

Dynamics of the peroxisomal import cycle of PpPex20p: ubiquitin-dependent localization and regulation

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We characterize the peroxin PpPex20p from *Pichia pastoris* and show its requirement for translocation of PTS2 cargoes into peroxisomes. PpPex20p docks at the peroxisomal membrane and translocates into peroxisomes. Its peroxisomal localization requires the docking peroxin Pex14p but not the peroxins Pex2p, Pex10p, and Pex12p, whose absence causes peroxisomal accumulation of Pex20p. Similarities between Pex5p and Pex20p were noted in their protein interactions and dynamics during import, and both contain a conserved NH₂-terminal domain. In the absence of

the E2-like Pex4p or the AAA proteins Pex1p and Pex6p, Pex20p is degraded via polyubiquitylation of residue K19, and the K19R mutation causes accumulation of Pex20p in peroxisome remnants. Finally, either interference with K48-branched polyubiquitylation or removal of the conserved NH₂-terminal domain causes accumulation of Pex20p in peroxisomes, mimicking a defect in its recycling to the cytosol. Our data are consistent with a model in which Pex20p enters peroxisomes and recycles back to the cytosol in an ubiquitin-dependent manner.

Introduction

Peroxisome biogenesis is a complex process involving >20 conserved peroxins (Titorenko and Rachubinski, 2001). Improper assembly of peroxisomes results in metabolic defects, such as the inability to perform fatty-acid oxidation, impairment in development, lethality in plants and mammals, and severe diseases in humans (Wanders, 2004).

Import of matrix proteins (cargoes) occurs by two pathways, depending on the type of peroxisomal targeting signal (PTS) present on the cargo (Subramani, 1998). Most cargoes are targeted by a COOH-terminal tripeptide, the PTS1. An unrelated signal, the PTS2, is an NH₂-terminal nonapeptide with a loose consensus sequence used by a smaller subset of proteins including the β -oxidation enzyme β -ketoacyl CoA thiolase (Fox3p) in yeasts (Petřiv et al., 2004). Targeting of PTS1 and PTS2 proteins to peroxisomes requires binding to soluble receptors, Pex5p and

Pex7p, respectively, in the cytosol. Evidence supports an “extended shuttle” mechanism, where the soluble receptors are translocated together with the cargo and then recycled back to the cytosol after cargo unloading in the peroxisomal lumen (Dammai and Subramani, 2001; Nair et al., 2004).

After receptor–signal interaction in the cytosol, both pathways converge by binding to the “docking complex” at the peroxisomal membrane (Pex13p, Pex14p, and Pex17p). E3-like peroxins (Pex2p, Pex10p, and Pex12p) containing really interesting new gene (RING) domains are also necessary for cargo import (Chang et al., 1999). Two AAA ATPases (Pex1p and Pex6p) and, in lower eukaryotes, an E2-like protein (Pex4p) are required for later steps of import (van der Klei et al., 1998; Collins et al., 2000). Finally, in lower eukaryotes, an intraperoxisomal peroxin (Pex8p) was proposed to bridge the docking and the RING subcomplexes in a larger structure, the importomer (Agne et al., 2003).

In higher eukaryotes, targeting of PTS2 proteins by Pex7p requires the long isoform of the PTS1 receptor Pex5L (Braverman et al., 1998; Matsumura et al., 2000; Otera et al., 2000). In yeasts and fungi, PTS2 import does not involve Pex5p but requires other PTS2 auxiliary proteins. *Saccharomyces cerevisiae* possesses redundant auxiliary proteins (Pex18p and

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Abbreviations used in this paper: 3-AT, 3-aminotriazole; BFP, blue fluorescent protein; DIC, differential interference contrast; mPTS, membrane PTS; mRFP, monomeric red fluorescent protein; PNS, postnuclear supernatant; PTS, peroxisomal targeting signal; RADAR, receptor accumulation and degradation in the absence of recycling; RING, really interesting new gene; UPS, ubiquitin–proteasome system.

The online version of this article contains supplemental material.

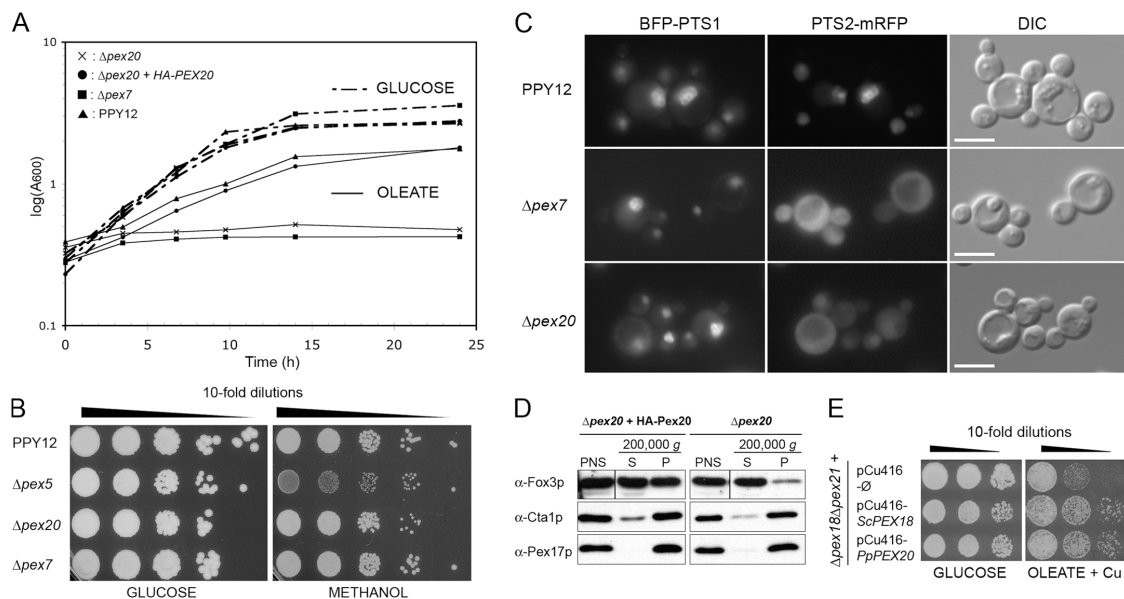


Figure 1. Involvement of PpPex20p in peroxisome biogenesis and import of PTS2 cargo. (A) Growth of $\Delta pex20$, PPY12 (wild type), $\Delta pex7$, or $\Delta pex20 + HA-Pex20$ strains in glucose (dashed lines) or oleate (solid lines) liquid media. (B) Growth of serial dilutions of PPY12 (wild type), $\Delta pex5$, $\Delta pex20$, or $\Delta pex7$ cultures ($1 A_{600}$ U/ml) on methanol or glucose (control) plates. (C) Fluorescence and differential interference contrast (DIC) microscopy pictures of methanol-grown PPY12 (wild type), $\Delta pex7$, or $\Delta pex20$ strains expressing BFP-PTS1 and PTS2-mRFP. Bars, 5 μm . (D) Differential centrifugation samples of oleate-grown $\Delta pex20 + HA-Pex20$ or $\Delta pex20$ cells (S200, supernatant; P200, pellet) were immunoblotted for the indicated antibodies. (E) Heterologous complementation of *S. cerevisiae* $\Delta pex18 \Delta pex21$ by PpPEX20. Cells were transformed with pCu416 containing no insert (\emptyset), ScPEX18, or PpPEX20, and serial dilutions of cultures ($1 A_{600}$ U/ml) were spotted on control (glucose) or oleate medium containing copper.

Pex21p; Purdue et al., 1998), but other organisms (*Yarrowia lipolytica*, *Neurospora crassa*, and *Hansenula polymorpha*; Titorenko et al., 1998; Sichtung et al., 2003; Otzen et al., 2005) contain only one, named Pex20p. These auxiliary proteins are not involved in PTS1 import but share with mammalian Pex5L a common motif for interaction with Pex7p, suggesting a replacement of these auxiliary proteins in higher organisms by Pex5L through domain swapping (Dodt et al., 2001; Einwächter et al., 2001).

The necessity and the equivalence of these auxiliary proteins in PTS2 protein targeting to peroxisomes is poorly understood and varies among organisms. *Y. lipolytica* Pex20p interacts directly with thiolase in a PTS2-independent fashion and helps in its oligomerization before translocation (Titorenko et al., 1998), whereas *H. polymorpha* Pex20p binds PTS2 sequences but does not assist thiolase oligomerization (Otzen et al., 2005). None of these interactions is observed for the *S. cerevisiae* homologues Pex18p and Pex21p (Stein et al., 2002). In addition, there are conflicting reports concerning the ability of the latter to dock at the peroxisomal membrane. Finally, in view of the ability of Pex7p to enter peroxisomes, it is unclear whether the auxiliary peroxins are translocated during the import process. Overall, both the function and the properties of Pex20p-like proteins required further study.

We functionally characterized Pex20p from *Pichia pastoris* and studied its subcellular localization and the regulation of its dynamics. Our results suggest that Pex20p behaves as a cycling peroxin. We propose a model for the dynamics of Pex20p during its import cycle involving a ubiquitin-dependent recycling mechanism.

Results

Identification of PpPex20p and cloning of the PEX20 gene

Putative PTS2 auxiliary peroxins of *P. pastoris* were investigated using a functional Pex7p-tandem affinity purification (TAP) construct. Pex7p-TAP was purified from oleate-grown cells after treatment of the extract with 0.5% digitonin. Mass spectrometry on the purified fraction and comparison of the data to the draft genome sequence of *P. pastoris* from Integrated Genomics revealed several proteins. These included the PTS2 protein Fox3p, the docking peroxin Pex14p, and a protein encoded by the ORF RPPA09328 (16% of sequence covered), with 25% overall identity to *Y. lipolytica* Pex20p (Titorenko et al., 1998). Cloning and sequencing of the gene showed a 969-nt ORF encoding a predicted protein of 323 residues (available from GenBank/EMBL/DDJB under accession no. AY768943).

Alignments of the predicted protein with Pex20p from other species revealed several conserved motifs (Fig. S1, available from <http://www.jcb.org/cgi/contents/full/jcb.200508096/DC1>), including the putative Pex7p interaction domain found in other Pex20p-like proteins and in the long isoform of human PTS1 receptor (Pex5L), and three diaromatic pentapeptide motifs: Wxxx(F/Y) (Dodt et al., 2001; Einwächter et al., 2001). Based on these homologies and the data in Figs. 1–3, the RPPA09328 gene is referred to as PEX20 and its product as Pex20p.

Characterization of $\Delta pex20$

The involvement of Pex20p in peroxisome biogenesis was analyzed by gene deletion. Although the $\Delta pex20$ strain grew at the

wild-type level on glucose, it failed to do so on oleate (Fig. 1 A), suggesting its involvement in peroxisome biogenesis. This also indicates that no other genes of redundant function with *PEX20* exist in *P. pastoris*. Growth of *P. pastoris* in methanol medium also requires functional peroxisomes; however, because the β -oxidation enzyme Fox3p is the only known PTS2 cargo in *P. pastoris* and is not required for methanol degradation, the PTS2 receptor mutant $\Delta pex7$ grows in methanol (Elgersma et al., 1998). Growth of $\Delta pex20$, like that of $\Delta pex7$ cells, was unaltered on methanol (Fig. 1 B), indicating that Pex20p is dispensable for import of PTS1 cargoes.

The ability of the $\Delta pex20$ strain to import proteins into peroxisomes was monitored by fluorescence microscopy. The blue fluorescent protein (BFP) fused to a COOH-terminal PTS1 and a PTS2-targeted monomeric red fluorescent protein (mRFP) served as markers for each pathway. $\Delta pex20$ cells displayed a defect in PTS2 protein import because PTS2-mRFP remained cytosolic, whereas targeting of BFP-PTS1 to peroxisomes was not affected (Fig. 1 C). In agreement with data presented later indicating lack of direct interaction between Pex20p and thiolase, we suggest that Pex20p has a general role in peroxisomal import of PTS2-containing proteins, including but not limited to thiolase.

After subcellular fractionation of a postnuclear supernatant (PNS), only a small amount of Fox3p was found in the organelle pellet (P200) of $\Delta pex20$ cells (Fig. 1 D), whereas it was more abundant in the P200 of the mutant strain complemented with a tagged version of Pex20p ($\Delta pex20$ + HA-Pex20p). The subcellular location of the PTS1 cargo catalase was not affected. Neither overexpression of Pex20p in $\Delta pex7$, nor of Pex7p in $\Delta pex20$, restored growth in oleate (unpublished data), suggesting that these peroxins, although involved in the same pathway, have nonoverlapping functions.

Finally, growth of the *S. cerevisiae* $\Delta pex18 \Delta pex21$ mutant on oleate was complemented by PpPex20p to the same extent as it was by the endogenous ScPex18p (Fig. 1 E). Therefore, Pex20p functionally substitutes for the PTS2 auxiliary proteins Pex18p and Pex21p. Collectively, these data demonstrate that Pex20p is the PTS2 auxiliary protein from *P. pastoris*.

Pex20p interacts with Pex7p and peroxins of the docking subcomplex

Because most interactions of Pex20p-like proteins were studied in artificial heterologous systems (Einwächter et al., 2001; Sichtung et al., 2003), we investigated these in a homologous context. HA-Pex7p coimmunoprecipitated with some Pex20p (Fig. 2 A). The amount of Pex20p interacting with Pex7p in wild-type cells was lower than that in $\Delta pex14$ cells (Fig. 2 A), indicating that the Pex20p–Pex7p complex accumulates when docking is prevented. However, because PTS2 cargoes are also cytosolic in this docking mutant, this observation may reflect a role of the cargo in stabilizing the Pex20p–Pex7p interaction.

Interactions of Pex20p with various proteins were examined by coimmunoprecipitation using an NH₂-terminal 3xHA-tagged version of Pex20p that complemented the $\Delta pex20$ strain on oleate. Coimmunoprecipitation of thiolase with HA-Pex20p was dependent on the presence of Pex7p (Fig. 2 B), suggesting

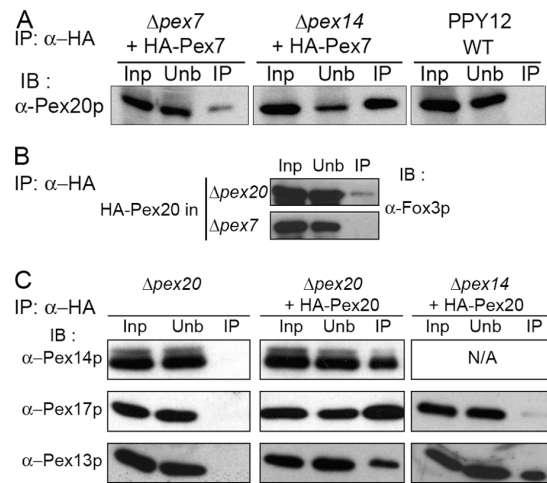


Figure 2. In vivo interaction of Pex20p with known peroxins and Fox3p. Immunoprecipitations (IP) were performed with the HA antibody (α -HA) on the indicated lysates and were immunoblotted (IB) with the indicated antibody. (A) Lysates of $\Delta pex7$ and $\Delta pex14$ cells expressing HA-Pex7p or of wild-type (WT; PPY12) cells. (B) Lysates of $\Delta pex20$ or $\Delta pex7$ cells expressing HA-Pex20p. (C) Lysates from $\Delta pex20$ cells or from HA-Pex20p-expressing $\Delta pex20$ and $\Delta pex14$ cells.

that Pex7p mediates the Pex20p–thiolase interaction. In this respect, PpPex20p behaves like ScPex18p, which had no direct interaction with thiolase (Stein et al., 2002). The amount of thiolase recovered in the immunoprecipitate was higher in the absence of Pex14p (unpublished data), presumably because the Pex20p–Pex7p–thiolase complex accumulates in the cytosol in the absence of docking to peroxisomes.

We then investigated potential interactions with members of the docking complex, as the ability of Pex20p-like proteins to dock to peroxisomes is poorly documented. HA-Pex20p coimmunoprecipitated with members of the docking complex (Pex13p, Pex14p, and Pex17p; Fig. 2 C). The interaction was particularly strong with Pex14p and Pex17p. In $\Delta pex14$ cells, Pex17p did not interact with HA-Pex20p (Fig. 2 C), showing that the Pex20p–Pex17p interaction observed was mediated by Pex14p. Interestingly, the interaction of HA-Pex20p with Pex13p was unaffected by the absence of Pex14p (Fig. 2 C).

The interactions identified in this paper demonstrate that Pex20p is found in the cytosol as a complex with Pex7p and thiolase, with Pex20p interacting indirectly with thiolase through Pex7p. Also, *P. pastoris* Pex20p docks at the peroxisomal membrane through independent interactions with Pex13p and Pex14p.

As judged by the yeast two-hybrid technique, Pex7p interacted with the full-length Pex20p construct (Fig. 3 A) as well as with its COOH-terminal half (residues 146–323), predicted to contain the Pex7p binding site (Fig. S1). Mutation of the conserved Ser residue present within this region (S280F) disrupted the interaction with Pex7p (Fig. 3 A) as described earlier (Matsumura et al., 2000; Dodt et al., 2001; Einwächter et al., 2001). Further truncations (constructs spanning either aa 146–260 or 260–323) abolished the interaction (unpublished data), suggesting that residues 276–296 are insufficient for Pex7p binding.

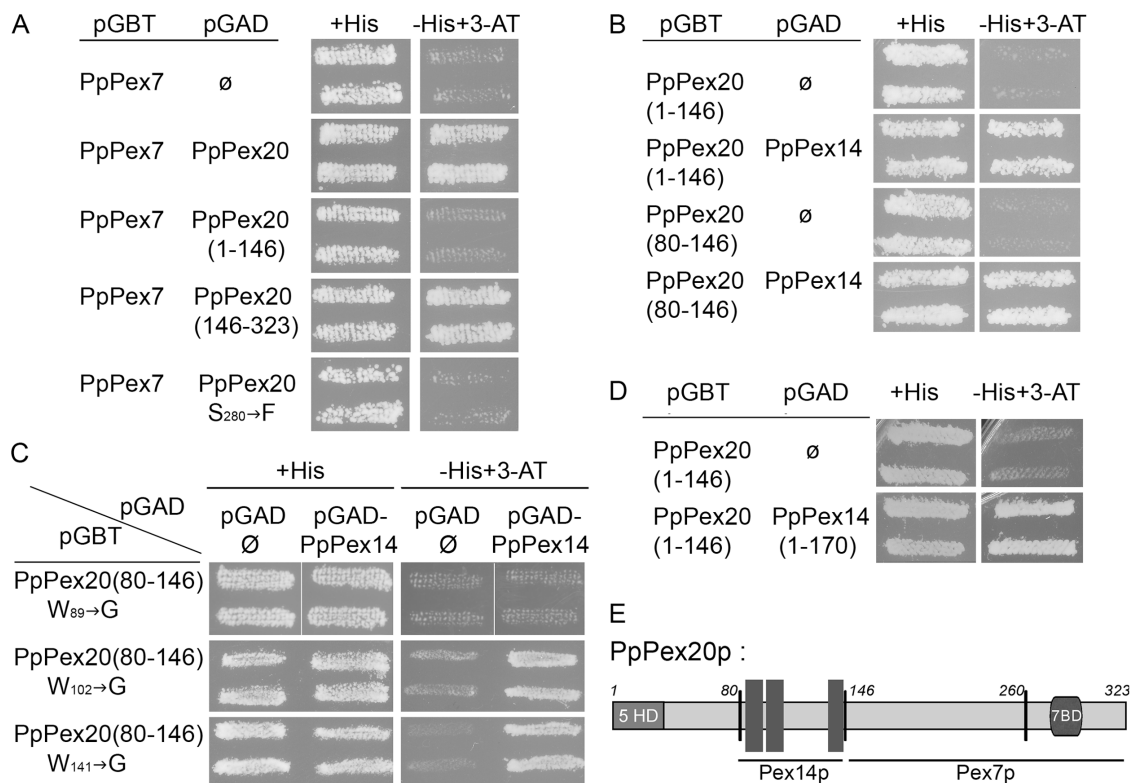


Figure 3. **Mapping of interaction domains of Pex20p with other peroxins by yeast two hybrid.** Interactions were determined by growth on medium lacking histidine supplemented with 50 mM 3-AT. Cells were cotransformed with the indicated vectors to map the following interactions: Pex7p binding domains of Pex20p (A); Pex14p binding domains of Pex20p (B); ability of point mutants of Pex20p (W89G, W102G, or W141G) to bind Pex14p (C); and Pex20p binding domain of Pex14p (D). (E) Summary of the interaction domains within Pex20p. 5HD, Pex5p homology domain (Fig. 7); 7BD, Pex7p binding domain (Einwächter et al., 2001; Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200508096/DC1>). The dark rectangles represent Wxxx(F/Y) motifs.

Although none of the Pex13p constructs were suitable for yeast two hybrid (unpublished data), Pex14p interacted with Pex20p(1–146) and Pex20p(80–146) (Fig. 3 B). Interaction between Pex20p and Pex14p has been described, but this was bridged by Pex7p (Stein et al., 2002; Sichtung et al., 2003). This is not the case here because Pex14p and Pex7p interact through different regions of Pex20p.

The diaromatic pentapeptide (Wxxx[F/Y]) repeats present in Pex5p from various organisms bind to Pex14p and Pex13p (Schliebs et al., 1999; Otera et al., 2002). The Pex14p-interacting region (aa 1–146 of PpPex20p) contains three such motifs (aa 89–93, 102–106, and 141–145). Site-directed mutagenesis was performed on each of these sites (W89G, W102G, and W141G). The construct Pex20p(80–146; W89G) failed to interact with Pex14p, whereas Pex20p(80–146; W102G) and Pex20p(80–146; W141G) still interacted with Pex14p (Fig. 3 C). Therefore, only the first Wxxx(F/Y) motif of PpPex20p is crucial for Pex14p interaction, with the other motifs being either not involved or redundant. Only the NH₂-terminal fragment (aa 1–170) of Pex14p interacted with Pex20p (Fig. 3 D). Interestingly, this region is involved in Pex5p binding (Schliebs et al., 1999), suggesting that the same region of Pex14p interacts with Wxxx(F/Y)-containing proteins, such as Pex5p or Pex20p.

Finally, although Pex7p interacted with Fox3p, there was no interaction between Pex20p and Fox3p (unpublished data). Although interactions between *S. cerevisiae* peroxins Pex18p and

Pex21p and Fox3p are mediated by Pex7p in the yeast two-hybrid system (Purdue et al., 1998; Stein et al., 2002), Pex20p interacts with Fox3p in *Y. lipolytica* (Titorenko et al., 1998; Smith and Rachubinski, 2001) and presumably in *H. polymorpha* (Otzen et al., 2005). As indicated in Fig. 2 B, thiolase was coimmunoprecipitated with HA-Pex20p but only in the presence of Pex7p.

The interaction data (Fig. 3 E) indicate a strong resemblance between Pex20p and Pex5p interaction maps because both proteins interact with Pex14p through the same motifs. Furthermore, the domain involved in Pex7p binding possesses strong similarities with that of human Pex5L.

Pex20p functions in thiolase translocation into peroxisomes, but Pex7p translocates independently

Pex20p and Pex7p functions appear to be tightly linked, but the precise function of Pex20p in PTS2 import is still unclear. We assessed its role in the various steps leading to thiolase import into the peroxisome, namely receptor–cargo binding, receptor docking to the peroxisomal membrane, and receptor–cargo translocation. Thiolase coimmunoprecipitated with HA-Pex7p independently of Pex20p (Fig. 4 A), indicating that Pex20p is not essential for Pex7p–thiolase interactions.

From the aforementioned data (Figs. 2 and 3) showing interactions of Pex20p with members of the docking complex and work on Pex18p (Stein et al., 2002), we assumed that Pex20p

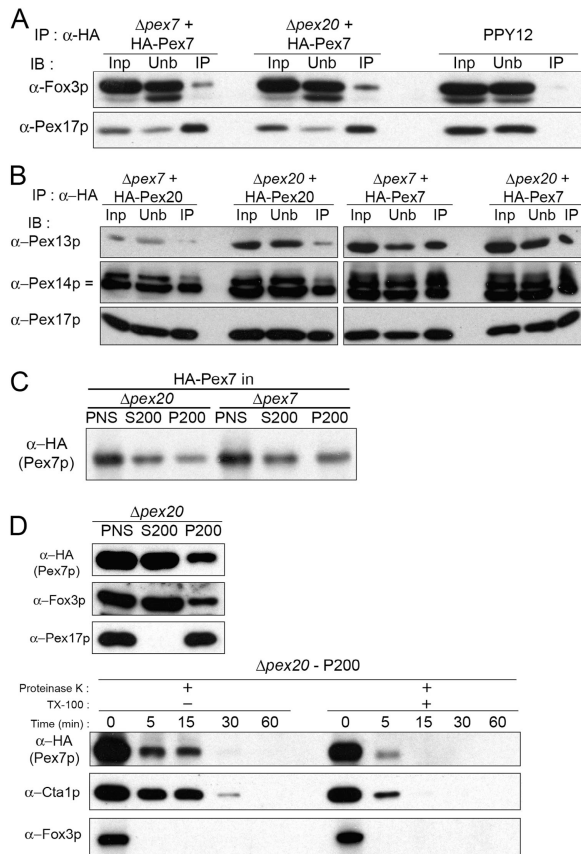


Figure 4. Functional analysis of Pex20p. (A) Lysates from $\Delta pex7$ or $\Delta pex20$ cells expressing HA-Pex7p were immunoprecipitated with the HA antibody, and samples were immunoblotted for thiolase (Fox3p) and Pex17p. IP lane represents 2 OD equivalents. (B) Lysates from $\Delta pex7$ or $\Delta pex20$ cells expressing HA-Pex7p or HA-Pex20p were immunoprecipitated with the HA antibody, and samples were immunoblotted for members of the docking complex (Pex13p, Pex14p, and Pex17p). (C) Differential centrifugation fractions of HA-Pex7p-expressing $\Delta pex7$ or $\Delta pex20$ cells were immunoblotted with the HA antibody. (D) Differential centrifugation fractions of HA-Pex7p-expressing $\Delta pex20$ strain and protease protection analysis of the P200 fraction were immunoblotted with the indicated antibodies.

might help Pex7p in its docking to peroxisomes. Instead, we observed that Pex13p, Pex14p, and Pex17p coimmunoprecipitated with HA-Pex7p, regardless of the presence of Pex20p (Fig. 4 B). A similar conclusion was made in *S. cerevisiae* where Pex7p still interacted with the docking peroxins even in the absence of Pex18p or Pex21p (Stein et al., 2002). However, contrary to a previous study (Stein et al., 2002), the interaction between Pex20p and the docking subcomplex was independent of Pex7p (Fig. 4 B). In addition, the subcellular distribution of Pex7p between the cytosol and the organelle pellet did not change drastically in the absence of Pex20p (Fig. 4 C). Conversely, the distribution of Pex20p in the supernatant and pellet fractions was not altered significantly by the presence or absence of Pex7p (unpublished data). Collectively, these data show for the first time that association of Pex7p or Pex20p with the peroxisomal docking subcomplex can be independent of the other and that each protein does not significantly affect the peroxisomal localization of the other.

Surprisingly, the protease protection assay performed on the P200 fraction from $\Delta pex20$ cells showed that Pex7p was

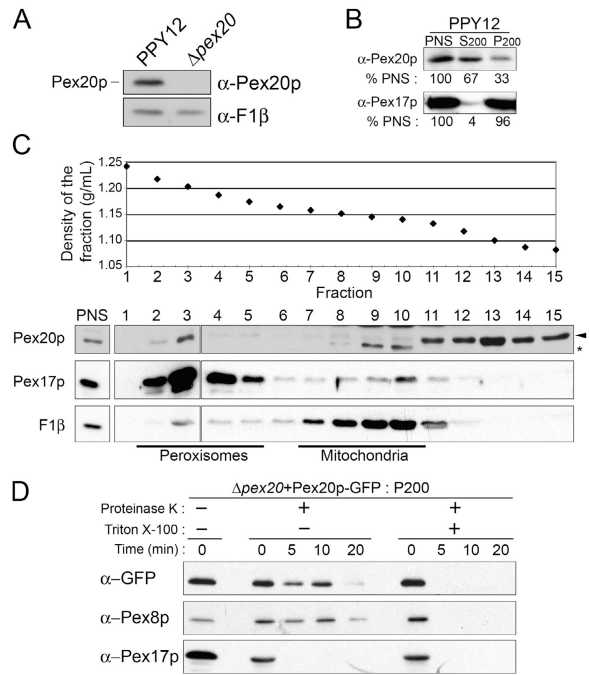


Figure 5. Subcellular localization of PpPex20p. (A) Specificity of the Pex20p antibody against crude extracts of oleate-grown PPY12 (wild type) and $\Delta pex20$ cells. (B) Differential centrifugation fractions of oleate-grown wild-type (PPY12) cells were immunoblotted with anti-PpPex20p or anti-Pex17p. (C) Histodenz density gradient analysis of a PNS from oleate-grown PPY12 cells immunoblotted with antibodies against Pex20p (arrowhead), Pex17p (peroxisomal), and F1β (mitochondrial). Density of each fraction (top) was determined by refractometry. The asterisk indicates a cross-reacting band. (D) Protease protection of a P200 fraction isolated from a PNS of oleate-grown $\Delta pex20$ expressing Pex20p-GFP. The indicated proteins were detected by immunoblotting.

protease resistant, as was the PTS1 enzyme catalase (Fig. 4 D). This suggests that Pex7p translocates into peroxisomes independently of Pex20p, a feature that has not been described previously and whose physiological role is unknown. Our results contrast with those obtained in *S. cerevisiae* where Pex7p depends on Pex18p and Pex21p for its peroxisomal localization (Purdue et al., 1998).

Both $\Delta pex7$ and $\Delta pex20$ are deficient in thiolase import and unable to grow on oleate. We investigated whether the organelle-associated fraction of thiolase in $\Delta pex20$ cells (Fig. 2) reflects a low efficiency, or rather an absence, of import. As shown in Fig. 4 D, thiolase was protease sensitive. Therefore, although Pex7p was imported into the peroxisomes of $\Delta pex20$ cells, thiolase remained on the surface of peroxisomes. Because $\Delta pex7$ also fails to import thiolase into peroxisomes (Elgersma et al., 1998), we conclude that Pex20p functions in the translocation of the Pex7p-cargo binary complex, although Pex7p alone does not require Pex20p to go into peroxisomes.

Pex20p is mostly cytosolic and partially intraperoxisomal

A polyclonal antibody to Pex20p (Fig. 5 A) was used to study Pex20p distribution after cell fractionation. One third of the cellular Pex20p pool could be pelleted, whereas two thirds were cytosolic (Fig. 5 B). This membrane-associated Pex20p sedimented at

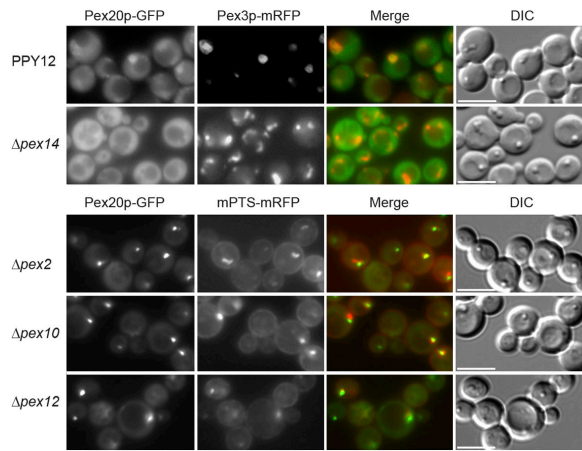


Figure 6. **Subcellular localization of Pex20p-GFP in selected *pex* mutants.** Fluorescence and DIC microscopy pictures of methanol-grown cells (wild-type, $\Delta pex14$, $\Delta pex2$, $\Delta pex10$, or $\Delta pex12$) expressing Pex20p-GFP and either Pex3p-mRFP or mPTS-mRFP as peroxisomal markers. Bars, 5 μ m.

the same density as peroxisomes in a density gradient (Fig. 5 C), whereas the rest of it was cytosolic and at the top of the gradient. A protease protection assay performed on the P200 fraction from $\Delta pex20$ + Pex20p-GFP cells showed that Pex20p-GFP behaved like the intraperoxisomal peroxin Pex8p, which is protected from external protease, unless detergent was added (Fig. 5 D). Therefore, Pex20p behaves as both a cytosolic and peroxisomal peroxin, similar to the cycling peroxins Pex5p and Pex7p (Dodt and Gould, 1996; Dammai and Subramani, 2001; Nair et al., 2004).

Peroxisomal localization of Pex20p requires Pex14p, but the RING peroxins are involved in the relocation of Pex20p to the cytosol

We established the requirements of Pex20p peroxisomal localization using a functional, COOH-terminal GFP-tagged version of Pex20p, driven by its own promoter. As shown in Fig. 6, much of the Pex20p-GFP was cytosolic in wild-type cells, although a signal was detected in structures that colocalized with a peroxisomal membrane marker (Pex3p-mRFP). Absence of Pex14p led to mislocalization of Pex20p-GFP to the cytosol (Fig. 6), suggesting that the presence of the docking complex is a prerequisite for the peroxisomal localization of Pex20p-GFP. Surprisingly, in any of the RING peroxin mutants ($\Delta pex2$, $\Delta pex10$, and $\Delta pex12$), Pex20p-GFP accumulated in a bright dot that colocalized with a marker containing a peroxisomal membrane PTS (mPTS)-mRFP (Fig. 6). These data strongly indicate that Pex20p-GFP does not require the RING complex to locate to peroxisomes; instead, it appears that the RING peroxins are involved in relocating Pex20p from the peroxisome to the cytosol.

A nonessential lysine of Pex20p, conserved also in Pex5p, is required for Pex20p degradation in receptor recycling mutants

While the localization of Pex20p was being addressed in mutants of the late steps of import (namely $\Delta pex1$, $\Delta pex6$, and $\Delta pex4$),

we were surprised to observe that no Pex20p was detectable in these strains (Fig. 7 A) as previously described for Pex5p (Koller et al., 1999; Collins et al., 2000). This down-regulation of Pex5p is conserved between *P. pastoris*, plants (Zolman and Bartel, 2004), and humans (Dodt and Gould, 1996; Yahraus et al., 1996), but the underlying mechanism is unknown. Other *pex* mutants contained amounts of Pex20p that were comparable to those of wild-type cells, although a decrease of Pex20p levels in the $\Delta pex13$ strain was noted, which remains unexplained. The low steady-state level of Pex20p in recycling mutants was not affected by further deletions affecting the vacuolar proteases Pep4p and Prb1p (unpublished data).

Therefore, Pex20p and Pex5p are regulated through similar mechanisms during the import cycle. To explain these common regulatory features, we hypothesized that sequence similarities in both proteins would confer a similar regulation. Alignment of Pex20p and Pex5p sequences revealed a conserved domain involving 25 out of 35 amino acids at their NH₂ termini (Fig. 7 B), including a conserved lysine residue. Because no function was assigned to this domain, we assessed its importance using truncated proteins. NH₂-terminal deletions of Pex20p were expressed in $\Delta pex20$ cells under the control of the endogenous promoter, and the ability to grow on oleate was checked. Deletion of the first 16 residues did not affect growth, but an effect was observed for further deletions ($\Delta 1-19$, $\Delta 1-22$, and $\Delta 1-31$; Fig. 7 C), although the proteins were expressed (not depicted). Mutation of the conserved lysine present in this domain (Pex20p-K19R and Pex5p-K22R) had no effect on the protein function (Fig. 7 D), suggesting that other residues within this domain may be essential or that the protein structure is affected when the whole region is missing.

To finally address whether the NH₂-terminal domain shared by Pex5p and Pex20p is the basis for the common down-regulation observed in late-steps mutants, we expressed Pex20p-K19R in the $\Delta pex20 \Delta pex4$ double mutant strain and observed that this mutation rendered the protein stable (Fig. 7 E), whereas Pex20p was undetectable in $\Delta pex4$ cells. Also, when Pex20p-GFP was expressed in $\Delta pex1$, $\Delta pex6$, or $\Delta pex4$, only small amounts of the fusion protein were detected, showing that Pex20p and Pex20p-GFP behave similarly. However, the steady-state level was comparable to that of the wild-type strain when Pex20(K19R)-GFP was expressed instead (Fig. 7 F). Therefore, K19 is essential for Pex20p down-regulation in $\Delta pex1$, $\Delta pex6$, or $\Delta pex4$ mutants.

In the recycling mutants of *S. cerevisiae*, ubiquitylated species of Pex5p are detected (Platta et al., 2004; Kiel et al., 2005; Kragt et al., 2005). We investigated whether the down-regulation of Pex20p observed in these mutants of *P. pastoris* results from an unusually fast degradation by the ubiquitin-proteasome system (UPS). Because a nearly complete absence of Pex20p is noticed after overnight induction in oleate (Fig. 7 A), we studied the steady-state level of Pex20p at earlier time points. Interestingly, after only 6 h of induction (Fig. 7 G), no change in Pex20p-GFP steady-state level was noted in the $\Delta pex4$ strain, but higher molecular mass bands were detected. Noticeably, these bands depended on the presence of the K19 residue (Fig. 7 G). These results define a new, essential NH₂-terminal

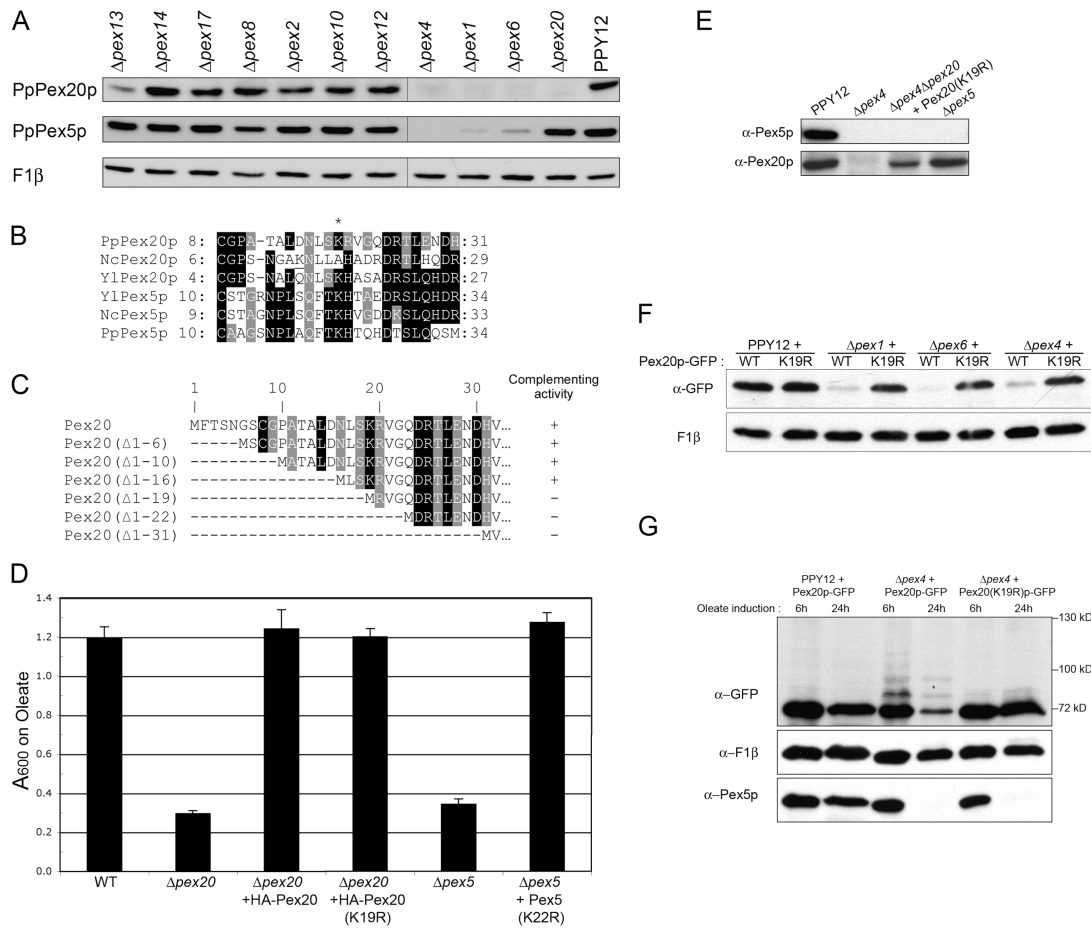


Figure 7. Pex20p steady-state levels in *pex* mutants and the role of the conserved K19 residue. (A) Immunoblotting of cell lysates from oleate-grown Δpex cells with the indicated antibodies. (B) Sequence alignment of Pex20p and Pex5p NH₂ termini from *P. pastoris* (Pp), *N. crassa* (Nc), and *Y. lipolytica* (Yl) showing the conserved lysine residue (indicated by asterisk). A lysine residue is present in NcPex20p (underlined), but its position is not conserved. (C) Truncated Pex20p were expressed from the endogenous *PEX20* promoter in the $\Delta pex20$ strain. Expression of the truncated protein was confirmed (not depicted) and tested for its ability to restore growth of the $\Delta pex20$ strain on oleate. (D) Growth of the indicated strains after overnight culture on oleate. (E) Cell lysates of oleate-grown wild-type (WT; PPY12), $\Delta pex4$, $\Delta pex4 \Delta pex20 + p_{PEX20}:PEX20(K19R)$, or $\Delta pex5$ immunoblotted for Pex5p or Pex20p. (F) Cell lysates of oleate-grown PPY12, $\Delta pex1$, $\Delta pex6$, and $\Delta pex4$ strains expressing either $p_{PEX20}:PEX20-GFP$ or $p_{PEX20}:PEX20(K19R)-GFP$ were immunoblotted with the indicated antibodies. (G) Cell lysates of PPY12 or $\Delta pex4$ cells expressing $p_{PEX20}:PEX20-GFP$ or $p_{PEX20}:PEX20(K19R)-GFP$ were prepared after 6 h of growth in oleate and immunoblotted with the indicated antibodies.

domain in Pex20p, which is conserved in Pex5p and whose conserved lysine is essential for Pex20p down-regulation in recycling mutants, likely via the UPS.

Overexpression of Ub (K48R) triggers polyubiquitylation of Pex20p-GFP and accumulation in peroxisome remnants

Because K48-branched polyubiquitylation of a protein acts as a signal for its degradation by the UPS, we investigated whether constitutive overexpression of the ubiquitin mutant, Ub (K48R), in wild-type cells would affect Pex20p regulation. First, we observed that it affected the ability of the strain to grow on oleate medium but not on glucose medium (Fig. 8 A). This suggests that polyubiquitylation is essential for peroxisome biogenesis, perhaps by interference with the action of Pex4p.

Surprisingly, we also observed higher molecular mass species of Pex20p in crude extracts from this strain, whose presence was dependent on the K19 residue (Fig. 8 B), mim-

icking the situation obtained in $\Delta pex4$ cells in the early stages of induction (Fig. 7 G). We assessed whether these species were polyubiquitylated forms of Pex20p. Denatured extracts of wild-type cells coexpressing Pex20p-GFP and myc-tagged Ub(K48R) were immunoprecipitated with a monoclonal anti-GFP antibody. This allowed recovery of Pex20p but also of higher molecular mass species that were also immunodecorated with the anti-myc antibody, indicating they are truly ubiquitylated forms of the protein (Fig. 8 C). Also, omission of the proteasome inhibitor MG-132 and the isopeptidase inhibitor *N*-ethylmaleimide during sample preparation did not allow detection of these species (unpublished data), supporting the idea that these bands represent ubiquitin conjugates of Pex20p-GFP that would normally be degraded by proteasomes.

When we addressed the subcellular localization of polyubiquitylated species of Pex20p-GFP by differential centrifugation, they were found exclusively in the pellet (P200) fraction (Fig. 8 D). Overexpression of Ub(K48R) in wild-type cells led to a dramatic accumulation of Pex20p-GFP in the organelle

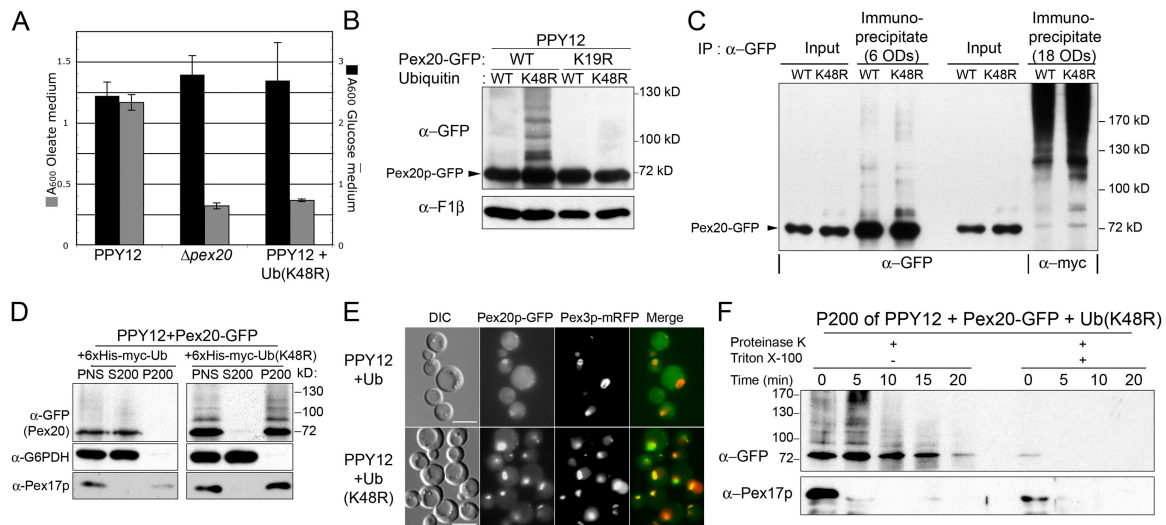


Figure 8. Effect of overexpression of ubiquitin K48R mutant on Pex20p localization. (A) Growth of PPY12, $\Delta pex20$, and PPY12 + $p_{GAP}::His_6\text{-myc-Ub(K48R)}$ strains on oleate (shaded bars) or glucose (filled bars) media ($n = 3$). (B) Crude extracts of oleate-grown PPY12 cells coexpressing either Pex20p-GFP or Pex20p(K19R)-GFP and either $p_{GAP}::His_6\text{-myc-Ub}$ or $p_{GAP}::His_6\text{-myc-Ub(K48R)}$ were immunoblotted with anti-GFP and anti-F1 β (loading control) antibodies. (C) Wild-type (WT) strain coexpressing Pex20p-GFP and either $p_{GAP}::His_6\text{-myc-Ub}$ or $p_{GAP}::His_6\text{-myc-Ub(K48R)}$ was lysed in 10% TCA and immunoprecipitated with anti-GFP antibody. Samples of the input and immunoprecipitate fractions were loaded and immunoblotted with anti-GFP or anti-myc monoclonal antibodies as indicated. (D) Differential centrifugation fractions of oleate-grown wild-type (PPY12) cells coexpressing Pex20p-GFP and either $p_{GAP}::His_6\text{-myc-Ub}$ or $p_{GAP}::His_6\text{-myc-Ub(K48R)}$ were immunoblotted with anti-GFP, anti-G6PDH (cytosolic marker), or anti-Pex17p (peroxisomal membrane marker) antibodies. (E) Fluorescence and DIC microscopy pictures of methanol-grown PPY12 + $p_{GAP}::His_6\text{-myc-Ub}$ or PPY12 + $p_{GAP}::His_6\text{-myc-Ub(K48R)}$ strains expressing Pex20p-GFP and Pex3p-mRFP as a peroxisomal marker. Bar, 5 μm . (F) A protease protection assay was performed on a P200 fraction isolated from oleate-grown PPY12 cells strain coexpressing Pex20p-GFP and $p_{GAP}::His_6\text{-myc-Ub(K48R)}$. Samples were immunoblotted with anti-Pex17p and anti-GFP antibodies.

pellet. This observation was confirmed by fluorescence microscopy experiments, where Pex20p-GFP colocalized with peroxisome remnants when Ub(K48R) was overexpressed (Fig. 8 E). At least part of the pelletable Pex20p was protected from external protease, unlike Pex17p (Fig. 8 F).

We conclude that protein polyubiquitylation is essential for peroxisome biogenesis. Alteration of polyubiquitylation with Ub(K48R) does not prevent peroxisomal import of Pex20p but instead causes it to accumulate in peroxisomes, mimicking a recycling defect.

Absence of recycling peroxins or deletion of residues 1–19 of Pex20p-GFP triggers peroxisomal accumulation of Pex20p-GFP

We exploited the apparent lack of down-regulation of Pex20p(K19R) in recycling mutants (Fig. 7, E and F) to study its subcellular localization. Differential centrifugation analysis and fluorescence microscopy experiments showed a sharp increase in the amount of peroxisome-associated Pex20p(K19R)-GFP (Fig. 9, A and B) in these mutants as compared with wild type. Pex1p, Pex6p, and Pex4p are thus essential for the proper distribution of Pex20p between the organelles and the cytosol, analogous to their proposed role in recycling of Pex5p (van der Klei et al., 1998; Collins et al., 2000; Platta et al., 2005).

It was recently proposed that the NH₂ terminus of human Pex5p is required for its recycling to the cytosol (Costa-Rodrigues et al., 2004). We therefore investigated the effect of an NH₂-terminal truncation of Pex20p-GFP on its subcellular local-

ization. Pex20($\Delta 1-19$)p-GFP, the longest truncated construct that fails to complement (Fig. 7 C), accumulated nearly exclusively in the organelle pellet as determined by differential centrifugation (Fig. 9 C) and colocalized in fluorescence microscopy with Pex3-mRFP (Fig. 9 D). Deletion of this domain might abolish the function because the cytosolic redistribution of Pex20p is affected.

Discussion

Pex20p is involved in thiolase translocation

Our characterization of PpPex20p confirms the necessity of this class of proteins for Pex7p-mediated peroxisomal import of PTS2 cargoes because $\Delta pex20$ cells fail to grow on oleate and have a PTS2 import defect (Fig. 1). However, the few studies (Einwächter et al., 2001; Sichtung et al., 2003) on the Pex20p from other species were either limited or done in artificial heterologous systems. Our systematic studies of the location, interactions, and steps of thiolase import into peroxisomes reveal new insights regarding the role of PpPex20p (Fig. 4, A and D). Pex20p might stabilize a thiolase–Pex7p complex before import or, more likely, act as a chaperone to facilitate its translocation across the peroxisomal membrane. Interestingly, Pex7p was translocated into peroxisomes even in $\Delta pex20$ cells (Fig. 4 D). This raises the existence of futile cycles in which Pex7p could be translocated without cargo and is consistent with our previous conclusion that cargo-binding mutants of Pex7p were partially peroxisomal like wild-type Pex7p (Elgersma et al., 1998).

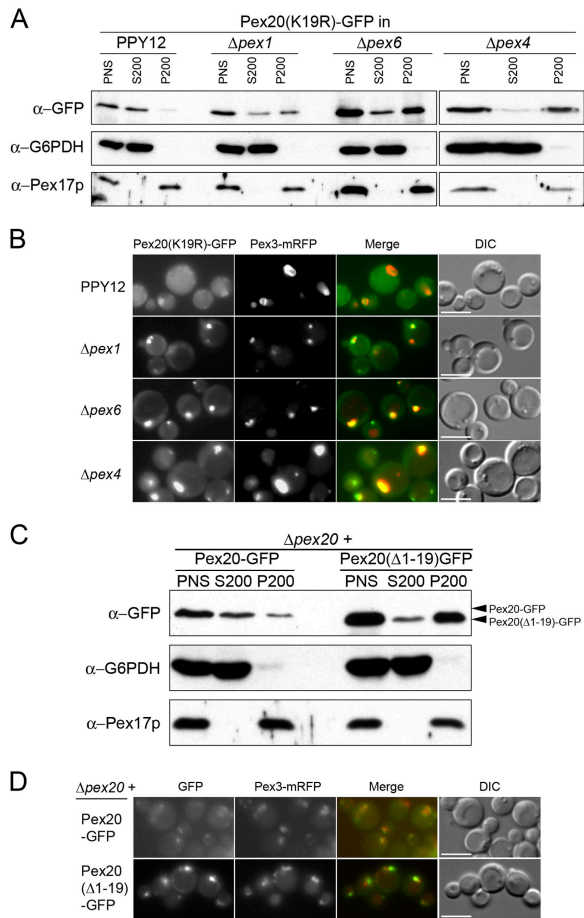


Figure 9. Role of Pex1p, Pex6p, and Pex4p in the subcellular localization of Pex20p-GFP and role of the NH₂-terminal domain of Pex20p-GFP. (A) Differential centrifugation fractions of oleate-grown wild-type (PPY12), $\Delta pex1$, $\Delta pex6$, or $\Delta pex4$ cells expressing Pex20p(K19R)-GFP were immunoblotted with the indicated antibodies. (B) Fluorescence and DIC microscopy pictures of methanol-grown cells (wild type, $\Delta pex1$, $\Delta pex4$, and $\Delta pex6$) coexpressing Pex20p(K19R)-GFP and Pex3p-mRFP as a peroxisomal marker. (C) Differential centrifugation fractions of oleate-grown $\Delta pex20$ cells expressing either Pex20p-GFP or Pex20p($\Delta 1-19$)-GFP were immunoblotted with the indicated antibodies. (D) Fluorescence and DIC microscopy pictures of methanol-grown cells (wild type, $\Delta pex1$, $\Delta pex4$, and $\Delta pex6$) coexpressing either Pex20p-GFP or Pex20p($\Delta 1-19$)-GFP and Pex3p-mRFP as a peroxisomal marker. Bars, 5 μ m.

Pex20p docks at the peroxisomal membrane and is translocated into peroxisomes

Our experiments support a model in which the peroxisomal import of PTS2 is mediated by the docking, import, and recycling steps of Pex20p itself (Fig. 10; discussed on the next page). Pex20p interactions with other peroxins resemble those of the PTS receptors. It interacts with members of the docking complex (Figs. 3 and 4), especially Pex14p, through its Wxxx(F/Y) repeats whose presence was noted in PTS2 auxiliary proteins (Einwächter et al., 2001; Schäfer et al., 2004; Otzen et al., 2005), but we show for the first time their actual involvement in docking to peroxisomes. This provides a structural clue to the question of why both PTS pathways converge at this docking site: they possess related motifs allowing interactions with the same peroxins.

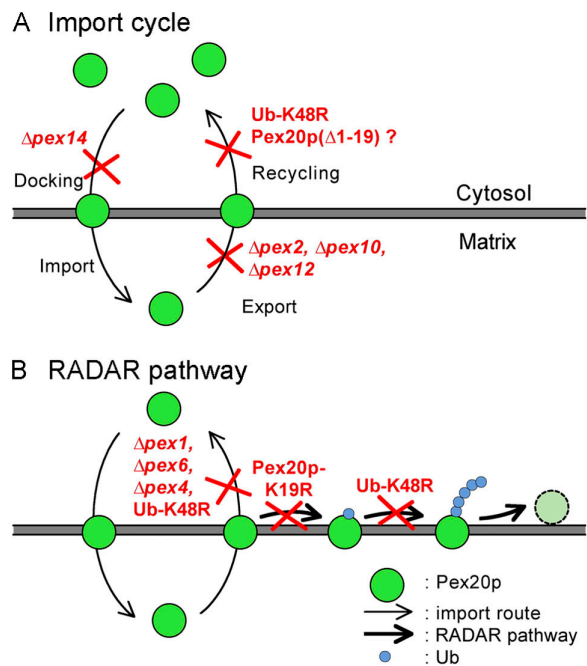


Figure 10. Working model for the dynamics of *P. pastoris* Pex20p during the import cycle. (A) Steps of the import cycles were studied with the use of deletion or point mutants (red) revealing the requirement of the docking peroxin Pex14p but not of Pex2p, Pex10p, or Pex12p for the peroxisomal localization of Pex20p (Fig. 6). Pex20p recycling to the cytosol is also affected when residues 1–19 of Pex20p are missing (Fig. 9, C and D). (B) When recycling is prevented, a quality-control system that we call the peroxisomal RADAR pathway is observed (Fig. 7 A; and Fig. 9, A and B). This depends on the presence of the Lys19 of Pex20p (Fig. 7, E and F) for subsequent degradation by the UPS (Fig. 7 G). Overexpression of Ub(K48R) affects at least two ubiquitin-dependent steps, one mediated by Pex4p and possibly the E3-like RING peroxins, involved in the completion of the import cycle (Fig. 8), and another by an undefined ubiquitin-conjugating enzyme distinct from Pex4p, involved in RADAR (Fig. 7 G).

Lack of Pex14p did not prevent interaction with Pex13p (Fig. 2 C), nor did it prevent docking of Pex20p to organelles (unpublished data). Thus, Pex20p possesses two docking sites on peroxisome membranes, as noted for Pex7p and Pex5p (Girzalsky et al., 1999; Otera et al., 2002; Fig. 4 B). Although Pex18p and Pex21p interact with Pex13p and Pex14p in a Pex7p-dependent fashion (Stein et al., 2002), our data show that Pex20p docks to peroxisomes independently of the PTS2 receptor (Fig. 4 B).

Several lines of evidence indicate that a fraction of Pex20p is peroxisome associated, with some of it being present inside peroxisomes (or fully embedded in the membrane; Fig. 5). Interestingly, Pex20p also interacts with the intraperoxisomal protein Pex8p (unpublished data), as described in *Y. lipolytica* (Smith and Rachubinski, 2001). In the context of the extended receptor shuttling model, the dual localization of Pex20p to both the cytosol and peroxisomes suggests that it too is a shuttling peroxin, like Pex5p and Pex7p (Dammai and Subramani, 2001; Nair et al., 2004).

Mutations of the NH₂ terminus of Pex20p or the RING peroxins affect its relocation to the cytosol

Interestingly, the E3-like RING peroxins (Pex2p, Pex10p, and Pex12p) were not required for the peroxisomal localization of

Pex20p (Fig. 6). Their absence or deletion of the NH₂-terminal 19 amino acids of Pex20p (Fig. 9, C and D; and Fig. 10) led to an increase in peroxisome-associated Pex20p, and the protein was inaccessible to the (cytosolic) ubiquitin-dependent degradation pathway. This indicates a role for the RING peroxins and this NH₂-terminal sequence in Pex20p relocation to the cytosol (Fig. 10 A), rather than in Pex20p translocation to the matrix.

Pex20p recycling to the cytosol

Similarly, the absence of peroxins involved in the late steps of protein import (the E2 Pex4p and the AAA ATPases Pex1p and Pex6p) caused a mostly peroxisomal localization of Pex20p (Fig. 9, A and B) when ubiquitin-dependent degradation was abolished by the Pex20p-K19R mutation (Fig. 7, F and G). However, in these same mutants, peroxisome-associated Pex20p was susceptible to ubiquitylation and degradation (Fig. 7, E–G), most likely on the cytosolic side of the peroxisomal membrane. Therefore, Pex1p, Pex4p, and Pex6p are not involved in Pex20p import into peroxisome but rather in its recycling from peroxisomes to the cytosol (Fig. 10 B). Epistasis analysis of Pex20p stability is consistent with the action of RING peroxins before that of recycling peroxins (unpublished data). This dependence of Pex20p recycling on Pex4p, Pex1p, and Pex6p is remarkably similar to that for Pex5p (van der Klei et al., 1998; Collins et al., 2000). During completion of this paper, a study was published that indicates a role of Pex1p and Pex6p in the recycling of ubiquitylated Pex5p from the peroxisomal membrane (Platta et al., 2005), in agreement with our data on Pex20p. Additionally, both Pex5p (Costa-Rodrigues et al., 2004) and Pex20p (Fig. 9) need their NH₂-terminal regions for recycling. This underlines the many similarities between Pex5p and Pex20p dynamics during the import cycle.

Pex20p regulation and subcellular localization depend on K48-branched polyubiquitylation

Pex20p steady-state level, like that of Pex5p (Koller et al., 1999; Collins et al., 2000), decreases in recycling mutants cultured overnight in oleate medium (Fig. 7). Among yeasts, this down-regulation of Pex5p is peculiar to *P. pastoris*. Instead, ubiquitylated species of Pex5p accumulate in these mutants of *S. cerevisiae* (Platta et al., 2004; Kiel et al., 2005; Kragt et al., 2005). At an earlier time point (6 h after induction; Fig. 7 G) higher molecular mass species (likely ubiquitin conjugates) of Pex20p-GFP are actually detected. K19R mutation in Pex20p prevents both the appearance of these additional Pex20p species and Pex20p down-regulation (Fig. 7, E–G). Therefore, in *P. pastoris*, this degradation is also likely to happen through the UPS. In conclusion, Pex20p is probably degraded by a quality-control mechanism triggered by the absence of recycling (Fig. 10 B), as suggested for ScPex5p (Kiel et al., 2005; Kragt et al., 2005). We call this the peroxisomal receptor accumulation and degradation in the absence of recycling (RADAR) pathway (Fig. 10).

We observed that interfering with K48-branched polyubiquitylation phenocopies the absence of the late-steps peroxins (Fig. 7 G; and Fig. 8). It was intriguing to see polyubiquitylated species of Pex20p appear when Ub(K48R) was overexpressed

(Fig. 8, B–D), a condition that should reduce polyubiquitylation. However, because Pex20p degradation in these mutant backgrounds is likely to happen via the UPS (Fig. 7 G), Ub(K48R) slows down this process and causes the accumulation of ubiquitylated species less susceptible to proteasome degradation, leading to a balance between the generation of ubiquitylated species by the RADAR pathway and their stabilization after interference with K48-branched polymerization. This allowed us to detect polyubiquitylated species of Pex20p, with K19 being the target residue (Fig. 8). In these conditions, both Pex20p and its ubiquitin conjugates were in and on peroxisomes (Fig. 8, D and E), with its ubiquitylated forms being more susceptible to protease than the nonubiquitylated form (Fig. 8 F). These observations are summarized in our working model (Fig. 10). Understanding the links between ubiquitin-mediated degradation and the import of peroxisomal proteins will be required for a better understanding of peroxisome biogenesis.

Materials and methods

Yeast strains and cultures

The strains used included *P. pastoris* PPY12 (*his4 arg4*; Gould et al., 1992), *pex* mutants (Collins et al., 2000; Hazra et al., 2002), and Δ *pex20* (this study). Cells were routinely grown in YPD (1% yeast extract [YE], 2% Bacto Peptone, and 2% glucose), YPM (1% YE, 2% Bacto Peptone, and 0.5% methanol), YNB (0.17% YNB, 0.5% [NH₄]₂SO₄, and 2% glucose), or YNO (0.05% YE, 0.25% [NH₄]₂SO₄, 1 mM MgSO₄, 20 mM NaH₂PO₄, 4 mM KH₂PO₄, 0.02% Tween 40, and 0.2% oleic acid). Oleate induction was overnight (18 h) unless otherwise indicated in the figures. Media were supplemented with 20 μ g/ml of histidine and arginine as needed. *S. cerevisiae* Δ *pex18* Δ *pex21* (UTL7 α : Mata, *ura3-52*, *trp1*, *leu2-3/112*, *PEX18::loxP*, and *PEX21::loxP*) was a gift of W.-H. Kunau (Ruhr-Universität, Bochum, Germany) and was grown on YNB medium.

PEX20 gene deletion

Oligonucleotides used are presented in Table S1 (available at <http://www.jcb.org/cgi/content/full/jcb.200508096/DC1>). The *KanMX6* G418 resistance cassette was amplified (KanMX.d/KanMX.r) from pFA Δ -KanMX6 and cloned at KpnI-BamHI in pBluescript II KS+ (Stratagene), creating pSEB44. The 5' flanking region of the *PEX20* ORF was amplified from PPY12 genomic DNA (pPEX20.d/5'20.r, KpnI-blunt) and cloned at KpnI-SmaI sites of pSEB44, resulting in pSEB46; the 3' flanking region of the *PEX20* ORF was amplified (3'20.d/3'20.r, XhoI-blunt) and was further cloned in pSEB46 (XhoI-EcoRV) to create pSEB47. The disruption cassette was amplified (pPEX20.d/3'20.r) from pSEB47 and transformed into the PPY12 strain. G418^r clones were screened by PCR and product size analysis.

Yeast two-hybrid analysis

The GAL4-based Matchmaker yeast two-hybrid system (CLONTECH Laboratories, Inc.) was used. The cloning strategy involved PCR amplification of *P. pastoris* peroxins from genomic DNA (oligonucleotides with XmaI-Sall sites; Table S1), in-frame cloning at the XmaI-Sall sites of pGAD-GH or pGBT9 and sequencing. *S. cerevisiae* AH109 strain was cotransformed and selected on complete synthetic medium lacking Leu and Trp (CSM-Leu-Trp). Independent cotransformants were patched on CSM-Leu-Trp and replica plated on CSM-Leu-Trp, CSM-Leu-Trp-His, and CSM-Leu-Trp-His + 50 mM 3-aminotriazole (3-AT; Sigma-Aldrich). Site-directed mutagenesis was performed with primers (20W89.d/20W89.r, 20W102.d/20W102.r, 20W141.d/20W141.r, and 20S280F.d/20S280F.r), sequencing, and excision/recloning into the original vector. Interactions were judged by the transcriptional activation of the *HIS3* gene (growth on CSM-Leu-Trp-His + 3-AT).

Functional complementation of *S. cerevisiae* Δ *pex18* Δ *pex21* strain

P. pastoris *PEX20* coding sequence was amplified (Y2H20.d/Y2H20.r, XmaI-Sall) and cloned at the XmaI-Sall sites of pCu416, creating pSEB41. *S. cerevisiae* *PEX18* was amplified from genomic DNA (CuPex18.d/CuPex18.r, XhoI-XbaI) and cloned (XhoI-XbaI) in pCu416, creating pSEB49. Serial dilution of YNB-grown cells were spotted on YNO agar plates.

Subcellular fractionation

Cells were grown overnight on YPD medium, precultured on YPD for 10 h, and transferred overnight into YNO. Cells were homogenized as described previously (Faber et al., 1998), except that the last centrifugation was performed at 200,000 g to ensure pelleting of peroxisome remnants in *pex* mutants (Harper et al., 2002).

Protease protection assay

Cells were broken as for subcellular fractionation but without protease inhibitors. Pellets of a 200,000-g centrifugation (see previous section) were resuspended in ice-cold Dounce buffer (Faber et al., 1998) to a protein concentration of 1 mg/ml, and 8 aliquots of 50 µg were taken. Freshly prepared proteinase K (Sigma-Aldrich) was added to all tubes (20 µg) after addition of Triton X-100 (0.125% final concentration) where specified in the figures and incubated for the indicated times. Trichloroacetic acid (10% final concentration) was added to stop the reaction. Proteins were precipitated overnight in ice, washed three times with acetone, and resuspended in lysis buffer, and 10 µg of each reaction was loaded.

Fluorescence microscopy

The *PEX20* ORF was amplified with its promoter (pPex20.d/20-GFP.r, KpnI-PstI) and cloned upstream the GFP coding sequence of pW3 (a gift of W. Dunn, University of Florida, Gainesville, FL) at KpnI-PstI sites, creating pSEB48. The vector was linearized with Sall and inserted at the *HIS* locus. Pex20(Δ1–19)-GFP was constructed by mutagenesis on pSEB48 using the primer pair 20G(Δ1–19).d/20G(Δ1–19).r, resulting in pSEB149. Cells were grown on YPD and switched to YNM or YNO when in exponential phase. Other constructs included p_{AOX}:BFP-PTS1 (a gift of W. Dunn), p_{CUP1}:PTS2-mRFP (pKSN39) and p_{CUP1}:mPTS-mRFP (pKSN7; gifts of K. Noda, University of California, San Diego, La Jolla, CA), and p_{PEX3}:PEX3-mRFP (pJCF215; a gift of J.C. Farré, University of California, San Diego). Copper induction was with CuSO₄ (100 µM final concentration) 2 h before observation. Images were captured on a motorized fluorescence microscope (AxioSkop 2 plus; Carl Zeiss MicroImaging, Inc.) coupled to a cooled charge-coupled device monochrome camera (AxioCam MRM; Carl Zeiss MicroImaging, Inc.) and processed using the AxioVision software.

Immunoprecipitations

The *PEX20* coding sequence was amplified (5SHA20/3PHA20, SacI-PstI) and cloned at SacI-PstI sites in a pIB2-based vector (constitutive GAP promoter; Sears et al., 1998) containing a triple HA tag (pIB2-HA), creating pIB2-HA-PEX20 (a gift from I. Suriapranata, University of California, San Diego). HA-Pex7p construct was obtained from W. Snyder (University of California, San Diego). Vectors were cut with Sall and integrated at the *HIS* locus. Transformants were grown overnight on YPD and transferred for 5 h on YNO before extraction. Immunoprecipitation of HA-tagged proteins was performed as follows. Cells (8 ODs) were broken with glass beads in 200 µl IP lysis buffer (50 mM Hepes-KOH, pH 7.5, 0.5 M NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, and protease inhibitor cocktail) and centrifuged twice (14,000 g, 10 min). Monoclonal anti-HA antibody (Covance) was added to the supernatant (6 µl/ml of lysate) and incubated overnight with the extract. 25 µl of GammaBind beads (GE Healthcare) were added and incubated for 2 h. Beads were washed twice (1 ml) with the lysis buffer for 10 min and three times (1 ml) with the wash buffer (50 mM Hepes-KOH, pH 7.5, 150 mM NaCl, and 1 mM EDTA) and finally boiled in 50 µl SDS loading buffer. Loading was as follows: input and unbound fraction, 0.2 OD equivalent; immunoprecipitate, 1 OD equivalent unless otherwise indicated.

Immunoprecipitations of TCA-lysed cells for the detection of ubiquitinated Pex20p were performed as described in Kragt et al. (2005) using the anti-GFP monoclonal antibody from Roche Applied Sciences.

Pex20p antibody

A chicken polyclonal antibody was generated against aa 1–146 of Pex20p. Affinity-purified antibodies were further purified by incubation with crude acetone powder extract of methanol-grown *Δpex20* cells and used at 1:1,000 dilution for immunoblotting.

Peroxisome steady-state levels

Cells were grown overnight on YPD, precultured on YPD for 10 h, and transferred overnight in YNO. Cells were disrupted with glass beads as for immunoprecipitations. The supernatant of the 14,000 g centrifugation was considered the raw extract. Proteins were assayed, and 20 µg were loaded on each lane.

Miscellaneous constructs

The *PEX20* promoter was amplified from genomic DNA (pPex20.d/r, KpnI-SmaI) and cloned in pIB1 (Sears et al., 1998), creating pSEB95. Sequences encoding truncated versions of Pex20p were amplified using a forward primer (Pex20-7.d, -11.d, -16.d, -22.d, and -31.d; SmaI) in combination with the reverse primer Y2H20.r (Sall) and cloned (SmaI-XhoI) downstream of p_{PEX20} in pSEB95 (pSEB101, -103, -105, -122, and -108, respectively). Myc-Ub(K48R) was a mutagenized version of pTK132 (p_{GAP}:myc-Ub from A. Koller, University of California, San Diego) and was provided by I. Suriapranata. 6xHis-myc-Ub and 6xHis-myc-Ub(K48R) were created by PCR amplification of myc-Ub from YEp105 or pTK132, respectively (6xHis-Myc.d/Ub.r, EcoRI-KpnI), and cloning into pJCF215 (pIB2-based vector containing the GAPDH promoter, in which the *HIS4* marker gene was replaced by *ARG4*; a gift from J.-C. Farré) was done to create pSEB127 and -128.

Online supplemental material

Fig. S1 shows an alignment of PpPex20p with its homologues from various organisms. Table S1 shows the oligonucleotides used. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200508096/DC1>.

We thank Dr. R. Tsien (University of California, San Diego, La Jolla, CA) for the mRFP construct and Dr. W.-H. Kunau (Ruhr-Universität, Bochum, Germany) for providing us with the Fox3p antibody and the *S. cerevisiae Δpex18 Δpex21* strain. The authors are indebted to many members of the Subramani laboratory for strains, constructs, and stimulating discussions. We thank Amar Patel for technical assistance.

This work was supported by National Institutes of Health grants to S. Subramani (DK41737 and DK59844) and J. Cregg (DK067371).

Submitted: 15 August 2005

Accepted: 22 November 2005

Note added in proof. While this manuscript was under review, an article appeared on the polyubiquitination of Pex5p from *Hansenula polymorpha* (Kiel, J.A., M. Otzen, M. Veenhuis, and I.J. van der Klei. 2005. *Biochim. Biophys. Acta.* 1745:176–186). Our findings on the RADAR pathway may also apply to HpPex5p.

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