Ubiquitination of the Peroxisomal Targeting Signal Type 1 Receptor, Pex5p, Suggests the Presence of a Quality Control Mechanism during Peroxisomal Matrix Protein Import

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PEX genes encode proteins (peroxins) that are required for the biogenesis of peroxisomes. One of these peroxins, Pex5p, is the receptor for matrix proteins with a type 1 peroxisomal targeting signal (PTS1), which shuttles newly synthesized proteins from the cytosol into the peroxisomal matrix. We observed that in various Saccharomyces cerevisiae pex mutants disturbed in the early stages of PTS1 import, the steady-state levels of Pex5p are enhanced relative to wild type controls. Furthermore, we identified ubiquitinated forms of Pex5p in deletion mutants of those PEX genes that have been implicated in recycling of Pex5p from the peroxisomal membrane into the cytosol. Pex5p ubiquitination required the presence of the ubiquitin-conjugating enzyme Ubc4p and the peroxins that are required during early stages of PTS1 protein import. Finally, we provide evidence that the proteasome is involved in the turnover of Pex5p in wild type yeast cells, a process that requires Ubc4p and occurs at the peroxisomal membrane. Our data suggest that during receptor recycling a portion of Pex5p becomes ubiquitinated and degraded by the proteasome. We propose that this process represents a conserved quality control mechanism in peroxisome biogenesis.

Peroxisomes are vital cell organelles and may contain highly variable sets of enzymes that control many important cellular processes. Their importance is demonstrated by the discovery of a number of inherited human metabolic disorders, with the prototype being Zellweger syndrome, that have been associated with peroxisomal defects, varying from the non-functioning of a single peroxisomal enzyme to complete absence of the organelle (reviewed in Ref. 1). Peroxisomal enzymes are synthesized in the cytosol and delivered post-translationally to their target organelle. To enable this sorting, these enzymes contain specific peroxisomal targeting signals (PTS),1 most of which fall into two categories (reviewed in Ref. 2). The vast majority of proteins contains a signal (PTS1) that is located at the carboxyl terminus and has a consensus sequence related to the canonical -S-K-L-COOH sequence observed in firefly luciferase (3). So far, only a few proteins have been discovered that utilize a PTS2 to enter peroxisomes. This signal, first described for rat 3-ketoacyl-CoA thiolase (4), is located at the amino terminus of proteins and has the consensus sequence (R/K)-(L/A)-(V/I). Additionally, certain proteins contain neither a PTS1 nor a PTS2, but are sorted via still unidentified signals. Finally, because folded and multimeric proteins are also imported into peroxisomes, an alternative strategy that is used by certain proteins is to hitch-hike into the organelle by binding to a protein containing a PTS (see e.g. Refs. 5 and 6).

For both peroxisomal targeting signals, separate cytosolic receptor molecules have been discovered, Pex5p for the PTS1, and Pex7p for the PTS2 (reviewed in Ref. 2), which appear to have a shuttling function. During the receptor cycle, Pex5p binds cargo proteins in the cytosol, sorts these to the surface of the organelle, and, subsequently, assists in transporting them across the membrane in a hitherto unknown fashion. Recent evidence suggests that the PTS1 receptor may actually accompany the cargo protein into the peroxisome, prior to its release into the lumen of the organelle (7). Finally, the receptor is brought back to the cytosol for a new import cycle, a step that has been demonstrated to require ATP hydrolysis (8).

Previous investigations have identified a variety of other proteins directly involved in the biogenesis of peroxisomes (termed peroxins; see Ref. 9; reviewed in Ref. 2). Based on these data, specific peroxins have been suggested to function at distinct steps during peroxisome biogenesis (cf. 10). Peroxins that are required for the formation/maintenance of the peroxisomal membrane are Pex3p and Pex19p (11). Furthermore, a docking/translocation complex at the peroxisomal membrane has been proposed that initiates binding of cargo-loaded Pex5p molecules and, subsequently, facilitates translocation of the cargo into the peroxisomal matrix (1, 2). This large complex has been shown to consist of two subcomplexes: a docking complex, comprising Pex13p, Pex14p, and Pex17p, and a putative translocation complex consisting of Pex2p, Pex10p, and Pex12p. These complexes are presumed to be held together by protein-protein interactions via the peroxins Pex3p or Pex8p (12, 13). The intraperoxisomal peroxin Pex8p, which has so far only been discovered in yeast species, appears also to have a more direct role in matrix protein import by releasing PTS1-carrying cargo from Pex5p molecules (14, 15). Finally, a number of interacting proteins have been suggested to play a role in recycling of the empty receptor to the cytosol. These include two interacting ATPases of the AAA family, Pex1p and Pex6p. In Saccharomyces cerevisiae, these ATPases are bound to the peroxisomal membrane by a...
physical interaction between Pex5p and the peroxisomal membrane protein Pex15p (see Refs. 16 and references therein). In humans, a protein only structurally related to Pex15p, which was designated Pex26p, appears to perform a similar function (17). Additionally, two physically interacting peroxins that have so far only been found in yeast species, the ubiquitin-conjugating enzyme Pex4p/Ubc10p and the integral membrane protein Pex22p (18), are thought to play a role in Pex5p recycling (10, 19). However, so far the target protein to which Pex4p actually conjugates ubiquitin has remained elusive.

Despite a wealth of knowledge about which factors are involved in PTS1 matrix protein import, little is actually known what happens to the receptor Pex5p during this process. Recently, it was observed in the yeast Pichia pastoris, in human cell lines, and in plant cells that the loss of one of the peroxins proposed to function in receptor recycling, Pex1p, Pex4p, Pex6p, or Pex22p, leads to strongly reduced steady-state levels of Pex5p (18, 20, 21). This phenomenon has actually been used to determine the order in which a number of peroxins supposedly act in matrix protein import (10). To investigate whether this process also occurs in S. cerevisiae, we determined the steady-state levels of Pex5p in various pex mutant strains of this yeast species. Surprisingly, we observed that in mutants of those PEX genes that are directly implicated in PTS1 import, steady-state Pex5p levels are enhanced as compared with wild type cells. Furthermore, we found that the PTS1 receptor is ubiquitinated when one of the peroxins proposed to be required for Pex5p recycling was absent. Our data suggest the presence of a possible quality control mechanism at the Pex5p export site that determines whether the receptor will be recycled to the cytosol to perform another round of import or will be turned over by the proteasome.

**Experimental Procedures**

**Strains, Media, and Growth Conditions—**Yeast strains used in this study are listed in Table I, and are derivatives of *S. cerevisiae* wild type UTL7A unless indicated otherwise (22). Deletion mutants were constructed using the KanMX-Marker (23). Strains in which the genomic copy of the PEX5 gene was replaced by a PEX5-ProtA fusion gene were obtained by transforming haploid yeast cells with a PCR product according to Knop et al. (24). The sequences of oligonucleotide primers used for the construction of these strains are available upon request. Complete (YPD) and glucose minimal media (SD) used for yeast culturing have been described previously (25). 0.1 mM CuSO4 was added for the construction of these strains are available upon request.

**Biochemical Methods—**Cell extracts were prepared as follows: Cells were grown to the end of the logarithmic growth phase on glucose minimal medium, galactose minimal medium, or oleate minimal medium. Subsequently, the OD600 was determined and 3 OD600 units of trichloroacetic acid precipitated yeast cells for each reaction. The pellet was suspended vigorously in 100 μl of urea cracking buffer (50 mM Tris-Cl, pH 7.5, 6 M urea, 1% SDS) and incubated for 10 min at 65 °C. Subsequently, 1 ml of Tween-20 IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA) and 10 μl of 100 mg/ml bovine serum albumin were added. After pelleting undissolved material, α-Pex5p antiserum was added, and the mixture was incubated under continuous swirling for 4 h at 4 °C. Subsequently, 75 μl of pre-swollen Protein A-Sepharose beads were added, and the mixture was further incubated for 1 h at 4 °C. The immunoprecipitated material was subsequently pelleted, washed two times with Tween-20 IP buffer, once with Tween-20-ura buffer (100 mM Tris-Cl, pH 7.5, 200 mM NaCl, 1% Tween 20), and once with Tween-20 buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl). Finally, the beads were boiled in 50 μl of IP-sample buffer (125 mM Tris-Cl, pH 6.8, 6.5% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue) and prepared for Western blotting.

**Differential Centrifugation—**Spheroplasts of yeast cells, homogenization, and differential centrifugation at 100,000 × g of homogenates to remove mucilages and other membraneous structures from cytosolic proteins was performed essentially according to Erdmann et al. (25).

**Purification of Pex5p-protA from S. cerevisiae pex5Δ mutant—**To investigate the steady-state levels of Pex5p in *S. cerevisiae* wild type and pex mutant cells by Western blotting, we constructed a strain in which the PEX5 gene was replaced by a fusion gene consisting of the proteasome subunit Pex5p to which Pex4p actually conjugates ubiquitin has role in Pex5p recycling (10, 19). However, so far the target protein to which Pex4p actually conjugates ubiquitin has remained elusive.

**Experimental Procedures—**

**Strains, Media, and Growth Conditions—**Yeast strains used in this study are listed in Table I, and are derivatives of *S. cerevisiae* wild type UTL7A unless indicated otherwise (22). Deletion mutants were constructed using the KanMX-Marker (23). Strains in which the genomic copy of the PEX5 gene was replaced by a PEX5-ProtA fusion gene were obtained by transforming haploid yeast cells with a PCR product according to Knop et al. (24). The sequences of oligonucleotide primers used for the construction of these strains are available upon request. Complete (YPD) and glucose minimal media (SD) used for yeast culturing have been described previously (25). 0.1 mM CuSO4 was added for the construction of these strains are available upon request.

**Biochemical Methods—**Cell extracts were prepared as follows: Cells were grown to the end of the logarithmic growth phase on glucose minimal medium, galactose minimal medium, or oleate minimal medium. Subsequently, the OD600 was determined and 3 OD600 units of cells were precipitated with 12.5% trichloroacetic acid. After overnight precipitation at −80 °C, the cells were pelleted by centrifugation and resuspended twice with 80% acetone of 20 °C. After thorough drying, the pellet was solubilized in 4 ml of solubilization buffer (1% SDS, 0.1% NaOH) by vigorous mixing with the aid of glass beads. Subsequently, the mixture was neutralized by the addition of 1 ml of 5× boiling buffer (250 mM Tris-Cl, pH 7.5, 10% SDS) and incubated for 5 min at 99 °C. After cooling to room temperature, 25 ml of the Tween 20-IP buffer was added. The mixture was centrifuged for 15 min at 6,000 × g to remove undissolved material. The resulting pellet was resuspended in 10 ml of Tween 20-IP buffer and, after thorough mixing, again centrifuged. Subsequently, both supernatants were combined, and the volume was brought up to 50 ml with Tween 20-IP buffer. The resulting extract contains fully denatured proteins and can if necessary be stored overnight at 4 °C.

Subsequently, the extract was loaded on a 0.25-column of IgG-Sepharose 6 Fast Flow (Amersham Biosciences; pre-washed in wash buffer 1 (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.05% Tween 20)). After loading, the column was washed with 40 volume columns of wash buffer 1, followed by washing with 12 volume columns of wash buffer 2 (5 mM NH4Ac, pH 5.0). Finally, the bound protA-containing proteins were eluted from the column using elution buffer (0.5 M acetic acid, pH 3.4). Eluate fractions of 0.5 ml were lyophilized and analyzed by SDS/PAGE and Western blotting.

**Mass Spectrometry—**For mass spectrometry, fractions with protA-containing proteins were separated by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue G250, and the protein bands were excised from the gel. Excised bands were digested with trypsin at 37 °C overnight. The tryptic peptides were extracted and spotted on a target for MALDI mass fingerprint analysis. Measurements were done on a Bruker Reflex IV instrument. Fingerprint mass analysis was performed using the ProFound algorithm. For tandem mass spectrometry analysis the same instrument was employed acquiring the post-source delay spectrum from the desired parent ion.

**RESULTS**

**Pex5p Is Modified in a Specific Set of S. cerevisiae pex Mutants—**To investigate the steady-state levels of Pex5p in *S. cerevisiae*, we grew cells to the end of the logarithmic growth phase on glucose medium and determined Pex5p levels in wild type and pex mutant cells by quantification of immuno...
### Table 1

**S. cerevisiae strains used in this study**

| Strain   | Characteristics                                                                 | Reference |
|----------|---------------------------------------------------------------------------------|-----------|
| UTL7A    | MATa, ura3-52, trp1, leu2-3,112                                                  | W. Dunz, Bochum, Germany |
| pex1     | UTL7A pex1::loxP                                                                   | This study |
| pex2     | UTL7A pex2::LEU2                                                                   | 11        |
| pex3     | UTL7A pex3::KanMX4                                                                | 11        |
| pex4     | UTL7A pex4::LEU2                                                                   | 29        |
| pex5     | UTL7A pex5::KanMX4                                                                | 11        |
| pex6     | UTL7A pex6::KanMX4                                                                | 11        |
| pex7     | UTL7A pex7::KanMX4                                                                | 11        |
| pex8     | UTL7A pex8::LEU2                                                                   | 14        |
| pex10    | UTL7A pex10::loxP                                                                   | 33        |
| pex11    | UTL7A pex11::LEU2                                                                  | 34        |
| pex12    | UTL7A pex12::LEU2                                                                  | 34        |
| pex13    | UTL7A pex13::KanMX4                                                               | 35        |
| pex14    | UTL7A pex14::KanMX4                                                               | 11        |
| pex15    | UTL7A pex15::CreloxP::KanMX4                                                       | This study |
| pex17    | UTL7A pex17::LEU2                                                                  | 35        |
| pex18    | UTL7A pex18::KanMX4                                                               | 36        |
| pex19    | UTL7A pex19::CreloxP::KanMX4                                                       | This study |
| pex21    | UTL7A pex21::CreloxP::KanMX4                                                       | This study |
| pex22    | UTL7A pex22::CreloxP::KanMX4                                                       | This study |
| pex1 pex2| UTL7A pex1::loxP::pex2::CreloxP::KanMX4                                            | This study |
| pex1 pex4| UTL7A pex1::loxP::pex4::KanMX4                                                     | This study |
| pex1 pex5| UTL7A pex1::loxP::pex5::CreloxP::KanMX4                                            | This study |
| pex1 pex6| UTL7A pex1::loxP::pex6::CreloxP::KanMX4                                            | This study |
| pex1 pex7| UTL7A pex1::loxP::pex7::CreloxP::KanMX4                                            | This study |
| pex1 pex8| UTL7A pex1::loxP::pex8::CreloxP::KanMX4                                            | This study |
| pex1 pex9| UTL7A pex1::loxP::pex9::CreloxP::KanMX4                                            | This study |
| pex1 pex10| UTL7A pex1::loxP::pex10::CreloxP::KanMX4                                          | This study |
| pex1 pex11| UTL7A pex1::loxP::pex11::CreloxP::KanMX4                                          | This study |
| pex1 pex12| UTL7A pex1::loxP::pex12::CreloxP::KanMX4                                          | This study |
| pex1 pex15| UTL7A pex1::loxP::pex15::CreloxP::KanMX4                                          | This study |
| pex4 pex2| UTL7A pex4::loxP::pex2::CreloxP::KanMX4                                           | This study |
| pex4 pex3| UTL7A pex4::loxP::pex3::CreloxP::KanMX4                                           | This study |
| pex4 pex5| UTL7A pex4::loxP::pex5::CreloxP::KanMX4                                           | This study |
| pex4 pex6| UTL7A pex4::loxP::pex6::CreloxP::KanMX4                                           | This study |
| pex4 pex7| UTL7A pex4::loxP::pex7::CreloxP::KanMX4                                           | This study |
| pex4 pex8| UTL7A pex4::loxP::pex8::CreloxP::KanMX4                                           | This study |
| pex4 pex9| UTL7A pex4::loxP::pex9::CreloxP::KanMX4                                           | This study |
| pex4 pex10| UTL7A pex4::loxP::pex10::CreloxP::KanMX4                                          | This study |
| pex4 pex11| UTL7A pex4::loxP::pex11::CreloxP::KanMX4                                          | This study |
| pex4 pex12| UTL7A pex4::loxP::pex12::CreloxP::KanMX4                                          | This study |
| pex4 pex13| UTL7A pex4::loxP::pex13::CreloxP::KanMX4                                          | This study |
| pex4 pex15| UTL7A pex4::loxP::pex15::CreloxP::KanMX4                                          | This study |
| pex4 pex22| UTL7A pex4::loxP::pex22::CreloxP::KanMX4                                         | This study |
| ubc1     | UTL7A ubc1::CreloxP::KanMX4                                                        | This study |
| ubc2     | UTL7A ubc2::CreloxP::KanMX4                                                        | This study |
| ubc4     | UTL7A ubc4::CreloxP::KanMX4                                                        | This study |
| ubc5     | UTL7A ubc5::CreloxP::KanMX4                                                        | This study |
| ubc6     | UTL7A ubc6::CreloxP::KanMX4                                                        | This study |
| ubc7     | UTL7A ubc7::CreloxP::KanMX4                                                        | This study |
| ubc8     | UTL7A ubc8::CreloxP::KanMX4                                                        | This study |
| ubc9     | UTL7A ubc9::CreloxP::KanMX4                                                        | This study |
| ubc11    | UTL7A ubc11::CreloxP::KanMX4                                                       | This study |
| ubc12    | UTL7A ubc12::CreloxP::KanMX4                                                       | This study |
| ubc13    | UTL7A ubc13::CreloxP::KanMX4                                                       | This study |
| pex1 ubc1| UTL7A pex1::loxP::ubc1::CreloxP::KanMX4                                            | This study |
| pex1 ubc2| UTL7A pex1::loxP::ubc2::CreloxP::KanMX4                                            | This study |
| pex1 ubc4| UTL7A pex1::loxP::ubc4::CreloxP::KanMX4                                            | This study |
| pex1 ubc5| UTL7A pex1::loxP::ubc5::CreloxP::KanMX4                                            | This study |
| pex1 ubc6| UTL7A pex1::loxP::ubc6::CreloxP::KanMX4                                            | This study |
| pex1 ubc7| UTL7A pex1::loxP::ubc7::CreloxP::KanMX4                                            | This study |
| pex1 ubc9| UTL7A pex1::loxP::ubc9::CreloxP::KanMX4                                            | This study |
| pex1 ubc11| UTL7A pex1::loxP::ubc11::CreloxP::KanMX4                                           | This study |
| pex1 ubc12| UTL7A pex1::loxP::ubc12::CreloxP::KanMX4                                           | This study |
| pex1 ubc13| UTL7A pex1::loxP::ubc13::CreloxP::KanMX4                                           | This study |
| pex4 ubc1| UTL7A pex4::LEU2 ubc1::CreloxP::KanMX4                                            | This study |
| pex4 ubc2| UTL7A pex4::LEU2 ubc2::CreloxP::KanMX4                                            | This study |
| pex4 ubc4| UTL7A pex4::LEU2 ubc4::CreloxP::KanMX4                                            | This study |
| pex4 ubc5| UTL7A pex4::LEU2 ubc5::CreloxP::KanMX4                                            | This study |
| pex4 ubc6| UTL7A pex4::LEU2 ubc6::CreloxP::KanMX4                                            | This study |
| pex4 ubc7| UTL7A pex4::LEU2 ubc7::CreloxP::KanMX4                                            | This study |
| pex4 ubc8| UTL7A pex4::LEU2 ubc8::CreloxP::KanMX4                                            | This study |
| pex4 ubc11| UTL7A pex4::LEU2 ubc11::CreloxP::KanMX4                                           | This study |
| pex4 ubc12| UTL7A pex4::LEU2 ubc12::CreloxP::KanMX4                                           | This study |
| pex4 ubc13| UTL7A pex4::LEU2 ubc13::CreloxP::KanMX4                                           | This study |
| PEX5-protA| UTL7A::PEX5-protA::KanMX4                                                          | This study |
| pex4 ubc10| UTL7A pex4::LEU2 ubc10::CreloxP::KanMX4                                          | This study |
| cim3-1 pex4| UTL7A::Cim3-1 pex4::KanMX4                                                         | This study |
| cim3-1 pex10| UTL7A::Cim3-1 pex10::KanMX4                                                        | This study |
| cim3-1 ubc4| UTL7A::Cim3-1 ubc4::CreloxP::KanMX4                                                | This study |
Essentially identical results were observed when the cells were
are apparently not observed in
certain
pex22
and
pex21
mutant cells that display no PTS1 import defect (e.g.
creased compared with wild type control cells. In contrast, in
Notably, the modification pattern of these
were also seen with oleate-induced cells (data not shown).
These bands were most prominent in
A
). The results indicate that in mutant cells affected in
pex13, pex11
, and
pex2
mutants proposed
Synthesis of Myc-
and
pex15

ubiquitin (8.5 kDa) becomes conjugated to a target protein, a
forms of Pex5p. However, dephosphorylation of immunopre-
possibility that the additional bands represent phosphorylated
of Pex5p in wild type cells. Furthermore, overproduction of
tagged ubiquitin did not affect Pex5p levels nor its modifi-
Uracil. These data are indicative of Pex5p ubiquitination in
ubiquitin did not result in any detectable modification
Tagged ubiquitin did not result in any detectable modification

Notably, in five
pex
mutants (pex1, pex4, pex6, pex15, and
pex22
), we observed, in addition to the normal Pex5p protein band,
a-Pex5p immunoreactive bands of higher
(Fig. 1A). The results indicate that in mutant cells affected in
either the formation of the peroxisomal membrane (pex3 and
pex9
), or in peroxisomal matrix protein import (pex2, pex8,
pex10, pex12, pex13, and pex14), Pex5p levels were much increased compared with wild type control cells. In contrast, in
mutant cells that display no PTS1 import defect (e.g. pex11,
Figs 1, pex14, and pex27), Pex5p levels remained rather similar to
those observed in wild type cells. Also in
pex
mutants proposed
to be disturbed in receptor recycling (pex1, pex4, pex6, pex15,
and pex22), Pex5p levels were rather close to wild type levels.
Therefore, the strong reductions in Pex5p levels observed in
certain
P. pastoris
, human, and plants
pex mutants (18, 20, 21)
are apparently not observed in
S. cerevisiae
pex mutants. Es-
esentially identical results were observed when the cells were
grown on oleate (data not shown).

Table II
Plasmids used in this study

| Plasmid          | Characteristics                                      | Reference        |
|------------------|------------------------------------------------------|------------------|
| pEMBLyex4        | E. coli/S. cerevisiae shuttle plasmid, contains GAL promoter; Amp<sup>R</sup>, URA3 | 37               |
| pEMBLyex4-PAS2   | pEMBLyex4 expressing the wild type PEX4 gene         | 29               |
| pEMBLyex4-PAS2<sup>a</sup> | pEMBLyex4 expressing the inactive PEX4-C115S gene | 29               |
| pRS416           | E. coli/S. cerevisiae shuttle vector, CEN, URA3, Amp<sup>R</sup> | 38               |
| pRSps1-Glu-744   | pRS416 expressing PEX1 K744E                         | 28               |
| pRSpas1-Glu-744  | pRS416 expressing PEX1 K744E                         | 28               |
| pRSpas1-Glu-744  | pRS416 expressing PEX1 K744E                         | 39               |
| YEp105           | YEp46 derivative; contains expression                 | T. Sommer, MDC<sup>a</sup>, Berlin, Germany            |
| YEp105           | Cassette Pcp1-MYC-UB-Tcry1; 2-μm, Amp<sup>R</sup>, TRP1 | T. Sommer, MDC<sup>a</sup>, Berlin, Germany            |
| pBKK8            | YEp352 with expression cassette Pcp1-MYC-UB-Tcry1    | This study       |
| pBKK8            | YEp46 derivative; contains expression                 | This study       |
| pBKK9            | YEp352 with expression cassette Pcp1-UB.K48R/Tcry1   | This study       |

<sup>a</sup> MDC, Max-Delbrück Center.

m; Amp, Ampicillin; 

Pex5p is Ubiquitinated in pex1 and pex4 Mutants—We set
out to identify the nature of the modifications of Pex5p ob-
erved in these specific
pex
mutants. First, we analyzed the
possibility that the additional bands represent phosphorylated
forms of Pex5p. However, dephosphorylation of immunopre-
cipitated Pex5p did not affect the presence of the bands (data
not shown). Also, a drastic change in conformation of the PTS1
receptor Pex5p may influence its behavior on SDS/polyacryl-
amide gels. However, iodoacetamide treatment of samples,
which should result in a more efficient unfolding of proteins,
had no effect on the presence of the modifications (data not
shown). To investigate whether Pex5p was modified by ubiqui-
tination, we transformed cells of selected
pex
mutants with a
plasmid that enables synthesis of an N-terminally Myc epitope-
tagged form of ubiquitin. When a Myc-tagged ubiquitin moiety
(calculated molecular mass of 10 kDa) instead of wild type
ubiquitin (8.5 kDa) becomes conjugated to a target protein, a
shift toward a higher molecular weight is observed. Analysis of
the transformants indicated that this indeed occurred with the
modified Pex5p bands (shown for
pex1
and
pex4
in Fig. 2,
compare lanes 1 and 4 with lanes 2 and 5). Synthesis of Myc-
tagged ubiquitin did not result in any detectable modification

![Fig. 1](http://www.jbc.org)
followed by analysis of the precipitates by Western blotting using monoclonal antibodies raised against bovine ubiquitin. α-Pex5p-specific antibodies were used as control. The results (Fig. 3, right panel) show that in α-Pex5p precipitates from pex1, pex6, and pex15 cells an identical pattern of proteins was detected with the ubiquitin antibodies, indicating the presence of ubiquitinated Pex5p species in these mutants. Similarly, in α-Pex5p precipitates from pex4 and pex22 cells a set of ubiquitinated proteins was observed, albeit with a different protein pattern than observed for pex1, pex6, and pex15 cells. These patterns were reminiscent of the Pex5p modification patterns seen in the immunoblotting experiments (cf. Fig. 1B). However, the most abundant modified Pex5p species in pex4 and pex22 cells observed in those experiments (see Fig. 1B) was hardly detectable in the α-Pex5p precipitates.

To confirm that all modification bands in the pex4 mutant indeed represented ubiquitinated species of Pex5p, we further analyzed these bands by mass spectroscopy. In baker’s yeast, Pex5p is a low abundance protein that is highly unstable in vitro. To enable its purification and subsequent analysis, we constructed S. cerevisiae strains producing Pex5p carboxylterminally tagged with Staphylococcus aureus protein A. We first investigated whether addition of the tag had an effect on the function of Pex5p. Growth analysis indicated that a wild-type strain producing Pex5p-protA instead of wild-type Pex5p was equally capable of growing on oleate plates as wild type. Additionally, Western blot analysis demonstrated that in pex4 cells in which PEX5 had been replaced by PEX5-protA the additional Pex5p-immunoreactive protein bands were still being synthesized (Fig. 4B). Subsequently, we purified denatured protein A-containing proteins from the pex4 PEX5-protA strain using IgG-Sepharose affinity chromatography (see “Materials and Methods”). The isolated proteins were separated by SDS-PAGE, and gels were stained with colloidal Coomassie. This revealed the expected pattern of three protein bands, with the lowest of the three being the most prominent (cf. Fig. 4B, lane 3), which were analyzed by mass spectroscopy (see “Materials and Methods”). The resulting mass fingerprint list identified Pex5p in all three bands. Moreover, in both minor bands of higher molecular weight, five additional parent masses (1523.8, 1345.9, 1267.7, 1067.2, and 1039.5 Da) were observed belonging to tryptic peptides from ubiquitin. To prove the identity of this post-translational modification the parent mass (M+H)1 at 1523.8 Da was further characterized by MALDI-post-source delay analysis, and the resulting fragment spectrum was analyzed by the SEQUEST algorithm. This procedure identified the amino acid sequence 30–42 (IQDKEGI-)

**Fig. 2.** Overproduction of modified forms of ubiquitin demonstrates ubiquitination of Pex5p in S. cerevisiae pex1 and pex4 mutants. S. cerevisiae pex1 and pex4 cells were transformed with plasmids pBKKS, expressing MYC-UB, and pBBK9, expressing UB.K48R using vector YEp352 as a control. Transformants were grown to the end of the exponential growth phase in selective glucose minimal medium in the presence of 0.1 mM CuSO4, to induce expression of the ubiquitin genes by the COP1 promoter. Subsequently, Western blotting was prepared from trichloroacetic acid extracts, with equal amounts of protein loaded per lane. The blots were decorated with specific antibodies against S. cerevisiae Pex5p (top panels) or monoclonal antibodies against bovine ubiquitin to demonstrate ubiquitin overproduction (lower panels). Lane 1, pex1 (YEp352); lane 2, pex1 (pBBK9); lane 3, pex1 (pBBK9); lane 4, pex4 (YEp352); lane 5, pex4 (pBBK9); and lane 6, pex1 (pBBK9). The values to the left of the blots indicate sizes of marker proteins (in kilodaltons).

**Fig. 3.** Immunoprecipitation of Pex5p from cells of specific S. cerevisiae pex mutants demonstrates ubiquitination of the PTS1 receptor. S. cerevisiae wild type cells and cells of the indicated pex mutants were grown on glucose minimal medium to the late exponential growth phase. Cells were harvested, and Pex5p was immunoprecipitated as described under “Materials and Methods.” Equal volumes of immunoprecipitates were loaded on SDS/polyacrylamide gels and Western blots were prepared. Blots were decorated with specific antibodies against S. cerevisiae Pex5p (left panel) or monoclonal antibodies against bovine ubiquitin (right panel). The left panel shows that Pex5p can indeed be immunoprecipitated. It must be noted that some of the α-Pex5p specific bands of higher molecular weight than Pex5p can only be visualized upon extreme overexposure (not shown). Nevertheless, in pex1, pex4, pex6, pex15, and pex22 mutants the ubiquitin antibodies recognize specific protein bands in the α-Pex5p immunoprecipitates. WT, wild type; the numbers under the blots refer to the S. cerevisiae pex1, pex4, pex5, pex6, and pex22 mutants, respectively.

**Fig. 4.** Analysis of Pex5p-protA species. A, addition of the protein A tag to Pex5p does not significantly affect peroxisome biogenesis in S. cerevisiae. Cells of the indicated strains were grown overnight on glucose minimal media. Subsequently, dilutions were prepared, and 2 μl of each dilution was spotted onto oleate plates. The plates were then incubated for 3–5 days at 30 °C and scored for the appearance of colonies. B, addition of the protein A tag to Pex5p has no effect on the formation of modified Pex5p molecules. Cells of wild type (lane 1), PEX5-protA (lane 2), and pex4 PEX5-protA (lane 3) were grown on glucose mineral medium to the late exponential growth phase and harvested. Trichloroacetic acid extracts were prepared and immunoblotted for Pex5p, using equal amounts of protein per lane. The values to the left of the blots indicate sizes of marker proteins (in kilodaltons). The asterisk indicates a minor degradation product of Pex5p-protA that is not observed in extracts of wild type cells.
form of Pex5p represents a mono-ubiquitinated species. It has been observed before that mono-ubiquitinated proteins are poorly recognized by monoclonal antibodies against bovine ubiquitin (cf. Ref. 26), which may explain why this prominent ubiquitinated species was hardly detectable in the immunoprecipitation experiment (see Fig. 3).

To analyze the Pex5p ubiquitination in somewhat more detail, in addition to transforming pex1 and pex4 cells with a plasmid that results in the synthesis of Myc-tagged ubiquitin (Fig. 2, lanes 2 and 5), we also transformed the same cells with a plasmid that enables synthesis of a mutant ubiquitin (Ub.Lys48Arg; Fig. 2, lanes 3 and 6). In cells that also produce wild type ubiquitin, synthesis of this form of ubiquitin will reduce the conjugation of multiple ubiquitin molecules to target proteins via the lysine 48 residue thereby interfering with their turnover by the proteasome (see Ref. 27). As a result, lowly ubiquitinated target proteins are expected to accumulate. In the pex1 mutant the ubiquitination pattern of Pex5p changed upon expression of Ub.Lys48Arg, with a clear increase in the amount of ubiquitinated Pex5p molecules (Fig. 2, compare lanes 1 and 3). Also in cells of the pex4 mutant, overproduction of Ub.Lys48Arg resulted in an increase of ubiquitinated Pex5p, especially of forms containing an estimated two to four ubiquitin moieties (Fig. 2, compare lanes 4 and 6).

Ubiquitinated Pex5p Is Pelletable—Pex5p is a cycling receptor that has a dual location in the wild type cell, i.e. it is present in the cytosol and in a peroxisome-bound form. In pex1 and pex4 mutants, peroxisomal matrix proteins are mislocalized to the cytosol, whereas the peroxisomal membrane proteins remain present in so-called peroxisomal membrane remnants. To obtain information as to the function of the observed ubiquitination of Pex5p in peroxisome biogenesis, we analyzed the subcellular location of the ubiquitinated species. Thus, post-nuclear supernatants of gently lysed protoplasts were prepared from pex1 and pex4 cells and separated by centrifugation at 100,000 × g into an organellar pellet fraction, which also contains the peroxisomal membrane remnants, and a cytosolic fraction. Western blot analysis demonstrated that in pex1 and pex4 cells unmodified Pex5p was located in both organelar and cytosolic fractions (Fig. 5). It must be noted, however, that in vitro Pex5p is a highly unstable protein and especially the cytosolic pool of Pex5p is subject to proteolysis. Therefore, the relative amount of unmodified Pex5p in the various fractions does not represent the physiological state. Nevertheless, the lowly abundant ubiquitinated forms of Pex5p, that apparently represent a rather stable fraction of the protein, co-fractionated exclusively with the organelar fraction, suggesting that these forms of Pex5p are localized at peroxisomal remnants rather than present in the cytosol.

Ubiquitination of Pex5p in pex1 and pex4 Cells Is Dependent on Ubc4p—The ubiquitination of Pex5p observed in pex4 cells is obviously not the result of the action of Pex4p, so far the only known ubiquitin-conjugating enzyme (also known as Ubc10p) to be involved in peroxisome biogenesis. To understand which Ubc protein is responsible for the ubiquitination of Pex5p in pex1 and pex4 cells, we constructed null mutants for UBC1, UBC2, UBC4, UBC5, UBC6, UBC7, UBC8, UBC11, UBC12, and UBC13 in wild type, pex1, and pex4 backgrounds and analyzed their effect on the formation of ubiquitinated Pex5p. The resulting data (Fig. 6, A and B) indicate that, for both pex1 and pex4 cells, introduction of the ubc4 mutation affected Pex5p ubiquitination severely. In contrast, none of the other ubc mutations had any significant effect on the Pex5p ubiquitination patterns. Notably, when the indicated ubc mutations were introduced in the wild type background no significant effect was observed on steady-state levels of Pex5p, and modified Pex5p bands were not observed (data not shown).
When Inactive Forms of Pex1p and Pex4p Are Present—

To ascertain that the ubiquitination of Pex5p observed in pex1 and pex4 mutants results from the absence of the activity of the peroxins Pex1p and Pex4p, rather than being a direct consequence of the absence of the proteins, we introduced in pex1 and pex4 cells versions of the corresponding genes mutated in the active sites of their gene products (Fig. 7). Thus, pex1 cells were transformed with a plasmid expressing the inactive gene PEX1-K744E, mutated in the second ATP binding site of the two AAA modules of Pex1p, which blocks its function in peroxisome biogenesis (28). Similarly, a plasmid carrying an inactive variant of the PEX4 gene (PEX4-C115S (29)) was introduced in the pex4 mutant. Notably, these mutant proteins have the same subcellular location as the wild type proteins (28, 29). In both cases, in specific cases the ubiquitination pattern changed to a certain extent (Fig. 8), indicating that ubiquitination of Pex5p in trichloroacetic acid extracts was determined by immunoblotting using α-Pex5p-specific antibodies. Equal amounts of protein were loaded per lane. A, a similar analysis for the S. cerevisiae pex1 mutant. The lower part of panel B shows a short exposure of the same blot to demonstrate that in certain double mutants (i.e. pex1 pex2, pex1 pex8, and pex1 pex15) Pex5p levels have increased as compared with the parental strain. The indicated numbers refer to the PEX gene deleted in the pex1 (A) or pex1 (B) backgrounds. The asterisks indicate the presence of a faint α-Pex5p immunoreactive protein band that is occasionally observed on Western blots.

Ubiquitination of Pex5p in pex1 and pex4 Cells Depends on the Presence of Other Peroxins—Recently, Collins et al. (10) studied steady-state Pex5p levels in pex mutants of P. pastoris and observed that the strongly decreased Pex5p levels observed in certain mutants (e.g. pex4 and pex22) could be restored to wild type levels when these were combined with mutations in those PEX genes that were directly required for peroxisomal protein import. To understand whether the ubiquitination of Pex5p observed in S. cerevisiae pex4 mutants is also dependent on other peroxins, we created double mutants by separately deleting selected PEX genes in the pex4 background. Subsequently, we studied Pex5p ubiquitination in the constructed strains. The results (Fig. 8A) indicate that ubiquitination of Pex5p can no longer be observed when the pex4 null mutation was combined with deletions in PEX genes that are required for the formation of the peroxisomal membrane (PEX3) or for peroxisomal matrix protein import (PEX2, PEX8, PEX10, and PEX13). No effect was observed on Pex5p ubiquitination when the pex4 null allele was combined with deletions in genes not involved in PTS1 import (PEX7 and PEX11). Also when two pex mutations in genes proposed to be involved in recycling of Pex5p were combined (pex4 pex1, pex4 pex6, pex4 pex15, and pex4 pex22), the ubiquitination remained present, although in specific cases the ubiquitination pattern changed to a certain extent (pex4 pex1, pex4 pex6, and pex4 pex15).

We have performed a similar analysis for the pex1 mutant (Fig. 8B) and obtained essentially the same results. Notably, in the double mutants that no longer have ubiquitinated forms of Pex5p, the steady-state levels of this protein increased as compared with the parental strain (shown for pex1 double mutants in Fig. 8B, lower blot), confirming that the PEX genes deleted in these mutants act prior to PEX1 and PEX4 (cf. Ref. 10). Thus, we conclude that ubiquitination of Pex5p in the pex1 and pex4 mutants depends on the presence/function of those peroxins that are required for functional PTS1 import.

Inhibition of Proteasome Function Results in the Accumulation of Ubiquitinated Pex5p—Our data suggested that the lower levels of Pex5p in wild type cells, as compared with pex mutant cells affected in PTS1 import, may actually result from proteasomal degradation of a portion of the PTS1 receptor during its recycling to the cytosol. Therefore, we analyzed Pex5p in a mutant disturbed in the function of the proteasome, i.e. cim3-1, which carries a mutant allele of the gene encoding the proteasomal ATPase Rpt6p (30). If a significant amount of
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Pex5p indeed becomes degraded by the proteasome, inhibition of proteasome function should result in the accumulation of ubiquitinated Pex5p molecules. Fig. 9 indicates that in the cim3-1 background α-Pex5p-specific proteins of higher molecular weight than Pex5p are indeed present. Notably, when the cim3-1 mutation was combined with a deletion of either UBC4 or PEX10, these modified forms of Pex5p were absent. In contrast, a cim3-1 pex4 mutant still contained (possibly even somewhat enhanced levels of) these modified Pex5p molecules. Taken together, these results strongly suggest that the modified forms of Pex5p observed here represent ubiquitinated species of the PTS1 receptor.

**DISCUSSION**

We describe the identification of ubiquitinated forms of Pex5p in mutants of PEX genes that encode two groups of physically interacting proteins (Pex4p/Pex22p and Pex1p/Pex6p/Pex15p). The identification of ubiquitinated Pex5p in a specific set of *S. cerevisiae* pex mutants and its dependence on other PEX genes is consistent with the role ascribed to certain peroxins in peroxisome biogenesis (2). Our data indicate that these ubiquitinated forms of Pex5p are exclusively synthesized in mutants lacking the peroxins Pex1p, Pex4p, Pex6p, Pex15p, and Pex22p, all of which have been implicated in Pex5p recycling. Modification of Pex5p was never observed in those pex mutants that lack one of the peroxins thought to be involved in the formation of the peroxisomal membrane (Pex5p and Pex19p) or in docking/translocation of PTS1 proteins (Pex2p, Pex6p, Pex10p, Pex12p, Pex13p, Pex14p, and Pex17p). Rather, the formation of ubiquitinated Pex5p molecules in pex1 and pex4 cells appeared to depend on the presence of these peroxins, which all act in the steps prior to receptor recycling. Such dependence implies that these Pex5p molecules have actually followed most of the translocation route at the peroxisomal membrane and have become blocked at a stage where Pex5p is normally recycled to the cytosol. Presumably, at this membrane-bound stage of the receptor cycle ubiquitination of Pex5p has occurred. Indeed, we demonstrated that the ubiquitinated forms of Pex5p were exclusively present in the organellar pellet upon differential centrifugation of lysed pex1 and pex4 spheroplasts, suggesting that these molecules are located at the peroxisomal membrane. Thus, our data seem to be consistent with previous experiments that suggested a limited import of PTS1 matrix proteins in *P. pastoris* pex4 and pex22 (10), *Hansenula polymorpha* pex4 (19), and *Arabidopsis thaliana* pex6 (21) cells.

Apparently, the inactivation of one of the peroxins in the Pex4p-Pex22p or Pex1p-Pex6p-Pex15p complexes, results in Pex5p ubiquitination, a process that we show to be dependent on the ubiquitin-conjugating enzyme Ubc4p. Clearly, this ubiquitination is not related to the alleged function of Pex4p (Ubc10p), which in a hitherto unknown fashion is involved in Pex5p recycling. Moreover, *ube4* mutants grow normally on oleate plates, precluding a direct role for the observed Pex5p ubiquitination in peroxisome biogenesis. But if the ubiquitination of Pex5p is not directly involved in recycling of the receptor to the cytosol, what then is the function of this protein modification? We propose that this function is related to quality control of Pex5p at the peroxisomal membrane (Fig. 10). In such a scheme, when peroxisomal import occurs normally, a receptor cycle, including Pex5p binding, import, and recycling, results in a highly efficient import of PTS1 proteins into the peroxisome matrix. However, occasionally the recycling of Pex5p may not function optimally. Under such conditions, the obstructing receptor molecules should be removed from the import/export site, which could be achieved by ubiquitination of Pex5p followed by its degradation via the proteasome. Such a process would be highly enhanced when recycling of Pex5p becomes blocked in specific pex mutants. This scenario is consistent with the reduced Pex5p levels in *P. pastoris* pex1, pex4, pex6, and pex22 cells, in human *pex1* and *pex6* cell lines and in *A. thaliana* pex6 cells (18, 20, 21, 31).

At first sight, such a scheme does not seem to fit completely with the phenotypes observed here for *S. cerevisiae*. In this yeast species, pex1, pex4, pex6, pex15, and pex22 mutants show steady-state levels of Pex5p rather similar to those observed in wild type cells. Furthermore, although ubiquitinated forms of Pex5p are visible in these mutants, and are thought to be located at peroxisomal remnants, these are apparently not removed with high efficiency by the proteasome. There is, however, one major difference between our data and those described for *P. pastoris*. Although in *P. pastoris* pex mutants affected in docking/translocation of PTS1 proteins the steady-state levels of Pex5p do not differ significantly from those in wild type cells (10), we have observed that *S. cerevisiae* mutants lacking these peroxins have significantly higher Pex5p levels than wild type cells. We interpret this phenomenon as an indication that already in *S. cerevisiae* wild type cells a significant number of Pex5p molecules may actually be degraded. This view is confirmed by the observation that in a mutant blocked in proteasome functioning (cim3-1) ubiquitinated forms of Pex5p accumulate, in a Ubc4p-dependent manner. Notably, also deletion of the peroxisomal membrane protein Pex10p prevented formation of modified Pex5p species in the cim3-1 mutant. This implies that also here the observed modification indeed takes place at the peroxisomal membrane and does not represent ubiquitination of wrongly folded Pex5p mol-
When this manuscript was under review, the group of Erdmann (40) reported that Pex5p is ubiquitinated in a Ube4p-dependent manner in S. cerevisiae pex mutants disturbed in receptor recycling. In many respects the data in both manuscripts are in line with each other and complementary. However, a significant difference between both reports concerns the steady-state level of Pex5p in pex mutants, which is an essential component of the model shown in Fig. 10. In the present communication, we show the results of a careful analysis of Pex5p levels in multiple independent samples that demonstrates unequivocally that Pex5p levels in a specific set of pex mutants is 2- to 3-fold higher than in wild type cells (Fig. 1A). Platta and co-workers do not show any data regarding Pex5p steady-state levels in their report. Nevertheless, they indicate that all pex mutants have identical Pex5p levels. To obtain reproducible data on Pex5p levels, we have cultivated the relevant pex mutant strains on medium containing either glucose or oleate as carbon source and utilized multiple exposures of Western blots to enable reliable quantification of Pex5p levels. In contrast, in their report Platta and co-workers have only analyzed oleate grown cells, which contain extremely high levels of Pex5p. Furthermore, their report concentrates on the detection of the very minor amounts of ubiquitinated Pex5p present in a specific set of pex mutant cells, which in our hands required significant overexposure of blots (cf. Fig. 1B). We feel that, because of continuous overexposure of their blots, Platta and co-workers may have missed the 2- to 3-fold difference in steady-state Pex5p levels that we have consistently observed.

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