Ubiquitination of the peroxisomal import receptor Pex5p

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Proteins harbouring a peroxisomal targeting signal of type 1 (PTS1) are recognized by the import receptor Pex5p in the cytosol which directs them to a docking and translocation complex at the peroxisomal membrane. We demonstrate the ubiquitination of Pex5p in cells lacking components of the peroxisomal AAA (ATPases associated with various cellular activities) or Pex4p–Pex22p complexes of the peroxisomal protein import machinery and in cells affected in proteasomal degradation. In cells lacking components of the Pex4p–Pex22p complex, mono-ubiquitinated Pex5p represents the major modification, while in cells lacking components of the AAA complex polyubiquitinated forms are most prominent. Ubiquitination of Pex5p is shown to take place exclusively at the peroxisomal membrane after the docking step, and requires the presence of the RING-finger peroxin Pex10p. Mono- and poly-ubiquitination are demonstrated to depend on the ubiquitin-conjugating enzyme Ubc4p, suggesting that the ubiquitinated forms of Pex5p are targeted for proteasomal degradation. Accumulation of ubiquitinated Pex5p in proteasomal mutants demonstrates that the ubiquitination of Pex5p also takes place in strains which are not affected in peroxisomal biogenesis, indicating that the ubiquitination of Pex5p represents a genuine stage in the Pex5p receptor cycle.

Key words: Pex5p, peroxin, peroxisome, protein import, ubiquitination.

INTRODUCTION

Peroxisomes are membrane-bound organelles that play important roles in lipid metabolism in virtually all eukaryotes (reviewed in [1]). So far, 32 *PEX* genes have been demonstrated to be indispensable for peroxisome biogenesis. Most of their gene products, collectively named peroxins, are required for protein transport across the peroxisomal membrane from the cytoplasm into the peroxisomal matrix (reviewed in [2–4]). Two well-characterized peroxisomal targeting signals, PTS1 and PTS2, and the corresponding import receptors Pex5p and Pex7p, have been identified (reviewed in [5,6]). Pex5p consists of a C-terminal domain of six TPRs (tetratricopeptide repeats), which provides the binding site for the tripeptide PTS1. The PTS2 receptor is characterized by multiple WD40 repeats. Both receptors are predominantly localized in the cytosol, with a minor portion being associated with the peroxisomal membrane. The receptors are supposed to bind their cargo proteins in the cytosol and target them to the peroxisomal membrane. Whereas Pex5p is able to perform its role in PTS1 protein targeting on its own, the PTS2 receptor Pex7p needs the auxiliary proteins Pex18p and Pex21p [7]. Three peroxins, Pex13p, Pex14p and Pex17p, have been demonstrated to contribute to the docking of the receptors to the *cis*-side of the peroxisomal membrane [8–15]. According to the model of shuttling receptors [16], the import receptors release their cargo at the membrane or into the peroxisomal lumen and cycle back to the cytosol [16,17].

Downstream of the docking complex, a second complex comprising three RING-finger peroxins, Pex2p, Pex10p and Pex12p [18], has been implicated in cargo translocation [5,19]. Most interestingly, peroxisomes accommodate folded, even oligomeric proteins; however, the mechanism of how these proteins traverse the membrane is still unknown. The peroxins Pex1p and Pex6p are AAAs (ATPases associated with various cellular activities), which are required for matrix protein import and which are likely to be responsible for the overall ATP-dependence of the import process [20–23]. Pex4p and Pex22p have been proposed to act at later steps of the import process [18]. Pex4p, also referred to as Ubc10p, is anchored to the peroxisomal membrane by Pex22p and belongs to the Ub (ubiquitin)-conjugating enzyme family [24,25]. Even though the target of Pex4p has not been identified, Pex18p, which is involved in the peroxisomal import of PTS2 proteins, has been demonstrated to be constitutively degraded in a Ub-dependent manner [26].

In the present paper, we demonstrate the Ubc4p-dependent mono- and poly-ubiquitination of the PTS1 receptor Pex5p at the peroxisomal membrane of cells lacking components of the peroxisomal AAA or Pex4p–Pex22p complexes, or of proteasomal mutants. The ubiquitination of Pex5p takes place after the docking event and requires the presence of the RING-finger peroxin Pex10p. We also discuss the functional relevance of the ubiquitination of Pex5p for peroxisome biogenesis.

EXPERIMENTAL

Strains and culture conditions

Saccharomyces cerevisiae strains used in the present study are listed in Table 1. Deletion strains were generated by the 'short flanking homology' method using the removable *loxP-kanMX4-loxP* marker as described in [27]. Yeast complete (YPD) and minimal media (SD) have been described previously [28]. YNO medium contained 0.1 % (w/v) oleic acid, 0.05 % (v/v) Tween 40, 0.1 % (w/v) yeast extract and 0.67 % (w/v) yeast nitrogen base without amino acids, adjusted to pH 6.0. When necessary, auxotrophic requirements were added according to [29]. For induction of the *CUP1* promoter, $CuSO_4$ was added according to [16].

Abbreviations used: AAA, ATPases associated with various cellular activities; CIM, co-lethal in mitogenesis; GFP, green fluorescent protein; PTS, peroxisomal targeting signal; Ub, ubiquitin.

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Table 1 Yeast strains used in the present study

Strain	Description	Source of reference	Oligonucleotides
UTL-7a	MATa, ura3-52, trp1, leu2-3/112	[28]	
*pex1*Δ	*pex1*::loxP	Present study	KU681/KU682
*pex1*Δ *pex4*Δ	*pex1*::loxP, *pex4*::*kanMX4*	Present study	KU394/KU395
*pex1*Δ *ubc1*Δ	*pex1*::loxP, *ubc1*::CreloxP-*kanMX4*	Present study	KU990/KU991
*pex1*Δ *ubc2*Δ	*pex1*::*loxP*, *ubc2*::CreloxP-*kanMX4*	Present study	KU993/KU994
*pex1*Δ *ubc4*Δ	*pex1*::*loxP*, *ubc4*::CreloxP-*kanMX4*	Present study	KU996/KU997
*pex1*Δ *ubc5*Δ	*pex1*::*loxP*, *ubc5*::CreloxP-*kanMX4*	Present study	KU999/KU1000
*pex1*Δ *ubc6*Δ	*pex1*::*loxP*, *ubc6*::CreloxP-*kanMX4*	Present study	KU938/KU939
*pex1*Δ *ubc7*Δ	*pex1*::*loxP*, *ubc7*::CreloxP-*kanMX4*	Present study	KU941/KU942
*pex1*Δ *ubc8*Δ	*pex1*:*loxP*, *ubc8*::CreloxP-*kanMX4*	Present study	KU944/KU945
*pex1*Δ *ubc11*Δ	*pex1*::*loxP*, *ubc11*::CreloxP-*kanMX4*	Present study	KU947/KU948
*pex1*Δ *ubc12*Δ	*pex1*::*loxP*, *ubc12*::CreloxP-*kanMX4*	Present study	KU917/KU918
*pex1*Δ *ubc13*Δ	*pex1*::*loxP*, *ubc13*::CreloxP-*kanMX4*	Present study	KU1002/KU1003
*pex4*Δ	*pex4*::*LEU2*	[24]	
*pex4*Δ *ubc1*Δ	*pex4*::*LEU2*, *ubc1*::CreloxP-*kanMX4*	Present study	KU990/KU991
*pex4*Δ *ubc2*Δ	*pex4*::*LEU2*, *ubc2*::CreloxP-*kanMX4*	Present study	KU993/KU994
*pex4*Δ *ubc4*Δ	*pex4*::*LEU2*, *ubc4*::CreloxP-*kanMX4*	Present study	KU996/KU997
*pex4*Δ *ubc5*Δ	*pex4*::*LEU2*, *ubc5*::CreloxP-*kanMX4*	Present study	KU999/KU1000
*pex4*Δ *ubc6*Δ	*pex4*::*LEU2*, *ubc6*::CreloxP-*kanMX4*	Present study	KU938/KU939
*pex4*Δ *ubc7*Δ	*pex4*::*LEU2*, *ubc7*::CreloxP-*kanMX4*	Present study	KU941/KU942
*pex4*Δ *ubc8*Δ	*pex4*::*LEU2*, *ubc8*::CreloxP-*kanMX4*	Present study	KU944/KU945
*pex4*Δ *ubc11*Δ	*pex4*::*LEU2*, *ubc11*::CreloxP-*kanMX4*	Present study	KU947/KU948
*pex4*Δ *ubc12*Δ	*pex4*::*LEU2*, *ubc12*::CreloxP-*kanMX4*	Present study	KU917/KU918
*pex4*Δ *ubc13*Δ	*pex4*::*LEU2*, *ubc13*::CreloxP-*kanMX4*	Present study	KU1002/KU1003
*pex5*Δ	*pex5*::*kanMX4*	[35]	
*pex6*Δ	*pex6*::*LEU2*	[58]	
*pex10*Δ	*pex10*::*kanMX4*	[43]	
*pex13*Δ	*pex13*::URA3	[35]	
*pex15*Δ	*pex15*::LEU2	[58]	
*pex4*Δ*pex10*Δ	*pex4*::*kanMX4*, *pex10*::CreloxP-*kanMX4*	Present study	KU562/KU699
cim5-1	*cim5-1*, *ura3-52*, *leu2Δ1*, *his3Δ200*	[53]	
*cim5-1pex5*Δ	*pex5*::CreloxP-*kanMX4*, *cim5-1*	Present study	KU301/KU302
*cim5-1pex1*Δ	*pex1*::CreloxP-*kanMX4*, *cim5-1*	Present study	KU681/KU682

Oligonucleotides and plasmids

Oligonucleotides used are listed in Table 2. Ub and *myc*Ub were expressed under the control of the copper-inducible *CUP1* promoter [30] from plasmid YEp96 and YEp105 [31] respectively. For expression of Ub-K48R plasmid YEp110 was used [32].

Yeast cell extracts

Yeast cells were grown on 0.3 % SD medium to late exponential phase and subsequently for 15 h in YNOD [0.1 % (w/v) dextrose, 0.1 % (w/v) oleic acid, 0.05 % (v/v) Tween 40, 0.1 % (w/v) yeast extract and 0.67 % (w/v) yeast nitrogen base]. Cells were harvested and aliquots of 30 mg of cells were resuspended in 300 μl of potassium phosphate buffer (pH 7.4) containing 20 % trichloroacetic acid. The samples were frozen at -80 °C for at least 30 min. Samples were sedimented, washed twice with ice-cold 50 % acetone and resuspended in 80 μl of 10 % (w/v) SDS/0.1 M NaOH and 20 μl of SDS loading buffer [5 % (w/v) 2-mercaptoethanol, 15 % (v/v) glycerol and 0.01 % (w/v) Bromophenol Blue].

Membrane sedimentation

Oleic-acid-induced cells were washed with water and 1 g was used per sedimentation. A volume of 3 ml of buffer A (0.2 M Hepes, 1 M potassium acetate and 50 mM magnesium acetate, pH 7.5), 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 Mannheim), 5 mM NaF and 3 g of glass beads (0.5 mm) was added to the cells. Breakage was achieved by vortex-mixing for 12 min (twelve 60 s bursts with breaks of at least 60 s on ice) [33]. Samples were transferred to Corex tubes and were centrifuged at 1500 ***g*** for 10 min. Supernatants were normalized for protein and volume, and membranes were sedimented at 40 850 rev./min for

Table 2 Oligonucleotides used in the present study

Oligonucleotide	Sequence
KU0394	5′-TGGAGACAACATAAAATATACATAATCATCGCCTTTATACATAATCGTACGCTGCAGGTCGAC-3′
KU0395	5′-GAGGGCCCATTGTTTGCCATTCGAACACATCCATCCTACGTGGTAATCGATGAATTCGAGCTCG-3′
KU0562	5′-GAGGGCGAAGTAGGTATTAGCCGTTTACATTAGAAAATAAGGTAGCGTACGCTGCAGGTCGAC-3′
KU0681	5′-GGACGGCAGTAACAAGAAACACCTGAGGAACTGCTCTTTCAACAGCTGAAGCTTCGTACGCT-3′
KU0682	5′-CAGCCGCATTTTTTGCCCTTTAAAGGGAAACGCGCTCCGTTCATAGGCCACCAGTGGATCTG-3′
KU0699	5′-GGCCTGTGGACAATGCTAAAAGAGTAGTCAAATTATTGACCAATAGGCCAGTAGTGGATCTG-3′
KU0990	5′-AGTCAATTGAAGCAAGTGTGACGACATAAGTATCGTAATTCAGCTGAAGCTTCGTACGCT-3′
KU0991	5′-TGATCATCAAGAACACGGATGCACAATGAAACATCCTCGCATAGGCCACTAGTGGATCTG-3′
KU0993	5′-ACTATCAAGTTGCAGAGTTGATGACATAAATAGTAGAGTGCAGCTGAAGCTTCGTACGCT-3′
KU0994	5′-GGTAATCGAATTCATAATATCGGCTCGGCATTCATCATTAATAATAGGCCACTAGTGGATCTG-3′
KU0996	5′-ATTTCACTGACTATAGAGTACATACATAAACAAGCATCCACAGCTGAABCTTCGTACGCT-3′
KU0997	5′-ACTGAAATATATGCGGGATATACCCGCGCTTGCGTGCATGATAGGCCACTAGTGGATCTG-3′
KU0999	5′-TCCAAGGTCAGGACTGCTTATTGACTACCATCTTGAAAAGCAGCTGAAGCTTCGTACGCT-3′
KU1000	5′-GCGCTGAGGAAGGTAAGTCTACACAATTTATCCGTTAGCCATAGGCCACTAGTGGATCTG-3′
KU0938	5′-TAGGAGCCGTGATAAAGAAGACTACCATCGCATATCGCAGCAGCTGAAGCTTCGTACGCT-3′
KU0939	5′-TGTCTATATAATACTATTGATTTCTATTCTGTGTTGTCAAATAGGCCACTAGTGGATCTG-3′
KU0941	5′-GGAACTTCCCTAGTAATAGTGTAATTTGGAAGGGCATAGCCAGCTGAAGCTTCGTACGCT-3′
KU0942	5′-GTTAAAAGGAAGACCAAATGATCATTAACCTGCTACCTGCATAGGCCACTAGTGGATCTG-3′
KU0944	5′-GTGAGCACCGGAAACAGCTATCTGCGCTTAAGTATCACTACAGCTGAAGCTTCGTACGCT-3′
KU0945	5′-TTGTAATTATAGTCGTACGTGTGGTATAGTATGCCATCATATAGGCCACTAGTGGATCTG-3′
KU0947	5′-AGGTGCTACAAAACCTGTATGATTCGAGGCTTGCTAGTATCAGCTGAAGCTTCGTACGCT-3′
KU0948	5′-CGCCGTAGAAAAATAGCATTGGAGTTTGAGAGTCTGTGGCATAGGCCACTAGTGGATCTG-3′
KU0917	5′-GTACGTAGATGCAACTATATCTTTCCGTTTCTCATTGGCGGTCAGCTGAAGCTTCGTACGCT-3′
KU0918	5′-AGCAAGATGTTACAAGGCGACAACTCGCCTAGTATGTCATAGGCCACTAGTGGATCTG-3′
KU1002	5′-GGATAAGTGATTCATTGAAAGATGGCGCGCCACCACGAACCAGCTGAAGCTTCGTACGCT-3′
KU1003	5′-ACATTAGAGTAGGACGGTCGATCCCGGCTTCCATGGAACGATAGGCCACTAGTGGATCTG-3′

30 min in a Sorvall AH650 rotor. The resulting pellet was resuspended in buffer A corresponding to the volume of the supernatant. Aliquots of the samples were analysed by SDS/PAGE.

Immunoblotting

Immunoblot analysis was performed according to standard protocols [34]. Immunoreactive complexes were visualized using horseradish-peroxidase-conjugated anti-rabbit or anti-mouse IgG in combination with the ECL® (enhanced chemiluminescence) system from Amersham Biosciences (Uppsala, Sweden). Polyclonal rabbit antibodies were against Pex5p [35], Pex13p [35] and fructose-1,6-bisphosphatase [36].

Microscopy

Analysis of live cells for GFP (green fluorescent protein) fluorescence was performed with a Zeiss Axioplan microscope and AxioVision 4.1 software (Zeiss, Jena, Germany). Before inspection, cells were grown for 2 days on solid minimal medium containing oleic acid as a sole carbon source.

Cell fractionation

Spheroplasting of yeast cells, homogenization and differential centrifugation at 25000 ***g*** of homogenates were performed as described previously [28].

RESULTS

Pex5p is modified in specific *pex* mutants

Previous studies have demonstrated that the steady-state concentration of Pex5p is influenced by the presence or absence of certain peroxins. Defects in either one of the human AAA peroxins Pex1p or Pex6p, as well as the Ub-conjugation enzyme Pex4p or its membrane anchor protein Pex22p of *Pichia pastoris*, resulted in a massive decrease of Pex5p level in these cells [17,18,37,38]. In order to analyse these observations in *S. cerevisiae*, we examined the abundance of Pex5p in 27 null mutant strains, each deleted for one of the known peroxins. Wild-type and mutant strains were cultured on oleic acid medium, and whole-cell lysates were prepared and analysed for the presence of Pex5p by immunoblotting. Although *pex* mutants cannot grow on oleic acid medium, induction of β-oxidation and proliferation of peroxisomes still takes place. In contrast with the above-mentioned observations for human and *P. pastoris* cells, the Pex5p amount within the different mutant strains of *S. cerevisiae* was not reduced, and Pex5p was indistinguishable from the wild-type protein in its size (results not shown). However, we noted that in *pex1*Δ, *pex6*Δ, *pex15*Δ, *pex4*Δ and *pex22*Δ mutant strains, additional higher-molecular-mass forms of Pex5p were labelled with the anti-Pex5p serum (Figure 1). Most interestingly, the mutants could be divided into two groups with respect to the Pex5p-modification pattern. The first group comprises the *pex1*Δ, *pex6*Δ and *pex15*Δ mutants, which were characterized by the presence of three additional protein bands for Pex5p. Group II consists of mutant *pex4*Δ and *pex22*Δ, which exhibit two additional bands, the higher one with the same migration behaviour as one of the bands of group I (Figure 1).

Pex5p is ubiquitinated in *pex1*Δ and *pex4*Δ mutant cells

The observed ubiquitination of Pex18p in yeast and the rapid degradation of Pex5p in other organisms tempted us to analyse whether the higher-molecular-mass species of Pex5p form by ubi-

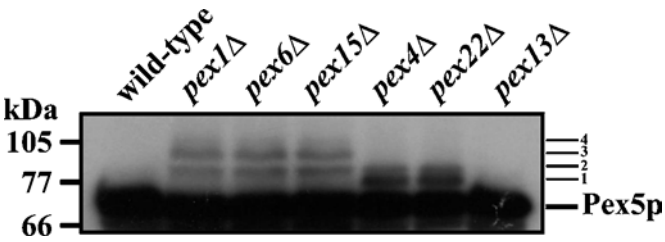


Figure 1 Pex5p-modification in *pex* mutants

Equal amounts of whole-cell trichloroacetic acid lysates of oleic-acid-induced wild-type, *pex1*Δ, *pex6*Δ, *pex15*Δ, *pex4*Δ, *pex22*Δ and *pex13*Δ cells were separated by SDS/PAGE and blotted on to a nitrocellulose filter. The filter was probed for the presence of Pex5p, and immunoreactive complexes were visualized with the ECL® system. Additional higher-molecular-mass forms of Pex5p are labelled 1–4.

quitination of the protein. In support of this assumption, the higher-molecular-mass species could be labelled with anti-Ub serum by immunoblot analysis (results not shown). For the ultimate proof of Pex5p ubiquitination, we also analysed yeast strains expressing either the wild-type Ub or the *myc*Ub fusion gene under the control of the yeast copper metallothionein (*CUP1*) promoter [31]. The epitope tag consists of a ten-residue sequence encoding a portion of the c-*myc* proto-oncogene product recognized by the monoclonal antibody 9E10 [39]. The *myc*Ub variant is about 1.5 kDa larger than wild-type Ub, but is indistinguishable from wild-type Ub in its ability to be enzymically conjugated to and cleaved from acceptor molecules [31]. If the high-molecular-mass species of Pex5p were Ub conjugates, *in vivo* substitution of the Ub with the larger *myc*Ub should lead to a decrease in electrophoretic mobility of these species. Wild-type, as well as *pex1*Δ and *pex4*Δ strains expressing either Ub or *myc*Ub, were grown on oleic acid medium with $CuSO_4$, which leads to an expression of plasmid-encoded Ub and *myc*Ub of up to 50–100 times greater than the endogenously produced levels of Ub [30]. Whole-cell lysates were prepared, and equal amounts of protein from each strain were separated by SDS/PAGE and processed for immunoblotting. Samples were probed with anti-Pex5p antibodies and slower migrating Pex5p species appeared (Figure 2). Comparison of samples from *pex1*Δ and *pex4*Δ expressing either Ub or *myc*Ub showed that the putative ubiquitinated species of Pex5p were replaced by slightly larger new bands. These data showed that overexpression of *myc*Ub was accompanied by an increase in size of all higher-molecular-mass Pex5p-species. Thus our data clearly demonstrate that Pex5p is ubiquitinated *in vivo*. In the following, the ubiquitinated Pex5p is referred to as Ub-Pex5p.

Ubiquitination of Pex5p only takes place at the peroxisomal membrane after receptor docking

Pex5p is predominantly cytosolic, with a portion also being localized at the peroxisomal membrane (for review see [4,40,41]). In order to analyse where in the cell the Pex5p ubiquitination takes place, we tested subcellular fractions of *pex1*Δ and *pex4*Δ and *pex1*Δ*pex4*Δ cells for the presence of Ub-Pex5p. The cells were lysed by treatment with glass beads, and the resulting crude extract was separated into a 100000 ***g*** supernatant (containing soluble proteins) and a membranous pellet fraction. These fractions were analysed for the presence of Pex5p and Pex13p, as well as cytosolic fructose-1,6-bisphosphatase (Fbp1p; [42]) as a control for proper separation. Pex13p, but not Fbp1p, did sediment, indicating the complete sedimentation of cytosol-free peroxisomal membranes (Figure 3A). In all strains (wild-type, *pex1*Δ and

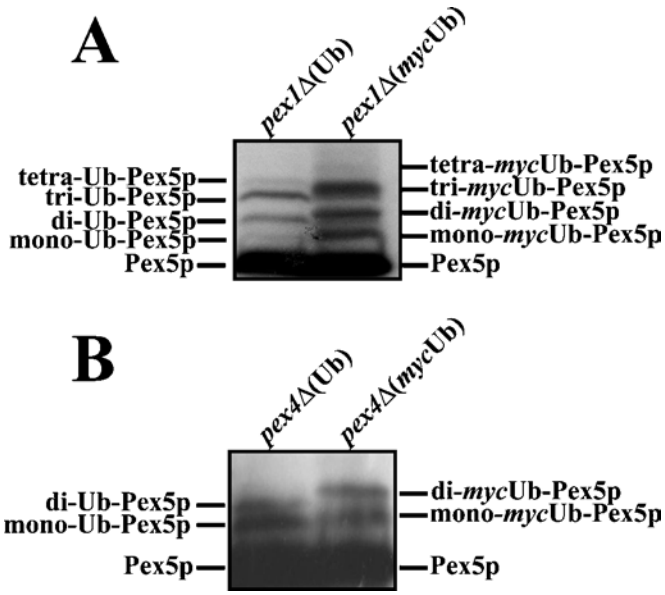


Figure 2 Ubiquitination of Pex5p

Cells of (**A**) *pex1*Δ or (**B**) *pex4*Δ bearing plasmids YEp96 and YEp105 [31] containing the genes coding for normal Ub or tagged Ub (*myc*Ub) respectively were grown in the presence of $CuSO_4$ to induce their expression from the *CUP1* promoter. Whole-cell trichloroacetic acid lysates were separated by SDS/PAGE, blotted on to nitrocellulose filters and labelled with anti-Pex5p antibodies. The shift of Pex5p forms in cells upon expression of *myc*Ub proves Pex5p to be ubiquitinated.

*pex4*Δ, as well as the *pex1*Δ*pex4*Δ double-mutant strain), Pex5p was localized to the 100000 ***g*** supernatant and pellet fraction. Ub-Pex5p found in the homogenate of these cells was totally recovered in the 100000 ***g*** sediment fraction. We conclude that Pex5p ubiquitination of Pex5p does not occur in the cytosol, but only takes place at the peroxisomal membrane.

Previously, it has been reported that the receptor docking complex consisting of Pex13p, Pex14p and Pex17p is associated in a Pex8p-dependent state with the three RING-finger proteins Pex2p, Pex10p and Pex12p, which also form a complex [43]. To investigate whether the ubiquitination of Pex5p already takes place after docking and whether it requires the presence of the RING-finger peroxins, we tested whether Ub-Pex5p is present in cells lacking Pex10p, an essential component of the RING-finger complex. Deletion of either one of the RING-finger proteins is known to dissociate the RING-finger complex, but docking of the receptors remains unaffected [44]. As shown in Figure 3(B), ubiquitinated Pex5p was not visible in whole-cell extracts of the *pex10*Δ strain (Figure 3B). The same was true for the combination of *pex4*Δ and *pex10*Δ. Taken together, our data demonstrate that Pex5p is ubiquitinated after receptor docking, and ubiquitination depends on the presence of Pex10p.

Ubiquitination of Pex5p in *pex1*Δ and *pex4*Δ cells depends on Ubc4p

The enzymic cascade for Ub conjugation is organized hierarchically. There is one Ub-activating enzyme (E1), a significant, but limited, number of Ub-conjugating enzymes (E2), and a much larger number of Ub ligases (E3). Each E3 recognizes a set of substrates that shares one or more ubiquitination signals, and co-operates with one or a few E2s (for review see [45]). In *S. cerevisiae*, there are 13 E2s known, one of which is Pex4p, also known as Ubc10p [24].

In order to analyse whether one of the known Ubc proteins is responsible for the Pex5p ubiquitination, combinations of *pex1*Δ or *pex4*Δ mutants with deletions of one of the *UBC* genes were constructed. Due to the fact that *ubc3*Δ and *ubc9*Δ exhibit a lethal

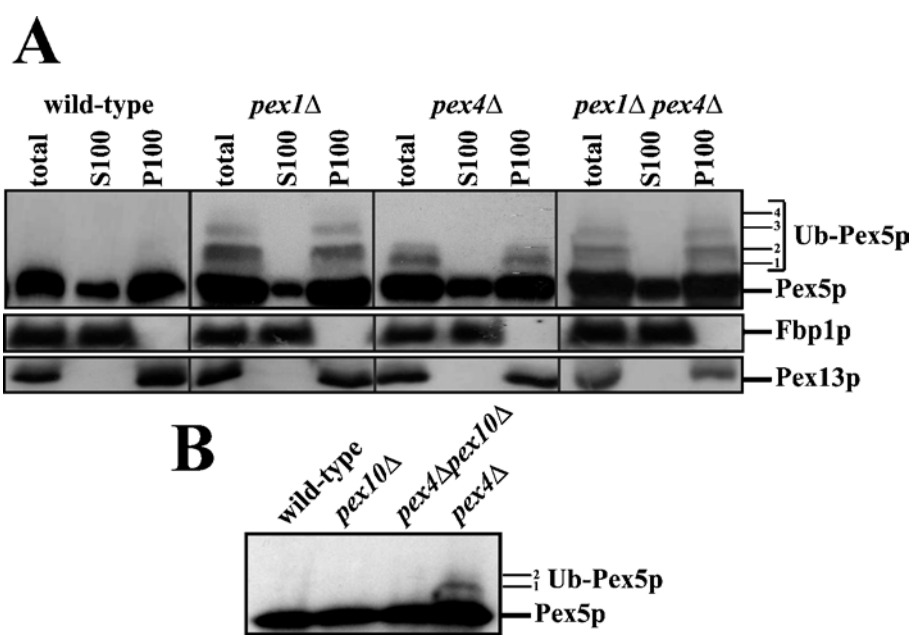


Figure 3 Pex5p ubiquitination takes place at the peroxisomal membrane after the docking step

(**A**) Homogenates of oleic-acid-induced cells derived from wild-type, *pex1*Δ, *pex4*Δ and *pex1*Δ*pex4*Δ were separated by differential centrifugation into a 100000 ***g*** supernatant (S100) and a pellet (P100) fraction. Equal portions of each fraction were analysed by immunoblotting. Pex13p, but no portion of the cytosolic fructose-1,6-bisphosphatase (Fbp1p), was found in the membrane sediment, indicative of proper separation. All Ub-Pex5p found in the homogenate (labelled 1–4) was recovered in the 100000 ***g*** pellet. (**B**) Whole-cell trichloroacetic acid lysates of wild-type, *pex10*Δ, *pex4*Δ and *pex4*Δ*pex10*Δ double-mutant strains were analysed for Pex5p ubiquitination by immunoblotting with anti-Pex5p antibodies. No Ub-Pex5p was visible in a *pex10*Δ or a *pex4*Δ*pex10*Δ strain, indicating that the presence of Pex10p is required for Pex5p ubiquitination.

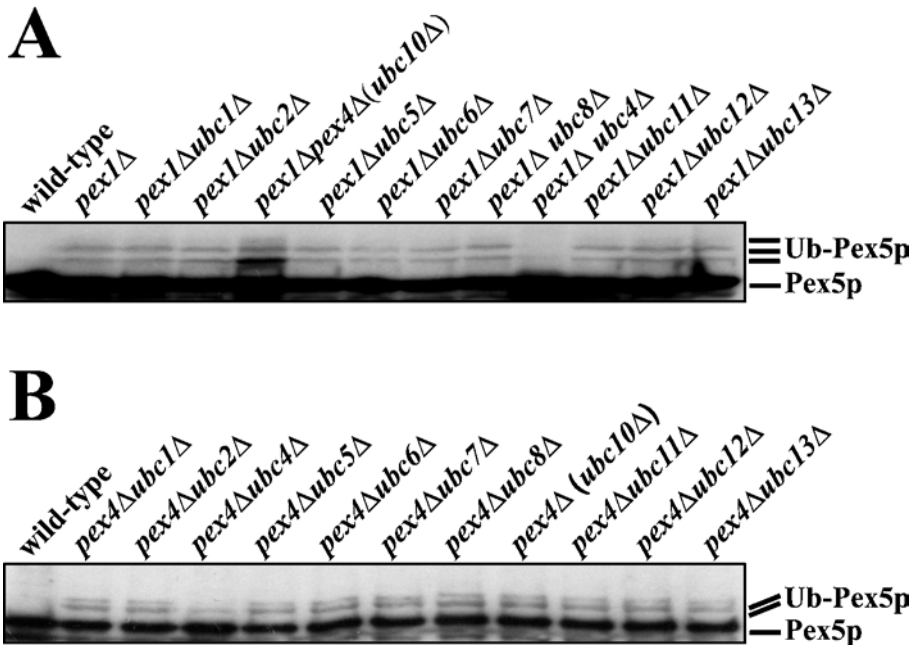


Figure 4 Pex5p ubiquitination depends on Ubc4p

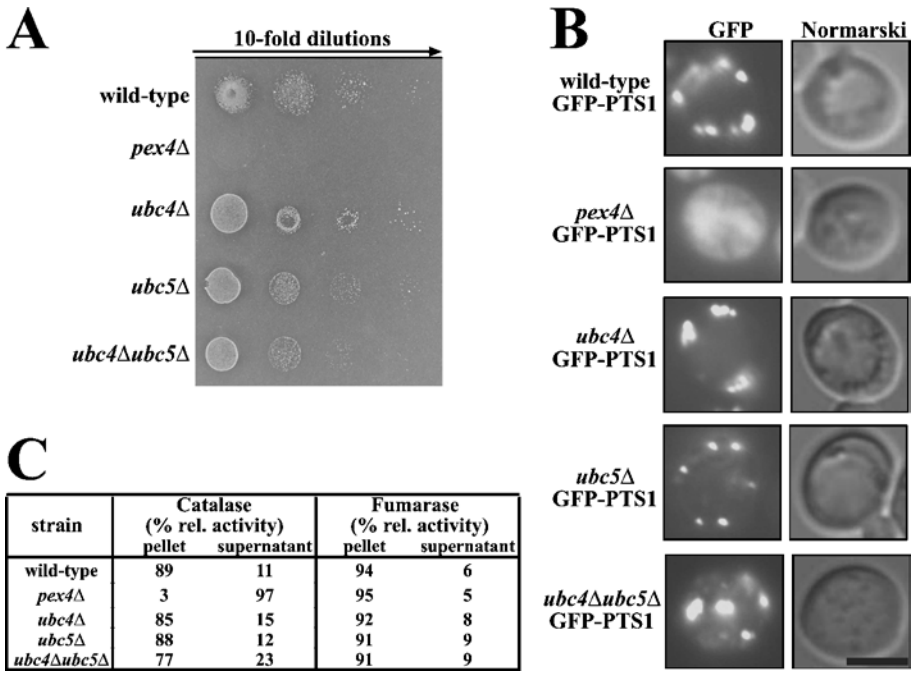
Analysis of (**A**) *pex1Δubc Δ* and (**B**) *pex4 Δubc Δ* double-mutants. Whole-cell lysates of indicated mutant strains were analysed for Pex5p ubiquitination by immunological detection with anti-Pex5p antibodies. Ub-Pex5p was significantly decreased in strains with deletions of *PEX1* or *PEX4* combined with deletion of *UBC4*.

phenotype [32,46,47], these combinations were omitted. Whole-cell lysates of the wild-type, *pex1Δ* and *pex1Δubc Δ* or *pex4Δ* and *pex4Δubc Δ* mutants were subjected to SDS/PAGE and immunologically probed for the presence of Pex5p and its ubiquitinated forms. The ubiquitinated Pex5p was clearly visible in samples derived from the *pex1Δ* or *pex4Δ* single mutant. With one exception, the combinations of *pex1Δ* and *pex4Δ* with the different *ubc Δ* did exhibit the same modification pattern as the corresponding single mutant strains of *pex1Δ* (Figure 4). Most interestingly, the modification pattern observed in the *pex1Δpex4Δ* strain appeared to be an assembly of the different patterns observed in the single mutants. However, deletion of either *pex1Δ* or *pex4Δ* combined with *ubc4Δ* reduced the Ub-Pex5p drastically. These data indicate that Ubc4p is required for Pex5p ubiquitination. One of the ubiquitinated forms of Pex5p was still visible in the *pex4Δubc4Δ* strain, although with a significantly reduced intensity. It has been reported that Ubc4p often exhibits overlapping function with Ubc5p [48]. However, deletion of both Ubcs together in a *pex4Δ* background did not result in a complete vanishing of the Ub-Pex5p (results not shown). This might be due to the fact that Ubc1p is also able to substitute for the lack of Ubc4p and Ubc5p [49].

Our data demonstrate that Ubc4p is involved in Pex5p ubiquitination. To determine whether deletion of *UBC4* also results in a loss of peroxisomal function, we analysed the growth behaviour of wild-type and different deletion strains on oleic acid as the sole carbon source. In contrast with wild-type cells, cells deficient in *PEX4* were unable to grow on oleic acid, as typical for *S. cerevisiae* mutant strains that are defective in peroxisome metabolism or biogenesis [28]. Strains *ubc4*Δ and *ubc5*Δ grew at the wild-type rate (Figure 5A). Notably, the *ubc4*Δ*ubc5*Δ strain demonstrated a slightly reduced growth on oleic acid, indicating that both proteins play a role in peroxisomal function (Figure 5A).

To elucidate further the basis of the reduced growth on oleic acid of the *ubc4*Δ*ubc5*Δ strain, we investigated the intracellular localization of peroxisomal matrix proteins. GFP–SKL (Ser-Lys-Leu) was used for analysing the PTS1- and therefore Pex5p-dependent import by fluorescence microscopy analysis. The protein was imported into peroxisomes of wild-type cells as demonstrated by the peroxisome-characteristic punctate staining (Figure 5B). As expected, GFP–SKL caused diffuse staining in the *pex4*Δ mutant, which is defective in PTS1- and PTS2-dependent import. Strains *ubc4*Δ and *ubc5*Δ were indistinguishable from wild-type cells in terms of their fluorescence pattern. However, when *ubc4*Δ and *ubc5*Δ were combined in a double-mutant strain, GFP–SKL caused a punctate staining like that in wild-type cells, but also a cytosolic fluorescence pattern (Figure 5B). These data suggest that a partial import defect is a consequence of combined deletion of *UBC4* and *UBC5*.

To quantify this import defect, we analysed the subcellular distribution of the peroxisomal matrix protein catalase (PTS1-containing peroxisomal matrix protein) by cell fractionation analysis of wild-type, *pex4*Δ, *ubc4*Δ, *ubc5*Δ and *ubc4*Δ*ubc5*Δ double-mutant strain. The different strains were grown on oleic acid and were subjected to subcellular fractionation to give rise to a 25000 ***g*** pellet enriched for peroxisomes and mitochondria, and a 25000 ***g*** supernatant enriched for cytosol. As expected, the majority of the peroxisomal matrix protein catalase as well as mitochondrial fumarase were detected in the 25000 ***g*** pellet of wild-type cells (Figure 5C). In contrast, in *pex4*Δ cells, catalase was preferentially localized to the 25000 ***g*** supernatant consistent with its mislocalization to the cytosol. Again, *ubc4*Δ and *ubc5*Δ cells were similar to wild-type strain in terms of the distribution of mitochondrial and peroxisomal marker proteins. However, deletion of both *UBC4* and *UBC5* led to a doubling in the amount of cytosolic catalase (Figure 5C). As in the *ubc4*Δ*ubc5*Δ strain, the distribution of mitochondrial fumarase between organellar pellet and cytosol was indistinguishable from samples derived from wild-type, indicating that the observation was not due to a higher breakage of organelles. Consequently, the sedimentation



C

strain	Catalase (% rel. activity) pellet	Catalase (% rel. activity) supernatant	Fumarase (% rel. activity) pellet	Fumarase (% rel. activity) supernatant
wild-type	89	11	94	6
pex4Δ	3	97	95	5
ubc4Δ	85	15	92	8
ubc5Δ	88	12	91	9
ubc4Δubc5Δ	77	23	91	9

Figure 5 Ubc4p and Ubc5p are required for proper protein import into peroxisomes

(**A**) Growth behaviour of *S. cerevisiae* strains on oleic acid as the sole carbon source. Serial dilutions of wild-type, *pex4*Δ, *ubc4*Δ, *ubc5*Δ and *ubc4*Δ*ubc5*Δ were spotted on to plates containing oleic acid, and incubated for 2–7 days at 30 °C. (**B**) Localization of a PTS1-reporter protein GFP–PTS1. Strains expressing GFP–PTS1 were examined for GFP fluorescence as indicated. Structural integrity of the cells is documented by bright-field microscopy. Scale bar, 5 μm. (**C**) Subcellular distribution of peroxisomal and mitochondrial marker enzymes in oleic-acid-induced strains. After centrifugation of cell-free homogenates at 25 000 ***g***, sediments and supernatants were assayed for peroxisomal catalase as well as for mitochondrial fumarase relative (rel.) activities.

data corroborate the fluorescence microscopic analysis supporting the notion that the lack of Ubc4p and Ubc5p results in a partial mislocalization of peroxisomal matrix proteins to the cytosol.

Polyubiquitination of Pex5p

Recognition of proteins destined for the Ub-mediated proteolysis pathway requires formation of a polyubiquitin chain. The Ub molecules are linked within this chain by isopeptide bonds connecting the C-terminal Gly^{76} of one Ub moiety to the ε-amino group of Lys^{48} of the adjacent Ub molecule [45]. Although Lys^{29} and Lys^{63} also have been found to function as sites for polyubiquitination [50], Lys^{48} is the primary site of this process. Ub with a substitution of Lys^{48} by an arginine (Ub-K48R) can still be conjugated to other proteins, but fails to function as an acceptor within the polyubiquitin chain [51]. Therefore Ub-K48R can serve as a probe for monitoring the presence of a Lys^{48}-linked polyubiquitin chain in a protein of interest. The appearance of several ubiquitinated Pex5p species suggested that these represent different states of ubiquitination. To investigate whether the observed modifications are generated by multiple mono-ubiquitination or by polyubiquitination, we compared the Pex5p pattern of *pex1*Δ and *pex4*Δ cells expressing either plasmid-encoded wild-type-Ub or Ub-K48R. The Pex5p pattern in both, *pex1*Δ and *pex4*Δ cells expressing wild-type Ub was indistinguishable from untransformed mutant cells. However, expression of Ub-K48R resulted in a significant decrease of the upper bands of Pex5p in *pex1*Δ cells (Figure 6A) and *pex4*Δ cells (Figure 6B), whereas the lower band was still visible or increased. The fact that the polyubiquitinated forms did not completely disappear upon expression of the Ub-R48K is explained by the fact that the endogenous wild-type Ub is still expressed, albeit much lesser than the mutant form. From these data, we conclude that linkage

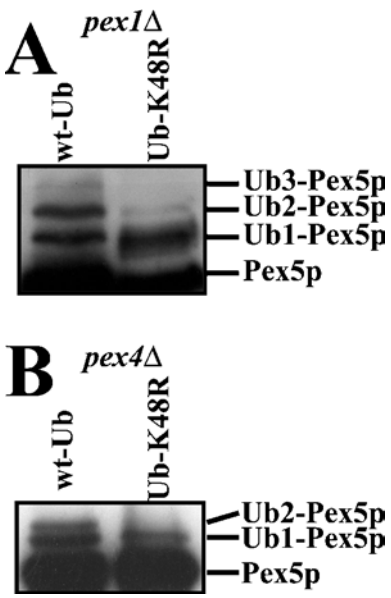


Figure 6 Polyubiquitination of Pex5p

(**A**) *pex1*Δ or (**B**) *pex4*Δ cells transformed with either a plasmid encoding wild-type Ub (wt-Ub) or mutant Ub (Ub-K48R) were grown in the presence of $CuSO_4$. Whole-cell lysates of the strains were analysed for the ubiquitination of Pex5p by immunological detection with anti-Pex5p antibodies. Expression of mutant Ub results in a disappearance of the upper Ub-Pex5p band(s), indicative of polyubiquitination of Pex5p.

of a polyubiquitin chain containing a Lys^{48} linkage is necessary to form the upper Pex5p-modification, which thus represents polyubiquitinated Pex5p, while the lower band most likely represents mono-ubiquitinated Pex5p.

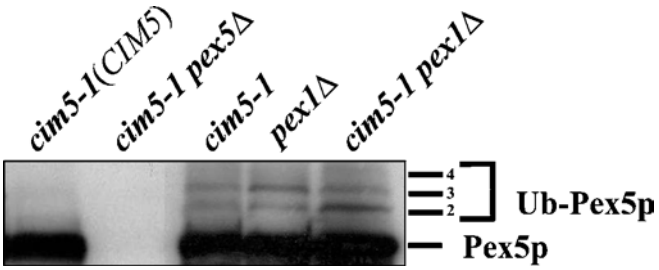


Figure 7 Ubiquitinated Pex5p accumulates in the proteasomal-mutant strain *cim5-1*

Strains were grown on oleic acid at the non-permissive temperature of 37 °C. Whole-cell trichloroacetic acid lysates were prepared and equal amounts thereof were separated by SDS/PAGE and blotted on to a nitrocellulose filter. The filter was probed for the presence of Pex5p and immunoreactive complexes were visualized with the ECL® system. Ubiquitinated forms of Pex5p are labelled 2–4.

Proteasomal degradation of ubiquitinated Pex5p

Lys[48]-dependent polyubiquitinated proteins are thought to be recognized and degraded by the proteasome, an essential high-molecular-mass protease present in the cytoplasm and the nucleus of all eukaryotic cells. Studies with proteasome inhibitors have shown that cellular proteins and their ubiquitinated species could be stabilized, when proteasome function, most likely that of the 20 S protease, which is the central protease in the Ub-dependent degradation process, is inhibited (reviewed in [52]). In order to elucidate whether Ub-Pex5p is also present in wild-type cells and thus reflects a physiologically relevant stage of the PTS1 receptor, we made use of a strain carrying a temperature-sensitive mutation in the *CIM5* (co-lethal in mitogenesis) gene. This gene encodes a regulatory subunit of the 26 S proteasome [53]. *Cim5-1* mutant cells arrest the cell cycle at non-permissive temperature and accumulate ubiquitinated proteins [54]. Whole-cell lysates of oleic-acid-induced *cim5-1* cells, as well as double mutants of *cim5-1* and either *pex1*Δ or *pex5*Δ, all grown at non-permissive temperature (37 °C), were analysed for the presence of modified Pex5p. No ubiquitinated Pex5p was labelled in samples derived from the *cim5-1* strain functionally complemented with a plasmid harbouring the *CIM5* gene, representing the wild-type situation (Figure 7). Accumulation of ubiquitinated Pex5p was observed in *cim5-1* and *cim5-1 pex1*Δ cells when grown at non-permissive temperature (Figure 7). When grown at the permissive temperature of 30 °C, Ub-Pex5 was not visible in *cim5-1* cells, but remained at the same level of intensity in *cim5-1/pex1*Δ cells (results not shown).

These data underline the involvement of the proteasome in the degradation of ubiquitinated Pex5p. Moreover, the data demonstrate that Pex5p-modification also takes place in strains which are not affected in peroxisomal biogenesis, and thus the Pex5p-ubiquitination is supposed to reflect a physiologically relevant stage in the Pex5p receptor cycle.

DISCUSSION

Different modifications of proteins involved in peroxisomal biogenesis have been reported previously. Pex14p has been shown to be phosphorylated in both *Hansenula polymorpha* and *P. pastoris* [55,56]. Farnesylation occurs to *S. cerevisiae* Pex19p [57], whereas mono- and di-ubiquitination of Pex18p is associated with a constitutive degradation of the protein in *S. cerevisiae* [26].

In the present paper, we demonstrate that the PTS1 receptor Pex5p is ubiquitinated at the peroxisomal membrane in cells lacking Pex1p, Pex6p, Pex15, Pex4p or Pex22p. Pex1p and Pex6p are AAAs that form a complex supposed to be anchored to the peroxisomal membrane via Pex15p [58]. In support of a close functional relationship of the three proteins, deficiency in one of the components leads to the same pattern of Pex5p modifications representing the di- and tri-ubiquitinated forms, as well as minor amount of mono- and tetra-ubiquitinated forms of Pex5p (Figure 1). This Pex5p pattern differed from the one obtained upon deletion of Pex4p or Pex22p, which mainly consists of the mono- and di-ubiquitinated forms. Pex4p and Pex22p also form a functional complex, with Pex4p being a putative Ub-conjugating protein, which is anchored to the peroxisomal membrane via Pex22p [38]. Given that the usual consequence of polyubiquitination is degradation, Pex5p should be less stable in the above mentioned mutants. We did not observe such an instability of Pex5p in *S. cerevisiae*; however, it has been reported to occur in exactly these mutants in human and *P. pastoris* cells [17,37,38]. Thus it also seems likely that in *S. cerevisiae*, the ubiquitination of Pex5p might prime the protein for degradation, but in contrast with human and *P. pastoris* cells, in the *S. cerevisiae pex* mutants, Pex5p degradation is not brought to completion.

We found that mono- and poly-ubiquitination of Pex5p depends on the presence of the Ub-conjugating enzyme Ubc4p, which is known to mediate selective proteasomal degradation of short-lived and abnormal proteins [48]. The involvement of the proteasome in the turnover of ubiquitinated Pex5p is indicated by the accumulation of ubiquitinated Pex5p in proteasomal mutants (Figure 7).

Our observation of the Pex5p-modification to be present in *pex4*Δ strain, but not in a *pex4*Δ*pex10*Δ double-mutant, indicates that Pex10p performs its function in the Pex5p-dependent protein import pathway before Pex4p. This is in line with the observation of Collins et al. [18] who used the instability of Pex5p to determine the epistatic relationships among several groups of *pex* mutants. Accordingly, the AAA- and the Pex4p-complexed proteins were to act late in the peroxisomal import pathway after docking and after the so far unspecified step in the import process performed by the RING-finger proteins. Deletion of either one of the RING-finger proteins is known to dissociate the RING-finger complex, but docking of Pex5p remains unaffected [43,44]. As ubiquitination of Pex5p was neither visible in whole-cell extracts of the *pex10*Δ mutant, nor in the extracts of the *pex4*Δ*pex10*Δ double-mutant, our data demonstrate that the ubiquitination of Pex5p takes place after receptor docking and that it depends on the presence of Pex10p. The requirement for Pex10p can be explained in two ways. As E3-ligases, RING finger proteins may play the primary role in determining substrate specificity for Ub conjugation [45]. Thus Pex5p could be a *bona fide* substrate for a putative E3 function of the RING finger peroxins. On the other hand, in cells lacking RING-finger peroxins, Pex5p remains associated with the docking complex [43]. The lack of Pex5p ubiquitination in these cells might therefore be due to the fact that Pex5p accumulates at an early stage of the import pathway. Thus Pex5p might not yet have reached a stage in the import cascade which requires or leads to dislocation and proteasomal disposal of the protein.

Pex5p is supposed to cycle between the cytosol and the peroxisomal compartment. According to the shuttle model of peroxisomal protein import, Pex5p binds its cargo in the cytosol, delivers it to the peroxisomal membrane and releases its cargo at a putative translocation complex [16,17]. According to the extended shuttle model, Pex5p enters the peroxisome in tandem with its cargo and releases the cargo in the peroxisomal lumen [59,60]. After cargo release, Pex5p is transported back to the cytosol in both scenarios. Gouveia et al. [61] reported that Pex5p exits the peroxisomal compartment in an ATP-dependent manner. We found

a significant amount of Pex5p to be membrane-associated in cells lacking components of the AAA complex (Figure 3). Thus the AAAs are good candidates for being responsible for the ATP-dependent recycling of Pex5p to the cytosol. Accordingly, components of the AAA complex as well as the Pex4p–Pex22p complex have been reported to be required for the late steps in the import process [18].

What is the functional role of the observed ubiquitination of Pex5p? First, ubiquitination of Pex5p might represent an intrinsic step in the peroxisomal protein import pathway. Although this might well be true and Pex5p might be the long sought *bona fide* substrate for Pex4p, our data do not provide conclusive evidence of this. At first sight, the major appearance of mono- and di-ubiquitinated forms of Pex5p in cells lacking Pex4p or Pex22p, but the nearly complete lack of the mono-ubiquitinated form and the appearance of tri- and tetra-ubiquitinated forms in the cells lacking components of the AAA complex, might suggest that Pex4p is specifically required for the polyubiquitination of Pex5p. In this respect, it is interesting to note that Pex4p has recently been reported to be required for di-ubiquitination of Pex18p [26]. However, the Pex5p-ubiquitination pattern in a *pex1Δpex4Δ* double-mutant (Figure 4) resembles a mixture of both pattern types. This, however, might be explained by the assumption that deficiency in the different components leads to the ubiquitination of different sites within Pex5p. In this case, Pex4p could be responsible for the polyubiquitination of Pex5p at one site in cells lacking components of the AAA complex, while in the absence of Pex4p, the protein is polyubiquitinated at another site. It will be of great interest to follow up this possibility in terms of the function of Pex4p in peroxisomal protein import. However, our observation also demonstrates that polyubiquitination of Pex5p can, in principle, also take place in the absence of Pex4p. Consequently, we have to consider that the observed Pex5p ubiquitination in mutants affected late in the import pathway is due to the fact that Pex5p might get stuck in the import pathway, leading to an aberrant accumulation of membrane-associated Pex5p. This would also be in line with the observed involvement of Ubc4p, which is known to be responsible for the proteasomal disposal of abnormal proteins. This, however, leads to the question of why ubiquitinated Pex5p is only observed in import mutants affected in late steps of the import pathway. In this respect, it is interesting to note that Gouveia et al. [61] distinguished three different stages of Pex5p association with the peroxisomal membrane, with stage 3 being predominantly detected under ATP-limiting conditions. Under these conditions, Pex5p has been reported to be a target for an ATP-utilizing component mediating its release from the peroxisomal compartment. Although the identity of this ATPase is not known, the AAA-peroxins are good candidates to perform this function. Based on these observations, we propose that the lack of the late components of the import cascade leads to a membrane accumulation of Pex5p at a stage which sets the signal for its ubiquitination and intended proteasomal degradation. Ubc4p has also been implicated in ubiquitination and constitutive degradation of Pex18p, a PTS2-specific peroxin, the function of which in peroxisome biogenesis is accompanied by its rapid turnover [26]. The mechanistic interplay of Pex18p ubiquitination and its function in protein import into peroxisomes, however, has not yet been elucidated. The observation that both Pex5p and Pex18p, two peroxins which are supposed to cycle between the cytosol and the peroxisomal membrane, are ubiquitinated gives rise to the idea that their turnover might be of importance for their functional role in peroxisomal protein import. Moreover, the Ub-Pex5p accumulation in proteasomal mutants demonstrates that the Pex5p-modification also takes place in strains that are not affected in peroxisomal biogenesis. Consequently, we propose that the ubiquitination of Pex5p represents a genuine stage in the Pex5p receptor cycle. The data presented here are clear in that Pex5p is modified by ubiquitination at the peroxisomal membrane when peroxisomal import is blocked at late stages or upon inhibition of proteasomal degradation; however, additional work will be needed to clarify the physiological relevance of this modification.

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