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 (Petriv 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 (Dammari 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...
Pex21p; Purdue et al., 1998), but other organisms (Yarrowia lipolytica, Neurospora crassa, and Hansenula polymorpha; Titorenko et al., 1998; Sichting 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/content/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 ∆pex20

The involvement of Pex20p in peroxisome biogenesis was analyzed by gene deletion. Although the ∆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 Δpex7 grows in methanol (Elgersma et al., 1998). Growth of Δpex20, like that of Δpex7 cells, was unaltered on methanol (Fig. 1 B), indicating that Pex20p is dispensable for import of PTS1 cargoes.

The ability of the Δpex20 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. Δpex20 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 Δpex20 cells (Fig. 1 D), whereas it was more abundant in the P200 of the mutant strain complemented with a tagged version of Pex20p (Δpex20 + HA-Pex20p). The subcellular location of the PTS1 cargo catalase was unaffected. Neither overexpression of Pex20p in Δpex7, nor of Pex7p in Δpex20, 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 Δpex18 Δpex21 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; Sichting et al., 2003), we investigated these in a homologous context. HA-Pex7p communoprecipitated with some Pex20p (Fig. 2 A). The amount of Pex20p interacting with Pex7p in wild-type cells was lower than that in Δpex14 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 communoprecipitation using an NH2-terminal 3xHA-tagged version of Pex20p that complemented the Δpex20 strain on oleate. Communoprecipitation of thiolase with HA-Pex20p was dependent on the presence of Pex7p (Fig. 2 B), suggesting 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 communoprecipitated with members of the docking complex (Pex13p, Pex14p, and Pex17p; Fig. 2 C). The interaction was particularly strong with Pex14p and Pex17p. In Δpex14 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.
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; Sichting 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 PpPex20p was involved in the interaction (Fig. 3 D). Interestingly, this region is involved in Pex5p binding (Schliebs et al., 1999), suggesting that the same region of Pex14p interacts with WxxxF(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-Pex7 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...
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 Δpex20 cells showed that Pex7p was 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).

**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 sediments at the cytosolic layers of the 200,000 g supernatant fraction, consistent with its partial intraperoxisomal localization.
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 Δpex20 + 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 (Δpex2, Δpex10, and Δpex12), 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 Δpex1, Δpex6, and Δpex4), 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 Δpex13 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 Δpex20 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 (Δ1–19, Δ1–22, and Δ1–31; 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 Δpex20 Δpex4 double mutant strain and observed that this mutation rendered the protein stable (Fig. 7 E), whereas Pex20p was undetectable in Δpex4 cells. Also, when Pex20p-GFP was expressed in Δpex1, Δpex6, or Δpex4, 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 Δpex1, Δpex6, or Δpex4 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 Δpex4 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
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-GFP in crude extracts from this strain, whose presence was dependent on the K19 residue (Fig. 8 B), mimicking the situation obtained in Δpex4 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 the indicated antibodies. (G) Cell lysates of oleate-grown PPY12 or Δpex4 cells expressing pPEX20:PEX20-GFP or pPEX20:PEX20(K19R)-GFP were immunoblotted 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.

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 Δpex20 strain. Expression of the truncated protein was confirmed (not depicted) and tested for its ability to restore growth of the Δpex20 strain on oleate. (D) Growth of the indicated strains after overnight culture on oleate. (E) Cell lysates of oleate-grown wild-type (WT; PPY12), Δpex4, Δpex6, Δpex20 + pPEX20:PEX20(K19R), or Δpex5 immunoblotted for Pex5p or Pex20p. (F) Cell lysates of oleate-grown PPY12, Δpex1, Δpex6, and Δpex4 strains expressing either pPEX20:PEX20-GFP or pPEX20:PEX20(K19R)-GFP were immunoblotted with the indicated antibodies. (G) Cell lysates of PPY12 or Δpex4 cells expressing pPEX20:PEX20-GFP or pPEX20:PEX20(K19R)-GFP were prepared after 6 h of growth in oleate and immunoblotted with the indicated 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 localization. Pex20p(Δ1–19)-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 Pex3p-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 Δpex20 cells fail to grow on oleate and have a PTS2 import defect (Fig. 1). However, the few studies (Einwächter et al., 2001; Sichting 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 Δpex20 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).
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.

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 peroxisomal associated, with some of it being present inside peroxisomes (or fully embedded in the membrane; Fig. 5). Interestingly, Pex20p 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). Pex20p recycling to the cytosol is also affected when residues 1–19 of Pex20p are missing (Fig. 9, C and D). 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).

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], Δpex1, Δpex6, or Δpex4 cells expressing Pex20p(K19R)-GFP were immunoblotted with the indicated antibodies. (B) Fluorescence and DIC microscopy pictures of methanol-grown cells (wild type, Δpex1, Δpex4, and Δpex4Δcoexpressing Pex20p(K19R)-GFP and Pex3p-mRFP as a peroxisomal marker. (C) Differential centrifugation fractions of oleate-grown Δpex20 cells expressing either Pex20p-GFP or Pex20p(Δ1–19)-GFP were immunoblotted with the indicated antibodies. (D) Fluorescence and DIC microscopy pictures of methanol-grown cells (wild type, Δpex1, Δpex4, and Δpex4coexpressing either Pex20p-GFP or Pex20p(Δ1–19)-GFP and Pex3p-mRFP as a peroxisomal marker. Bars, 5 μm.
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 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 PBY12 (his4 arg4; Gould et al., 1998), pex20 mutants (Collins et al., 2000; Hazra et al., 2002), and ∆pex20 (this study). Cells were routinely grown in YPD [1% yeast extract (YE), 2% Bacto Peptone, and 2% glucose], SD (0.67% yeast nitrogen base without amino acids, 2% Bacto Peptone, and 2% glucose), or YPM (1% YE, 2% Bacto Peptone, and 2% glucose), or YNO (0.05% YE, 0.25% [NH₄]₂SO₄, 1 mM MgSO₄, 20 mM NaH₂PO₄, 4 mM KH₂PO₄, 0.02% (w/v) 40, and 0.2% oleic acid). Olate induction was overnight (16 h) unless otherwise indicated in the figures. Media were supplemented with 20 μg/ml of histidine and arginine as needed. S. cerevisiae ∆pex18 ∆pex21 (UT174: 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 KanMX G418 resistance cassette was amplified (KanMX ΔKanMXix from pFA6a-KanMXvi and cloned at KpnI–BamHI in pBluescript II KS+; Table S1), in-frame cloning at the XmaI–SalI sites of pGAD-GH (Stratagene), creating pSEB44. The 5′ flanking region of the PEX20 ORF was amplified from PPY12 genomic DNA (pPEX20.d/5′20.o, KpnI– blunt) and cloned at KpnI–SalI sites of pSEB44, resulting in pSEB46; the 3′ flanking region of the PEX20 ORF was amplified (3′20.d/3′20.o, XhoI–EcoRV) and was further cloned in pSEB46 (XhoI–EcoRV) to create pSEB47. The disruption cassette was amplified (pEX20.d/3′20.o) from pSEB47 and transformed into the PEX20 strain. G418 colonies 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–SalI flanking region of the ORF) from genomic DNA (oligonucleotides with XmaI–SalI flanking region of the ORF) and cloned at the XmaI–SalI sites of pSEB44, creating pSEB46. The 5′ flanking region of the PEX20 ORF was amplified (3′20.d/3′20.o, XhoI–EcoRV) and was further cloned in pSEB46 (XhoI–EcoRV) to create pSEB47. The disruption cassette was amplified (pEX20.d/3′20.o) from pSEB47 and transformed into the PEX20 strain. G418 colonies were screened by PCR and product size analysis.

Functionality complementation of S. cerevisiae ∆pex18 ∆pex21 strain

P. pastoris PEX20 coding sequence was amplified (YPH20.d/YPH20.o, Xmal–Sall) and cloned at the Xmal–Sall sites of pCu416, creating pSEB41. S. cerevisiae PEX20 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 protease 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 protein was loaded.

Fluorescence microscopy

The PEX20 ORF was amplified with its promoter (pPex20-2/0-GFP, rKpn-Pst) and cloned upstream the GFP coding sequence of pPD3 (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(A1–19)GFP was constructed by mutagenesis on pSEB48 using the primer pair 20G(A1–19).d/20G(A1–19.r, resulting in pSEB149. Cells were grown on YPD and switched to YNM or YNO when in exponential phase. Other constructs included pKExBFPPTS1 (a gift of W. Dunn), pDIP2:PTS2-mRFP (pKS39) and pDIP2:MTS5-mRFP (pKS7, gifts of K. Noda, University of California, San Diego, La Jolla, CA), and pPREX3:mRFP [pCF215; 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 (5SHEA20/3PHEA20, 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). 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 MR; Carl Zeiss Microimaging, Inc.) and processed using the AxioVision software.

Miscellaneous constructs

The PEX20 promoter was amplified from genomic DNA (pPex20-2/0, rKpn-Smal) and cloned in pBl1 (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; Small] in combination with the reverse primer Y2H20.r (Sall) and cloned (Sall–XhoI) downstream of Ptrs20 in pSEB95 (pSEB101, -103, -105, -127, and -108, respectively). Myc-Ub(K48R) was a mutagenized version of pTK132 [pGAP: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.c.Ub.r, EcoRI-KpnI], and cloning into pCF215 (pIB2-based vector containing the cDNA coding sequence) where indicated. The CM4 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.

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Note added in proof. While this manuscript was under review, an article appeared on the polyubiquitylation of Pex20p from Hansenula polymorpha (Kiel, J.A., M. Otzen, M. Veenhuis, and I.J. van der Klei. 2005. Biochem. Biophys. Acta. 1745:176–186). Our findings on the RADAR pathway may also apply to HpPex5p.

References

Agne, B., N.M. Meindl, K. Niederhoff, H. Einwächter, P. Rehling, A. Sickmann, H.E. Meyer, W. Girzalsky, and W.H. Kunau. 2003. Pep18p. An intraperoxisomal organizer of the peroxisomal import machinery. Mol. Cell. 11:635–646.

Braverman, N., G. Dodt, S.J. Gould, and D. Vallee. 1998. An isoform of Pex5p, the human PTS1 receptor, is required for the import of PTS2 proteins into peroxisomes. Hum. Mol. Genet. 7:1195–1205.

Chang, C.C., D.S. Warren, K.A. Sacksteder, and S.J. Gould. 1999. PEX12 interacts with PEX3 and PEX10 and acts downstream of receptor docking in peroxisomal matrix protein import. J. Cell. Biol. 20:7516–7526.

Collins, C.S., J.E. Kalish, J.C. Morrell, J.M. McCaffery, and S.J. Gould. 2000. The peroxisome biogenesis factors Pex4p, Pex22p, Pex1p, and Pex6p act in the terminal steps of peroxisomal matrix protein import. Mol. Biol. Cell. 20:7516–7526.

Costa-Rodríguez, J., A.F. Carvalho, A.M. Gouveia, M. Fransen, C. Sa-Miranda, and J.E. Azevedo. 2004. The N terminus of the peroxisomal receptor, Pex5p, is required for redirecting the peroxisome-associated peroxin back to the cytosol. J. Biol. Chem. 279:46573–46579.

Dammai, V., and S. Subramani. 2001. The human peroxisomal targeting signal receptor, Pex5p, is translated into the peroxisomal matrix and recycled to the cytosol. Cell. 105:187–196.

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.

Dodt, G., D. Warren, E. Becker, P. Rehling, and S.J. Gould. 2001. Domain mapping of human PEX5 reveals functional and structural similarities to Saccharomyces cerevisiae Pex18p and Pex21p. J. Biol. Chem. 276:41769–41781.

Einwächter, H., S. Sowinski, W.H. Kunau, and W. Schliebs. 2001. Yarrowia lipolytica Pex20p, Saccharomyces cerevisiae Pex18p/Pex21p and mammalian Pex5p fulfill a common function in the early steps of the peroxisomal PTS2 import pathway. EMBO Rep. 2:1035–1039.

Elgersma, Y., M. Elgersma-Hooisma, T. Wenzel, J.M. McCaffery, M.G. Farqu
har, and S. Subramani. 1998. A mobile PTS2 receptor for peroxisomal protein import in Pichia pastoris. J. Cell Biol. 140:807–820.

Faber, K.N., J.A. Heyman, and S. Subramani. 1998. Two AAA family peroxins, 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. 18:930–943.

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.

Gould, S.J., D. McCollum, A.P. Spong, J.A. Heyman, and S. Subramani. 1992. Development of yeast P. pastoris as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast. 8:613–628.

Harper, C.C., S.T. South, J.M. McCaffery, and S.J. Gould. 2002. Peroxisomal membrane protein import does not require Pex17p. J. Biol. Chem. 277:16498–16504.

Hazra, P.P., I. Suriaprannata, W.B. Snyder, and S. Subramani. 2002. Peroxisome remnant in pex3Δ cells and the requirement of Pex3p for interactions between the peroxisomal docking and translocation subcomplexes. Traffic. 3:560–574.

Kiel, J.A., K. Emmrich, H.E. Meyer, and W.H. Kunau. 2005. Ubiquitination of the peroxisomal targeting signal type 1 receptor, Pex5p, suggests the presence of a quality control mechanism during peroxisomal matrix protein import. J. Biol. Chem. 280:1921–1930.

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.

Kragt, A., T.M. Voorn-Brouwer, M. Van den Berg, and B. Distel. 2005. The Saccharomyces cerevisiae peroxisomal import receptor Pex5p is monoubiquitinated in wild type cells. J. Biol. Chem. 280:7867–7874.

Matsumura, T., H. Otera, and Y. Fujiki. 2000. Disruption of the interaction of the longer isoform of Pex5p, Pex5pL, with Pex7p abolishes peroxisomal targeting signal type 2 protein import in mammals. Study with a novel Pex5-impaired Chinese hamster ovary cell mutant. J. Biol. Chem. 275:21715–21721.

Nair, D.M., P.E. Purdue, and P. Lazaroaz. 2004. Pex7p translocates in and out of peroxisomes in Saccharomyces cerevisiae. J. Cell Biol. 167:599–604.

Otera, H., T. Harano, M. Honsho, K. Ghaedi, S. Mukai, A. Tanaka, A. Kawai, N. Shimizu, and Y. Fujiki. 2000. The mammalian peroxin Pex5pL, the longer isoform of the mobile peroxisomal targeting signal (PTS) type 1 transporter, translocates the Pex7p.PTS2 protein complex into peroxisomes via its initial docking site, Pex14p. J. Biol. Chem. 275:21703–21714.

Otera, H., K. Setoguchi, M. Hamasaki, T. Kumashiro, N. Shimizu, and Y. Fujiki. 2002. Peroxisomal targeting signal receptor Pex5p interacts with cargoes and import machinery components in a spatiotemporally differentiated manner: conserved Pex5p WXXXF/Y motifs are critical for matrix protein import. Mol. Cell. Biol. 22:1639–1655.

Otzen, M., D. Wang, M.G. Lunenborg, and I.J. van der Klei. 2005. Hansenula polymorpha Pex20p is an oligomer that binds the peroxisomal targeting signal 2 (PTS2). J. Cell Sci. 118:3409–3418.

Petriv, O.I., L. Tang, V.I. Titorenko, and R.A. Rachubinski. 2004. A new definition for the consensus sequence of the peroxisome targeting signal type 2. J. Mol. Biol. 341:119–134.

Platta, H.W., W. Girzalsky, and R. Erdmann. 2004. Ubiquitination of the peroxisomal import receptor Pex5p. Biochem. J. 384:37–45.

Platta, H.W., S. Grunau, K. Rosenkrantz, W. Girzalsky, and R. Erdmann. 2005. Functional role of the AAA peroxins in dislocation of the PTS1 receptor back to the cytosol. Nat. Cell Biol. 7:817–822.

Purdue, P.E., X. Yang, and P.B. Lazarow. 1998. Pex18p and Pex21p, a novel pair of related peroxins essential for peroxisomal targeting by the PTS2 pathway. J. Cell Biol. 143:1839–1849.

Schliebs, W., J. Sadowsky, B. Agianian, G. Dott, F.W. Herberg, and W.H. Kunau. 1999. Recombinant human peroxisomal targeting signal receptor PEX5. Structural basis for interaction of PEX5 with PEX14. J. Biol. Chem. 274:5666–5673.

Schäfer, A., D. Kerssen, M. Veenhuis, W.H. Kunau, and W. Schliebs. 2004. Functional similarity between the peroxisomal PTS2 receptor binding protein Pex18p and the N-terminal half of the Pts1p receptor Pex5p. Mol. Cell. Biol. 24:8905–8906.

Sears, I.B., J. O’Connor, O.W. Rossanese, and B.S. Glick. 1998. A versatile set of vectors for constitutive and regulated gene expression in Pichia pastoris. Yeast. 14:783–790.

Sichting, M., A. Schell-Steven, H. Proksch, R. Erdmann, and H. Rottensteiner. 2003. Pex7p and Pex20p of Neurospora crassa function together in PTS2-dependent protein import into peroxisomes. Mol. Biol. Cell. 14:810–821.

Smith, J.J., and R.A. Rachubinski. 2001. A role for the peroxin Pex5p in Pex20p-dependent thiolase import into peroxisomes of the yeast Yarrowia lipolytica. J. Biol. Chem. 276:1618–1625.

Stein, K., A. Schell-Steven, R. Erdmann, and H. Rottensteiner. 2002. Interactions of Pex7p and Pex18p/Pex21p with the peroxisomal docking machinery: implications for the first steps in PTS2 protein import. Mol. Cell. Biol. 22:6086–6089.

Subramani, S. 1998. Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement. Physiol. Rev. 78:171–188.

Titorenko, V.I., and R.A. Rachubinski. 2001. The life cycle of the peroxisome. Nat. Rev. Mol. Cell Biol. 2:357–368.

Titorenko, V.I., J.J. Smith, R.K. Szilard, and R.A. Rachubinski. 1998. Pex20p of the yeast Yarrowia lipolytica is required for the oligomerization of thiolase in the cytosol and for its targeting to the peroxisome. J. Cell Biol. 142:403–420.

van der Klei, L.J., R.E. Hilbrands, 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.

Wanders, R.J. 2004. Metabolic and molecular basis of peroxisomal disorders: a review. Am. J. Med. Genet. A. 126:355–375.

Yahraus, T., N. Braverman, G. Dott, 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.

Zolman, B.K., and B. Bartel. 2004. An Arabidopsis indole-3-butyric acid-response mutant defective in PEROXIN6, an apparent ATPase implicated in peroxisomal function. Proc. Natl. Acad. Sci. USA. 101:1786–1791.