The Saccharomyces cerevisiae Peroxisomal Import Receptor Pex5p Is Monoubiquitinated in Wild Type Cells*

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Pex5p is a mobile receptor for peroxisomal targeting signal type 1-containing proteins that cycles between the cytoplasm and the peroxisome. Here we show that Pex5p is a stable protein that is monoubiquitinated in wild type cells. By making use of mutants defective in vacuolar or proteasomal degradation we demonstrate that monoubiquitinated Pex5p is not a breakdown intermediate of either system. Monoubiquitinated Pex5p is localized to peroxisomes, and ubiquitination requires the presence of functional docking and RING finger complexes, which suggests that it is a late event in peroxisomal matrix protein import. In pex1, pex4, pex6, pex15, and pex22 mutants, all of which are blocked in the terminal steps of peroxisomal matrix protein import, polyubiquitinated forms of Pex5p accumulate, ubiquitination being dependent on the ubiquitin-conjugating enzyme Ubc4p. However, Ubc4p is not required for Pex5p ubiquitination in wild type cells, and cells lacking Ubc4p are not affected in peroxisome biogenesis. These results indicate that Pex5p monoubiquitination in wild type cells serves to regulate rather than to degrade Pex5p, which is supported by the observed stability of Pex5p. We propose that Pex5p monoubiquitination in wild type cells is required for the recycling of Pex5p from the peroxisome, whereas Ubc4p-mediated polyubiquitination of Pex5p in mutants blocked in the terminal steps of peroxisomal matrix protein import may function as a disposal mechanism for Pex5p when it gets stuck in the import pathway.

Peroxisomes are ubiquitous eukaryotic organelles bounded by a single membrane. Two of their well conserved functions are β-oxidation of fatty acids and detoxification of hydrogen peroxide. To date 32 genes encoding proteins involved in peroxisome biogenesis (called peroxins, see Ref. 1) have been identified, and about half of them are directly required for the posttranslational import of peroxisomal matrix proteins (for review, see Ref. 2). The import of matrix proteins into peroxisomes is mediated by the mobile receptors Pex5p and Pex7p, which bind to proteins with a peroxisomal targeting signal type 1 (PTS1) or a type 2 (PTS2), respectively. Both receptors are predominantly soluble proteins that are thought to cycle between the cytoplasm and the peroxisome. For Pex5p the following steps in the receptor cycle have been proposed; 1) Binding to a PTS1-containing protein in the cytoplasm, 2) docking of the PTS1-Pex5p complex on the peroxisomal membrane, 3) dissociation of this complex and translocation of the PTS1 protein into the peroxisomal matrix, and 4) recycling to the cytoplasm. Recent evidence suggests that Pex5p may enter the peroxisomal matrix with its PTS1 cargo, a process referred to as the extended receptor shuttle (Ref. 3; for review, see Ref. 4). About a dozen different peroxins present on the peroxisomal membrane have been implicated to function directly in peroxisomal matrix import. The exact roles of most of these proteins in the import process are still unclear, however. Exceptions are Pex13p and Pex14p, which interact with Pex5p and form, together with Pex17p, the membrane-associated docking complex (4–12). Another complex consists of the integral membrane proteins Pex2p, Pex10p, and Pex12p, which all contain a RING (really interesting new gene) finger domain (13). Pex5p, the only known peroxin localized to the matrix side of the membrane (14), joins the docking complex (Pex13p, Pex14p, Pex17p) and the RING finger complex (Pex2p, Pex10p, Pex12p) into a large complex called Importomer, which may mediate the translocation of PTS proteins (15). Two peroxins, Pex1p and Pex6p, are known as ATPase associated with various cellular activities (AAA) ATPases (16, 17). In Saccharomyces cerevisiae they partially associate with the membrane via the integral membrane protein Pex15p (18, 19). AAA ATPases have been suggested to function in the dissociation of protein complexes and protein unfolding (for review, see Refs. 20 and 21). Pex4p, which is attached to the membrane by the integral membrane protein Pex22p, has been identified as the ubiquitin-conjugating enzyme Ubc10p (22, 23). Pex4p and Pex22p together with the AAA ATPases Pex1p and Pex6p may act late in peroxisomal matrix protein import, possibly in the recycling of the receptor to the cytoplasm (24, 25).

The concept of a cycling Pex5p implies sequential binding of the receptor to different proteins or protein complexes at the peroxisome. A way to regulate these interactions is by reversible posttranslational modification such as phosphorylation or ubiquitination. Indeed, the integral membrane proteins Pex14p and Pex15p appear to be phosphorylated (19, 26, 27). However, the physiological roles of phosphorylation in peroxisomal matrix protein import are unknown. Two peroxins have been demonstrated to be ubiquitinated; Pex18p, a protein involved in the PTS2 pathway, is constitutively degraded in a ubiquitin-dependent manner (28), whereas two groups recently reported polyubiquitination of Pex5p in cells lacking components of the AAA or AAA, ATPases associated with various cellular activities; Ubc, ubiquitin-conjugating enzyme (E2); RING, really interesting new gene; E3, ubiquitin ligase.
Table 1

| Strain       | Yeast strains                                                                 |
|--------------|-------------------------------------------------------------------------------|
| BJ1991       | MATa; leu2, ura3–251, trp1, prb1-1122, pep4-3, gal2                           |
| BY4741       | MATa; his3Δ1, leu2–Δ0, met15–Δ0, ura3–Δ0                                   |
| BY4742       | MATα; his3Δ1, leu2–Δ0, lys2–Δ0, ura3–Δ0                                    |
| ubc1Δ        | BY4741; ubc1::loxP-KanMX4-loxP                                              |
| ubc2Δ        | BY4741; ubc2::KanMX4                                                        |
| ubc3Δ        | BY4741; ubc3::KanMX4                                                        |
| ubc5Δ        | BY4741; ubc5::KanMX4                                                        |
| ubc6Δ        | BY4741; ubc6::loxP-KanMX4-loxP                                              |
| ubc7Δ        | BY4741; ubc7::KanMX4                                                        |
| ubc8Δ        | BY4741; ubc8::KanMX4                                                        |
| ubc10Δ (pex4Δ) | BY4741; ubc10 (pex4::KanMX4)                                               |
| ubc11Δ       | BY4741; ubc11::KanMX4                                                       |
| ubc12Δ       | BY4741; ubc12::KanMX4                                                       |
| ubc13Δ       | BY4741; ubc13::KanMX4                                                       |
| ubc1Δ pex4Δ  | BY4741; ubc1::loxP-KanMX4-loxP                                              |
| ubc2Δ pex4Δ  | BY4741; ubc2::KanMX4                                                        |
| ubc4Δ pex4Δ  | BY4741; ubc4::KanMX4                                                        |
| ubc5Δ pex4Δ  | BY4741; ubc5::KanMX4                                                        |
| ubc6Δ pex4Δ  | BY4741; ubc6::loxP-KanMX4-loxP                                              |
| ubc7Δ pex4Δ  | BY4741; ubc7::KanMX4                                                        |
| ubc8Δ pex4Δ  | BY4741; ubc8::KanMX4                                                        |
| ubc11Δ pex4Δ | BY4741; ubc11::KanMX4                                                       |
| ubc12Δ pex4Δ | BY4741; ubc12::KanMX4                                                        |
| ubc13Δ pex4Δ | BY4741; ubc13::KanMX4                                                        |
| pex4Δ        | BY4741; ubc6::loxP-URA3-loxP                                                |
| ubc4Δ pex6Δ  | BY4741; ubc4::loxP-LEU2-loxP                                                |
| ubc4Δ pex6Δ  | BY4741; ubc4::loxP-LEU2-loxP                                                |
| ubc5Δ        | BY4741; ubc5::loxP-URA3-loxP                                                |
| ubc6Δ        | BY4741; ubc6::loxP-URA3-loxP                                                |
| ubc7Δ        | BY4741; ubc7::loxP-URA3-loxP                                                |
| ubc8Δ        | BY4741; ubc8::loxP-URA3-loxP                                                |
| ubc4Δ ubc6Δ  | BY4741; ubc4::loxP-URA3-loxP                                                |
| ubc4Δ ubc6Δ  | BY4741; ubc4::loxP-URA3-loxP                                                |
| ubc4Δ ubc6Δ  | BY4741; ubc4::loxP-URA3-loxP                                                |
| ubp14Δ       | BY4742; ubp14::KanMX4                                                       |
| WCG4a        | MATα; his3–11, leu2–3,112, ura3                                             |
| pre1-1       | WCG4a; pre1-1                                                               |
| pre1-1 pre2-1| WCG4a; pre1-1 pre2-1                                                        |
| pex1Δ        | BJ1991; pex1::KanMX4                                                        |
| pex2Δ        | BJ1991; pex2::KanMX4                                                        |
| pex3Δ        | BJ1991; pex3::KanMX4                                                        |
| pex4Δ        | BJ1991; pex4::KanMX4                                                        |
| pex5Δ        | BJ1991; pex5::KanMX4                                                        |
| pex6Δ        | BJ1991; pex6::KanMX4                                                        |
| pex7Δ        | BJ1991; pex7::KanMX4                                                        |
| pex8Δ        | BJ1991; pex8::KanMX4                                                        |
| pex10Δ       | BJ1991; pex10::KanMX4                                                       |
| pex11Δ       | BJ1991; pex11::KanMX4                                                       |
| pex12Δ       | BJ1991; pex12::LEU2                                                         |
| pex13Δ       | BJ1991; pex13::LEU2                                                         |
| pex14Δ       | BJ1991; pex14::LEU2                                                         |
| pex15Δ       | BJ1991; pex15::LEU2                                                         |
| pex17Δ       | BJ1991; pex17::KanMX4                                                       |
| pex21Δ       | BJ1991; pex19::KanMX4                                                       |
| pex22Δ       | BJ1991; pex22::loxP-KanMX4-loxP                                             |
| pex4Δ pex5Δ  | BJ1991; pex3::KanMX4 pex4::loxP-URA3-loxP                                   |
| pex6Δ pex7Δ  | BJ1991; pex3::KanMX4 pex6::loxP-URA3-loxP                                   |

Pex5p/Pex22p complexes (29, 30). Also for Pex5p, it was proposed that ubiquitination results in proteasomal degradation.

Ubiquitination of proteins requires the sequential action of at least three types of enzymes; they are a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (for review, see Ref. 31). In the final step of the cascade an isopeptide bond between ubiquitin and the lysine residue of the substrate is formed, a reaction that is catalyzed by the E2 enzyme, usually in conjunction with the E3 ligase.

The length of the ubiquitin chain conjugated to a substrate plays an important role. Polyubiquitinated proteins (i.e. with a chain of at least four ubiquitin molecules) are usually recognized and degraded by the proteasome (32). In contrast, monoubiquitination, i.e. attachment of a single ubiquitin moiety, regulates cellular processes such as endocytosis, sorting into multivesicular bodies and virus budding in a proteasome-independent way (for review, see Ref. 33).

Here we show for the first time that Pex5p is a monoubiquitinated and stable protein in wild type cells. Pex5p monoubiquitination takes place at the peroxisome and is blocked in cells lacking functional docking or RING finger complexes, suggesting that Pex5p monoubiquitination is a late event in peroxisomal matrix protein import. Furthermore, we show that polyubiquitinated forms of Pex5p accumulate in certain pex mutants in an Ubc4p-dependent manner, an observation that is in line with recent reports (29, 30). However, monoubiquitination of Pex5p in wild type cells is not dependent on Ubc4p, and peroxisome biogenesis is not affected in cells lacking Ubc4p. On the basis of these findings we propose distinct functions for Pex5p monoubiquitination and polyubiquitination.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Reagents—**Yeast strains used in this study are listed in Table I. Deletion strains were either obtained from the EUROSCARF consortium (www.uni-frankfurt.de/fb15/mikro/eroscarf/index.html) or generated by one-step PCR-mediated gene disruption using KanMX, HIS5, LEU2, or URA3 as a selectable marker (34). Yeast
Transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO; Difco), 2% glucose, and amino acids (20–30 µg/ml) as needed. The liquid media used for culturing of the cells for total protein isolation, subcellular fractionation, and immune precipitation contained 0.5% potassium phosphate buffer, pH 6.0, 0.3% yeast extract, 0.5% peptone, 0.1% (v/v) Tween 40, 3% Triton X-100 containing 0.5% potassium chloride, and amino acids as needed. Antibodies used for immunoprecipitation or immune precipitation contained 0.5% potassium phosphate buffer, pH 6.0, 0.3% yeast extract, 0.5% peptone, 0.1% (v/v) Tween 40, and amino acids as needed. Antibodies used for immunoprecipitation or immune precipitation were prepared by breaking the cells with glass beads and acid precipitation as described (8). SDS-PAGE and immunoblotting were performed as described (6). Antibody binding was detected using ECL reagents from Amersham Biosciences.

RESULTS

**Pex5p Is Posttranslationally Modified and Stable with Time**—The prevailing model for protein import into peroxisomes predicts that Pex5p can mediate multiple rounds of import and, thus, should be a relatively stable protein. To address this, we carried out pulse-chase experiments and monitored the stability of Pex5p in wild type yeast cells grown on oleate (Fig. 1). The levels of Pex5p remained constant during the chase for up to 5 h, indicating that Pex5p turnover was very slow. The estimated half-life of Pex5p of more than 5 h by far exceeds the calculated import rates of most PTS1 proteins (36). Thus, Pex5p is stable with time, supporting the shuttling model in which Pex5p can mediate multiple rounds of PTS1 import. Interestingly, a slower migrating Pex5p band was detected in experiments with 200 µl of protein A-Sepharose (Amersham Biosciences). Precipitates were washed 3 times with buffer B1 (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, and 0.2% SDS), twice with buffer B2 (50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.5% SDS, and 0.5% sodium deoxycholate), twice with buffer B3 (50 mM Tris, pH 7.5, 2 mM EDTA, 500 mM NaCl, and 1% Triton X-100), and once with buffer B4 (50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl) and analyzed by SDS-PAGE and immunoblotting. Immunoprecipitations on homogenate, pellet, and supernatant fractions, obtained by subcellular fractionation, were performed in an identical way. Half of the 1-ml fractions was trichloroacetic acid-precipitated by adding an equal volume of 20% trichloroacetic acid followed by a 1-h incubation at 4 °C. Trichloroacetic acid precipitates were used for immunoprecipitation as described above.

**Miscellaneous**

Protein Extracts—Protein extracts were prepared by breaking the cells with glass beads and acid precipitation as described (8). SDS-PAGE and immunoblotting were performed as described (6). Antibody binding was detected using ECL reagents from Amersham Biosciences.

**Table II. Myc-tagged ubiquitin was expressed under the control of the CUP1 promoter from plasmid YEP105 (35).** The plasmid pTetEr2 expressing a mutant form of ubiquitin in which all lysines are replaced by arginine was a generous gift from Dr. Elliot (University of Alberta, Edmonton, Canada).

**Oligonucleotides**—Oligonucleotides used are listed in Table II. Myc-tagged ubiquitin was expressed under the control of the CUP1 promoter from plasmid YEP105 (35). The plasmid pTetEr2 expressing a mutant form of ubiquitin in which all lysines are replaced by arginine was a generous gift from Dr. Elliot (University of Alberta, Edmonton, Canada).

**Pulse-Chase Experiments**—Cells growing exponentially on rich oleate medium were harvested, and 20 A600 units were resuspended in fresh minimal oleate medium. Cells were allowed to grow for 1.5 h at 28 °C and subsequently concentrated in 1 ml of 20% trichloroacetic acid followed by an hour of incubation at 4 °C. Trichloroacetic acid precipitates were used for immunoprecipitation with 5 µl of Pex5p rabbit polyclonal antiserum and 50 µl of protein A-Sepharose (Amersham Biosciences). Precipitates were washed 3 times with buffer B1 (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, and 0.2% SDS), twice with buffer B2 (50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.5% SDS, and 0.5% sodium deoxycholate), twice with buffer B3 (50 mM Tris, pH 7.5, 2 mM EDTA, 500 mM NaCl, and 1% Triton X-100), and once with buffer B4 (50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl) and analyzed by SDS-PAGE and immunoblotting. Immunoprecipitations on homogenate, pellet, and supernatant fractions, obtained by subcellular fractionation, were performed in an identical way. Half of the 1-ml fractions was trichloroacetic acid-precipitated by adding an equal volume of 20% trichloroacetic acid followed by a 1-h incubation at 4 °C. Trichloroacetic acid precipitates were used for immunoprecipitation as described above.

**Miscellaneous**—Protein extracts were prepared by breaking the cells with glass beads and acid precipitation as described (8). SDS-PAGE and immunoblotting were performed as described (6). Antibody binding was detected using ECL reagents from Amersham Biosciences.
Monoubiquitination of Pex5p—The process of monoubiquitination occurs through the conjugation of ubiquitin to a single lysine residue of the ubiquitination acceptor protein. However, the interpretation of these results was not straightforward since a cross-reacting band was present in pex5Δ cells with the same apparent molecular mass as the slower migrating Pex5p species in wild type cells. To unambiguously demonstrate that Pex5p is ubiquitinated, Pex5p was immunoprecipitated with anti-Pex5p antibodies from wild type cells expressing Myc-Ub, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc and anti-Pex5p antibodies (Fig. 2B). In the blot probed with anti-Myc antibodies, a single band with an apparent molecular mass of about 90 kDa was detected. In a control experiment with pex5Δ cells, this band was absent. The estimated molecular mass difference between the primary Pex5p species (70 kDa) and the Myc-Ub-conjugated Pex5p (90 kDa) is consistent with the presence of two Myc-ubiquitin moieties (Myc-Ub, ~9.5 kDa).

To distinguish between Pex5p monoubiquitination at two lysines and diubiquitination at one site, we overexpressed a mutant Myc-Ub (Myc-Ub-K0), in which all seven lysine residues were replaced by arginine. Myc-Ub-K0 can still be conjugated to other proteins but cannot function as an acceptor for ubiquitin-chain elongation. Myc-Ub- and Myc-Ub-K0-transformed wild type cells were analyzed by immunoprecipitation and immunoblotting as described above (Fig. 2C). Again, a single band representing Pex5p attached to two Myc-Ub moieties was present in Myc-Ub-expressing cells. Importantly, the intensity of this band did not decrease in Myc-Ub-K0-overexpressing cells nor did we observe faster migrating bands, which would be indicative of blocked ubiquitin-chain elongation. As shown in Fig. 2C, bottom panel, equal amounts of Pex5p were immunoprecipitated in each case. From these data we conclude that Pex5p is monoubiquitinated on two different lysine residues.

Monoubiquitinated Pex5p Does Not Represent a Breakdown Intermediate of Proteasome- or Vacuole-mediated Degradation—To rule out the possibility that the observed ubiquitinated Pex5p species represents a proteasomal breakdown intermediate, we analyzed the ubiquitinated state of Pex5p in the ubp14Δ mutant, which is impaired in proteasome-mediated degradation due to the accumulation of free ubiquitin chains (37). Myc-Ub-transformed ubp14Δ and the isogenic wild type strain BY4741 were grown on oleic acid and analyzed by immunoprecipitation as described above (Fig. 2D). The amount of Myc-Ub-conjugated Pex5p in the ubp14Δ cells was comparable to that of the isogenic wild type cells, and no slower-migrating ubiquitinated Pex5p species were detected in the proteasome-defective mutant. Essentially the same results were obtained with the pre1-1 and pre1-1/pre2-1 mutants, which are defective in the proteolytic activity of the proteasome (38) (data not shown). Notably, comparable amounts of Myc-Ub-conjugated Pex5p were recovered from the wild type strain BY4741 and the non-isogenic wild type strain BJ1991. The latter strain is defective in protease activities of the vacuole due to a mutation in the PEP4 gene encoding the vacuolar protease A (39). Taken together, these data demonstrate that monoubiquitinated Pex5p in wild type cells does not represent a breakdown intermediate of proteasomal or vacuolar degradation and suggest that the Pex5p-ubiquitination event serves a regulatory role in the Pex5p receptor cycle.

Ubiquitinated Pex5p Is Associated with the Peroxisome—We have previously shown that Pex5p in yeast is a predominantly cytosolic, partly peroxisomal protein (8). To determine the distribution of the ubiquitinated Pex5p species, wild type cells expressing Myc-Ub were subjected to subcellular fractionation. As a control, wild type cells transformed with empty vector were fractionated in parallel. For both strains a homogenate (H) was prepared and fractionated into an organelle pellet (P) consisting of peroxisomes and mitochondria and a cytosolic supernatant (S). Equal proportions of these fractions were immunoprecipitated with anti-Pex5p and analyzed by immunoblotting with anti-Myc and anti-Pex5p antibodies (Fig. 3A). As a control each fraction was also analyzed directly by anti-Pex5p immunoblotting. Consistent with previous studies, the unmodified Pex5p was predominantly localized in the cytosolic supernatant, and only a small portion was associated with the organelle pellet. In contrast, the ubiquitinated Pex5p was almost exclusively present in the organelle pellet fraction. The controls showed that overexpression of Myc-Ub did not influence the distribution of unmodified Pex5p and that unmodified Pex5p had the same subcellular distribution before and after immunoprecipitation. These results suggest either that Pex5p was ubiquitinated in the cytoplasm followed by rapid association with the peroxisome and subsequent slow recycling or that Pex5p was ubiquitinated at the peroxisome and rapidly deubiquitinated after its return to the cytoplasm. To distinguish between both possibilities Pex5p ubiquitination was analyzed in a strain deficient in functional Pex3p. Pex3p is required for synthesis of peroxisome membranes, and pex3Δ mutant cells are devoid of peroxisome-like structures (40). Myc-Ub-transformed pex3Δ cells were analyzed by immunoprecipitation and immunoblotting as described before. As controls, Myc-Ub-transformed wild type and pex5Δ cells were analyzed in parallel (Fig. 3B). The ubiquitinated Pex5p species was absent in the pex3Δ cells, whereas it was clearly discernable in wild type cells. The reduction of the ubiquitinated Pex5p species in pex3Δ cells was not caused by inefficient immunoprecipitation since equal amounts of unmodified Pex5p were immunoprecipitated in each case (Fig. 3B, bottom panel). These results demonstrate that in the absence of peroxisome membranes, Pex5p is not efficiently ubiquitinated.

Pex5p Ubiquitination Takes Place Late in Peroxisomal Matrix Protein Import—Of the more than 30 peroxins identified, about 12 seem to be directly involved in peroxisomal matrix protein import (21). Based on biochemical approaches and genetic studies, the order of action of these 12 peroxins has been suggested (24). Our observation that Pex5p is ubiquitinated allowed us to use this modification as a marker and address the question at which step of the import cycle the ubiquitination event occurs. First we asked whether docking of Pex5p at the peroxisome, one of the earliest steps in peroxisomal matrix protein import, is required for ubiquitination. This process is mediated by the major docking factor Pex14p (5, 7, 12) and may also involve Pex13p and Pex17p (8–11). Fig. 4 shows that the ubiquitinated Pex5p species is significantly reduced in cells deleted for Pex14p. Again, the controls showed that equal amounts of unmodified Pex5p were immunoprecipitated from pex14Δ cells and wild type cells. These results demonstrate that Pex5p monoubiquitination requires the docking factor Pex14p.
Fig. 2. Pex5p is monoubiquitinated in wild type cells. A, wild type (WT) and pex5Δ cells carrying a plasmid expressing Myc-tagged ubiquitin (Myc-Ub) or a control vector were grown on oleic acid-containing medium and treated with 100 μM copper sulfate to induce ubiquitin overexpression. Cell lysates were prepared and analyzed by immunoblotting (IB) with Pex5p antiserum. B, wild type cells and pex5Δ cells carrying the Myc-Ub plasmid were grown and treated as in A. Cell lysates were prepared, and Pex5p was immunoprecipitated (IP) with Pex5p antiserum. The immunoprecipitates were analyzed by anti-Myc and anti-Pex5p immunoblotting. C, wild type cells overexpressing wild type Myc-Ub or mutant Myc-Ub (Myc-UbK0, ubiquitin with all lysine residues replaced by arginine) were grown on oleate and analyzed as described in B. D, isogenic PEX5 (BJ1991) and pex5Δ (BJpex5Δ) cells and isogenic UBP14 (BY7441) and ubp14Δ (BYubp14Δ) cells overexpressing Myc-Ub were grown on oleic acid-containing medium. Lysates were prepared and analyzed by immunoprecipitation and immunoblotting as in B.

Fig. 3. Monoubiquitinated Pex5p is associated with peroxisomes and ubiquitination of Pex5p requires peroxisomal membranes. A, wild type (WT) cells carrying the Myc-Ub plasmid or a control vector was grown on oleate. A cell-free homogenate (H) was fractionated into a 17,500 × g pellet (P) and a 17,500 × g supernatant (S), and each fraction was split into two equal aliquots. One aliquot of each fraction was used directly for anti-Pex5p immunoblotting (IB; bottom panel), whereas the second aliquot was precipitated with trichloroacetic acid. Trichloroacetic acid precipitates were solubilized by boiling in SDS, diluted in immunoprecipitation buffer, and subjected to anti-Pex5p immunoprecipitation (IP). The immunoprecipitates were analyzed by immunoblotting with anti-Myc (top panel) or anti-Pex5p (middle panel) antibodies. B, wild type, pex5Δ, and pex3Δ cells overexpressing Myc-Ub were grown on oleate. Cell lysates were prepared and analyzed by immunoprecipitation and immunoblotting as described in the legend to Fig. 2B.

The ubiquitinated Pex5p form was observed in pex2Δ and pex12Δ cells (data not shown). Cells lacking the peroxins Pex11p or Pex7p, neither of which is involved in the Pex5p receptor cycle, showed wild type levels of the ubiquitinated Pex5p species, demonstrating the specificity of the observed Pex5p ubiquitination defect in the other pexΔ mutants. Taken together, these results demonstrate that Pex5p ubiquitination requires the association of functional RING finger and docking complexes and place the ubiquitination event late in peroxisomal matrix protein import.

Pex Mutants That Act in the Terminal Steps of Peroxisomal Matrix Protein Import Accumulate Ubiquitinated Forms of Pex5p—Epistasis analysis in Pichia pastoris has suggested that the peroxins Pex1p, Pex4p, Pex6p, and Pex22p act in the terminal steps of peroxisomal matrix protein import after receptor docking and matrix protein translocation (24). Pex4p (Ubc10p) belongs to the E2 family of ubiquitin-conjugating enzymes and is a peripherally-associated peroxisomal membrane protein that is anchored via interaction with the integral membrane protein Pex22p (22, 23). Given our observation that Pex5p ubiquitination occurs at the peroxisomal membrane at a late step in peroxisomal matrix protein import, we examined whether Pex4p and Pex22p are involved in this process (Fig. 5). Immunoblot analysis with anti-Pex5p antibodies of whole cell extracts revealed that Pex5p ubiquitination is not blocked in pex4Δ or pex22Δ cells. Instead, two slower migrating bands

The controls showed that wild type levels of unmodified Pex5p were present in both strains. Similarly, a strong reduction of Pex5p—Epistasis analysis in Pichia pastoris has suggested that the peroxins Pex1p, Pex4p, Pex6p, and Pex22p act in the terminal steps of peroxisomal matrix protein import after receptor docking and matrix protein translocation (24). Pex4p (Ubc10p) belongs to the E2 family of ubiquitin-conjugating enzymes and is a peripherally-associated peroxisomal membrane protein that is anchored via interaction with the integral membrane protein Pex22p (22, 23). Given our observation that Pex5p ubiquitination occurs at the peroxisomal membrane at a late step in peroxisomal matrix protein import, we examined whether Pex4p and Pex22p are involved in this process (Fig. 5). Immunoblot analysis with anti-Pex5p antibodies of whole cell extracts revealed that Pex5p ubiquitination is not blocked in pex4Δ or pex22Δ cells. Instead, two slower migrating bands...
were detected in both mutants in addition to the primary Pex5p species, an observation that is in line with results from other groups (29, 30). Overexpression of Myc-Ub in these pex mutants confirmed that the slower-migrating species represent ubiquitinated forms of Pex5p. The second group of mutants, comprising pex1Δ, pex6Δ, and pex15Δ showed three, and occasionally four, slower migrating Pex5p bands, all of which represent ubiquitinated species with an increasing number of ubiquitins attached to Pex5p (Fig. 5). The levels of ubiquitinated Pex5p in the mutant strains appeared to be higher than in wild type cells, suggesting that the mutants accumulate ubiquitinated Pex5p.

Pex5p Ubiquitination in the pex4Δ Mutant Depends on Ubc4p—The observation that Pex5p is ubiquitinated in the absence of Pex4p, a proven E2 enzyme, is exactly opposite of what one would expect if Pex5p were the cognate substrate of Pex4p. One possible explanation for this observation could be that in the absence of Pex4p, another E2 enzyme takes over as a ubiquitin donor. To test the involvement of other E2s in Pex5p ubiquitination, we constructed double deletion strains of pex4 with each of the nonessential, ubiquitin-specific E2s and analyzed by immunoblotting with anti-Pex5p antibodies. The deletion of ubc1Δ or ubc4Δ single and double mutants showed wild type growth rates on oleate indicating that these mutant strains have fully functional peroxisomes (Fig. 7B). Together, these results demonstrate that deletion of UBC4 (or UBC4 and UBC1) in wild type cells does not result in loss of peroxisomal function nor does it affect the pattern or level of Pex5p ubiquitination. Thus, these data support the notion that Ubc4p (and Ubc1p) is not required for Pex5p ubiquitination in wild type cells.

DISCUSSION

Ubiquitination is best known for its role in proteasome-mediated protein degradation, which requires the conjugation of a polyubiquitin chain of at least four ubiquitin moieties to the target protein (32). In contrast, the attachment of a single ubiquitin moiety, referred to as monoubiquitination, regulates protein function in a proteasome-independent way (33). Here we report that the PTS1 receptor Pex5p is monoubiquitinated on two different lysine residues in wild type cells (Fig. 2, A–C). We show that the monoubiquitinated Pex5p species is not a breakdown intermediate of the vacuolar or proteasomal degradation system, indicating that it represents a functional intermediate in the Pex5p receptor cycle (Fig. 2D). In line with this notion, we have found that Pex5p is a stable protein with an estimated half-life of >5 h in wild type cells (Fig. 1). Furthermore, we present evidence that Pex5p monoubiquitination takes place at the membrane after receptor docking and requires the function of the RING finger complex (Figs. 3 and 4).

How the RING finger complex functions in the import process of peroxisomal matrix proteins is poorly understood. Genetic and biochemical analyses suggest a role for the RING finger complex in a step after docking of cargo-bound receptor to the membrane either in translocation of cargo across the membrane (41, 42) or, as proposed recently, in recycling of the receptor to the cytoplasm (15). A molecular function for the RING finger complex, however, is so far lacking. The fact that all three RING finger peroxins are required for ubiquitination of Pex5p may suggest a direct role for the RING finger complex in this process. An attractive possibility is that the RING finger peroxins function as a multisubunit E3 ligase complex that ubiquinates Pex5p. The following observations are in line with this suggestion. First, the RING domain of Pex10p displays a significant sequence similarity to the RING domain of human c-Cbl, a proven E3 ligase (43). Second, Eckert and Johnson (44) have shown that the RING finger of Pex10p interacts with Pex4p, the peroxisomal membrane-associated ubiquitin-conjugating enzyme. Finally, two subunits of the RING finger complex, Pex10p and Pex12p, physically interact with Pex5p, the putative substrate (13, 41, 45). The proposed role for the RING finger complex, although attractive, remains hypothetical at this stage.
Monoubiquitination of Pex5p

Fig. 7. Pex5p monoubiquitination in wild type cells does not depend on Ubc4p and cells lacking Ubc4p have no peroxisome biogenesis defects. A, wild type (WT), pex5Δ, ubc8Δ, ubc1Δ, ubc4Δ, ubc5Δ, and ubc4Δubc1Δ cells overexpressing Myc-Ub were grown on oleate. Cell lysates were prepared and analyzed by immunoprecipitation (IP) and immunoblotting (IB) as described before. B, serial dilutions of wild type, pex4Δ, ubc1Δ, ubc6Δ, and ubc4Δubc1Δ cells were spotted onto plates containing oleic acid or glucose as sole carbon source and incubated for 7 and 2 days at 28 °C, respectively. All strains except the pex4Δ strain can efficiently metabolize oleic acid as shown by the clearance zone around the colonies.

The poor growth phenotype of Pex5p mutant used in our studies (30) have a pleiotropic phenotype and grow very slowly on most culture media (46). In vitro reconstitution of the ubiquitination reaction using purified proteins will be required to address this point.

Cells lacking Pex1p, Pex4p, Pex6p, Pex15p, or Pex22p do not display the wild type pattern of Pex5p ubiquitination but show an accumulation of different ubiquitinated forms of Pex5p (Fig. 5). Among these five mutants, two groups can be distinguished based on the pattern of ubiquitinated Pex5p, and the peroxins deficient within a group are functionally related (18, 22). The first group consists of the pex1Δ and pex22Δ mutants, whereas the second group comprises pex1Δ, pex6Δ, and pex15Δ. Ubiquitination of Pex5p in these mutant strains also occurs at the peroxisome membrane and depends on Ubc4p (Fig. 6 and data not shown), an observation that is in concordance with recent reports (29, 30). However, our data demonstrate that Ubc4p does not play a role in Pex5p ubiquitination in wild type cells since a wild type pattern and level of Pex5p ubiquitination was observed in both the ubc4Δ and ubc4Δubc1Δ mutants (Fig. 7A). Moreover, the ubc4Δ and ubc4Δubc1Δ strains showed wild type growth rates on oleate, indicating that Ubc4p-dependent Pex5p ubiquitination is not essential for the formation of functional peroxisomes (Fig. 7B). These latter results are in line with the data of Kiel et al. (29) but are slightly different from those reported by Platta et al. (30). They noticed a small growth defect of a ubc4Δubc5Δ double mutant on oleic acid and a partial mislocalization of peroxisomal matrix proteins, whereas the ubc4Δ single mutant showed no peroxisome biogenesis defects. Based on these observations they have suggested a role for Ubc4p and Ubc5p in peroxisome biogenesis in wild type cells. However, it should be noted that ubc4Δubc5Δ strains have a pleiotropic phenotype and grow very slowly on most culture media (46). The poor growth phenotype of ubc4Δubc5Δ cells could easily explain the observed, minor, peroxisome biogenesis defect (30). In contrast, the ubc4Δubc1Δ mutant used in our studies showed wild type growth rates on both oleate and glucose, and monoubiquitination of Pex5p appeared to occur at wild type levels (Fig. 7). These data indicate that Pex5p monoubiquitination in the ubc4Δubc1Δ double mutant is mediated by another Ubc. Because Ubc1p, Ubc4p, and Ubc5p constitute a subfamily of ubiquitin-conjugating enzymes with overlapping functions, Pex5p monoubiquitination in the ubc4Δubc1Δ mutant may be the result of Ubc5p activity. However, Pex4p is a more likely candidate to carry out this function not only in the ubc4Δubc1Δ mutant but also in wild type cells; Pex4p is the only known peroxisome-associated Ubc, and cells lacking Pex4p are deficient in import of peroxisomal matrix proteins. Pex4p has also been implicated in ubiquitination of Pex18p, a peroxin involved in the import of PTS2-containing proteins (28). However, in contrast to Pex5p, Pex18p is a relatively unstable protein in wild type cells. These observations suggest that ubiquitination may regulate both PTS1- and PTS2-dependent protein import by modification of a pathway-specific peroxin.

What could be the function of the different types of Pex5p ubiquitination? Our analysis shows that Pex5p monoubiquitination is a late event in the peroxisomal protein import pathway occurring, most likely after receptor docking and matrix protein translocation. A possible late step in the receptor cycle that could be regulated by ubiquitination is recycling of the receptor to the cytoplasm. A number of observations that have been reported previously are consistent with such a scenario. First, in Hansenula polymorpha and P. pastoris mutants lacking the ubiquitin-conjugating enzyme Pex4p, it was found that Pex5p accumulates in or at the peroxisome (24, 25), a phenotype that is consistent with a role of Pex4p in receptor recycling. Second, two studies have suggested that Pex5p exits the peroxisomal compartment by a process that requires ATP (42, 47). Although the ATPase involved in this step is currently unknown, the AAA ATPases Pex1p and Pex6p are likely candidates to carry out this function. Also epistasis analysis in P. pastoris has indicated that Pex4p, Pex22p, Pex1p, and Pex6p act in the terminal steps of peroxisomal matrix protein import, after receptor docking and matrix protein translocation (24). Thus, Pex4p-Pex22p and Pex1p-Pex6p (and Pex15p in S. cerevisiae) are, directly or indirectly, implicated in Pex5p recycling. Based on these observations and our current finding that Pex5p is monoubiquitinated in a late step of the receptor cycle, we would like to propose a molecular function for these proteins. In this model the function of the Pex4p-Pex22p complex, possibly in conjunction with the RING finger complex as E3 ligase, is to monoubiquitinate Pex5p. The monoubiquitinated Pex5p is then recognized and bound by the Pex1p-
Pex5p has been shown recently that a complex of two RING finger proteins, RAD18 and RAD5, that function in DNA repair can recruit Pex5p instability in these mutants (data not shown and Refs. 22, 24, 51).

In summary, this study has demonstrated that Pex5p is rapidly deubiquitinated by one of the many deubiquitinating enzymes present within the cell (49).

In cells lacking components of the (putative) Pex5p recycling machinery (i.e. Pex4p, Pex22p, Pex1p, Pex6p, or Pex15p), the receptor is trapped at the peroxisome, resulting in the accumulation of Pex5p in the membrane. This event may signal Ubc4p-dependent polyubiquitination of Pex5p, which may involve the same RING finger complex as E3 ligase (29, 30).

Although polyubiquitinated Pex5p forms in pex mutants have also been reported in two recent studies (29, 30), the monoubiquitination of Pex5p in wild type cells has gone unnoticed thus far. This may be related to the fact that the steady state amounts of ubiquitinated Pex5p in the cell are rather low and can only be easily visualized after purification of Pex5p by immunoprecipitation. Moreover, Pex5p is rapidly deubiquitinated during preparation and subsequent processing of cell lysates, and stabilization of the monoubiquitinated form requires the addition of inhibitors of deubiquitinating enzymes such as N-ethylmaleimide. Also, the use of glucose-grown cells instead of oleate-grown cells may have hampered the detection of the monoubiquitinated Pex5p species (29).

During growth on oleic acid, peroxisomes proliferate, hampered the detection of the monoubiquitinated Pex5p significantly.

In steady state amounts of monoubiquitinated Pex5p in the cells (22, 24, 42, 51).

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