Receptor recognition by the peroxisomal AAA complex depends on the presence of the ubiquitin moiety and is mediated by Pex1p

Received for publication, May 11, 2018, and in revised form, July 23, 2018. Published, Papers in Press, August 10, 2018, DOI 10.1074/jbc.RA118.003936

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Edited by Phyllis I. Hanson

The receptor cycle of type I peroxisomal matrix protein import is completed by ubiquitination of the membrane-bound peroxisome biogenesis factor 5 (Pex5p) and its subsequent export back to the cytosol. The receptor export is the only ATP-dependent step of the whole process and is facilitated by two members of the AAA family of proteins (ATPases associated with diverse cellular activities, AAAs), namely Pex1p and Pex6p. To gain further insight into substrate recognition by the AAA complex, we generated an N-terminally linked ubiquitin–Pex5p fusion protein. This fusion protein displayed biological activity because it is able to functionally complement a PEX5-deletion in Saccharomyces cerevisiae. In vitro assays revealed its interaction at WT level with the native cargo protein Pcs60p and Pex14p, a constituent of the receptor docking complex. We also demonstrate in vitro deubiquitination by the deubiquitinating enzyme Ubp15p. In vitro pulldown assays and cross-linking studies demonstrate that Pex5p recognition by the AAA complex depends on the presence of the ubiquitin moiety and is mediated by Pex1p.

Peroxisomes are ubiquitous cell organelles with a soluble matrix surrounded by a single lipid bilayer membrane. These organelles are involved in a broad range of metabolic processes, most notably displayed by a wide phenotypic range of peroxisomal disorders caused by peroxisome malfunction (1, 2). A special feature of peroxisomes, which clearly distinguishes them from other cellular organelles, is their ability to import folded, oligomerized and even co-factor bound proteins into the peroxisomal matrix (3, 4). To this end, matrix proteins are equipped with a targeting sequence, either a C-terminal PTS1 (peroxisomal targeting signal 1) or an N-terminal PTS2, which are recognized and bound in the cytosol by the import receptor Pex5p or Pex7p, respectively (5, 6). The receptors ferry the cargoes to the peroxisomal membrane, where they bind to a docking complex and become part of a highly dynamic and transient translocation pore (7, 8). How cargo translocation occurs mechanistically is still unknown, but available data are clear in that the cargo-free receptors are exported back to the cytosol in an ATP-dependent manner with ubiquitin serving as an export signal (9–11).

Among the different peroxisomal matrix protein import pathways, the so far best understood is the PTS1 pathway with Pex5p as the related import receptor. Pex5p comprises two separated and functionally distinct domains. The C-terminal domain of the receptor consists of an array of tetratricopeptide repeat domains and directly binds the PTS1 motif (12). The N-terminal domain is intrinsically disordered and capable of mediating all transport steps of the receptor cycle, including docking and pore formation (13–15). Within the N-terminal region, Pex5p is modified by the attachment of ubiquitin moieties. Two kinds of receptor ubiquitination are known, namely mono- and polyubiquitination (16). Pex5p polyubiquitination occurs on conserved lysine residues of Pex5p (Lys18/Lys24 in Saccharomyces cerevisiae) and leads to receptor degradation via the proteasome (17–20). In contrast, monoubiquitin is attached as an exception to the rule via a thioester bond to a conserved cysteine of Pex5p (Cys6 in S. cerevisiae) and enables the receptor recycling for further rounds of matrix protein import (21). In the cytosol, the monoubiquitin moiety is removed either in a nonenzymatic manner by a nucleophilic attack of GSH or enzyme-catalyzed by a ubiquitin hydrolase to allow another import cycle (22–24).

Studies on yeast and human fibroblasts illustrated that extraction of ubiquitinated Pex5p from the peroxisomal membrane is carried out by Pex1p and Pex6p (10, 25). Both proteins display similar architecture and belong to the family of ATPases associated with diverse cellular activities (AAAs). Studies on Pex1p and Pex6p from S. cerevisiae revealed that both peroxins form a heterohexameric complex (26, 27). The complex is present in the cytosol, as well as attached to the peroxisomal membrane. Specifically, the AAA complex is dynamically recruited to the peroxisomal membrane via a nucleotide-dependent interaction of Pex6p with the cytosolic domain of the tail-
anchored membrane protein Pex15p or its orthologues in mammals (Pex26p) and plants (APM9) (28). Despite its crucial role for the dislocation, the functional relevance of the monoubiquitination of Pex5p, as well as the exact molecular mechanism of substrate recognition and extraction from the membrane, remains unclear.

Here we analyzed substrate recognition by the AAA complex by use of a linear N-terminal ubiquitin–Pex5p fusion protein. We demonstrate that this fusion functionally complements a PEX5-deletion in S. cerevisiae. Moreover, ubiquitin–Pex5p binds the cargo protein Pcs60p and Pex14p at the WT level. Based on in vitro pulldown assays and cross-linking studies, we conclude that the ubiquitination of Pex5p is a prerequisite for recognition by the AAA complex, with Pex1p representing the main binding partner.

Results
A main step of the peroxisomal import cycle is the release of the receptors from the peroxisomal membrane back to the cytosol. It was demonstrated for yeast and mammalian cells that the membrane release of the PTS1 receptor Pex5p is catalyzed by the AAA peroxins Pex1p and Pex6p (10, 25). Modification of the receptor with either mono- or polyubiquitin moieties on Pex5p turned out to be a prerequisite for this release from the membrane either for receptor recycling or for its degradation, respectively (16). It seems very likely that ubiquitin serves as an export signal, which primes the receptor molecule for the recognition by the AAA-type ATPase complex. To fulfill its function in Pex5p export, a direct or indirect interaction of the AAA complex with Pex5p and/or ubiquitin is required, the nature of which so far remains unknown.

To address this issue, we analyzed the ability of Pex1p and Pex6p for Pex5p binding by means of the yeast two-hybrid system. Gal4p fusions of the activation domain and the DNA-binding domain with various peroxins were co-expressed in different pairwise combinations in the S. cerevisiae host strain PJ69-4A (29), and interactions were monitored by histidine/adenine auxotrophy. In line with published results, Pex5p displayed clear interaction to the receptor docking constituent Pex14p as indicated by growth of the corresponding yeast reporter strain (Fig. 1 and Ref. 30). In addition, we confirmed Pex6p interaction with Pex1p as well as with the cytosolic part of its anchor protein Pex15p (Fig. 1 and Ref. 31). These results demonstrate that our Gal fusion proteins are expressed and properly folded to allow protein–protein interactions. The controls included show that expression of either of the fusion proteins alone did not support transcription activation of the reporter genes. However, when Gal fusions of the peroxisomal AAA proteins Pex1p or Pex6p were co-expressed with Pex5p, no growth of the reporter strain was observed in medium lacking histidine/adenine. The finding indicates an inability of Pex1p–Pex6p in binding of the PTS1 receptor under these conditions (Fig. 1).

**In vitro binding of Pex6p to ubiquitin**

One possible reason for the observed lack of interaction between Pex5p and Pex1p–Pex6p might be that ubiquitin itself mediates the contact of the modified receptor to the peroxisomal AAA complex. To address this question, we also analyzed the interaction of recombinant ubiquitin and Pex1p–Pex6p by in vitro binding studies. Recombinant yeast Pex1p and Pex6p both fused to an N-terminal hexahistidyl tag (His6) and Pex1p in addition to a C-terminal GST tag were expressed separately in Escherichia coli and purified according to (32). To obtain the Pex1p–Pex6p complex, suspensions of Pex1p- and Pex6p-expressing cells were mixed prior to cell disruption, and complex isolation comprised successive affinity- and size-exclusion chromatography steps (32). It is important to note that during the isolation procedure, the GST tag was removed from Pex1p by thrombin cleavage with the GST tail remaining on the affinity column. Ubiquitin was expressed and purified as a GST fusion protein (UbGST). The fusion protein was eluted from the column by addition of GSH, which was subsequently dialyzed out of the obtained eluate fraction.

For in vitro binding, purified UbGST was preincubated either with Pex1p or Pex6p alone or with the assembled Pex1p–Pex6p complex. Thereafter the samples were loaded onto GSH–agarose, unbound proteins were removed, and bound proteins eluted by addition of GSH. It turned out that Pex1p was bound neither to UbGST nor GST alone, which served as control for the experiments (Fig. 2A). In contrast, Pex6p alone, as well as the Pex1p–Pex6p complex, displayed clear binding to the ubiquitin fusion protein, but not to the GST control (Fig. 2, B and C). From these results we conclude that ubiquitin is bound to the peroxisomal AAA complex via direct binding to Pex6p. It is known that a high number of proteins is capable of ubiquitin binding but with different binding motifs. However, the majority of these binding motifs interact with a hydrophobic region within ubiquitin with isoleucine on position 44 as central amino acid residue (33). To analyze whether this “hydrophobic patch” around isoleucine 44 might also play an important role in the binding to the Pex1p–Pex6p complex, mutant ubiquitin bearing an I44A substitution (UbI44A) was

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**Table 1. Yeast two-hybrid interaction assay of Pex5p and Pex1p–Pex6p.**

| Gal4p-AD fused to | Gal4p-DB fused to | Pex5p | Pex1p | Pex6p |
|------------------|------------------|-------|-------|-------|
| Pex5p | - | - | - | - |
| Pex5p | - | Pex14p | - | - |
| Pex6p | Pex1p | - | - | - |
| Pex6p | - | Pex1p | - | - |
| Pex15p(1–315) | Pex6p | - | - | - |
| Pex15p(1–315) | - | Pex6p | - | - |
| Pex5p | Pex1p | - | - | - |
| Pex5p | Pex6p | - | - | - |
 included in our experiments. Because Pex1p did not bind to WT ubiquitin, it was not surprising that it also did not bind to the mutant ubiquitin (Fig. 2A). Binding capacity of Pex6p and the Pex1p–Pex6p complex to UbI44A was significantly reduced when compared with WT ubiquitin (Fig. 2, B and C), supporting the idea of a commune ubiquitin-binding motif most likely within Pex6p.

Biological activity of the N-terminal Ub(1–6/H9004)Pex5p

In vivo the PTS1 receptor Pex5p is modified by the attachment of a monoubiquitin moiety via a thioester bond at a conserved cysteine (Cys6 in S. cerevisiae) (17–20), which enables the receptor recycling for further rounds of matrix protein import (21). Because receptor ubiquitination is a prerequisite for Pex1p–Pex6p binding, we aimed to mimic and arrest this very dynamic and transient state of Pex5p for in vivo and in vitro analysis. To this end, we generated a construct in which the first six amino acid residues of Pex5p containing the conserved cysteine residue were replaced by ubiquitin (Fig. 3A, Ub(1–6/H9004)Pex5p). Biological activity of the fusion protein was analyzed by testing its capability to complement the peroxisomal mutant phenotype of a PEX5-deficient yeast deletion strain. The pex5/H9004 strain displays a growth defect on oleic acid as the sole carbon source caused by an import defect of a group of peroxisomal matrix proteins (PTS1 and non-PTS proteins) (34). We transformed WT and pex5/H9004 cells with a plasmid expressing the fluorescence marker mCherry fused to type 1 targeting signal Ser-Lys-Leu (SKL). Fluorescence microscopy analysis of oleic acid-induced WT cells producing mCherry-SKL revealed the presence of distinct fluorescent spots, indicative for a peroxisomal localization of the synthetic peroxisomal matrix protein (Fig. 3B). Such spots were missing in pex5Δ cells, which display in contrast to WT an overall cytosolic labeling, demonstrating the specific import defect of this mutant (34).

Although the import defect was functionally complemented by expression of WT Pex5p, Pex5p lacking the first six amino acid residues (1–6/H9004)Pex5p, including the conserved cysteine residue, was unable to restore the import defect of pex5Δ cells. Conversely, the fluorescence pattern of the mutant expressing the Ub–Pex5p fusion protein was indistinguishable from that of WT cells (Fig. 3B). Thus, the replacement of amino acids 1–6 of Pex5p by ubiquitin restored peroxisomal import of the pex5/H9004 mutant, demonstrating that the N-terminal tagging with ubiquitin does not interfere with the biological function of Pex5p. On the contrary, the presence of the ubiquitin moiety can account for the lack of the first six amino acids, especially the conserved cysteine, which otherwise is essential for Pex5p function in peroxisome biogenesis.

Ubiquitination does not alter Pex5p binding to cargo and Pex14p

In its function as PTS1 receptor, Pex5p binds peroxisomal matrix proteins harboring this type of the peroxisomal targeting signal. One representative of this group is Pcs60p, an oxalyl-CoA synthetase that belongs to the family of AMP-binding proteins (35, 36). The PTS1 of Pcs60p was demonstrated to be
crucial for peroxisomal targeting (35). Moreover, a direct binding to the PTS1 receptor was previously reported (37). To investigate the influence of the linear fusion of ubiquitin to the N terminus of Pex5p on receptor cargo recognition, we tested whether presence of the ubiquitin moiety alters the in vitro binding of Ub–Pex5p to Pcs60p. To this end, Pex5p, Ub(1–6)Pex5p, and Pcs60p were fused to an N-terminal GST tag. The genes coding for the different fusion proteins were expressed separately in E. coli, and soluble fractions of cells were loaded onto GSH–agarose. The GST–Pex5p variants were eluted with GSH and dialyzed to remove the GSH, whereas Pcs60p was removed by thrombin cleavage with the GST tag remaining on the agarose column. Equal portions of GST–Pex5p were bound to the agarose (**Fig. 4A**), which is in line with our previous reports of a direct Pex5p/Pcs60p interaction (37). Pcs60p was also co-eluted when GST–Ub(1–6)Pex5p was bound to the column. The binding efficiency was similar to WT Pex5p (**Fig. 4A**). Thus, the fusion to ubiquitin does not alter cargo binding to Pex5p in vitro.

Once the receptor is cargo-loaded, the receptor–cargo complex binds to the peroxisomal membrane. Because of its direct interaction with Pex5p and the increase of binding when Pex5p...
Ubiquitin-dependent receptor recognition mediated by Pex1p

Figure 5. In vitro deubiquitination of N-terminally ubiquitinated Ub(1–6)Pex5p. WT Ubp15p and the catalytically inactive mutant Ubp15pC214A were treated with or without NEM and incubated with purified GST-tagged Ub(1–6)Pex5p. Samples taken at the indicated time points were subjected to SDS–PAGE and Coomassie staining (upper panels) or immunoblot analysis (lower panels) using antibodies against GST (red) and Pex5p (green). The increase of Pex5p and free GST-tagged ubiquitin (GST-Ub) in the sample containing the catalytically active and non-NEM-treated Ubp15p shows that the N-terminally ubiquitinated Ub(1–6)Pex5p is deubiquitinated by Ubp15p in vitro.

is loaded with a PTS1-containing peptide, Pex14p has been proposed to serve as the docking site for the cytosolic receptor–cargo complex (30). Here we compared the Pex14p binding of the ubiquitin–Pex5p fusion and Pex5p. As described above, GST–Pex5p, GST–Ub(1–6)Pex5p, and as control GST alone were expressed in E. coli and purified. We generated a Pex14p variant fused to an N-terminal His6 tag, which was separately expressed in E. coli and purified by affinity chromatography with nickel–nitritotriacetic acid. As described above for Pcs60p, the different Pex5p variants were incubated with purified Pex14p, loaded on GSH–agarose, and eluted. As judged by Coomassie stain, Pex14p co-eluted with GST–Pex5p but also with Ub(1–6)Pex5p in equal amounts, indicative of an efficient interaction (Fig. 4B). Signal-intensity measurements of the Pex5p and Pex14p protein bands of the Coomassie-stained gel indicated that similar amounts of Pex14p bound to the column, independent of whether Pex5p or Ub(1–6)Pex5p served as bait (data not shown). Taken together, our results demonstrate that binding neither to Pcs60p nor to Pex14p is influenced by the exchange of the extreme N terminus of Pex5p by ubiquitin.

In vitro deubiquitination of the linear ubiquitin–Pex5p fusion by Ubp15p

Pex5p is mono- or polyubiquitinated in vivo, and the ubiquitin moiety has to be removed from Pex5p presumably during or after receptor export to allow another round of import. The cleavage of ubiquitin from a substrate protein is generally carried out by ubiquitin hydrolases also known as deubiquitinating enzymes (DUBs) (38). S. cerevisiae expresses more than 20 putative DUBs, which all except one are encoded by nonessential genes (39). Thus, individual deletions result in only subtle phenotypes, suggesting redundancy in their functions (40, 41).

Among the yeast DUBs, Ubp15p was identified as a component of the AAA complex, and cells affected in Ubp15p display a stress-related partial protein import defect of PTS1 proteins (22). Based on in vitro cleavage assays, it was demonstrated that Ubp15p is capable of removing ubiquitin from Pex5p. Here we addressed the question of whether Ubp15p exhibits its deubiquitinating activity also on the linear ubiquitin Pex5p fusion protein (Ub(1–6)Pex5p). To this end, we performed in vitro ubiquitin-cleavage assays. Heterologously expressed and isolated Ubp15p was incubated with GST-tagged Ub(1–6)Pex5p, and the reaction was stopped by freezing in liquid nitrogen after zero (control) or later time points as indicated. Cleavage was monitored by Coomassie-stained SDS gels and immunoblot analysis. In the absence of Ubp15p, the Pex5p fusion remained mainly intact; only a slight cleavage of the joint region between Pex5p and the GST–Ub moiety occurred even at time point 0 (Fig. 5, lanes 1–4). In the presence of Ubp15p, a time-dependent decrease of the fusion protein was observed (Fig. 5, lanes 5–8). An increase of cleavage products can be seen already at 0 min, indicating that a large portion of the fusion protein was cleaved even before the temperature was shifted to 37 °C (Fig. 5, lanes 9–12). Sequence alignment of Ubp15p with other UBP3 indicated that Cys214 of Ubp15p most likely represents an amino acid residue, which is crucial for the deubiquitinating activity (42). In fact, a Cys214 to Ala substitution introduced into the full-length Ubp15p (Ubp15pC214A) was shown to be enzy-
matically affected but not completely inactive (22). Accordingly, GST–Ub was slightly cleaved off from GST–Ub(H90041–6)Pex5p, but most of the fusion protein remained stable when incubated with Ubp15pC214A (Fig. 5, lanes 13–16). Taken together, our data are clear in that like the natively ubiquitinated Pex5p, the linear Ub(H90041–6)Pex5p fusion also displays a target for deubiquitination by Ubp15p.

**Ubiquitin-dependent binding of Pex5p to the Pex1p–Pex6p complex**

Our studies show that the Pex1p–Pex6p complex can bind ubiquitin (Fig. 2). However, the natural substrate for the Pex1p–Pex6p-dependent release is monoubiquitinated Pex5p, which in our study is mimicked by the linear Ub(H90041–6)Pex5p fusion. Therefore, in the following we tested the binding of Ub(H1α–6)Pex5p to Pex1p and/or Pex6p by in vitro pulldown assays. To this end, heterologously expressed and purified GST fusions of Pex5p or Ub(H90041–6)Pex5p, as well as GST alone (control), were incubated with isolated Pex1p, Pex6p, or the Pex1p–Pex6p complex. The samples were loaded onto GSH–agarose columns, and after extensive washing bound proteins were eluted with GSH. Neither Pex1p, Pex6p, nor the Pex1p–Pex6p complex co-eluted with GST as bait (Fig. 6). In contrast, the Pex1p–Pex6p complex co-eluted together with GST–Ub(H90041–6)Pex5p (Fig. 6C), indicating that Ub(H1α–6)Pex5p can physically interact with the Pex1p–Pex6p complex. Because the nonubiquitinated Pex5p did not interact with the AAA complex, the data also show that the interaction depends on the presence of ubiquitin. Also Pex1p alone did bind Ub(H1α–6)Pex5p (Fig. 6A). A small amount of Ub(H1α–6)Pex5p seems to associate with Pex6p; this, however, is only seen upon longer exposure (Fig. 6D). In contrast to Pex1p, which did bind to Ub(H1α–6)Pex5p with nearly the same efficiency as the Pex1p–Pex6p complex, binding of Pex6p alone was much less efficient (Fig. 6D). These findings indicate that both Pex1p and Pex6p are able to interact with Ub(H1α–6)Pex5p, but the data also suggest that Pex1p might be the primary binding partner for Ub(H1α–6)Pex5p.

**Site-specific in vitro cross-linking of the Pex1p–Pex6p–Ub(H1α–6)Pex5p complex**

To gain deeper insight into the recruitment of the ubiquitinated PTS1 receptor to the Pex1p–Pex6p complex, we aimed to stabilize the complex by use of site-specific in vitro photocross-linking (43). Our initial experiments demonstrate that the interaction of ubiquitin with the Pex1p–Pex6p complex is weakened when the isoleucine at position 44 of the ubiquitin is replaced by alanine (Fig. 2). This result indicates that ubiquitin binds to the AAA complex via a hydrophobic patch with Ile44 as central amino acid residue. Such an interaction is typical for binding of ubiquitin to many ubiquitin-binding proteins (33). For our cross-linking experiments, we genetically incorporated the photocross-linking amino acid para-benzoyl-phenylalanine (pBpa) into the corresponding ubiquitin part of recombinant Ub(H1α–6)Pex5p. The pBpa is encoded by the amber codon TAG, and its incorporation into the proteins takes place during

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**Figure 6. Ubiquitin-dependent in vitro interaction of Pex5p with Pex1p and Pex6p.** Purified GST and N-terminal GST fusion constructs of Pex5p and Ub(H1α–6)Pex5p were combined with HisPex1p (A), HisPex6p (B), or the assembled HisPex1p–HisPex6p complex (C) and loaded onto GSH–agarose. Bound proteins were eluted with buffer containing 50 mM reduced GSH. Equal volumes of each relevant step were subjected to immunoblot analysis using antibodies against Pex1p, Pex6p, and GST. For comparison, the immunoblot analysis of higher amounts of eluates is shown in D. Interaction of Pex1p and Pex6p with the receptor Pex5p strongly depends on the presence of ubiquitin fused to Pex5p. A maximum of 2.5% of loaded AAA protein was recovered in the eluates. L, load; Fl, flow through; W1–W5, wash 1–5; E, concentrated eluate.
translation, using an orthogonal tRNA/ami­noacyl-tRNA synthetase pair. The pBpa-containing proteins efficiently form sta­ble complexes with their partners upon UV irradiation at 365-nm wavelength (43, 44). In our case, the base triplets encoding amino acid residues 42–48 of ubiquitin were indi­vidually changed to amber codons. Expression of the Ub(1–6)pex5p variants was carried out in the presence of an evolved Methanococcus jannashii tRNA/ami­noacyl-tRNA synthetase pair, 0.02% arabinose and 1 mM pBpa in E. coli strain BL21 (DE3). Proteins were expressed as GST fusions, and their isolation was carried out by affinity chromatography on GSH–agarose with GSH elution. SDS–PAGE analysis showed no difference between the purified amber mutant Ub(1–6)pex5p and the WT protein (data not shown).

Isolated Ub(1–6)pex5p variants were incubated with the purified Pex1p–Pex6p complex for 1 h in the presence and absence of UV light. Subsequently, samples were subjected to SDS–PAGE followed by either Coomassie staining or immunoblot analysis with specific antibodies against GST (middle and bottom panels, green), Pex1p (middle panels, red), and Pex6p (bottom panels, red). All depicted lanes in the top, middle, and bottom panels, respectively, belong to the same gel/blot. The Leu43 and the Phe45 substitutions resulted in a cross-link product of Pex1p and GST–Ub(1–6)pex5p that appeared as a very thin band stained by Coomassie and was detected with GST and Pex1p antibodies, but not with Pex6p antibodies.

Figure 7. Site-specific photocross-linking of monoubiquitinated Pex5p and Pex1p. Purified GST-tagged Ub(1–6)pex5p constructs harboring different ubiquitin amino acid substitutions by the cross-linker pBpa were combined with purified HisPex1p–HisPex6p complex and irradiated with UV light (365 nm). Samples taken before and after UV treatment were subjected to SDS–PAGE and Coomassie staining (top panels) or immunoblot analysis with specific antibodies against GST (middle and bottom panels, green), Pex1p (middle panels, red), and Pex6p (bottom panels, red). All depicted lanes in the top, middle, and bottom panels, respectively, belong to the same gel/blot. The Leu43 and the Phe45 substitutions resulted in a cross-link product of Pex1p and GST–Ub(1–6)pex5p that appeared as a very thin band stained by Coomassie and was detected with GST and Pex1p antibodies, but not with Pex6p antibodies.

Discussion

In this study we report on the importance of Pex5p ubiquitination for its association with the AAA proteins Pex1p–Pex6p. We identify Pex1p as the main binding factor and demonstrate that ubiquitination does not affect Pex5p binding to its cargo or to the docking complex at the peroxisomal membrane.

A crucial step in the peroxisomal import cycle for PTS1 proteins is the release of the unloaded PTS1 receptor Pex5p from the peroxisomal membrane back to the cytosol to allow another round of import. This export step 1) is responsible for the ATP dependence of the overall process, 2) depends on monoubiquitination of the receptor, and 3) is performed by a complex of the peroxisomal AAAs Pex1p and Pex6p (21, 45). In contrast to conventional targets for ubiquitination, Pex5p is special, formed by ubiquitination of a lysine residue, a thioester bond is formed between ubiquitin and a conserved cysteine of Pex5p (21, 46, 47). This conserved cysteine residue proved to be essential for the biological function of Pex5p (49–51). In line with this finding, a Pex5p truncation of the first six amino acid residues, including the conserved cysteine, is incapable of restoring the PTS1 import deficiency of a pex5Δ strain (Fig. 3).

In addition to lysine or cysteine ubiquitination, ubiquitin can also be conjugated to the free α-amino group of the first residue of a target protein (52). The biological role of such an N-termi-
nal ubiquitination remains elusive because of a low number of examples. It is speculated that it may serve as a target for further polyubiquitination, which is a well-known degradation signal recognized by the proteasome (52, 53). With respect to Pex5p, it turned out that not the type of target residue but its position is of major relevance for the functionality of the receptor. For instance, Pex5p function was preserved upon replacement of the conserved cysteine by lysine (49, 51, 54). To mimic the monoubiquitinated version of the PTS1 receptor, we used here the biological template of a linear N-terminal ubiquitin fusion and replaced the extreme N terminus of Pex5p by ubiquitin. Expression of the linear ubiquitin–Pex5p fusion in pex5Δ restored the peroxisomal import defect, despite a reduced steady-state level of about 50% compared with the plasmid-encoded Pex5p WT (data not shown), indicating that the linear fusion of the receptor with ubiquitin fulfills all requirements for proper matrix protein import (Fig. 3). The result demonstrates that the N-terminal ubiquitin tagging can functionally replace the cystein-dependent monoubiquitination.

The monoubiquitination primes the PTS1 receptor for its export back to the cytosol, which is an essential step in the import of matrix proteins (55, 56). However, it is still an open question whether this is the only function of the monoubiquitination in the import process. According to the idea of the export-driven import model, the ubiquitin-mediated and ATP-dependent export of the receptor might be functionally interconnected to protein translocation across the peroxisomal membrane (57). This idea is contradicted by data from mammalian cells, suggesting that unloading of the receptor; thus release of the PTS1 cargo protein occurs prior to and thus independently of monoubiquitination of Pex5p (58–60). However, the time point when the native receptor is modified during the receptor cycle and import process is still not clearly defined. In principle, Pex5p monoubiquitination could coincide in time with any of the steps of the receptor cycle prior to its export back to the cytosol, an idea that was also proposed by Williams et al. (21). We demonstrate that binding of Pex5p to its cargo and to Pex14p is not affected by its ubiquitination (Fig. 4), indicating that Pex5p monoubiquitination alone seems not to play a crucial role in cargo binding in the cytosol, docking of the receptor–cargo complex to the peroxisomal membrane, or release of the cargo during the import process.

To explore the possibility that the ubiquitin moiety provides a protein interface, which triggers association with the receptor export machinery, we focused on Pex1p and Pex6p as potential binding partners of the ubiquitinated receptor. These peroxins belong to the family of AAA proteins (61–63), typically involved in processes such as protein unfolding and degradation or disassembly of protein complexes (64). They were shown to form a heterohexameric complex, organizing as a trimer of dimers (26, 27, 65). Pex1p and Pex6p have been implicated in the recycling of Pex5p, because they were found to act in the terminal steps of matrix protein import in the yeast Pichia pastoris (66) and ATP was reported to be indispensable for receptor export (10). Work with human cells as well as S. cerevisiae showed that indeed Pex1p and Pex6p are necessary for Pex5p export (10, 25). However, our knowledge about the mechanism of action is still scarce. Here we show that the Pex1p–Pex6p complex directly interacts with monoubiquitinated Pex5p in vitro. In line with recently published results, we found no direct interaction with unmodified Pex5p (67). This is in contrast to mammalian Pex1p, which exists as a homo-oligomer in the cytosol and a hetero-oligomer with Pex6p on peroxisome membranes (68). A surface plasmon resonance–based assay demonstrated that the mammalian Pex1p homo-oligomer binds directly to nonubiquitinated Pex5p, although at a low affinity (69). The study, however, did not investigate the contribution of ubiquitin to the interaction. In our study, the interaction of Pex5p to the AAA complex, in particular Pex1p, strongly depends on the presence of the ubiquitin moiety fused to the N terminus of Pex5p (Fig. 6). Furthermore, the hydrophobic patch around isoleucine 44 of ubiquitin seems to be involved in the interaction. The mutation of this amino acid to alanine in UbGST resulted in a decreased binding to the AAA proteins (Fig. 2), and site-specific photocross-linking was only successful when the cross-linker was inserted next to the essential Ile44 residue (Fig. 7). The involvement of the hydrophobic patch of ubiquitin for recognition is typical for many ubiquitin-binding proteins (33, 70). The N-terminal domains of both Pex1p and Pex6p have been shown to comprise two double-ψβ-barrel domains (65), which were previously identified as ubiquitin-binding domains in the related AAA protein p97 and its adapter protein Ufd1 (71).

Interestingly, our data suggest that Pex1p and Pex6p have distinct binding capabilities for ubiquitin with respect to the surrounding protein context. Although Pex6p was able to bind GST-tagged ubiquitin, Pex1p showed a higher specificity for the Ub–Pex5p fusion construct. The N-terminal domains of Pex1p are flexibly located above the double-ring structure of the AAA complex (26, 65), which makes them a likely candidate for the initial contact with the ubiquitinated receptor. Pex1p might therefore be able to specifically recognize the ubiquitin modification at the conserved cysteine of Pex5p. Pex6p, however, seems to have a more general affinity toward ubiquitin. It could be imagined that after initial binding by Pex1p, the ubiquitin moiety is handed over to Pex6p to position it for deubiquitination by Ubp15p, which is associated with the D1 domain of Pex6p (22), whereas Pex5p is directed to the pore of the AAA complex for further processing. Alternatively,
Expression of recombinant proteins was induced with 0.4 mM isopropyl/β-D-thiogalactopyranoside with expression conditions of 4 h at 30 °C for GSTPex5p, GSTPcs60p, His6Pex14p, Ub14A, and Ub14A GST, as well as 20 h at 20 °C for GSTUbp15p, GSTPex5p, GSTUbp15p, and GSTUbp15pC214A. For expression of cross-linking constructs of GSTUb−(1–63)Pex5p, the medium additionally contained 0.02% arabinose and 1 mM Bpa.

The cells were harvested after expression and suspended in buffer I (50 mM Tris, 100 mM NaCl, 1 mM DTT, pH 7.4) (GSTPex5p, GSTUb−(1–63)Pex5p, GSTUb−(1–63)Pex5p cross-linkers, GSTUbp15p, GSTUbp15pC214A), buffer II (50 mM Tris, 150 mM NaCl, 1 mM DTT, pH 7.4) (GST, GSTPcs60p, UbGST, Ub14A GST), or buffer III (20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 7.9) (His6Pex14p). All buffers for cell lysis contained a selection of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 8 mM antipain, 0.3 mM aprotinin, 1 mM bestatin, 10 mM chymostatin, 5 mM leupeptin, 15 mM pepstatin) and 25 μg/ml DNase I.

The cells were broken by sonication, and the homogenate was centrifuged at 14,000 rpm for 1 h (rotor SS-34; Thermo Scientific). Supernatants containing soluble GST fusion proteins were loaded onto a GST–agarose 4B matrix (Macherey-Nagel, Düren, Germany). After incubation for 1 h, unbound protein was washed off with 20-fold column volume of buffer. The proteins were eluted either with buffer containing 50 mM reduced GSH (GSTPex5p, GSTUb−(1–63)Pex5p, and GSTUb−(1–63)Pex5p cross-linkers, GSTUb15p, GSTUbp15pC214A), or after incubation with thrombin overnight to retain the GST tag on the column (GSTUb15p, GSTUbp15pC214A, GSTPcs60p). The homogenate containing His6Pex14p was loaded onto a HisTrap HP column (GE Healthcare) using an AKTA Prime system (GE Healthcare). After washing with buffer III containing 60 mM imidazole, the protein was eluted with a continuous imidazole gradient of up to 1 M imidazole.

**In vitro deubiquitination**

Purified GST-tagged Ub–Pex5p fusion protein was incubated with or without Ubp15p for 15 min at 37 °C. Samples

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### Table 2

| Plasmid     | Description                                | Source or reference | Oligonucleotides |
|-------------|--------------------------------------------|---------------------|------------------|
| pPC86–PEx5  | GalAD–PEx5                                 | Ref. 81             |                  |
| pPC96–PEx6  | GalAD–PEx6                                 | Ref. 74             |                  |
| pPC86–PEx15| GalAD–PEx15 (1–315)                        | Ref. 82             |                  |
| pPC97–PEx14 | GalBD–PEx14                                | Ref. 30             |                  |
| pBM5        | GalBD–PEx1                                 | Ref. 74             |                  |
| pBM12       | GalBD–PEx6                                 | Ref. 31             |                  |
| pGEX–AT3    | GST                                        | Invitrogen          |                  |
| pET–Ub–V–GST| UBIQUITIN–GST                              | Ref. 83             |                  |
| pET–Ubi44A–V–GST| UBIQUITIN(44A)–GST                      | Ref. 83             |                  |
| pRSFpdeo–HisPex1 | His6–Pex1X                       | Ref. 84             |                  |
| pRSFpdeo–HisPex1–GST| His6–Pex1X–GST                  | Ref. 84             |                  |
| pRSFpdeo–HisPex6 | His6–Pex6                                | Ref. 84             |                  |
| pGEX4T2–GST–Pex5 | GST–Pex5X                                | Ref. 81             |                  |
| pG42       | UbA1–Pex5X                                  | This study          | RE3176/3178      |
| pNH01      | GST–PCS60                                   | Ref. 37             |                  |
| pET9d–His6–PEx14 | HisPex14p                               | Ref. 48             |                  |
| pGEX4T2–GST–Ubi(R42amber)–Pex5 | GST–Ub(R42pBpa)–Pex5 | This study          | RE3589/3890      |
| pGEX4T2–GST–Ubi(L43amber)–Pex5 | GST–Ub(L43pBpa)–Pex5 | This study          | RE3591/3922      |
| pGEX4T2–GST–Ubi(H44amber)–Pex5 | GST–Ub(H44pBpa)–Pex5 | This study          | RE3587/3588      |
| pGEX4T2–GST–Ubi(F45amber)–Pex5 | GST–Ub(F45pBpa)–Pex5 | This study          | RE3593/3948      |
| pGEX4T2–GST–Ubi(A46amber)–Pex5 | GST–Ub(A46pBpa)–Pex5 | This study          | RE3595/3956      |
| pGEX4T2–GST–Ubi(G47amber)–Pex5 | GST–Ub(G47pBpa)–Pex5 | This study          | RE3597/3958      |
| pGEX4T2–GST–Ubi(K48amber)–Pex5 | GST–Ub(K48pBpa)–Pex5 | This study          | RE3599/3600      |
| pHP17      | Yeast expression vector, PEX5              | Ref. 20             |                  |
| pFMO3      | Yeast expression vector, UbA1–Pex5X         | This study          | RE3176/3178      |
| pFMO1      | Yeast expression vector, UbA1–Pex5X         | This study          | RE3013/3014      |
| pGEX–4T–2–UBP15 | GST–UBP15X                             | Ref. 22             |                  |
| pGEX–4T–2–UBP15(C214A) | GST–UBP15(C214A)                | Ref. 22             |                  |
| pEVOL–RS   | t-RNA and t-RNA–synthetase                 | P. Schultz, Scripps Research Institute, La Jolla, CA |                  |

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**Materials and methods**

### Strains, plasmids, and primers

The strains, plasmids, and sequences of oligonucleotides used are listed in **Tables 1–3**. The plasmids pIG26–pIG32 for expression of the cross-linker constructs of GSTUb−(1–63)Pex5p were generated by QuikChange site-directed mutagenesis according to the manufacturer’s instructions (Stratagene) using selected primer pairs and the template pIG24.

Yeast expression of Pex5p was performed using the plasmid pHP17 (45). For yeast expression of (1–63)Pex5p, truncated Pex5p was amplified from pHP17 using primer pair RE3177/RE3178 and introduced behind the Pex5p promoter in a pRS416 plasmid using BamHI and NotI restriction sites, resulting in the plasmid pFM01. For yeast expression of Ub−(1–63)Pex6p, synthetic ubiquitin was first amplified from YEp96 (25). The resulting plasmid pHP17 was used for yeast transformation. Truncated (1–63)Pex5p was amplified from pHP17 with primer pair RE3176/RE3178 and introduced into the ubiquitin construct, resulting in the plasmid pFM03.

### Protein purification

Protein expression and purification of Pex1p, Pex6p, or the Pex1p–Pex6p complex was performed according to Ref. 32. Expression of recombinant proteins was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside with expression conditions of 4 h at 30 °C for GSTPex5p, GSTPcs60p, His6Pex14p, UbGST, and Ub14A GST, as well as 20 h at 20 °C for GSTUbp15p, GSTPex5p, GSTUbp15p, and GSTUbp15pC214A. For expression of cross-linking constructs of GSTUb−(1–63)Pex5p, the medium additionally contained 0.02% arabinose and 1 mM Bpa.
taken every 5 min were analyzed via SDS–PAGE and subsequent Coomassie staining or immunoblotting. For control purposes, Ubp15p activity was inhibited by preincubation with NEM or by point mutation C214A.

**In vitro binding assay**

For binding assays purified GST-tagged bait proteins were premixed with other proteins and incubated with 50 μl of GSH–agarose 4B (Macherey-Nagel) for 1 h. Estimated amounts of 50 μg (Figs. 2 and 6), 30 μg (Fig. 4A), and 15 μg (Fig. 4E) of each protein were used in these assays. Unbound protein was washed off with 10-fold column volume of buffer, and bound components were eluted with buffer containing 100 mM reduced GSH. For assays containing Pex1p and Pex6p, the bait proteins were dialyzed against AAA buffer before the experiment.

**In vivo complementation**

The WT *S. cerevisiae* strain UTL-7A and the corresponding pex5Δ strain were transformed with a plasmid containing mCherry with C-terminally fused PTS1. Additionally, the pex5Δ strain was transformed with an empty pRS416 plasmid, as well as plasmid-encoded WT Pex5p, or Ub−(Δ1−6)Pex5p. The cells were precultured in YNBG medium containing 0.3% glucose. Peroxisome proliferation was induced of 50 μg/ml. Estimated amounts of 50 μg (Figs. 2 and 6), 30 μg (Fig. 4A), and 15 μg (Fig. 4E) of each protein were used in these assays. Unbound protein was washed off with 10-fold column volume of buffer, and bound components were eluted with buffer containing 100 mM reduced GSH. For assays containing Pex1p and Pex6p, the bait proteins were dialyzed against AAA buffer before the experiment.

**Antibodies and immunoblotting**

Immunoblot analysis was performed according to Harlow and Lane (73) with polyclonal rabbit antibodies raised against Pex1p (74), Pex6p (31), Pex5p (30), or monoclonal anti-GST (Signal). Primary antibodies were detected with an IRDye 800CW goat anti-rabbit IgG secondary antibody (LI-COR Bioscience, Bad Homburg, Germany) followed by detection using an IR imaging system (LI-COR Bioscience). Semiquantitative analyses of immunoblot signals were obtained using the IR Imaging System Application software version 3.0 (LI-COR Bioscience). Images were taken every 5 min via a Zeiss Axioskop50 fluorescence microscope (Zeiss). Images were processed with Photoshop CS5 (Adobe) and arranged in figures using Illustrator CS5 (Adobe). The applied two-hybrid assay was based on the described method by Fields and Sternglanz (75). Co-transformation of two-hybrid vectors into the strain PJ69-4A was performed according to Gietz and Woods (76). Transformed yeast cells were plated onto SD synthetic medium without tryptophan and leucine. β-Galactosidase filter assays were performed as described elsewhere (77). Plasmid expressing mCherry-SKL was kindly provided by B. Warscheid (Freiburg, Germany). All experiments shown here were performed at least two times with reproducible results.

**Fluorescence microscopy**

Wide-field fluorescence microscopy imaging was performed on a Zeiss Axioskop50 fluorescence microscope (Zeiss). Images were taken with a Princeton Instruments 1300Y digital camera. The mCherry fluorescence was visualized with a 546/12-nm band-pass excitation filter, a 560-nm dichromatic mirror, and a 575–640-nm band-pass emission filter.

**Miscellaneous**

Semiquantitative analyses of immunoblot signals were obtained using the IR Imaging System Application software version 3.0 (LI-COR Bioscience). Images were processed with Photoshop CS5 (Adobe) and arranged in figures using Illustrator CS5 (Adobe). The applied two-hybrid assay was based on the described method by Fields and Sternglanz (75). Co-transformation of two-hybrid vectors into the strain PJ69-4A was performed according to Gietz and Woods (76). Transformed yeast cells were plated onto SD synthetic medium without tryptophan and leucine. β-Galactosidase filter assays were performed as described elsewhere (77). Plasmid expressing mCherry-SKL was kindly provided by B. Warscheid (Freiburg, Germany). All experiments shown here were performed at least two times with reproducible results.

**Author contributions**—R. E. and W. G. conceptualization; R. E. and W. G. supervision; R. E. funding acquisition; R. E., D. P. S., I. G., and W. G. validation; R. E., D. P. S., I. G., and W. G. investigation; R. E., D. P. S., I. G., and W. G. methodology; R. E., D. P. S., and W. G. writing-original draft; D. P. S. and I. G. data curation; D. P. S. and I. G. formal analysis.

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**Table 3**

Oligonucleotides used in this study

| Designation  | Sequence (5′ → 3′)                                   |
|-------------|-----------------------------------------------------|
| RE3013      | CGGGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3014      | GTAGACCTTTTGATCTTTGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3176      | GGGGTCGGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3178      | GTAGACCTTTTGATCTTTGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3587      | GGGGTCGGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3588      | GTAGACCTTTTGATCTTTGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3589      | GGGGTCGGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3590      | GGGGTCGGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3591      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3592      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3593      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3594      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3595      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3596      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3597      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3598      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3599      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
| RE3600      | CACCGTCGCAAGATGACCTTCGCTTACGACTTTTACCCCGTTGTTGAGGTC |
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