYPP07	*arg4-1 pex4Δ::ARG4 pex6-1 his4Δ::ARG4*	This study
CYPP08	*arg4-1 pex2-2 pex4Δ::ARG4 his4Δ::ARG4*	This study
CYPP09	*arg4-1 pex4Δ::ARG4 pex12Δ::ARG4 his4Δ::ARG4*	This study
CYPP10	*arg4-1 pex4Δ::ARG4 pex13Δ::ARG4 his4Δ::ARG4*	This study
CYPP11	*arg4-1 pex4Δ::ARG4 pex14Δ::ARG4 his4Δ::ARG4*	This study
CYPP12	*arg4-1 pex4Δ::ARG4 pex17Δ::ARG4 his4Δ::ARG4*	This study
CYPP14	*arg4-1 pex3-1 pex4Δ::ARG4 his4Δ::ARG4*	This study
CYPP15	*arg4-1 pex4Δ::ARG4 pex22Δ::Zeocin his4*	This study
CYPP16	*arg4-1 pex10Δ::LEU2 pex22Δ::Zeocin his4*	This study
CYPP18	*arg4-1 pex1Δ::ARG4 pex22Δ::Zeocin his4*	This study
CYPP19	*arg4-1 pex6-1 pex22Δ::Zeocin his4*	This study
CYPP20	*arg4-1 pex14Δ::ARG4 his4Δ::ARG4*	Laboratory stock
CYPP21	*arg4-1 pex17Δ::ARG4 his4Δ::ARG4*	Laboratory stock

[a] The SGY55 strain was used in the wild type in the experiments described here.

lation medium (1% potassium chloride, 0.5% sodium acetate, 1% dextrose) was used to induce mating and sporulation. All cultures and plates were grown at 30°C.

Yeast strains. The yeast strains used in this study are presented in Table 1. The *pex his4*Δ strains were created by mating each *pex* mutant with SGY55 (4), sporulating the resultant diploids, and screening the haploid progeny for the *pex his* phenotype. The double-mutant strains created for this study were made as follows. The single-mutant strains in question were grown to saturation in YPD, and 0.5 ml of each was mixed and allowed to grow overnight on a YPD plate. On the following day, cells were scraped off the YPD plate, resuspended in 1 ml of sterile water, and plated on solid sporulation medium to induce allow mating. After 24 h, the sporulation plates were replica plated to minimal-methanol plates to select for diploids. Three days later, cells were transferred to fresh minimal-methanol plates and grown for another 3 days. The resulting diploid cells were then transferred to a 3-ml liquid YPD culture and grown overnight. The yeast cells from this culture were harvested, resuspended in 0.5 ml of sterile water, and seeded at high density onto solid sporulation medium. The cells were incubated for 5 days to allow the formation of a significant number of asci. The asci were then scraped from the plates, pelleted, and resuspended in 2 ml of sterile water. To separate asci, 650 μl of the resuspended pellet was incubated with 350 μl of 100% ethanol at room temperature for exactly 30 min. Cells were then plated at 10-fold serial dilutions to obtain single haploid spores. Spores were colony purified and assayed for growth on minimal-methanol medium. Spores that could not grow on methanol were then assayed by complementation analysis to distinguish the double-mutant progeny from the single-mutant progeny.

Plasmid construction and yeast transformations. The plasmids expressing the wild-type *pex4* gene and the *pex4-C133A* mutant have been described previously (5). All bacterial manipulations were carried out with the *Escherichia coli* DH10B host (18). Purified plasmid DNA was introduced into the yeast strains by electroporation (5).

Electron and immunoelectron microscopy. For transmission electron microscopy, strains were grown in YPD to an optical density at 600 nm (OD_{600}) of 1.0, diluted to an OD_{600} of 0.5, and incubated in YPM for 18 h to induce peroxisomal enzymes. Cells were fixed and processed as described previously (17). Samples were sectioned at 70 to 80 kV with a 35° angle diatome diamond knife and placed on Formvar-coated copper grids (Ted Pella, Inc.). The sections were poststained with 2% uranyl acetate and 0.3% lead citrate and observed on a Zeiss 10B transmission electron microscope.

For immunoelectron microscopy, log-phase cells were induced in minimal-methanol medium for 18 h and fixed in suspension for 15 min by adding an equal volume of freshly prepared 8% formaldehyde contained in 1× phosphate-buffered saline (PBS), pH 7.4. The cells were pelleted and resuspended in 4% formaldehyde contained in 1× PBS, pH 7.4, and fixed for an additional 18 to 24 h at 4°C. The cells were then washed briefly in PBS and resuspended in 1% low-temperature-gelling agarose. After cooling, the agarose blocks were trimmed into 1-mm^3 pieces, cryoprotected by infiltration with a mixture of 2.3 M sucrose–20% polyvinylpyrrolidone (10K; pH 7.4) for 2 h, mounted onto cryo-pins, and rapidly frozen in liquid nitrogen. Ultrathin cryosections were cut on a Leica UCT ultramicrotome equipped with an FC-S cryo-attachment and collected onto Formvar-carbon-coated nickel grids. The grids were washed with several drops of 1× PBS containing 2.5% fetal calf serum–10 mM glycine, pH 7.4.; blocked in 10% fetal calf serum for 30 min; and incubated overnight in affinity-purified anti-pex10 polyclonal antibody (diluted 1:50). After washing, the grids were incubated for 2 h in 5-nm gold donkey anti-rabbit conjugates (available from Jackson Immunoresearch Labs). The grids were then washed with several drops of PBS, followed by several drops of double-distilled H_2O, and subsequently embedded in an aqueous solution containing 3.2% polyvinyl alcohol (10K), 0.2% methylcellulose (400 centiposes), and 0.1% uranyl acetate. The grids were observed and photographed on a Philips 410 transmission electron microscope at 80 kV.

Differential centrifugation, subcellular fractionation, and enzyme assays. All fractionation experiments were performed as previously described (5). For differential centrifugation, yeast cells were grown in YPD to mid-logarithmic phase, diluted to an OD_{600} of 0.5, and shifted to rich oleic acid medium or minimal-methanol medium to induce proliferation of peroxisomal enzymes. After an 18- to 20-h incubation period, a postnuclear supernatant (PNS) of each strain was prepared. Organelles were then isolated by centrifugation of the PNS at 25,000 × *g*. Equal proportions of the organelle pellet and cytosolic supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed for the presence of the protein of interest by standard Western blot techniques. For subcellular fractionation, the 25,000 × *g* organelle pellet containing the mitochondria and peroxisomes (generated as described above) was fractionated over a 32 to 60% linear sucrose gradient as described previously (5). Fractions of 1 ml were collected from the bottom of the gradient and analyzed for catalase activity and succinate dehydrogenase activity.

RNA extraction and Northern blot analysis. The *pex4*Δ and *pex10*Δ strains were grown to an OD_{600} of 0.6 in YPD, pelleted, and shifted to minimal-methanol medium. The methanol-induced cultures were incubated overnight at 30°C with agitation. On the following day, approximately 250 OD_{600} units of cells were collected by centrifugation and total RNA was extracted as previously described (13). Poly(A)$^+$ RNA was extracted from the total RNA by magnetic bead separation (Dynabeads; Dynal Inc.) in accordance with the manufacturer's directions.

For Northern blot analysis, 0.6 μg of poly(A)$^+$ RNA was loaded per lane on a denaturing agarose gel (each sample was loaded twice to check for consistency). Following separation by electrophoresis, RNA was transferred to GeneScreen Plus filters (NEN Life Science Products). Probe hybridization and autoradiography were performed using standard protocols (35). Briefly, the filter was hybridized to a ^{32}P-labeled probe specific for the *PEX5* gene (GenBank accession no. U59222), washed, and exposed to X-ray film (Kodak X-Omat AR). The filter was then stripped (boiled for 15 min in 0.5% SDS) and hybridized to a ^{32}P-labeled probe specific for the *P. pastoris* actin gene (*ACT1*; GenBank accession no. AF216956). The probe for the *PEX5* gene was generated by PCR amplification of a plasmid containing the *PEX5* cDNA (forward primer, 5′-GTCCATGTTGAACAGTAAAACCC-3′; reverse primer, 5′-TCCTCCGGTTTGTTGTAATTAGC-3′). The resulting 809-bp fragment was labeled as described below. The *ACT1* probe consisted of a 1,090-bp segment generated by colony PCR using whole *P. pastoris* cells as a template (forward primer, 5′-CAATGGTTCCGGTATGTGTAAGG-3′; reverse primer, 5′-ACACTTGAGGTGCACAATGGATG-3′). The *PEX5* PCR product was purified using the QiaQuick PCR Purification System (Qiagen), and the *ACT1* PCR product was purified using the QiaQuick Gel Extraction System (Qiagen). Purified PCR products were radiolabeled using the Prime-It II kit (Stratagene) in accordance with the manufacturer's directions. The amount of signal produced in each lane was quantitated by analyzing the density of each band (from a scan of the original film) using MacBAS V2.5 software.

Antibodies, protein extracts, and Western blotting. We have previously described the polyclonal antibodies recognizing *P. pastoris* Pex4p (5), Pex5p (15), and Pex10p (23). Anti-thiolase antibodies were raised against recombinant *Saccharomyces cerevisiae* thiolase and affinity purified. Secondary horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Sigma and Jackson ImmunoResearch.

For the production of whole-cell lysates, yeast strains were grown in YPD to mid-logarithmic phase, pelleted, and resuspended in an equal volume of minimal-methanol medium. Cultures were incubated with agitation at 30°C for 14 h to allow the induction of peroxisomal proteins. Approximately five OD_{600} units of methanol-induced cells were pelleted and resuspended in 1 ml of ice-cold sterile water. To lyse the cells, 150 μl of an ice-cold 2 M NaOH–1.2 M β-mercaptoethanol solution (freshly made) was added to the resuspended cells. The cells were vortexed briefly to mix and incubated on ice for 10 min. To precipitate

cellular proteins, 150 μl of ice-cold 50% trichloroacetic acid was added to the lysate. Following a 15-min incubation on ice, the proteins were pelleted by centrifugation at 12,000 × *g* for 5 min. The supernatant was discarded, and the pellet was patiently resuspended in 100 μl of 5% SDS in TBS (25 mM Tris base [pH 8.0], 137 mM NaCl, 3 mM KCl). The protein concentration in each lysate was quantitated using the Micro BCA Protein Assay Reagent Kit (Pierce) in accordance with the manufacturer's directions.

To detect Pex5p levels, 30 μg of the total protein was separated by SDS–10% PAGE. Western blotting and chemiluminescent detection were performed as detailed by Crane et al. (5). Equal loading was confirmed on all blots by staining the protein remaining on the gel after transfer with Coomassie blue. The amount of Pex5p detected relative to the wild type was quantitated for each sample by analyzing the density of each band (from a scan of the original film) using MacBAS V2.5 software.

Detection of initial synthesis of Pex5p. To detect Pex5p synthesis, the *pex4*Δ and *pex10*Δ strains were grown to log phase in YPD and subsequently shifted to minimal-methanol medium for 14 to 18 h. Cells were harvested and resuspended in 0.5 ml of minimal-methanol medium per 2.5 OD_{600} units of cells. The resuspension was incubated at 30°C with agitation for 10 min, and the cells were then labeled by the addition of 75 μCi of [^{35}S]methionine per 0.5 ml (NEN EasyTag [^{35}S]methionine). After 2 to 5 min (2 min for the experiment shown), 20 μl of chase solution (0.5 M methionine, 0.5 M cysteine) was added per 0.5 ml. A 0.5-ml aliquot was then immediately transferred to 0.5 ml of ice-cold azide stop solution (0.04 M methionine, 0.04 M cysteine, 0.13% sodium azide). Cells were then pelleted and resuspended in 1 ml of ice-cold water. The cells were lysed, and total cellular protein was precipitated as outlined above. Protein pellets were resuspended in 30 μl of SDS-PAGE loading buffer (NaOH was added as needed to neutralize the sample) and boiled for 5 min to solubilize Pex5p. One milliliter of ice-cold dilution buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.5]) plus protease inhibitors (Boehringer Mannheim Complete Tablets) was added, and the samples were allowed to sit on ice for 40 min to assist solubility. At this point, any nonsoluble debris were removed and a saturating amount of polyclonal anti-Pex5p antibody was added (diluted into 0.5 ml of dilution buffer plus protease inhibitors per sample, distributed to each sample from a single mixture). After incubation overnight at 4°C, a saturating amount of protein A agarose beads (Santa Cruz Biotechnology) was added and samples were incubated for an additional 90 min at 4°C with end-over-end rotation. The beads were then pelleted and washed five times in ice-cold wash buffer (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.5]). The beads were resuspended in 15 μl of SDS-PAGE buffer and boiled for 5 min, and the entirety of the sample was separated by SDS-PAGE. The gel was then soaked in 0.5 M sodium salicylate for 30 min, dried, and exposed to film.

RESULTS

Reduced abundance of the PTS1 receptor in cells lacking Pex4p activity. We have previously demonstrated that *P. pastoris* Pex4p is highly similar to known ubiquitin-conjugating enzymes, forms an adduct with ubiquitin in vivo, and requires its active-site cysteine (C133) for activity (5). Its likely function is therefore to ubiquitinate one or more target polypeptides. Given that the usual consequence of ubiquitination of target proteins is their degradation, we screened for peroxins that were stabilized by loss of Pex4p. We have yet to identify such a protein, but we did observe that *pex4*Δ cells contain extremely low levels of Pex5p, the PTS1 receptor (Fig. 1A). This phenotype has been previously observed by our lab for human and yeast cells lacking *pex1* (7, 33) or *pex6* (49) and has also been reported by Koller et al. for both the *P. pastoris pex4* and *pex22* mutants (25). Reduced Pex5p abundance is not a common phenotype of *pex* mutants (7), and the *P. pastoris pex2-2*, *pex3-1*, *pex8-3*, *pex10*Δ, *pex12*Δ, and *pex13*Δ mutants all contain normal levels of Pex5p (Fig. 1B). Normal Pex5p abundance was also observed in the *pex14*Δ and *pex17*Δ strains (data not shown). Although the *pex1*Δ and *pex6-1* strains have reduced Pex5p levels, the phenotype is not as pronounced as in the *pex4*Δ or *pex22*Δ mutant. The average levels of Pex5p, compared to that of the wild type, were 35% for *pex1*Δ, 48% for *pex6-1*, 10% for *pex4*Δ, and 7% for *pex22*Δ. However, it was not unusual for the levels of Pex5p to vary by 20% of the wild-type level from one trial to the next.

The reduction of Pex5p levels in *pex4*Δ cells could be caused either by the absence of the Pex4p polypeptide or the absence of its enzymatic activity. To distinguish between these possibilities, we examined the steady-state levels of Pex5p in *pex4*Δ cells carrying plasmids that express either wild-type *PEX4* or a mutant form of *pex4* in which the active-site cysteine is replaced with alanine (*PEX4*-C133A [5]; the *PEX4*-C133A product is properly localized to the peroxisome [data not shown]). Total cellular protein was isolated from the two strains, equal amounts of the two protein samples were separated by SDS-PAGE, and the levels of Pex4p, Pex5p, and thiolase were determined by Western blot analysis. Although Pex4p and thiolase were present at similar levels in both strains, Pex5p levels were significantly reduced in the strain expressing *PEX4*-C133A, indicating that Pex4p enzyme activity is required for normal Pex5p abundance (Fig. 1C).

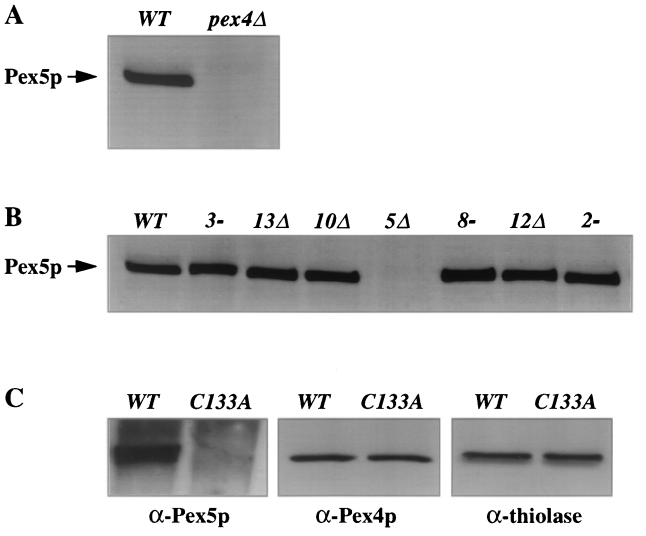

FIG. 1. Loss of Pex4p results in reduced steady-state levels of Pex5p. (A and B) Equal amounts of protein were extracted from methanol-induced cells, separated by SDS-PAGE, and blotted with anti-Pex5p antibodies. (A) Levels of Pex5p in wild-type (*WT*) and *pex4*Δ cells. (B) Normal Pex5p levels in *pex3*, *pex13*, *pex10*, *pex8*, *pex12*, and *pex2* strains. (C) Total cellular proteins extracted from *pex4*Δ strains carrying a wild-type *PEX4* expression plasmid and a plasmid that expresses an active-site mutant of *PEX4*, C133A. Levels of Pex5p (left), Pex4p (middle), and the peroxisomal matrix enzyme thiolase (right) present in these samples were determined by immunoblotting with specific antibodies.

Pex5p is synthesized normally in *pex4*Δ cells. To determine if the reduced level of Pex5p in the *pex4*Δ strain was due to reduced levels of *PEX5* transcript, we performed Northern blot analysis on *pex4*Δ and *pex10*Δ cells. Poly(A)$^+$ RNAs from *pex4*Δ and *pex10*Δ cells were separated by denaturing agarose gel electrophoresis, transferred to membranes, and sequentially hybridized to radiolabeled probes for the *P. pastoris PEX5* and *ACT1* (actin) genes (Fig. 2A). Quantitation of the *PEX5* and *ACT1* mRNA levels revealed that the *PEX5*:*ACT1* ratio in the *pex4*Δ strain was 1.3 times that in the *pex10*Δ strain. Similar results were obtained in two additional independent trials (data not shown). Thus, the reduction of Pex5p levels in the *pex4*Δ strain cannot be due to decreased levels of *PEX5* mRNA synthesis.

To determine whether *PEX5* mRNA is translated in the *pex4*Δ strain, we compared the initial synthesis of Pex5p in *pex4*Δ and *pex10*Δ cells. Methanol-induced cells were labeled with [^{35}S]methionine for 2 min, and total cellular protein was collected by alkaline lysis. Pex5p was immunoprecipitated from these lysates using excess anti-Pex5p antibodies, and the level of labeled Pex5p in each sample was determined by fluorography (Fig. 2B). The amounts of Pex5p synthesized in *pex4*Δ and *pex10*Δ cells were similar, with slightly higher levels of

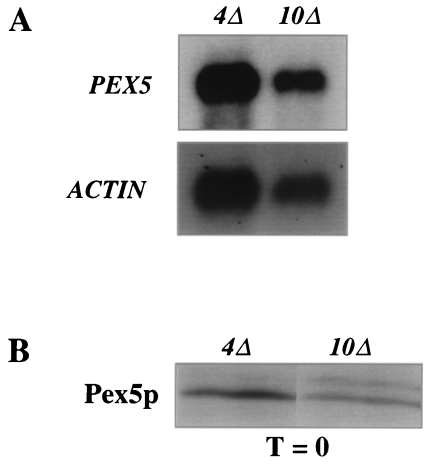

FIG. 2. *PEX5* mRNA and Pex5p are synthesized normally in *pex4*Δ cells. (A) Northern blot analysis of poly(A)$^+$ RNAs from *pex4*Δ and *pex10*Δ cells. The two RNA samples were separated by denaturing gel electrophoresis, transferred to membranes, and sequentially hybridized to radiolabeled probes specific for the *PEX5* (top) and *ACT1* (bottom) genes. Quantification revealed that the *PEX5: ACT1* hybridization signal was 1.3-fold higher in the *pex4*Δ strain than in the *pex10*Δ strain. Similar results were observed in two additional independent trials. (B) Synthesis of equivalent levels of Pex5p during pulse-labeling of *pex4*Δ and *pex10*Δ cells. Each strain was incubated in the presence of [^{35}S]methionine for 2 min and lysed in alkali, and the Pex5p present in each sample was immunoprecipitated using excess anti-Pex5p antibodies. The immunoprecipitates were separated by SDS-PAGE, and the amount of Pex5p was determined by fluorographic exposure to X-ray film. Similar results were observed in nine additional trials of this experiment.

translation in the *pex4*Δ strain. This experiment was repeated 10 times, with similar results in all of the trials. These data demonstrate that the reduced level of Pex5p in *pex4*Δ cells occurs posttranslationally, presumably via accelerated degradation of Pex5p. Consistent with this, the reduced level of Pex5p in human *pex1*- and *pex6*-deficient cells is known to result from accelerated Pex5p degradation (7, 49). Unfortunately, we were unable to measure the half-life of Pex5p in *pex4*Δ and *pex10*Δ cells due to technical difficulties in chasing the [^{35}S]methionine from the cells.

Epistasis analysis places Pex4p late in peroxisomal matrix protein import. The variance in Pex5p stability displayed by different *pex* mutants allowed us to examine epistatic relationships among various *pex* mutants. An array of studies with both yeast (19, 21, 46, 48) and human (38) cells have established that *PEX3* is required for synthesis of peroxisome membranes and that *pex3* mutants are devoid of peroxisome-like structures. To test whether the reduced Pex5p levels seen in *pex4*Δ cells require the presence of peroxisome membranes and *PEX3* function, we examined the levels of Pex5p in a *pex3-1 pex4*Δ double mutant. Total cellular protein was extracted from methanol-induced wild-type, *pex5*Δ, *pex3-1*, *pex4*Δ, and *pex3-1 pex4*Δ cells. The same amount of protein from each strain was then separated by SDS-PAGE and blotted with anti-Pex5p antibodies (Fig. 3A). In contrast to the low level of Pex5p detected in *pex4*Δ cells, the level of Pex5p observed in the *pex3-1 pex4*Δ double mutant was similar to those seen in the *pex3-1* single-mutant and wild-type cells. These results demonstrate that Pex3p acts upstream of Pex4p. To visualize equivalency in loading, the protein remaining on the gel after transfer was stained with Coomassie blue (Fig. 3, 4, and 5).

One of the earliest steps in peroxisomal matrix protein import is the docking of PTS receptors to the peroxisome membrane. This process is mediated by Pex13p (8, 9, 14, 15) and Pex14p (1, 11) and may involve Pex17p (22), a peroxin that has also been implicated in PMP import (37). To assess the epistatic relationship of *PEX4* to the *PEX* genes necessary for receptor docking, we examined Pex5p levels in the *pex4*Δ *pex13*Δ, *pex4*Δ *pex14*Δ, and *pex4*Δ *pex17*Δ double mutants, as well as in all relevant single mutants. In contrast to the *pex4*Δ mutant, the double mutants all contained high levels of Pex5p that were similar to those of the *pex13*Δ, *pex14*Δ, and *pex17*Δ single mutants (Fig. 3B). The fact that Pex13p, Pex14p, and Pex17p must act in order for the loss of Pex4p to cause destabilization of Pex5p indicates that these three peroxins act upstream of Pex4p.

The integral peroxisomal membrane proteins Pex8p, Pex10p, and Pex12p have been implicated in peroxisomal matrix protein import at a step downstream of receptor docking, most probably at matrix protein translocation (3, 32). Double mutants lacking *pex4* and *pex8*, *pex10*, or *pex12* were generated, and their Pex5p levels were examined. High Pex5p levels were detected in these double mutants, indicating that these matrix protein import factors act upstream of Pex4p (Fig. 4A and B). A specific role for *PEX2* has yet to be elucidated, but the phenotype of *pex2*-deficient cells suggests that they also participate in matrix protein import downstream of receptor docking (7). The *pex2-2 pex4*Δ double mutant was generated and found to have normal Pex5p levels, indicating that *PEX4* also acts after *PEX2* (Fig. 4B).

Pex1p, Pex6p, and Pex22p also act late in peroxisomal matrix protein import. The phenotype of Pex5p instability is also shared by the *pex22*Δ mutant and to lesser extents by the *pex1*Δ and *pex6-1* mutants. While a previous study has established that Pex22p interacts physically with Pex4p (25), there is no evidence linking *PEX4* or *PEX22* function with *PEX1* or *PEX6*

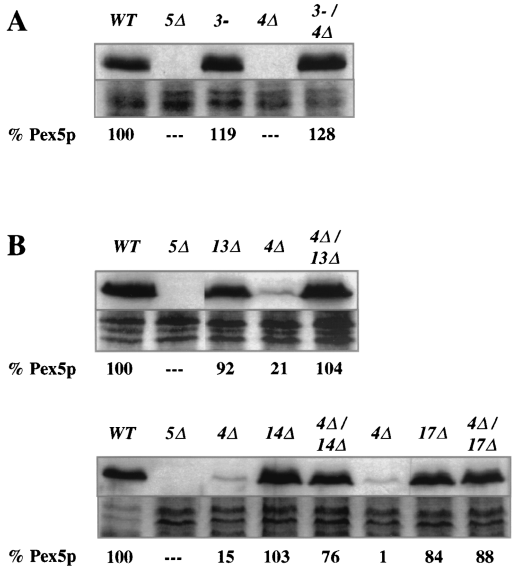

FIG. 3. The destabilization of Pex5p in the *pex4*Δ strain requires Pex3p, Pex13p, Pex14p, and Pex17p. For each panel, the strains were grown side by side and total cellular protein was extracted with alkali, separated by SDS-PAGE, and blotted with antibodies specific for Pex5p (top of each panel). Coomassie blue staining of each protein sample is also shown (bottom of each panel), and the quantity of Pex5p in each sample (as assessed by scanning densitometry of the resulting films) is shown below each lane. (A) Levels of Pex5p in wild-type (*WT*) cells; the *pex5*Δ, *pex3-1*, and *pex4*Δ mutants; and the *pex3-1 pex4*Δ double mutant. (B) Levels of Pex5p in wild-type cells; the *pex5*Δ, *pex13*Δ, and *pex4*Δ mutants; and the *pex4*Δ *pex13*Δ double mutant. The lower blot in panel B shows levels of Pex5p in wild-type cells; the *pex5*Δ, *pex4*Δ, and *pex14*Δ mutants; the *pex4*Δ *pex14*Δ double mutant; the *pex4*Δ and *pex17*Δ mutants; and the *pex4*Δ *pex17*Δ double mutant.

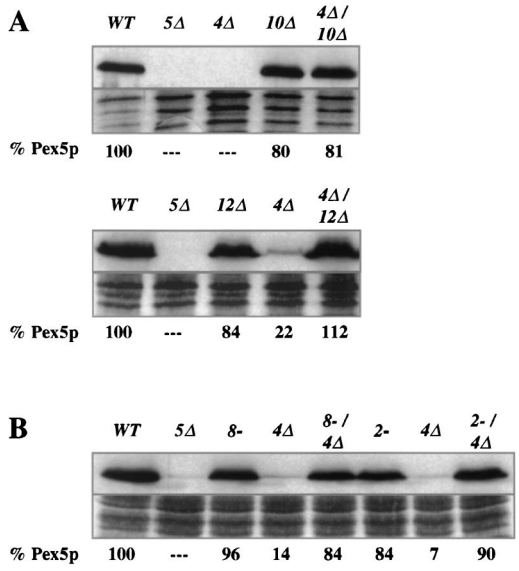

FIG. 4. Destabilization of Pex5p in the *pex4*Δ strain requires Pex10p, Pex12p, Pex8p, and Pex2p. For each panel, the strains were grown side by side and total cellular protein was extracted with alkali, separated by SDS-PAGE, and blotted with antibodies specific for Pex5p (top of each panel). Coomassie blue staining of each protein sample is also shown (bottom of each panel), and the quantity of Pex5p in each sample (as assessed by scanning densitometry of the resulting films) is shown below each lane. (A) Levels of Pex5p in wild-type (*WT*) cells; the *pex5*Δ, *pex4*Δ, and *pex10*Δ mutants; and the *pex4*Δ *pex10*Δ double mutant. The lower blot in panel A shows levels of Pex5p in wild-type cells; the *pex5*Δ, *pex12*Δ, and *pex4*Δ mutants, and the *pex4*Δ *pex12*Δ double mutant. (B) Levels of Pex5p in wild-type cells; the *pex5*Δ, *pex8-3*, and *pex4*Δ mutants; the *pex4*Δ *pex8-3* double mutant; the *pex2-2* and *pex4*Δ mutants; and the *pex2-2 pex4*Δ double mutant.

function. Therefore, we tested whether these mutants might also act late in peroxisomal matrix protein import. To do this, we generated double-mutant combinations of *pex1*Δ, *pex6-1*, and *pex22*Δ with *pex10*Δ, which blocks matrix protein import downstream of PTS receptor docking (3). Pex5p levels were examined in *pex1*Δ *pex10*Δ, *pex6-1 pex10*Δ, and *pex10*Δ *pex22*Δ double mutants, and in each case the levels of Pex5p were similar to that of the *pex10*Δ single mutant (Fig. 5A). The *pex4*Δ *pex22*Δ double mutant was also examined and found to have low Pex5p levels that were similar to those of both single mutants (Fig. 5A). The slight increase seen in the quantitation (6% for the double mutant versus undetectable for the single mutants) is not outside the sensitivity limit of the quantitation method.

The fact that *PEX10* is epistatic to *PEX1*, *PEX4*, *PEX6*, and *PEX22* indicates that the products of these four genes act late in peroxisomal matrix protein import. However, it was apparent from our studies that the *pex1*Δ and *pex6-1* mutants contain higher levels of Pex5p than the *pex4*Δ and *pex22*Δ mutants. To order these genes relative to one another, we examined Pex5p abundance in *pex1*Δ *pex4*Δ and *pex4*Δ *pex6-1* double mutants. Pex5p levels in the *pex1*Δ *pex4*Δ and *pex4*Δ *pex6-1* strains resembled those of the single *pex1*Δ and *pex6-1* mutants, indicating that Pex1p and Pex6p act upstream of Pex4p (Fig. 5B). We also examined Pex5p levels in *pex1*Δ *pex22*Δ and *pex6-1 pex22*Δ double mutants, and the Pex5p levels again resembled those of the *pex1*Δ and *pex6-1* single mutants (Fig. 5C). This indicates that Pex1p and Pex6p also act before Pex22p. As noted earlier, there is a noticeable variability in the relative amount of Pex5p present in a given strain. The range of variability is usually about 20% of the wild-type level, which likely explains why the double mutants often contained levels of Pex5p that were slightly higher or lower than that of either single mutant alone.

Peroxisomes of *pex4*Δ mutants contain residual levels of peroxisomal matrix proteins. Several lines of evidence indicate that peroxisomal matrix protein import involves the cycling of PTS receptors between the cytoplasm and peroxisome, suggesting that the terminal step in matrix protein import is the release of PTS receptors back to the cytoplasm (42). Cells defective in this step would be expected to contain recognizable peroxisomes, import PMPs normally, and import residual levels of peroxisomal matrix proteins.

Previous studies of *P. pastoris pex1-*, *pex6-*, and *pex22*-deficient strains have indicated that these mutants have precisely this phenotype (20, 25, 39). As our prior analysis of the *P. pastoris pex4*Δ mutant did not address its phenotype at that level of detail (5), we proceeded to characterize the peroxisomes of *pex4*Δ cells. Previous studies have established that the yeast *P. pastoris* has numerous large peroxisomes when grown on energy sources that require peroxisomal metabolic pathways, particularly methanol or fatty acids (17, 23, 29). In par-

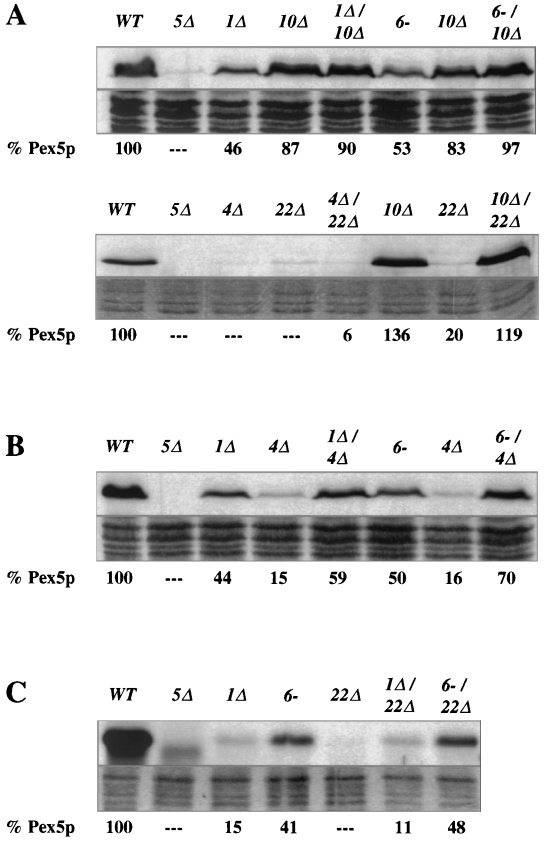

FIG. 5. Pex1p and Pex6p act downstream of Pex10p and upstream of Pex4p and Pex22p. For each panel, the strains were grown side by side and total cellular protein was extracted with alkali, separated by SDS-PAGE, and blotted with antibodies specific for Pex5p (top of each panel). Coomassie blue staining of each protein sample is also shown (bottom of each panel), and the quantity of Pex5p in each sample (as assessed by scanning densitometry of the resulting films) is shown below each lane. (A) Levels of Pex5p in wild-type (*WT*) cells; the *pex5*Δ, *pex1*Δ, and *pex10*Δ mutants; the *pex1*Δ *pex10*Δ double mutant; the *pex6-1* and *pex10*Δ mutants; and the *pex6-1 pex10*Δ double mutant. The lower blot in panel A shows levels of Pex5p in wild-type cells; the *pex5*Δ, *pex4*Δ, and *pex22*Δ mutants; the *pex4*Δ *pex22*Δ double mutant; the *pex10*Δ and *pex22*Δ mutants; and the *pex10*Δ *pex22*Δ double mutant. (B) Levels of Pex5p in wild-type cells; the *pex5*Δ, *pex1*Δ, and *pex4*Δ mutants; the *pex1*Δ *pex4*Δ double mutant; the *pex6-1* and *pex4*Δ mutants; and the *pex6-1 pex4*Δ double mutant. (C) Levels of Pex5p in wild-type cells; the *pex5*Δ, *pex1*Δ, *pex6-1*, and *pex22*Δ mutants; and the *pex1*Δ *pex22*Δ and *pex6-1 pex22*Δ double mutants.

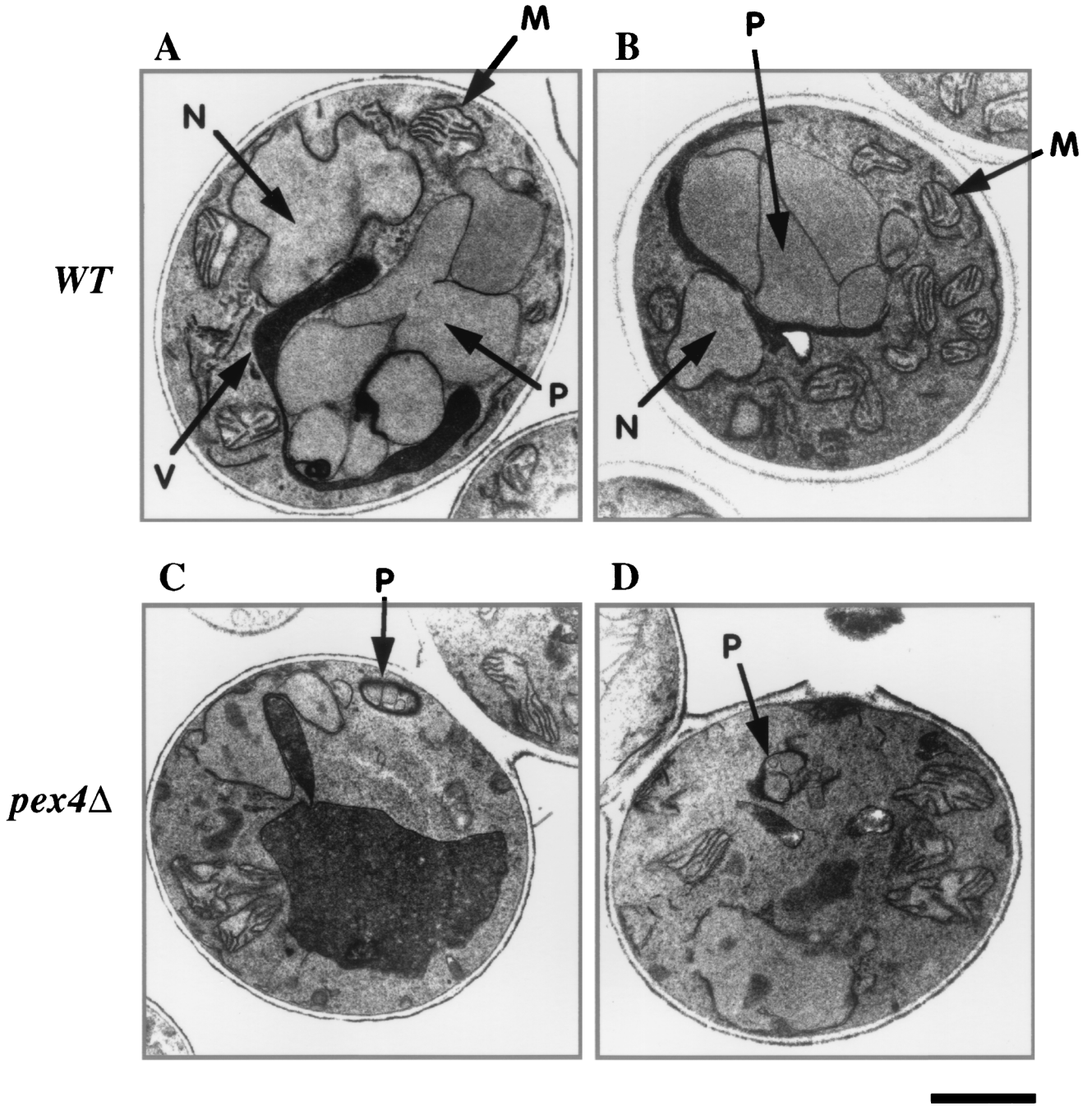

FIG. 6. Transmission electron microscopy reveals the presence of small, matrix-containing peroxisomes in *pex4*Δ cells. Wild-type (*WT*) (A and B) and *pex4*Δ (C and D) cells were induced in methanol medium and processed for transmission electron microscopy. M, mitochondria; N, nucleus; P, peroxisome; V, vacuole. Bar, 1.0 μm.

ticular, peroxisomes of methanol-grown cells can be easily detected in thin-section electron micrographs due to their large cuboidal shape, their electron-dense matrix, and the fact that they are typically attached to one another (Fig. 6A and B). Cells lacking Pex4p also contain peroxisomes with an electron-dense granular matrix and semicuboidal shape, though they are much smaller than peroxisomes of wild-type cells (Fig. 6C and D).

To confirm that these structures were indeed peroxisomes, we also performed immunoelectron microscopy on wild-type (Fig. 7A) and *pex4*Δ (Fig. 7B to F) cells. Cryosections of embedded cells were incubated with antibodies specific for Pex10p, a known integral PMP. The anti-Pex10p antibody was visualized by binding of a secondary gold particle conjugate. These experiments demonstrate that *pex4*Δ cells contain peroxisomes with a discernible electron-dense matrix but have only 1/10 of the diameter of normal peroxisomes.

The morphology of peroxisomes in *pex4*Δ cells suggested that they were able to import low but significant levels of peroxisomal matrix proteins into the peroxisome lumen. We tested this hypothesis by examining *pex4*Δ cells for the presence of peroxisomes of normal density. Wild-type, *pex4*Δ, and *pex10*Δ cells were induced in methanol-containing medium and harvested, and a PNS was generated from each strain. Peroxi-

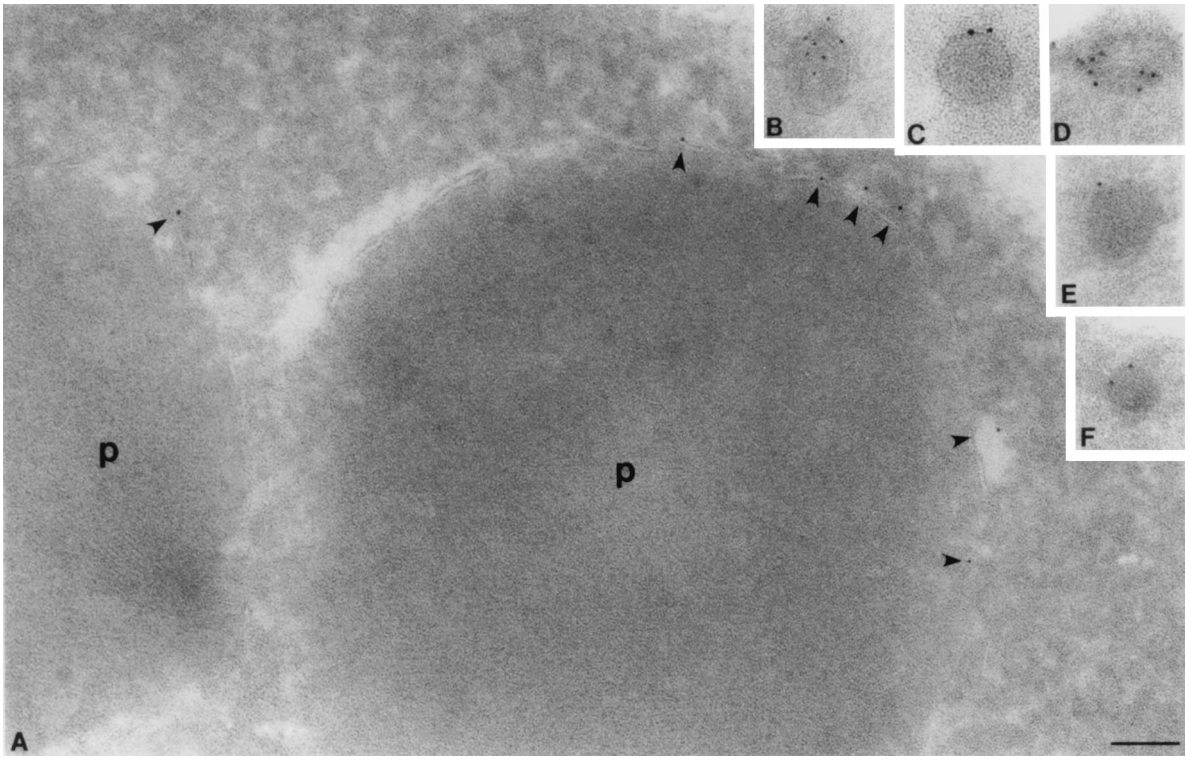

FIG. 7. Immunocryoelectron microscopy demonstrates that *pex4*Δ cells contain small, electron-dense peroxisomes. (A) Peroxisomes (p) of wild-type cells labeled with antibodies specific for Pex10p, shown here by immunogold labeling of the peroxisome membrane. Note the position of gold particles at the peroxisome membrane (shown by arrowheads) and the electron-dense nature of the protein-rich peroxisome matrix. (B to F) Peroxisomes of *pex4*Δ cells are also labeled with antibodies specific for Pex10p and contain an electron-dense, granular matrix. However, their radius is only 10% of the radius of wild-type peroxisomes, indicating that their volume may be only 1/1,000 of that of the wild-type peroxisome. Bar, 0.1 μm.

somes and other large organelles were collected by differential centrifugation and separated by sucrose density gradient centrifugation. The resulting fractions were assayed for the peroxisomal matrix protein catalase and the mitochondrial marker succinate dehydrogenase (Fig. 8). These experiments show that *pex4*Δ cells contain significant levels of catalase at approximately the same density as wild-type peroxisomes. Previous studies have shown that loss of Pex10p leads to a far more severe defect in peroxisomal matrix protein import (23), and this is reflected in the absence of a catalase peak at the normal density of peroxisomes. The low-density peak of catalase activity in each sample reflects enzyme that is nonspecifically trapped during collection of the organelle pellet, as well as enzyme that leaks from peroxisomes during manipulation of the organelle pellet.

To further address the ability of *pex4*Δ cells to import peroxisomal proteins, we assayed the import of a PTS2 protein and a PMP in wild-type, *pex4*Δ, *pex5*Δ, and *pex10*Δ cells. The PNS generated from each strain was separated into an organelle pellet and cytosolic supernatant by centrifugation at 25,000 × *g*, and the proportions of thiolase (PTS2 marker) and Pex10p (PMP marker) in the supernatant and pellet were quantitated. The *pex4*Δ cells import all of the detectable Pex10p, indicating that the strain has no defect in PMP import. The *pex4*Δ cells also imported 42% of the cellular thiolase, versus 75% for wild type, 69% for *pex5*Δ cells, and only 1% for *pex10*Δ cells (Fig. 9). The fact that *pex4*Δ cells import 1.5-fold less thiolase than *pex5*Δ cells indicates that the import defect of *pex4*Δ cells is not limited to the PTS1 pathway. This hypothesis is supported by the fact that overexpression of Pex5p is unable to suppress the growth defects of the *pex4*Δ strain (data not shown).

Subcellular distribution of Pex5p in the *pex4*Δ strain. A previous study has established that *P. pastoris* Pex5p is a predominantly cytosolic, partly peroxisomal protein in wild-type cells (15). The hypothesis that Pex4p plays an important role in the recycling of Pex5p back to the cytoplasm predicts that loss of Pex4p should result in accumulation of Pex5p at the peroxisome. We examined the distribution of Pex5p in wild-type, *pex4*Δ, *pex5*Δ, and *pex10*Δ cells. Strains were induced in methanol medium, and postnuclear supernatants (PNS) were prepared and separated into an organelle pellet and a cytosolic supernatant. Equal proportions of these fractions were assayed Pex5p by Western blot analysis (Fig. 10). Virtually all of the Pex5p present in *pex4*Δ cells was associated with the organelle pellet, a stark contrast to the predominantly cytosolic distribution in wild-type and *pex10*Δ cells.

DISCUSSION

Genetic studies of peroxisome biogenesis have led to the identification of more than 20 different peroxins, each required for normal peroxisome biogenesis. Studies of these factors and of peroxisomal protein import mechanisms have shown that PMP import and peroxisomal matrix protein import occur through separate pathways, with each process controlled by a distinct set of genes. As all peroxisomal matrix proteins are translated in the cytosol, their proper localization is dependent on the action of the PTS receptors. Studies on Pex5p, the PTS1 receptor, indicate that it is a predominantly cytoplasmic, partly

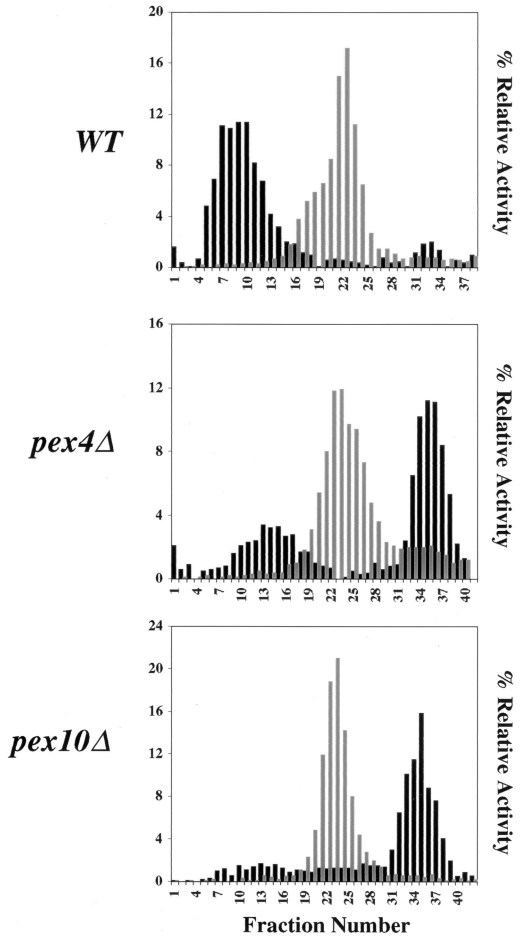

FIG. 8. The *pex4*Δ mutant contains peroxisomes of normal density. Wild-type (*WT*), *pex4*Δ, and *pex10*Δ cells were induced in methanol medium, a PNS was generated from each strain, and peroxisomes and mitochondria were collected by differential centrifugation. The resulting three organelle pellets were then separated by sucrose density gradient centrifugation. The same amount of each fraction was assayed for catalase (a PTS1-containing peroxisomal enzyme) activity (black bars), succinate dehydrogenase (mitochondrial enzyme) activity (grey bars), and density (data not shown). Enzyme activities are plotted as percentages of the total activity in the gradient. The densities of all of the gradient profile were similar, with the peak peroxisomal fraction of wild-type cells migrating at a density of 1.19 to 1.21 g/cm^3 and the peak peroxisomal fraction of *pex4*Δ cells migrating at a density of 1.18 to 1.20 g/cm^3.

peroxisomal protein (6, 8, 15, 45) and may cycle between the cytoplasm and the peroxisome (7). Such results have inspired models of peroxisomal matrix protein import in which peroxins are required not only for the translocation of proteins through the peroxisome membrane but also to facilitate the movement of PTS receptors through their cycles. Such a view of peroxisomal matrix protein import suggests the existence of PTS receptor docking factors, matrix protein translocation factors, and PTS receptor recycling factors.

Within the context of this model, determining the order of action for different peroxisome biogenesis factors should be extremely useful for determining their functions. Obviously, biochemical approaches can be used to determine the order of peroxin action. Such studies have led to current models in which Pex14p acts in PTS receptor docking in peroxisomal matrix protein import (42) and Pex8p (32), Pex10p, and Pex12p (3) act downstream of receptor docking in peroxisomal matrix protein import. However, epistasis analysis is ideally suited for determining the order of gene action, provided that

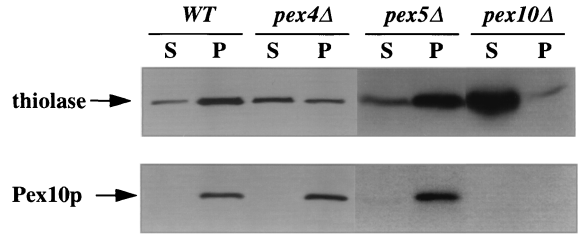

FIG. 9. The *pex4*Δ mutant imports significant amounts of the PTS2 marker enzyme thiolase but less than wild-type (*WT*) or *pex5*Δ cells. Wild-type, *pex4*Δ, *pex5*Δ, and *pex10*Δ cells were induced to proliferate peroxisomal proteins, and a PNS was generated from each strain. This was then separated into an organelle pellet (p) and a cytosolic supernatant (s) by centrifugation at 25,000 × *g*. The same proportion of each fraction was separated by SDS-PAGE and blotted with antibodies specific for the PTS2-targeted enzyme thiolase (top) and the integral PMP Pex10p (bottom).

there are defining subphenotypes that can be used as markers of different steps in the process being studied. In this study, we used the subphenotype of reduced Pex5p abundance to examine the epistatic relationships among 12 different *pex* genes.

We have previously established that reduced Pex5p abundance is a reproducible phenotype for human *pex1*- and *pex6*-deficient cell lines but is not observed in human *pex2*-, *pex7*-, *pex10*-, *pex12*-, and *pex16*-deficient cells (7, 49). Furthermore, these studies revealed that the reduced abundance of Pex5p in *pex1*- and *pex6*-deficient cells was due to an increased rate of Pex5p degradation. Koller et al. recently reported that Pex5p abundance is reduced in *P. pastoris pex4* and *pex22* mutants (25). Here we show that Pex5p abundance is normal in *P. pastoris pex2-2*, *pex3-1*, *pex8-3*, *pex10*Δ, *pex12*Δ, *pex13*Δ, *pex14*Δ, and *pex17*Δ mutants and that the reduction in Pex5p abundance is more severe in *pex4* and *pex22* mutants than in *pex1* and *pex6* mutants. Furthermore, we demonstrated that *pex4*Δ cells have normal *PEX5* mRNA abundance and are able to synthesize Pex5p. That *pex4* and *pex10* mutants have similar rates of Pex5p synthesis but different steady-state levels of Pex5p represents strong evidence that Pex5p is degraded at an accelerated rate in *pex4*Δ cells. These results are consistent with the increased rate of Pex5p degradation in human *pex1*- and *pex6*-deficient cells (7, 49).

Although technical difficulties in chasing labeled methionine from *P. pastoris* cells prevented us from measuring Pex5p stability in the *pex4*Δ and *pex10*Δ mutant strains, we were still able to use the variance in Pex5p abundance to order the *pex* mutants relative to one another by epistasis analysis. Our data show that Pex4p acts after the peroxisome membrane synthesis factor Pex3p, after the PTS receptor docking factors Pex13p and Pex14p, after the putative protein translocation factors Pex8p, Pex10p, and Pex12p, and after the other peroxins Pex2p and Pex17p. Furthermore, we found that Pex1p, Pex6p, and

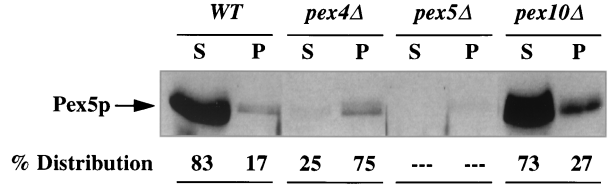

FIG. 10. Most Pex5p is present in the organelle fraction of *pex4*Δ cells. Wild-type (*WT*), *pex4*Δ, *pex5*Δ, and *pex10*Δ cells were induced in methanol medium, and a PNS was generated from each strain. This was then separated into an organelle pellet (p) and a cytosolic supernatant (s) by centrifugation at 25,000 × *g*. The same proportion of each fraction was separated by SDS-PAGE and blotted with antibodies specific for Pex5p.

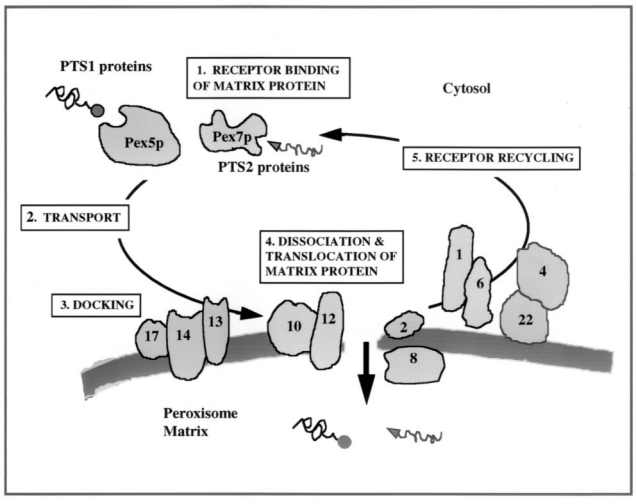

FIG. 11. Model of peroxisomal matrix protein import. PTS-containing proteins are bound by their receptor after translation in the cytoplasm (step 1). The mechanism by which the receptor-bound matrix protein is transported to the peroxisome membrane remains unclear (step 2). The receptor-bound matrix protein then docks on the surface of the peroxisome membrane via interactions with the docking factors Pex13p, Pex14p, and possibly Pex17p (step 3). Following docking, the matrix protein must dissociate from the receptor and be translocated across the peroxisome membrane into the matrix space (step 4). It is not clear whether the receptor follows the cargo into the lumen or remains on the cytosolic surface of the peroxisome. However, in either scenario, the receptor is subsequently released from the translocation pore and recycled back to the cytoplasm to undergo further rounds of import (step 5).

Pex22p also act downstream of Pex10p and that Pex1p and Pex6p act upstream of both Pex4p and Pex22p. This result suggests a model in which Pex1p, Pex4p, Pex6p, and Pex22p all act downstream of matrix protein translocation (Fig. 11).

The placement of Pex4p very late in peroxisomal matrix protein import is consistent with the cellular phenotypes of *pex4* mutants. Electron microscopy studies revealed that *pex4* cells still have peroxisomes and that these peroxisomes resemble those of wild-type cells in all respects but their overall size. Peroxisomes of *pex4*Δ cells had the clustered, cuboidal, electron-dense appearance of normal methanol-induced *P. pastoris* peroxisomes, but their diameter was 10 times less. Biochemical studies revealed that peroxisomes of *pex4*Δ cells import wild-type levels of the PMP Pex10p and import reduced but significant levels of both PTS1- and PTS2-targeted peroxisomal matrix proteins. In contrast, *pex10*Δ cells displayed more severe peroxisomal matrix protein import defects, indicating that the peroxisomal matrix protein import defects of the *pex4* mutant are unusually mild. The phenotypes of the *pex4* mutant are not what we would expect for loss of a peroxin that plays an essential role in peroxisome membrane synthesis, PMP import, PTS receptor docking, or peroxisomal matrix protein translocation. However, they are consistent with the loss of a peroxin that participates in PTS receptor recycling, which is thought to be the final step in peroxisomal matrix protein import. This hypothesis for Pex4p function predicts that Pex5p should be trapped at or in the peroxisome in *pex4*Δ cells, and it is interesting that 75% of the Pex5p in *pex4*Δ cells is present in the organelle fraction (compared to only 17% in wild-type cells).

An analysis of *Hansenula polymorpha* Pex4p also concluded that Pex4p participates in the recycling of Pex5p to the cytoplasm (44). That study also found that Pex5p accumulates on or in peroxisomes in the absence of Pex4p. However, they also found that the peroxisomal protein import defect of *pex4*Δ cells could be partially suppressed by overexpressing *PEX5* (44). Interestingly, *H. polymorpha pex4* mutants import PTS2 proteins normally (44) whereas *S. cerevisiae* and *P. pastoris pex4* mutants display a reproducible defect in PTS2 protein import (47). The mild PTS2 protein import defect and the severe reduction in Pex5p abundance in *P. pastoris pex4*Δ cells raise the possibility that the phenotypes of *pex4*Δ cells is simply due to reduced Pex5p abundance. However, the PTS2 protein import defect of *pex4*Δ cells is more severe than that of *pex5*Δ cells. Also, we tested whether *PEX5* overexpression could rescue the growth defects of *pex4*Δ cells but failed to see any evidence of phenotypic rescue. Thus, it is unlikely that reduced Pex5p abundance can explain all of the phenotypes of *pex4*Δ cells.

Koller et al. (25) have recently reported that Pex22p physically interacts with Pex4p, that *pex22* mutants show severely reduced Pex5p levels, and that Pex22p is required for Pex4p abundance. We previously established that Pex4p is peripherally associated with the outer surface of the peroxisome membrane (5), and Koller et al. (25) speculated that Pex22p may function as a docking site for Pex4p on the peroxisome membrane. Thus, it is was not surprising that our epistasis analysis placed Pex22p after Pex1p, Pex6p, and Pex10p and, by deduction, after all of the other peroxins we examined, except Pex4p. These multiple lines of evidence connect Pex22p and Pex4p at a terminal step in peroxisomal matrix protein import.

Our epistasis studies indicate that Pex1p and Pex6p also act late in peroxisomal matrix protein import, though upstream of Pex4p and Pex22p. The action of Pex1p and Pex6p at a common point in peroxisome biogenesis is altogether expected, given that genetic interactions have been described for *PEX1* and *PEX6* and that physical interactions have been reported for Pex1p and Pex6p (10, 12, 24). Furthermore, results that place Pex1p and Pex6p at a late step in peroxisomal matrix protein import are consistent with the phenotypes of *pex1* and *pex6* mutants in most species. In humans (33, 36, 49) and the yeasts *P. pastoris* (20, 39), *H. polymorpha* (24), and *S. cerevisiae* (19), cells lacking *pex1* or *pex6* contain numerous peroxisomes that are competent for PMP import. Studies of the yeasts *P. pastoris* (20, 39) and *H. polymorpha* (24) and human cells (33, 49) have also shown that peroxisomes of *pex1*- and *pex6*-deficient cells import residual levels of peroxisomal matrix proteins. Thus, the *pex1* and *pex6* mutants, like the *pex4* and *pex22* mutants, display phenotypes that we might expect from the loss of receptor recycling factors. The epistasis analysis presented here clearly places *P. pastoris* Pex1p and Pex6p downstream of Pex10p, a protein that acts downstream of receptor docking in peroxisomal matrix protein import, supporting the hypothesis that Pex1p and Pex6p act late in peroxisomal matrix protein import. However, studies of the yeast *Yarrowia lipolytica* have led Rachubinski and colleagues to propose a different model in which Pex1p and Pex6p participate in peroxisome membrane biogenesis (43). We have no model that can explain these differences in results or interpretation, and it may be that the roles of Pex1p and Pex6p are quite different in *Y. lipolytica*.

How the Pex1p-Pex6p step of peroxisome biogenesis relates to the subsequent step defined by Pex4p-Pex22p remains to be determined. However, the fact that reduced Pex5p abundance is observed in cells lacking any of these four factors indicates that there may be a functional connection between these two steps. Pex1p and Pex6p are a pair of interacting AAA ATPases, and it is well established that these proteins participate in the formation, organization, and/or dissociation of protein complexes (30). It is perhaps worthwhile to consider the possibilities that Pex1p and Pex6p can prepare or present substrates for ubiquitination by a Pex4p-Pex22p complex and that

the ubiquitination event has a positive effect on Pex5p stability. However, the possibilities for how Pex4 and Pex22p protect Pex5p from degradation are numerous. For example, it could involve the destruction of a protein that degrades Pex5p or a modification of Pex5p that protects it from degradation by some general proteolysis machinery. Elucidation of the molecular mechanisms by which Pex1p, Pex6p, Pex4p, and Pex22p impact the stability of Pex5p is clearly a challenge but must be pursued if we are to understand the roles of these peroxins and the process of PTS receptor recycling.

ACKNOWLEDGMENTS

We thank Yifei Liu for affinity purifying the anti-Pex10p antibody.

C.S.C. was partially supported by the Predoctoral Program in Human Genetics at The Johns Hopkins University. This work was supported by NIH grant DK45787 to S.J.G.

REFERENCES

1. **Albertini, M., P. Rehling, R. Erdmann, W. Girzalsky, J. A. K. W. Kiel, M. Veenhuis, and W.-H. Kunau.** 1997. Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. Cell **89:** 83–92.
2. **Chang, C. C., S. South, D. Warren, J. Jones, A. B. Moser, H. W. Moser, and S. J. Gould.** 1999. Metabolic control of peroxisome abundance. J. Cell Sci. **112:**761–774.
3. **Chang, C. C., D. S. Warren, K. A. Sacksteder, and S. J. Gould.** 1999. PEX12 binds PEX5 and PEX10 and acts downstream of receptor docking in peroxisomal matrix protein import. J. Cell Biol. **147:**761–773.
4. **Crane, D. I., and S. J. Gould.** 1994. The *Pichia pastoris* HIS4 gene: nucleotide sequence, creation of a non-reverting his4 deletion mutant, and development of HIS4-based replicating and integrating plasmids. Curr. Genet. **26:**443–450.
5. **Crane, D. I., J. E. Kalish, and S. J. Gould.** 1994. The *Pichia pastoris PAS4* gene encodes a ubiquitin-conjugating enzyme required for peroxisome assembly. J. Biol. Chem. **269:**21835–21844.
6. **Dodt, G., N. Braverman, C. Wong, A. Moser, H. W. Moser, P. Watkins, D. Valle, and S. J. Gould.** 1995. Mutations in the PTS1 receptor gene, PXR1, define complementation group 2 of the peroxisome biogenesis disorders. Nat. Genet. **9:**115–124.
7. **Dodt, G., and S. J. Gould.** 1996. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. J. Cell Biol. **135:** 1763–1774.
8. **Elgersma, Y., L. Kwast, A. Klein, T. Voorn-Brouwer, M. van den Berg, B. Metzig, T. America, H. F. Tabak, and B. Distel.** 1996. The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import of PTS1 containing proteins. J. Cell Biol. **135:**97–109.
9. **Erdmann, R., and G. Blobel.** 1996. Identification of Pex13p, a peroxisomal membrane receptor for the PTS1 recognition factor. J. Cell Biol. **135:**111–121.
10. **Faber, K. N., J. A. Heyman, and S. Subramani.** 1998. Two AAA family proteins, PpPex1p and PpPex6p, interact with each other in an ATP-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes. Mol. Cell. Biol. **18:**936–943.
11. **Fransen, M., S. R. Terlecky, and S. Subramani.** 1998. Identification of a human PTS1 receptor docking protein directly required for peroxisomal protein import. Proc. Natl. Acad. Sci. USA **95:**8087–8092.
12. **Geisbrecht, B. V., C. S. Collins, B. E. Reuber, and S. J. Gould.** 1998. Disruption of a PEX1-PEX6 interaction is the most common cause of the neurologic disorders Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. Proc. Natl. Acad. Sci. USA **95:**8630–8635.
13. **Geisbrecht, B. V., D. Zhu, K. Schulz, K. Nau, J. C. Morrell, M. Geraghty, H. Schulz, R. Erdmann, and S. J. Gould.** 1998. Molecular characterization of *Saccharomyces cerevisiae* D^3,D^2-enoyl-CoA isomerase. J. Biol. Chem. **273:** 33184–33191.
14. **Girzalsky, W., P. Rehling, K. Stein, J. Kipper, L. Blank, W. H. Kunau, and R. Erdmann.** 1999. Involvement of Pex13p in Pex14p localization and peroxisomal targeting signal 2-dependent protein import into peroxisomes. J. Cell Biol. **144:**1151–1162.
15. **Gould, S. J., J. E. Kalish, J. C. Morrell, J. Bjorkman, A. J. Urquhart, and D. I. Crane.** 1996. PEX13p is an SH3 protein in the peroxisome membrane and a docking factor for the PTS1 receptor. J. Cell Biol. **135:**85–95.
16. **Gould, S. J., G. A. Keller, N. Hosken, J. Wilkinson, and S. Subramani.** 1989. A conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. **108:**1657–1664.
17. **Gould, S. J., D. McCollum, A. P. Spong, J. A. Heyman, and S. Subramani.** 1992. Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast **8:**613–628.
18. **Grant, S. G., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
19. **Hettema, E. H., W. Girzalsky, M. van Den Berg, R. Erdmann, and B. Distel.** 2000. Saccharomyces cerevisiae pex3p and pex19p are required for proper localization and stability of peroxisomal membrane proteins. EMBO J. **19:** 223–233.
20. **Heyman, J. A., E. Mononsov, and S. Subramani.** 1994. Role of the *PAS1* gene of *Pichia pastoris* in peroxisome biogenesis. J. Cell Biol. **127:**1259–1273.
21. **Höhfeld, J., M. Veenhuis, and W. H. Kunau.** 1991. PAS3, a *Saccharomyces cerevisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. J. Cell Biol. **114:**1167–1178.
22. **Huhse, B., P. Rehling, M. Albertini, L. Blank, K. Meller, and W. H. Kunau.** 1998. Pex17p of Saccharomyces cerevisiae is a novel peroxin and component of the peroxisomal protein translocation machinery. J. Cell Biol. **140:**49–60.
23. **Kalish, J. E., C. Theda, J. C. Morrell, J. M. Berg, and S. J. Gould.** 1995. Formation of the peroxisome lumen is abolished by loss of *Pichia pastoris* Pas7p, a zinc-binding integral membrane protein of the peroxisome. Mol. Cell. Biol. **15:**6406–6419.

23a. **Kalish, J. E., G. A. Keller, J. C. Morrell, S. J. Mihalik, B. Smith, J. M. Cregg, and S. J. Gould.** 1996. Characterization of a novel component of the peroxisomal protein import apparatus using fluorescent peroxisomal proteins. EMBO J. **15:**3275–3285.

24. **Kiel, J. A., R. E. Hilbrands, I. J. van der Klei, S. W. Rasmussen, F. A. Salomons, M. van der Heide, K. N. Faber, J. M. Cregg, and M. Veenhuis.** 1999. Hansenula polymorpha Pex1p and Pex6p are peroxisome-associated AAA proteins that functionally and physically interact. Yeast **15:**1059–1078.
25. **Koller, A., W. B. Snyder, K. N. Faber, T. J. Wenzel, L. Rangell, G. A. Keller, and S. Subramani.** 1999. Pex22p of Pichia pastoris, essential for peroxisomal matrix protein import, anchors the ubiquitin-conjugating enzyme, Pex4p, on the peroxisomal membrane. J. Cell Biol. **146:**99–112.
26. **Lazarow, P. B., and Y. Fujiki.** 1985. Biogenesis of peroxisomes. Annu. Rev. Cell Biol. **1:**489–530.
27. **Marzioch, M., R. Erdmann, M. Veenhuis, and W.-H. Kunau.** 1994. PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. EMBO J. **13:**4908–4918.
28. **Matsuzono, Y., N. Kinoshita, S. Tamura, N. Shimozawa, M. Hamasaki, K. Ghaedi, R. J. Wanders, Y. Suzuki, N. Kondo, and Y. Fujiki.** 1999. Human PEX19: cDNA cloning by functional complementation, mutation analysis in a patient with Zellweger syndrome, and potential role in peroxisomal membrane assembly. Proc. Natl. Acad. Sci. USA **96:**2116–2121.
29. **McCollum, D., E. Monosov, and S. Subramani.** 1993. The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells. The PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal and is a member of the TPR protein family. J. Cell Biol. **121:**761–774.
30. **Neuwald, A. F., L. Aravind, J. L. Spouge, and E. U. Koonin.** 1999. AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. **9:**27–43.
31. **Rehling, P., M. Marzioch, E. Wittke, M. Veenhuis, and W. H. Kunau.** 1996. The import receptor for the peroxisomal targeting signal 2 (PTS2) in Saccharomyces cerevisiae is encoded by the PAS7 gene. EMBO J. **15:**2901–2913.
32. **Rehling, P., A. Skaletz-Rorowski, W. Girzalsky, T. Voorn-Brouwer, M. M. Franse, B. Distel, M. Veenhuis, W. H. Kunau, and R. Erdmann.** 2000. Pex8p, an intraperoxisomal peroxin of Saccharomyces cerevisiae required for protein transport into peroxisomes, binds the PTS1 receptor pex5p. J. Biol. Chem. **275:**3593–3602.
33. **Reuber, B. E., E. Germain Lee, C. S. Collins, J. C. Morrell, R. Ameritunga, H. W. Moser, D. Valle, and S. J. Gould.** 1997. Mutations in PEX1 are the most common cause of the peroxisome biogenesis disorders. Nat. Genet. **17:** 445–448.
34. **Sacksteder, K. A., J. M. Jones, S. T. South, X. Li, Y. Liu, and S. J. Gould.** 2000. PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. J. Cell Biol. **148:**931–944.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **Slawecki, M., G. Dodt, S. Steinberg, A. B. Moser, H. W. Moser, and S. J. Gould.** 1995. Identification of three distinct peroxisomal protein import defects in patients with peroxisomal biogenesis disorders. J. Cell Sci. **108:** 1817–1829.
37. **Snyder, W. B., A. Koller, A. J. Choy, M. A. Johnson, J. M. Cregg, L. Rangell, G. A. Keller, and S. Subramani.** 1999. Pex17p is required for import of both peroxisome membrane and lumenal proteins and interacts with Pex19p and the peroxisome targeting signal-receptor docking complex in Pichia pastoris. Mol. Biol. Cell **10:**4005–4019.
38. **South, S. T., K. A. Sacksteder, X. Li, Y. Liu, and S. J. Gould.** 2000. Inhibitors of COPI and COPII do not block PEX3-mediated peroxisome synthesis. J. Cell Biol. **149:**1345–1360.

39. **Spong, A. P., and S. Subramani.** 1993. Cloning and characterization of *PAS5*: a gene required for peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris*. J. Cell Biol. **123:**535–548.
40. **Subramani, S.** 1993. Protein import into peroxisomes and biogenesis of the organelle. Annu. Rev. Cell Biol. **9:**445–478.
41. **Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski, and S. Subramani.** 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. EMBO J. **10:**3244–3262.
42. **Tabak, H. F., I. Braakman, and B. Distel.** 1999. Peroxisomes: simple function but complex in maintenance. Trends Cell Biol. **9:**447–453.
43. **Titorenko, V. I., H. Chan, and R. A. Rachubinski.** 2000. Fusion of small peroxisomal vesicles in vitro reconstructs an early step in the in vivo multistep peroxisome assembly pathway of Yarrowia lipolytica. J. Cell Biol. **148:** 29–44.
44. **van der Klei, I. J., R. E. Hibrands, J. A. Kiel, S. W. Rasmussen, J. M. Cregg, and M. Veenhuis.** 1998. The ubiquitin-conjugating enzyme Pex4p of Hansenula polymorpha is required for efficient functioning of the PTS1 import machinery. EMBO J. **17:**3608–3618.
45. **van der Klei, I. J., R. E. Hibrands, G. J. Swaving, H. R. Waterham, E. G. Vrieling, V. I. Titorenko, J. M. Cregg, W. Harder, and M. Veenhuis.** 1995. The *Hansenula polymorpha PER3* gene is essential for the import of PTS1 proteins into the peroxisome matrix. J. Biol. Chem. **270:**17229–17236.
46. **Waterham, H. R., V. I. Titorenko, G. J. Swaving, W. Harder, and M. Veenhuis.** 1993. Peroxisomes in the methyltrophic yeast *Hansenula polymorpha* do not necessarily derive from pre-existing organelles. EMBO J. **12:**4785–4794.
47. **Wiebel, F. F., and W.-H. Kunau.** 1992. The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. Nature **359:** 73–76.
48. **Wiemer, E. A. C., G. H. Luers, K. N. Faber, T. Wenzel, M. Veenhuis, and S. Subramani.** 1996. Isolation and characterization of Pas2p, a peroxisomal membrane protein essential for peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris*. J. Biol. Chem. **271:**18973–18980.
49. **Yahraus, T., N. Braverman, G. Dodt, J. E. Kalish, J. C. Morrell, H. W. Moser, D. Valle, and S. J. Gould.** 1996. The peroxisome biogenesis disorder group 4 gene, *PXAAA1*, encodes a cytoplasmic ATPase required for stability of the PTS1 receptor. EMBO J. **15:**2914–2923.