Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling

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Pex5p, which is the import receptor for peroxisomal matrix proteins harboring a type I signal sequence (PTS1), is mono- and polyubiquitinated in *Saccharomyces cerevisiae*. We identified Pex5p as a molecular target for Pex4p-dependent monoubiquitination and demonstrated that either poly- or monoubiquitination of the receptor is required for the ATP-dependent release of the protein from the peroxisomal membrane to the cytosol as part of the receptor cycle. Therefore, the energy requirement of the peroxisomal import pathway has to be extended by a second ATP-dependent step, namely receptor monoubiquitination.

Introduction

The peroxisomal import receptor Pex5p binds its cargo proteins in the cytosol and targets them to docking and translocation machinery at the peroxisomal membrane (for review see Lazarow, 2003), where the receptor releases the cargo proteins into the peroxisomal lumen and shuttles back to the cytosol. Dislocation of the yeast PTS receptor Pex5p from the peroxisomal membrane to the cytosol after cargo release is performed by the peroxisomal AAA proteins Pex1p and Pex6p (Miyata and Fujiki, 2005; Platta et al., 2005). At the peroxisomal membrane Pex5p is modified by mono- and polyubiquitination (Platta et al., 2004; Kiel et al., 2005a; Kragt et al., 2005), but the functional role of this modification was not known.

Results and discussion

The study of the role of the monoubiquitination of Pex5p observed in wild-type cells (Kragt et al., 2005) has been hampered by the polyubiquitination of Pex5p, which accumulates at the peroxisomal membrane in cells lacking components required for the late steps in the import pathway (Platta et al., 2004; Kiel et al., 2005a). To study Pex5p monoubiquitination, we followed two strategies to avoid polyubiquitination. For both strategies, we assumed the polyubiquitination site to reside within the N-terminal region of the protein because this region of Pex5p from human and rat is sufficient to carry out its docking to, as well as its consecutive dislocation from, the peroxisomal membrane to the cytosol (Costa-Rodrigues et al., 2004). First, we fused three *myc* epitopes to the N terminus of Pex5p, which for other proteins has been shown to prevent polyubiquitination. Second, we substituted arginine for the first conserved lysine residue (lysine 18) of Pex5p by site-directed mutagenesis. The corresponding lysine residue is required for polyubiquitination of Pex5p from *Hansenula polymorpha* (Kiel et al., 2005b) and of Pex20p from *Pichia pastoris*, which is the putative functional counterpart in the peroxisomal PTS2-dependent protein import pathway (Leon and Subramani, 2007). However, in our case, the single-mutant protein (Pex5pK18R) was still polyubiquitinated (unpublished data). Thus, we considered that an adjacent lysine might substitute for the loss, as is the case for many other proteins destined for degradation (Baldi et al., 1996), and accordingly, we also replaced lysine 24 of Pex5p with arginine. Both the *myc*-tagged Pex5p and Pex5pK18/24R restored the growth defect of a *PEX5* deletion strain on medium with oleate as the sole carbon source (not depicted) and imported GFP-PTS1 properly into peroxisomes (Fig. 1 A). Moreover, *myc*Pex5p and Pex5pK18/24R were normally bound and released from the peroxisomal membrane (Fig. 1 B). Thus, both variants behaved like the wild-type protein, thereby demonstrating that neither the tag nor the introduced mutations interfered with the physiological role of Pex5p in peroxisomal protein import. Significantly, when transformed into the *pex4*Δ*pex5*Δ or *pex1*Δ*pex5*Δ strains, no polyubiquitinated forms of the *myc*-tagged or mutated Pex5p could be detected in the cell lysates (Fig. 1 C). Thus, the exchange of the lysine residues deleted the target residues for ubiquitination. Similarily, the N-terminal *myc* tagging prevented polyubiquitination, possibly by interfering with polyubiquitin-specific factors such as Ubc4p/Ubc5p or the corresponding E3 enzyme, or by masking the target lysine residues for polyubiquitin chain formation.

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Abbreviations used in this paper: NEM, *N*-ethylmaleimide; RADAR, receptor accumulation and degradation in absence of recycling.

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The fact that *pex5Δ* cells harboring *myc*Pex5p or Pex5pK18/24R did not exhibit a growth defect on oleic acid medium and import GFP-PTS1 indicates that polyubiquitination is not a prerequisite for functional peroxisomal protein import in *Saccharomyces cerevisiae*. These data are in agreement with the idea that polyubiquitination is part of a quality control system that primes membrane-accumulated Pex5p for proteasomal degradation (Erdmann and Schliebs, 2005; Kiel et al., 2005a; Platta et al., 2005). A similar system (receptor accumulation and degradation in absence of recycling [RADAR]) has also been described for the quality control of membrane-associated Pex20p (Leon et al., 2006).

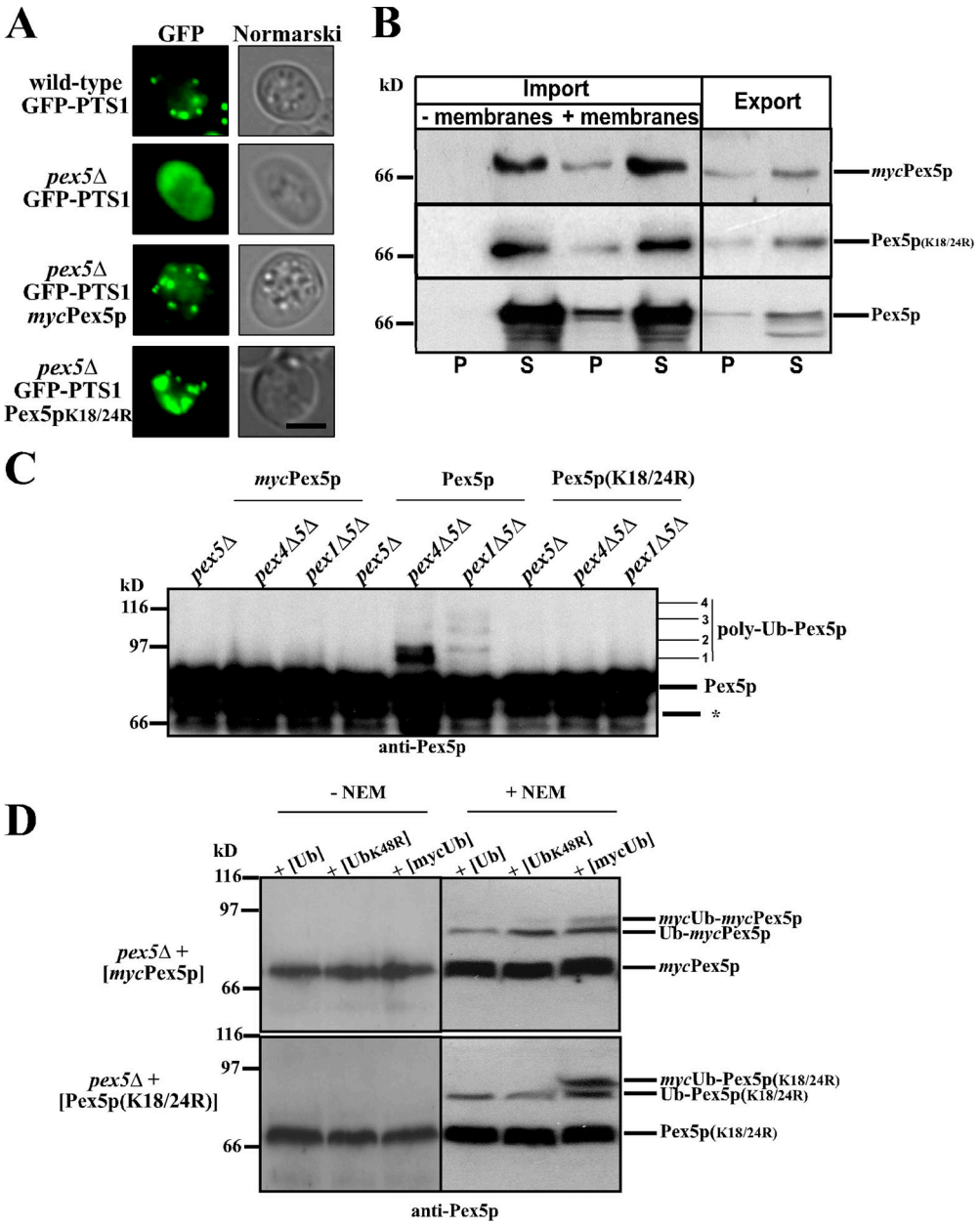

Figure 1. **The N-terminal *myc* tag and the K18/24R substitutions of Pex5p interfere with polyubiquitination, but do not hamper monoubiquitination.** (A) *myc*Pex5p and Pex5pK18/24R complement the protein import defect of *pex5*Δ cells. The strains indicated were examined for the intracellular localization of the GFP-PTS1 by fluorescence microscopy. Structural integrity of the cells is documented by bright-field microscopy. Bar, 5 μm. (B) Binding assays were performed with Pex5p-, *myc*Pex5p-, or Pex5pK18/24R-containing cytosol and membranes from *pex5*Δ cells. For the export reaction, Pex5p-containing membranes were incubated with *pex5*Δ cytosol in presence of an ATP-regenerating system. Samples were analyzed by immunoblot analysis with antibodies against Pex5p. P, membrane pellet; S, supernatant. (C) Polyubiquitinated forms of Pex5p were visualized in samples derived from trichloroacetic acid lysates of *pex5Δ*, *pex1Δpex5Δ*, and *pex4Δpex5Δ* mutant cells by immunoblot analysis. The pattern of higher molecular weight forms indicated the polyubiquitination of wild-type Pex5p that is typical for mutants that are affected in late stages of the import pathway. In contrast, neither *myc*Pex5p nor Pex5pK18/24R was polyubiquitinated in these mutants. The estimated number of ubiquitin moieties attached to both polyubiquitination sites is indicated on the right. (D) Monoubiquitinated Pex5p and *myc*Pex5p or Pex5pK18/24R was visualized by preparation of indicated cell lysates in the presence of NEM to prevent deubiquitination. Membrane-enriched fractions were subjected to TCA precipitation and immunoblot analysis. Coexpression of *myc*-Ub resulted in a shift of modified Pex5p, demonstrating that these bands represent ubiquitinated Pex5p. In contrast, no band shift to a lower molecular weight was observed when polyubiquitination was prevented by expression of UbK48R, thereby confirming the monoubiquitination.

Despite the lack of polyubiquitination, *myc*Pex5p and Pex5pK18/24R were still normally monoubiquitinated (Fig. 1 D). Preparation of membrane pellets in the presence of *N*-ethylmaleimide (NEM) to inhibit deubiquitinating enzymes results in the appearance of a more slowly migrating form of Pex5p, which has been shown to represent monoubiquitinated Pex5p (Kragt et al., 2005). The slower migrating form did shift to a higher molecular weight upon expression of *myc*Ub, demonstrating its ubiquitin nature. No change in molecular weight was observed when mutated ubiquitin (UbK48R) was expressed, which prevents the formation of polyubiquitin chains (Chau et al., 1989), confirming the monoubiquitination of the proteins. Thus, in respect to monoubiquitination, the myc-tagged and the K18R/K24R-double-mutated Pex5p behave like the endogenous protein.

It has been reported that monoubiquitination of Pex5p is independent of *UBC8*, *UBC5*, *UBC4*, and *UBC1*, and even takes place in a *UBC1/UBC4* double-deletion strain (Kragt et al., 2005). However, the observations on monoubiquitination were hampered in mutants lacking UBC10 (Pex4p) and other late peroxins like Pex1p and Pex6p because of the presence of polyubiquitinated Pex5p. We took advantage of the fact that *myc*Pex5p is not polyubiquitinated, but is still susceptible to monoubiquitination, enabling us to investigate which of the known UBCs is responsible for the monoubiquitination event. We isolated *myc*Pex5p via immunoprecipitation from chosen *UBC* deletion strains, leaving out the lethal deletion of *UBC3*, as well as of the SUMO-conjugating enzyme Ubc9p and the Nedd8/Rub1-conjugating enzyme Ubc12p. In all of the UBC deletion strains tested (*ubc1Δ*, *ubc2Δ*, *ubc4Δubc5Δ*, *ubc6Δ*, *ubc7Δ, ubc8Δ*, *ubc11Δ*, and *ubc13Δ*), formation of mono-Ub-*myc*Pex5p was visible, except in the case of Pex4p (Ubc10p) deletion (Fig. 2 A). These data clearly demonstrate the dependence of *myc*Pex5p monoubiquitination on the presence of Pex4p. To determine whether not only the presence but also the catalytic activity of Pex4p is essential for Pex5p monoubiquitination, we expressed an inactive Pex4p mutant protein, which carries a C–S point mutation at position 115. This amino acid residue is essential for the activity of ubiquitin-conjugating enzymes (Pickart, 2001). Although Pex4p(C115S) can be expressed to nearly wild-type levels and is properly targeted to peroxisomes (Wiebel and Kunau, 1992), monoubiquitination of the *myc*-tagged or point-mutated PTS1 receptor was completely abolished (Fig. 2 B and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200611012/DC1).

In addition to its catalytic activity, the peroxisomal localization of Pex4p also proved to be essential for Pex5p monoubiquitination. *myc*Pex5p or Pex5pK18/24R was expressed in a *pex22Δ* background, which lacks the peroxisomal membrane anchor for Pex4p (Koller et al., 1999). As shown in Fig. 2 B, monoubiquitination of Pex5p was not observed in the *pex22Δ* strain. The fact that the presence of an active ubiquitin-conjugating enzyme Pex4p attached to the peroxisomal membrane via Pex22p is indispensable for the formation of monoubiquitinated Pex5p indicates that the PTS1 receptor is a physiological substrate of Pex4p.

Next, we addressed the question of whether Pex4p is required up- or downstream of the AAA complex that is responsible for Pex5p release from the peroxisomal membrane (Miyata and Fujiki, 2005; Platta et al., 2005). Previous findings demonstrated that the receptor docking at the peroxisomal membrane and transfer to the RING-finger peroxins is prerequisite for

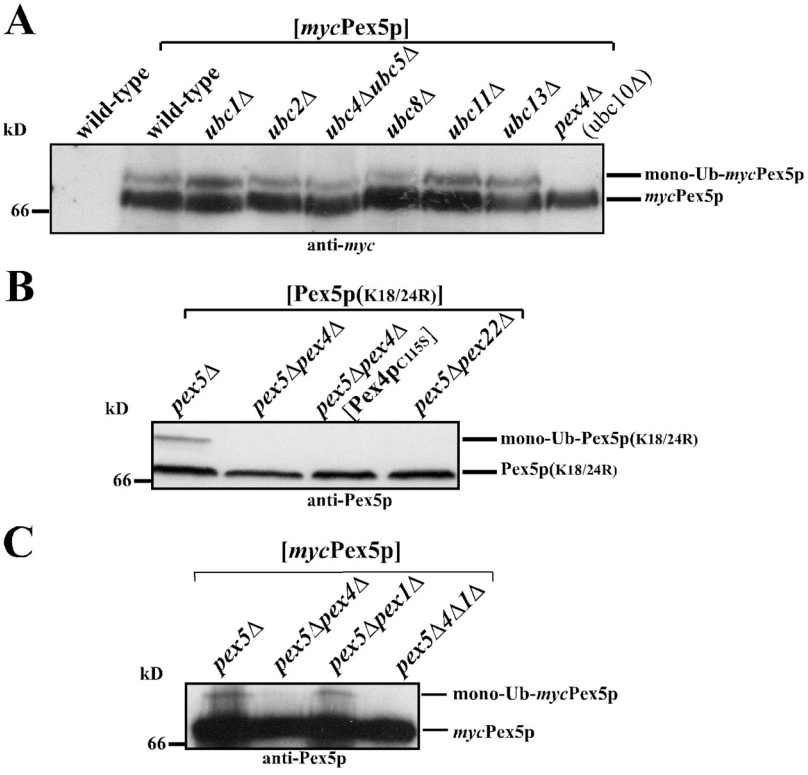

Figure 2. **Monoubiquitination of Pex5p depends on the presence, activity, and peroxisomal localization of the ubiquitin-conjugating enzyme Pex4p.** (A) *myc*Pex5p was transformed into UBC mutant strains and isolated by immunoprecipitation. In contrast to all other tested *ubc* deletion strains, no mono-Ub-*myc*Pex5p is observed in the *pex4Δ* strain. (B) Equally, monoubiquitination of Pex5pK18/24R was not observed in a strain expressing a catalytically inactive Pex4p or in *pex22Δ* cells. (C) Pex4p-mediated monoubiquitination of Pex5p occurs upstream of the AAA–ATPase complex. *myc*Pex5p isolated from indicated strains by immunoprecipitation was tested for Pex5p modification by immunoblot analysis. Monoubiquitination of *myc*-Pex5p still took place in the *pex1Δ* strain, but not in the *pex1Δ/4Δ* double-deletion strain, indicating that the presence of the AAA peroxin is not required for receptor modification.

monoubiquitination (Kragt et al., 2005). Another attempt to elucidate the order of events was made by Collins and co-workers in *P. pastoris* (Collins et al., 2000). They took advantage of a specific instability of Pex5p in mutant strains lacking components of the AAA and Pex4p–Pex22p complex. Based on the finding that the Pex5p level in a *pex1*Δ/*pex4*Δ strain was reduced to the level of the *pex1*Δ single-mutant strain, it was concluded that Pex4p acts downstream of the AAA peroxins (Collins et al., 2000). In *S. cerevisiae pex1*Δ cells, such a Pex5p instability is not observed, but the protein becomes polyubiquitinated and accumulates at the peroxisomal membrane. Thus, the observed Pex5p instability in other yeasts is likely to be a consequence of polyubiquitination and subsequent proteasomal degradation. In this case, the Pex5p polyubiquitination seems to be part of a quality control system that is not directly related to the import process. Instead of this pathological situation, we now took advantage of the physiological monoubiquitination that is also present in wild-type cells to study the epistasis. The analysis revealed that both *myc*Pex5p and Pex5pK18/24R, which are monoubiquitinated under wild-type conditions, but not in *PEX4*-affected cells, are still monoubiquitinated in a *pex1*Δ strain. In contrast, ubiquitination did not take place in a *pex4Δpex1Δ* double-deletion strain (Fig. 2 C and Fig. S1 B). This result demonstrates that Pex4p-dependent monoubiquitination occurs independently of the presence of the AAA peroxins. Thus, monoubiquitination of Pex5p takes place before the protein is released from the peroxisomal membrane in an AAA peroxin– and ATP-dependent manner. An explanation for the different conclusion drawn by Collins and co-workers is provided by the different nature of the Pex5p fraction analyzed. Collins and co-workers used the instability of Pex5p as an indicator, and thus, most probably analyzed the Pex5p form designated for proteasomal degradation (Collins et al., 2000), a process for which Pex4p has been demonstrated to be dispensable (Platta et al., 2004). Monoubiquitination requires Pex4p, and thus, is likely to represent an important step in the peroxisomal protein import process. Thus, the epistasis on the basis of monoubiquitination is expected to reflect the sequence of events in the Pex5p receptor cycle.

To investigate whether the Pex4p dependence of the monoubiquitination of Pex5p is indeed defined by a direct ubiquitination reaction between both proteins, we established a cell-free ubiquitination assay with recombinant GST-Pex4p. The functionality of the protein was tested by an in vitro autoubiquitination reaction in the presence of recombinant E1 and ubiquitin (Fig. 3 A). As the higher molecular weight forms of GST-Pex4p were resistant to β-mercaptoethanol, they likely represent conjugates of ubiquitin and Pex4p that are linked via

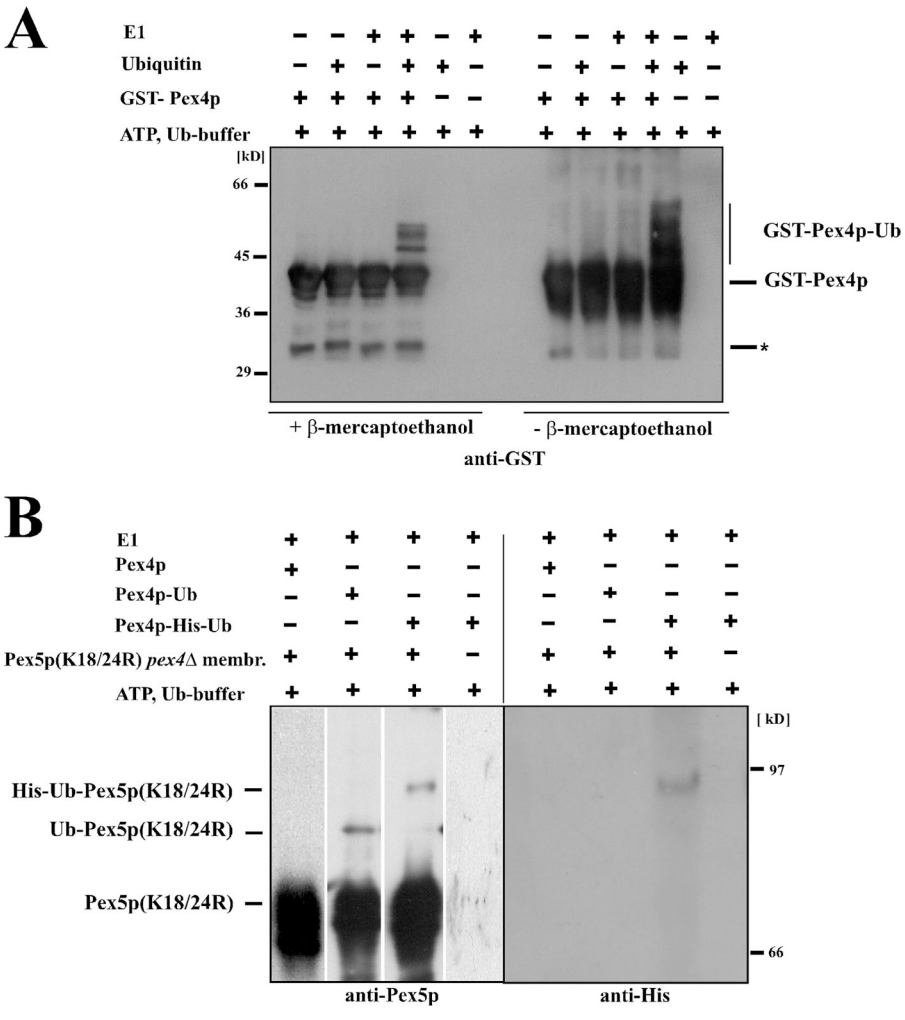

Figure 3. **Pex4p monoubiquitinates the PTS1 receptor in vitro.** (A) Recombinant and purified GST-Pex4p was subjected to an in vitro autoubiquitination reaction. The generation of higher molecular weight forms of GST-Pex4p did depend on the presence of both recombinant E1 and ubiquitin. The formation of the thioester conjugate of GST-Pex4p and Ub was visible in samples without β-mercaptoethanol. The asterisk refers to a degradation product of GST-Pex4p. (B) Membrane fractions prepared from *pex4*Δ + Pex5p(K18/24R) cells were incubated with E1, buffer, and the Ub- or His-Ub–charged GST-Pex4p forms. Only in the presence of Ub- or His-Ub–charged Pex4p- and Pex22p-containing membranes was the appearance of modified Pex5p was observed. His-Ub-Pex5p was also detected with anti-His antibodies. As the His-Ub moiety of Pex5p can only be derived from the charged Pex4p, this result identifies Pex5p as a molecular target for Pex4p-dependent ubiquitination.

a peptide bond to a lysine residue of the E2 enzyme. Ubiquitin-conjugating enzymes bind ubiquitin via a thioester bond to their catalytically relevant cysteine residue within the UBC- fold before they pass it to the lysine of a target protein. The formation of the Pex4p-ubiquitin thioester was monitored by omitting reducing agents. Under these conditions, slower migration species indicated the presence of the thioester linkage of ubiquitin. (Fig. 3 A). Alternatively, the conjugation of His-Ub to GST-Pex4p resulted in the appearance of slower-migrating GST-Pex4p (unpublished data).

In a second step, we prepared membranes from *pex4*Δ*pex5*Δ + Pex5pK18/24R cells, which are known to harbor the required E3 activity. To assay the ubiquitination, the samples were incubated with E1 and Pex4p alone or with Pex4p that has been charged with Ub or His-Ub. Pex5pK18/24R was found to be unmodified in the samples with uncharged Pex4p, while it was ubiquitinated, when Pex4p was preloaded with Ub (Fig. 3 B). Modified Pex5p species with higher molecular weights were observed when Pex4p had been charged with His-Ub. This form was also specifically recognized by the Penta-His antibody. As no additional His-Ub was added to the reaction, the His-Ub acquired by Pex5p had to originate from the Pex4p-His-Ub conjugate, demonstrating a direct ubiquitination reaction. These results identified Pex5p as a molecular target for Pex4p-dependent monoubiquitination.

We then asked whether ubiquitination of Pex5p is a prerequisite for the release of Pex5p from the peroxisomal membrane. Previously, we demonstrated that Pex5p can only be exported from membranes derived from a *pex1*Δ strain when incubated with either cytosol containing the AAA peroxins or the isolated AAA complex (Platta et al., 2005). To test for the ubiquitin requirement, we aimed to delete all possible ubiquitination sites of Pex5p. For the prevention of polyubiquitination, this was achieved by deletion of K18/24R. However, deletion of any of the 15 lysines within the N-terminal half of Pex5p did not abolish the monoubiquitination of the receptor, indicating that the absence of one ubiquitination site could be overcome by using another. Therefore, we took advantage of the fact that Pex5p is not monoubiquitinated in *pex4Δ* cells. The lack of Pex5p monoubiquitination in *pex4Δ* and the prevention of polyubiquitination of Pex5pK18/24R enabled us to separately investigate the contribution of mono- and polyubiquitination to Pex5p

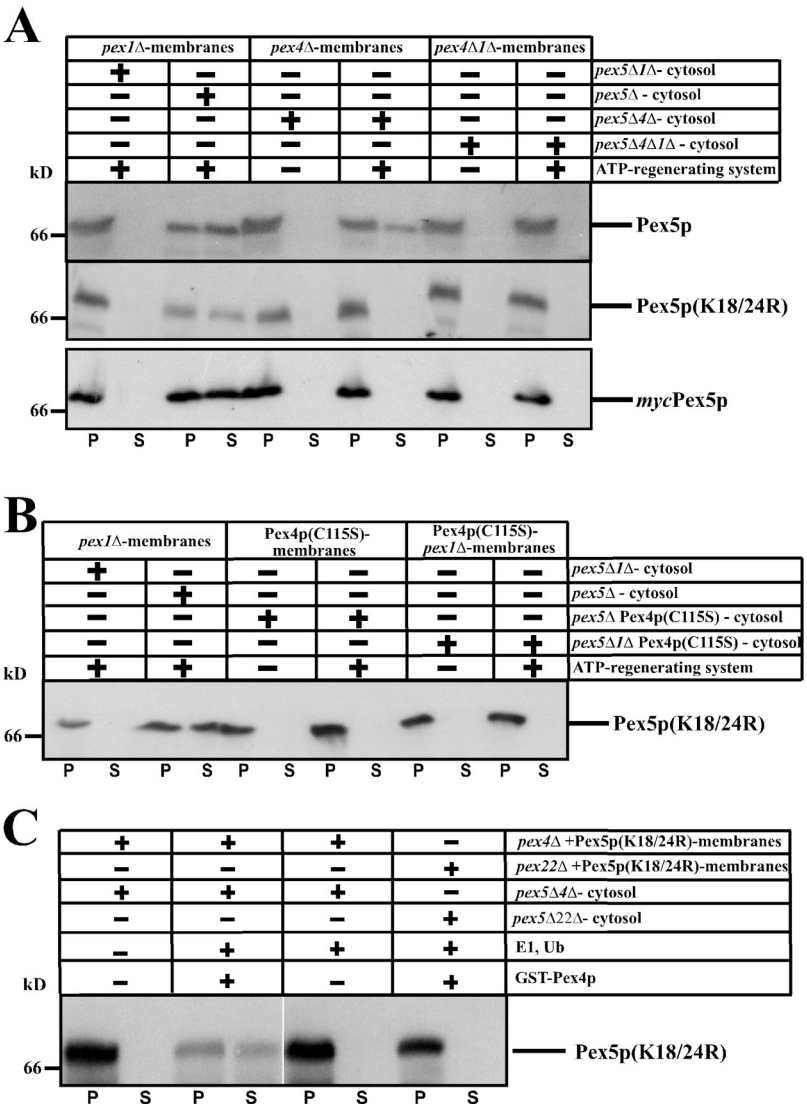

Figure 4. **Receptor release requires ubiquitination.** (A) In vitro export assays were performed with membranes from *pex1Δ*, *pex4Δ*, or *pex1Δ4Δ* cells expressing wild-type Pex5p, Pex5p(K18/24R), or *myc*Pex5p and cytosol fractions, as indicated. Cytosolic supernatant (S) and pellet (P) fraction were analyzed for the presence of Pex5p. Under *PEX4*-deficient conditions, the ATP and AAA peroxin–dependent export of wild-type Pex5p was significantly reduced. No export was observed when *PEX4* deficiency was combined with Pex5p foms lacking polyubiquitination. (B) Receptor release requires the catalytic activity of Pex4p. Pex4p-deficient cells were transformed with the catalytically inactive Pex4p(C115S), and the in vitro export reaction was performed as described in A. No release of the receptor was observed when the deletion of the target lysine residues for polyubiquitination was combined with the loss of the enzymatic activity of Pex4p. (C) Membranes containing Pex5p(K18/24R) were incubated with *pex5Δ4Δ* cytosol, recombinant E1 enzyme, and ubiquitin in the presence and absence of recombinant GST-Pex4p. Adding GST-Pex4p to the Pex22p-containing system did result in the reconstitution of the Pex5p release from the membrane.

release from the peroxisomal membrane. Fig. 4 A shows that Pex5pK18/24R is still exported from the peroxisomal membrane in an AAA peroxin and ATP-dependent manner. Similarily, in a Pex4p-deficient system reflected by *pex4*Δ membranes incubated with a *pex4*Δ*/pex5*Δ cytosol, a fraction of endogenously encoded Pex5p was released from the membrane. This liberation of Pex5p from cells lacking Pex4p still required the presence of ATP and the activity of the AAA peroxins. However, when Pex5pK18/24R was subjected to the export assay in a Pex4p-deficient system, release of the receptor from the membrane was completely blocked. Thus, the simultaneous loss of both polyubiquitination and monoubiquitination of the receptor prevented release of the receptor, demonstrating that Pex5p ubiquitination is required for its release from the membrane.

No release of the Pex5pK18/24R mutant protein was also observed when the wild-type Pex4p was replaced by Pex4p (C115S), demonstrating that the catalytic activity of Pex4p is required for release of the receptor from the membrane (Fig. 4 B). When recombinant GST-Pex4p was added to the Pex4p-deficient export system, as outlined in Fig. 4 C, the Pex5pK18/24R was released from the membrane, thereby unequivocally demonstrating the functional role of Pex4p in Pex5p export. When Pex22p-deficient membranes were subjected to the assay, no Pex5pK18/24R was released from the membrane, demonstrating that anchoring of the recombinant GST-Pex4p to Pex22p is required for its function in Pex5p release.

Our findings show that mono- or polyubiquitination are both sufficient to prepare Pex5p for the AAA-dependent release to the cytosol. The fate of the released mono- or polyubiquitinated Pex5p is proposed to be different, as outlined in the model depicted in Fig. 5. After its release, the polyubiquitinated Pex5p is directed to proteasomal degradation as part of a quality control system. In support of this assumption, prevention of polyubiquitination did not significantly interfere with Pex5p function, or with its AAA peroxin–dependent release from the peroxisomal membrane. The released monoubiquitinated Pex5p is supposed to be deubiquitinated and made available for further rounds of matrix protein import. Interestingly, in our experimental design, only part of the total membrane-bound Pex5p seems to be monoubiquitinated, and the released Pex5p no longer contained the ubiquitin moiety (Fig. 1 D and Fig. 3 A). This is supposed to reflect the situation that Pex5p is only transiently monoubiquitinated and that the ubiquitin is released during the export step. Future research will also reveal whether all subunits of the supposedly homooligomeric Pex5p may require ubiquitination for its release from the membrane or whether ubiquitination of only one subunit of the Pex5p might be sufficient for its release. Our data provide a plausible explanation for the PTS1 import defect of a *PEX4* deletion strain and for the previously observed accumulation of Pex5p in cells lacking Pex4p (van der Klei et al., 1998; Platta et al., 2004). As *pex4*Δ cells also exhibit a PTS2 import defect, one could assume a similar role of Pex4p in the cycle of the PTS2 receptor or the auxiliary proteins Pex18p/Pex21p.

An essential role for polyubiquitination in recycling of Pex5p and Pex18p/Pex21p orthologue Pex20p in *H. polymorpha* and *P. pastoris*, respectively, has also been reported based on an enhanced degradation of these receptors upon overexpression of Ub(K48R) (Kiel et al., 2005b; Leon et al., 2006). The authors suppose that the overexpression of Ub(K48R) might interfere with a constitutive degradation of a thus far unidentified target factor, which certainly is not Pex5p.

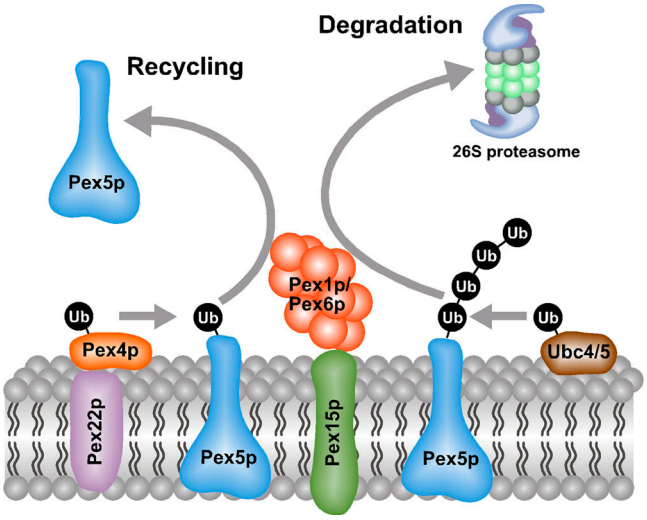

Figure 5. **Model for the ubiquitination and recycling of the PTS1 receptor Pex5p.** Proteins harboring a type 1 peroxisomal targeting signal are recognized by the import receptor Pex5p in the cytosol. Upon association of the cargo-loaded receptor with the docking complex subunits, at least a fraction of Pex5p inserts into the bilayer, and cargo is released into the peroxisomal lumen (not depicted). The membrane-bound Pex5p can be modified by poly- and monoubiquitination, with the polyubiquitination being dependent on the E2 enzymes Ubc4 and Ubc5. Polyubiquitinated Pex5p is recognized by the AAA peroxins Pex1p and Pex6p and directed to proteasomal degradation as a part of a quality control system. Monoubiquitination of Pex5p is mediated by the E2 enzyme Pex4p, which is then recognized by the AAA peroxins Pex1p and Pex6p and released from the peroxisomal membrane to the cytosol, where it is made available for further rounds of matrix protein import.

We have demonstrated that ubiquitination of the Pex5p is a prerequisite for its dislocation from the peroxisomal membrane by the AAA peroxins. Ubiquitination of Pex5p is expected to facilitate the recruitment of the AAA machinery. The functional role of Pex4p/Ubc10p, which is the only ubiquitin-conjugating enzyme known to be involved in the biogenesis of an organelle, has been a mystery for nearly 13 yr. We demonstrate that Pex5p is a molecular target for monoubiquitination by Pex4p and show a direct role for the protein in the membrane release of Pex5p at the end of the import cascade. It has been demonstrated that the ATP-consuming step in this process is not the binding and import of Pex5p, but the AAA peroxin–dependent export of the receptor (Oliveira et al., 2003; Miyata and Fujiki, 2005). As ubiquitination is essential for the recycling of the PTS1 receptor, we have expanded the energy requirement of the peroxisomal import pathway by a second ATP-dependent step, i.e., receptor monoubiquitination.

Materials and methods

Yeast strains and culture conditions

The *S. cerevisiae* strain UTL-7A (MATa, ura3-52, trp1, leu2-3/112; Erdmann et al., 1989) was used as an isogenic wild-type strain for the generation of

wild-type, *pex1Δ* (Platta et al., 2004), *pex1Δ/4Δ* (Platta et al., 2004), *pex1Δ/5Δ* (Platta et al., 2005), *pex4Δ* (Wiebel and Kunau, 1992), *pex4Δ/5Δ* (this study), *pex4Δ/5Δ /1Δ* (this study), *pex5Δ* (Girzalsky et al., 1999), and *pex22Δ5Δ* (this study), as well as *ubc1Δ*, *ubc2Δ*, *ubc4Δ/ubc5Δ*, *ubc6Δ*, *ubc7Δ*, *ubc8Δ*, *ubc11Δ*, and *ubc13Δ* strains (Platta et al., 2004). Deletion strains were generated by the "short flanking homology" method, as previously described (Güldener et al., 1996). Yeast media have been described in another work (Erdmann et al., 1989).

Plasmids and cloning strategies

Pex5p was expressed from a low-copy vector under the control of its own *PEX5* promotor (pHP17-PEX5). Point mutations in *PEX5* were introduced using overlap extension PCR, leading to the PEX5 K–R mutant collection used in this study.

Plasmids expressing *myc*Pex5p (pRS6*myc*$_3$; this study), Pex4p^{C115S} (pEMBL*yex*-PAS2^{Ser}; Wiebel and Kunau, 1992), GFP-PTS1 (Schäfer et al., 2004), ubiquitin (YEp96; Ecker et al., 1987), and ubiquitin (K48R; YEp110; Hochstrasser et al., 1991), as well as *myc*Ubiquitin (YEp105; Ellison and Hochstrasser, 1991), were used.

Recombinant proteins

GST-Pex4p was expressed in *E. coli* BL21(DE3). Cells were harvested, diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, and 1.4 mM KH_2PO_4, pH 7.3), containing protease inhibitors (8 μM antipain, 0.3 μM aprotinin, 1 μM bestatin, 10 μM chymostatin, 5 μM leupeptin, 1.5 μM pepstatin, 1 mM benzamidine, and 1 mM PMSF [Boehringer]) and broken using a French press. The 100,000 *g* supernatant containing the soluble GST-Pex4p was loaded on a glutathione–Sepharose 4B (Pharmacia). After intense washing with PBS buffer, GST-Pex4p was either eluted from the column with 10 mM glutathione or cleaved from the fusion tag with thrombin.

The GST-Pex4p–containing plasmid pGEX-4T-1-PEX4 was a gift of W.-H. Kunau (Ruhr-University Bochum, Bochum, Germany). Recombinant E1 enzyme, ubiquitin, and His-ubiquitin were purchased from Sigma-Aldrich.

In vitro ubiquitination assay

Recombinant Pex4p or GST-Pex4p was charged with recombinant ubiquitin by an in vitro autoubiquitination assay. The reaction mixture contained 0.1 μg E1, 3 μg Pex4p, 5 μg ubiquitin, 2 mM ATP, 2 mM $CaCl_2$, and 25 mM Tris/HCl, pH 7.6 (Ub buffer I). The reaction proceeded for 1.5 h at 30°C and was quenched by addition of SDS-gel sample buffer. The formation of the Pex4p–ubiquitin thioester forms was monitored by using SDS-gel sample buffer without β-mercaptoethanol.

To acquire in vitro monoubiquitination of Pex5pK18/24R in a cell-free system, Pex5pK18/24R-containing yeast membranes were isolated from a *pex4Δpex5Δ* strain, as previously described (Platta et al., 2004). The Pex5pK18/24R samples were dissolved in Ub buffer II (1 mM $ZnSO_4$, 1 mM DTT, 20 mM NEM, 2 mM ATP, 2 mM $CaCl_2$, and 25 mM Tris/HCl, pH 7.6). 0.1 μg E1 and 5 μg Ub- or His-Ub–charged or uncharged Pex4p were added to the reaction mixture. The reaction proceeded for 20 min at 37°C. After precipitation with trichloroacetic acid, the pellets were washed twice with 80% acetone and dissolved in SDS-gel sample buffer.

Miscellaneous

Membrane sedimentation, in vitro import, and export assays were performed according to Platta et al. (2005). If required, 0.1 μg E1, 5 μg ubiquitin, and 5 μg recombinant GST-Pex4p were added to the in vitro export reaction. Protein complexes were isolated by coimmunoprecipitation as described by Girzalsky et al. (1999). TCA lysates of cellular fractions were prepared as described by Platta et al. (2004). Immunoreactive complexes were visualized using anti–rabbit or –mouse IgG-coupled horseradish peroxidase in combination with the ECL system (GE Healthcare). Polyclonal rabbit antibodies were raised against Pex5p (Albertini et al., 1997), Pex13p (Girzalsky et al., 1999), and Fructose-1,6-bisphosphatase (Bigl and Escherich, 1994). Monoclonal mouse antibodies were raised against the C-*myc* epitope (Evan et al., 1985), GST (Sigma-Aldrich), and Penta-Histidin (QIAGEN). The direct fluorescence of GFP was recorded at room temperature in distilled water with an Axiophot microscope (Carl Zeiss MicroImaging, Inc.) and a 100×/1.4 NA oil immersion objective. Both fluorescence and optical photographs were taken by using the connected hardware in combination with the Spot RT software version 3.1 (Diagnostics Instruments). Adjustments of contrast and brightness were performed with Photoshop software version 7.0 (Adobe), and characteristic cells were cut out and copied to Micromedia Freehand software version 10.0.

Online supplemental material

Fig. S1 shows that monoubiquitination of *myc*Pex5p depends on the presence, activity and peroxisomal localization of the ubiquitin-conjugating enzyme Pex4p and occurs before the AAA peroxin Pex1p. The online version of this article is available at http://www.jcb.org/cgi/content/full/jcb.200611012/DC1.

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