A Conserved Cysteine Is Essential for Pex4p-dependent Ubiquitination of the Peroxisomal Import Receptor Pex5p*

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The peroxisomal protein import receptor Pex5p is modified by ubiquitin, both in an Ubc4p-dependent and -independent manner. Here we show that the two types of ubiquitination target different residues in the NH2-terminal region of Pex5p and we identify Pexp4p (Ubc10p) as the ubiquitin-conjugating enzyme required for Ubc4p-independent ubiquitination. Whereas Ubc4p-dependent ubiquitination occurs on two lysine residues, Pex4p-dependent ubiquitination neither requires lysine residues nor the NH2-terminal α-NH2 group. Instead, a conserved cysteine residue appears to be essential for both the Pex4p-dependent ubiquitination and the overall function of Pex5p. In addition, we show that this form of ubiquitinated Pex5p is susceptible to the reducing agent β-mercaptoethanol, a compound that is unable to break ubiquitin-NH2 group linkages. Together, our results strongly suggest that Pex4p-dependent ubiquitination of Pex5p occurs on a cysteine residue.

Conjugation of ubiquitin to a substrate protein is a well conserved process in eukaryotic cells, sequentially involving an ubiquitin-activating enzyme (E1),2 an ubiquitin conjugating enzyme (E2), and an ubiquitin ligase (E3) (1), whereas ubiquitin chain elongation sometimes requires the action of an additional conjugation factor called E4 (2). The effect of ubiquitination on a particular protein is, in part, determined by the length of the ubiquitin chain. “Poly-ubiquitination,” i.e. the attachment of 4 or more ubiquitin moieties, typically results in degradation of the substrate by the 26 S proteasome (3), whereas “monoubiquitination,” comprising the linkage of 1–3 ubiquitins, usually has a non-proteolytic function, e.g. inducing a change in activity or cellular location (or both) (4). Many important cellular processes, such as DNA repair, endoplasmic reticulum-retrotranslocation, endocytosis, cell division, and apoptosis are regulated by the poly- or monoubiquitination of participating proteins (for review, see Ref. 5). Not surprisingly, defective ubiquitination has been implicated in the etiology of important human diseases, such as neurodegenerative disorders and cancer.

In the large majority of cases, ubiquitin appears to be conjugated to the ε-NH2 group of a lysine residue in the substrate protein, whereas in a limited number of proteins the NH2-terminal α-NH2 group is used as a conjugation site (6–8). Recently, however, it was reported that the ubiquitination of a lysine-less COOH-terminal tail of the major histocompatibility complex class I heavy chain was dependent on the presence of a cysteine residue, suggesting that ubiquitin conjugation is not restricted to NH2 groups and that the SH group of a cysteine may also serve as a target (9). The frequency of this novel mode of ubiquitination and the functional and mechanistic differences (if any) with ubiquitination on NH2 groups remains to be established.

A recent addition to the list of processes potentially regulated by ubiquitination is that of the import of proteins into peroxisomes. Peroxisomes are eukaryotic organelles with a wide range of functions, two of which, β-oxidation of long chain fatty acids and H2O2 detoxification, are very well conserved throughout evolution (for review, see Ref. 10). Peroxisomes post-translationally import all their matrix enzymes with the aid of a peroxisomal targeting signal (PTS) types one, two, or three. Proteins that contain a PTS signal are recognized in the cytosol by their corresponding cycling receptor (Pex5p for PTS1/3 proteins and Pex7p for PTS2 proteins) and transported to the peroxisomal membrane, where docking takes place. The PTS protein is then released into the peroxisomal matrix and the receptor is recycled to the cytosol for another round of import (reviewed in Ref. 11). So far, 32 Pex proteins (called peroxins) have been identified, with around 12 being directly involved in protein import (12). Characteristic features of some of these 12 proteins seem to point at a role for ubiquitin in the import process. Pex4p, one of the first peroxins characterized (13), belongs to the E2 family of ubiquitin-conjugating enzymes. Pex4p is associated with the peroxisomal membrane (14, 15) and genetic experiments have suggested that it functions in the late steps of protein import (16). Additionally, there are three membrane-localized peroxins, Pex2p, Pex10p, and Pex12p each possessing a RING finger domain (17, 18), the hallmark of a specific class of E3 ligases (19).

Indeed, two ubiquitinated peroxins have recently been identified: Pex5p, the PTS1 receptor (20–22) and Pex18p/Pex20p, which act as co-receptors in the PTS2 pathway (23, 24). Two distinct forms of Pex5p ubiquitination have been reported, one of which is dependent on the E2 enzyme Ubc4p, whereas the other one is not. Ubc4p-dependent ubiquitination is only observed in certain pex deletion strains, namely pex4, pex22,

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2. The abbreviations used are: E1, ubiquitin-activating enzyme; Ubc (E2), ubiquitin-conjugating enzyme; E3, ubiquitin ligase; PTS, peroxisomal targeting signal; MALDI, matrix-assisted laser desorption ionization; K0N, lysine-less NH2-terminal fragment; WT, wild type; IP, immunoprecipitation; Sc, S. cerevisiae.
pex1, pex6, and pex15 (20–22). These mutants are blocked at a stage where Pex5p is normally recycled from the peroxisome membrane to the cytosol (16). Such a situation seems to trigger the ubiquitination of Pex5p, which accumulates at the peroxisomal membrane (20, 21). The available evidence suggests that Ubc4p-dependent ubiquitination serves a quality control function, priming Pex5p that is unable to recycle for proteasomal degradation (20, 21). For this reason, Ubc4-dependent Pex5p ubiquitination has been referred to as polyubiquitination, although in this particular case only one to four ubiquitin residues are attached. As mentioned above, this type of ubiquitination occurs on two lysines, Ubc4p-independent ubiquitination and the E2 enzyme responsible, however, remained enigmatic (22).

Here we report that the two forms of Pex5p ubiquitination target different amino acid residues within the NH2-terminal region of the protein. Whereas Ubc4p-dependent ubiquitination occurs on two lysines, Ubc4p-independent ubiquitination does not require lysine residues or the NH2-terminal α-NH2 group. Instead, a conserved cysteine residue in the NH2-terminal domain is absolutely essential for this modification. Mutation of this cysteine not only blocks Ubc4p-independent ubiquitination, but also results in a non-functional Pex5p. In addition, we show that the E2 enzyme Pex4p is involved in the Ubc4p-independent ubiquitination of Pex5p.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Culture Conditions—**The *Escherichia coli* strain DH5α ( recA, hisD, supE, endA, gyrA96, thi-α, relA1, lacZ) was used for all plasmid isolations. The following yeast strains were used in this study: *Saccharomyces cerevisiae* BJ1991 pex5Δ (MATa; pex5::KanMX4, leu2, ura3-251, trpl, prb1-1122, pep4-3, gal2), BJ1991 pex4Δpex5Δ (MATa, pex4::KanMX4, pex5::LEU2, leu2, trpl, ura3-251, prb1-1122, pep4-3, gal2) and BJ1991 pex5Δpex6Δ (MATa, pex5::KanMX4, pex6::LEU2, leu2, trpl, ura3-251, prb1-1122, pep4-3, gal2). Yeast transformations were performed as described in Ref. 25. Transformants were grown on minimal medium containing 0.67% yeast nitrogen base (YNB, Difco), 2% glucose, 2% agar, and amino acids (20 μg/ml) as required. For immunoprecipitations, trichloroacetic acid lysates and subcellular fractionations, cells were grown on 0.67% YNB containing 0.3% glucose for at least 24 h and then shifted to 0.1% oleate medium containing 0.5% potassium phosphate buffer, pH 6.0, 0.3% yeast extract, 0.5% peptone, and 0.2% Tween 40 and grown for 7–16 h. Trichloroacetic acid lysates for Pex5p (1–308) and controls (Fig. 1A) were prepared from cells grown overnight on 0.67% YNB containing 0.3% glucose. CuSO4 (100 μM final concentration) was added to cultures for expression of CUP1 promoter-controlled Myc-ubiquitin. Oleate plates contained 0.67% YNB, 0.1% oleate, 0.5% Tween 40, 2% agar, 0.1% yeast extract and amino acids (20 μg/ml) as required.

**Plasmids—**Plasmids used in this study are listed in Table 1. All plasmids, except the Myc-Ub expressing plasmids, are low-copy shuttle vectors that are maintained in 1–2 copies per cell. Further details of plasmids are available on request. Pex5p site-directed mutants were constructed using either the QuikChange® site-directed or multisite-directed mutagenesis kits (Stratagene) and confirmed by sequencing. The plasmid YEP105, expressing Myc-tagged ubiquitin was a generous gift from Dr. Ellison (26).

**Immunoprecipitation—**Oleate-grown cells (20 A600 units) co-expressing Myc-tagged ubiquitin and wild type or mutant forms of Pex5p were lysed with glass beads in 5% trichloroacetic acid and precipitates were resuspended in 175 μl of 50 mM Tris, pH 7.5, 6 M urea, and 1% SDS and heated to 65 °C for 10 min.

### TABLE 1

| Name          | Promoter | Comments                        | Ref. |
|---------------|----------|---------------------------------|------|
| pT98          | PEX5     | Wild type Pex5                  | 42   |
| YEP105        | CUP1     | Myc-tagged Ubiquitin            | 26   |
| pCW122        | PEX5     | Ubc5p                           | This study |
| pCW127        | CUP1     | Myc-tagged Ubiquitin            | This study |
| pCW131        | CUP1     | Myc-tagged Ubiquitin            | This study |
| pCW138        | PEX5     | Pex5-(1–308) HsPex5-C6R/K0N     | This study |
| pCW145        | PEX5     | Fusion of HsPex5p-(1–41) and ScPex5-(43–612) | This study |
| pCW80         | Catalase | Pex5-(1–308)–His6p              | This study |
| pMB23         | Catalase | Pex5-(1–308) HsPex5-C6R/K0N     | This study |
| pMB35         | PEX5     | Pex5 K18R/K24R                  | This study |
| pMB36         | PEX5     | Pex5 K18R/K24R                  | This study |
| pMB37         | PEX5     | Pex5 K31R/K46R/K81R             | This study |
| pMB38         | PEX5     | Pex5 K14R/K210R/K244R           | This study |
| pMB39         | PEX5     | Pex5 K210R/K213R/K227R          | This study |
| pMB40         | PEX5     | Pex5 K142R/K213R/K227R          | This study |
| pMB41         | PEX5     | Pex5 K18R/K24R                  | This study |
| pMB42         | PEX5     | Pex5 K31R/K46R/K81R             | This study |
| pMB74         | PEX5     | Pex5 K0N                        | This study |
| pMB94         | PEX5     | Pex5-(1–308) K0N                | This study |
| pMB95         | PEX5     | HsPex5 C11R/Sc                  | This study |
| pMB112        | PEX5     | HsPex5 K0N/Sc                   | This study |
| pMB113        | PEX5     | HsPex5 K0N/Sc                   | This study |
| pMB114        | PEX5     | HsPex5 C11R/Sc                  | This study |

*See pCW138.
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Undissolved material was pelleted and 1.75 ml of IP-Tween buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween 20, and 0.1 mM EDTA) was added, containing 0.1% bovine serum albumin, 20 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Sigma). Lysates were first pre-cleared with 20 μl of Protein-A Sepharose (Amersham Biosciences) and then incubated with 5 μl of rabbit polyclonal Pex5p antiserum and 50 μl of Protein A-Sepharose beads for 2 h at 4 °C. Precipitates were washed 3 times with IP-Tween buffer, twice with IP-urea buffer (100 mM Tris, pH 7.5, 2 M urea, 200 mM NaCl, and 0.5% Tween 20) and twice with TBS buffer (50 mM Tris, pH 7.5, and 150 mM NaCl) and elution was carried out by heating the beads in 25 μl of IP-elution buffer (125 mM Tris, pH 6.8, 1.5% SDS, 6 M urea, and 20% glycerol) for 10 min at 65 °C. Samples were analyzed by SDS-PAGE and immunoblotting. The antibodies used for immunoprecipitation or immunoblotting were anti-Pex5p (raised in our own laboratory, rabbit polyclonal) and anti-Myc (Cell Signaling technology, Inc., mouse monoclonal). For anti-Myc immunoblotting analysis, 10 μl of the elution fraction was used. For anti-Pex5p analysis, 1 μl of the elution fraction was diluted in 20 μl of IP-elution buffer containing 50 mM dithiothreitol and heated at 37 °C for 5 min. Subsequently, 5 μl of this sample was used for SDS-PAGE and immunoblotting.

Purification of Pex5p-(1–308) His₆—Olate-grown cells (300 A₄₀₀ units) expressing Pex5p-(1–308) with a COOH-terminal His₆ tag were lysed with glass beads in lysis buffer (75 mM Tris, pH 7.4, 200 mM NaCl, 15 mM NaF, 1.5 mM Na₃VO₄, 5 mM β-mercaptoethanol, 20 mM N-ethylmaleimide, 1% Triton X-100, 0.5% octyl β-D-glucopyranoside (Sigma), 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). Guanidine HCl was added to a final concentration of 6 M and undissolved material was removed by centrifugation at 10,000 × g. The lysate was passed over nickel-nitrolotriacetic acid resin (Qiagen) and sequentially washed with buffers W1 (lysis buffer containing 6 M guanidine HCl), W2 (lysis buffer containing 2 M guanidine HCl), and W3 (75 mM Tris, pH 7.4, 200 mM NaCl, 5 mM β-mercaptoethanol, 1% Triton X-100, 0.5% octyl β-D-glucopyranoside, and 1 mM phenylmethylsulfonyl fluoride). Elution from the resin was carried out using buffer W3 containing 330 mM imidazol and the sample was concentrated using an Amicon® Ultracentrifugal filter (Millipore) and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (Serva).

Mass Spectrometry—Coomassie-stained bands were excised from gel, treated with dithiothreitol and iodoacetamide to block the thiol groups on cysteine residues by carbamidomethylation, and digested overnight with sequence grade trypsin (Roche Applied Science). Peptides were extracted as described in Ref. 27 and analyzed by peptide mass fingerprinting and peptide sequencing, using a QSTAR-XL equipped with an MALDI interface (Applied Biosystems/MDS Scienx, Toronto, Canada). The resulting peptide spectra were used to search the Mascot search engine (www.matrixscience.com).

Treatment of Immunoprecipitates with Reducing Agent—Immunoprecipitation was performed as described above except that 150 μl of CNBr-activated Sepharose beads (Amersham Biosciences) conjugated with polyclonal Pex5p antiserum was used. Beads were treated for 10 min at 65 °C with 25 μl of IP-elution buffer without urea and either lacking reducing agent (control) or containing 10% β-mercaptoethanol. For anti-Myc immunoblotting, 10 μl of the elution was used. For anti-Pex5p immunoblotting, 1 μl of the elution was diluted in 20 μl of IP elution buffer without urea and 5 μl of this dilution was used.

RESULTS

The NH₂-terminal 308 Amino Acids of Pex5p Are Sufficient for Ubiquitination—The NH₂-terminal half of Pex5p contains the binding sites for the docking proteins Pex13p and Pex14p and, therefore, is required for the association of the protein with the peroxisomal membrane (28–30). As membrane association is also essential for the ubiquitination of Pex5p (20, 22), we examined whether a COOH-terminal truncated version of Pex5p, consisting of the first 308 amino acids (Pex5p-(1–308)) could still be ubiquitinated. To assess Ub4p-dependent ubiqui-
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Ubiquitination Is Not Reliant on Lysine Residues—Having identified the likely region involved in the ubiquitination of Pex5p, we reverted to using the full-length protein for further analysis, because the 1–308 construct does not complement the pex5Δ strain as it lacks the essential PTS1 binding region (31, 32). Because the conjugation of ubiquitin to a substrate is usually via a lysine residue, we mutagenized all 15 lysines present in the NH₂-terminal 308 amino acids of Pex5p to arginines, in combinations of two, three, or four lysine residues at a time. Next, the constructs were tested in their ability to undergo both forms of ubiquitination. Mutation of lysines 18 and 24 resulted in a severe reduction in the slower migrating Pex5p bands when expressed in a pex4Δ/pex5Δ (Fig. 2A) or pex5Δ/pex6Δ (not shown) strain, indicating that Ubc4p-dependent ubiquitination was inhibited. No other combinations of lysine mutations resulted in a loss of ubiquitination (Fig. 2A), suggesting that these two residues are the main targets. Construction of the individual mutants Pex5p K18R and K24R revealed that lysine 24 is the main target for Ubc4p-dependent ubiquitination, but that lysine 18 to a certain extent can also act as a target (not shown). This is in line with other recent work, in which the ubiquitination of Hansenula polymorpha Pex5p and Pichia pastoris Pex20p in a pex4Δ strain was shown to be dependent on lysine residues present in the NH₂-terminal region of the proteins (24, 33).

Remarkably, Ubc4p-independent ubiquitination was not blocked in the K18R/K24R mutant form of Pex5p (Fig. 2B) or in all the other lysine mutants tested (not shown). All of these Pex5p lysine mutants were functional, as they could rescue the growth of a pex5Δ strain on oleate, a carbon source requiring peroxisomes for its metabolism (not shown). To test whether any of the remaining lysine residues in the NH₂-terminal region could act as a target for Ubc4p-independent ubiquitination, we made a version of Pex5p in which all the lysines in this domain were mutated to arginines (K0N). Surprisingly, this mutant was still ubiquitinated, although at a lower level than the wild type Pex5p (Fig. 2C, upper panels) and was able to complement the pex5Δ phenotype, indicating that the K0N Pex5p is functional (Fig. 2C, lower panel). Because it cannot be ruled out that the lysines still present in the COOH-terminal region of the Pex5p construct that was used in Fig. 2C may have become targets for ubiquitin conjugation, we checked the extent of ubiquitination of a lysine-less NH₂-terminal fragment (1–308 K0). The 1–308

ubiquitination, wild type (WT) Pex5p or Pex5-(1–308) constructs were expressed in a pex4Δ/pex5Δ strain containing either a vector expressing Myc-tagged ubiquitin or a control vector. Next, total protein lysates were prepared and analyzed by immunoblotting with anti-Pex5p antibodies (Fig. 1A). In cells expressing a control vector, two slower migrating bands could be observed in addition to the major Pex5p and Pex5p-(1–308) species (lanes 1 and 5), patterns that are characteristic for Ubc4p-dependent ubiquitination in a pex4Δ strain (20–22). Indeed, these slower migrating bands shifted upon overexpression of Myc-ubiquitin in the cell (lanes 2 and 6), confirming that the Pex5-(1–308), like wild type Pex5p, is ubiquitinated.

Previously, we have shown that in wild type cells Pex5p is transiently ubiquitinated in an Ubc4p-independent manner (22). To detect this low abundance ubiquitin-ated form of Pex5p, an immunoprecipitation assay was developed using cells expressing Myc-tagged ubiquitin (22). pex5Δ cells expressing wild type Pex5p, Pex5-(1–308), or a control vector and Myc-tagged ubiquitin were subjected to immunoprecipitation with anti-Pex5p antibodies and analyzed by anti-Pex5p and anti-Myc immunoblotting (Fig. 1B). In the blot probed with anti-Myc antibodies, a single band typical for Ubc4p-independent ubiquitination was detected in cells expressing either wild type Pex5p (lanes 2 and 3) or Pex5-(1–308) (lane 4), but not in the empty vector control (lane 1). Together, these results show that the first 308 amino acids of Pex5p contain the target residues of both Ubc4p-dependent and -independent ubiquitination.
K₀ fragment was ubiquitinated to a level comparable with that of the lysine-containing 1–308 control construct (Fig. 2D), indicating that the Ubc4p-independent ubiquitination of Pex5p does not occur on lysine residues.

**A Conserved Cysteine Residue Near the NH₂ Terminus Is Essential for Ubc4p-independent Ubiquitination and Function of Pex5p**

Conjugation of ubiquitin to the NH₂ group on the NH₂ terminus of many proteins has been observed, providing a clear example of a non-lysine linkage (34). In principle, Ubc4p-independent ubiquitination of Pex5p could also be targeted to the NH₂-terminal NH₂ group. However, analysis of Pex5p using the TerminVator program (www.isv.cnrs-gif.fr/Terminator) predicts the NH₂-terminal NH₂ group to be acetylated, a modification that would prevent ubiquitin conjugation on the α-NH₂ group. To analyze its acetylation status, we purified Pex5p-(1–308) using a COOH-terminal His₆ tag and excised the most prominent band from the gel for trypsin digestion followed by mass spectrometry analysis (Fig. 3A). Data base searches and peptide sequencing revealed that the majority of peptides recovered corresponded to Pex5p-(1–308)-His₆ with a total coverage of 80%. Nevertheless, an NH₂-terminal peptide with an unmodified α-NH₂ group was not detected. Instead, a peptide was found that corresponded to a carbamidomethylated NH₂-terminal peptide, but with an extra 42 Da (Fig. 3A, inset). Carbamidomethylation (see Fig. 3B) is the result of treatment of the peptides with iodoacetamide, whereas the 42 Da increase in mass is consistent with an extra acetyl group being attached to the peptide. Peptide sequencing analysis revealed that this additional mass is present on the first methionine residue, as the b-ion series (NH₂-terminal containing fragments), but not the y-ion series (COOH-terminal containing fragments) show the 42-Da increase (Fig. 3B). These data indicate that the α-NH₂ group on the first methionine residue of Pex5p is acetylated, effectively blocking it for ubiquitin conjugation.

Recently, ubiquitination on a non-NH₂ group of a protein was reported, which appeared to target the SH group of a cysteine residue (9). Interestingly, sequence alignment analysis of Pex5p from various species shows a well-conserved cysteine residue in the NH₂-terminal region of the protein, whereas the NH₂ terminus of all members of the Pex20p family also harbors a conserved cysteine (Fig. 4A). To assess the importance of this cysteine (Cys⁶ in S. cerevisiae Pex5p) in Ubc4p-independent ubiquitination and Pex5p function, we replaced it with an arginine (Fig. 4, B and C), alanine, tryptophan, or a serine (not

![FIGURE 3. The NH₂ terminus of Pex5p is acetylated.](image-url)

**FIGURE 3. The NH₂ terminus of Pex5p is acetylated.** A, MALDI-time of flight spectrum of a tryptic digest of purified Pex5p-(1–308)-His₆ (inset). The molecular masses (monoisotopic MH⁻) of abundant Pex5p peaks are indicated. The acetylated, carbamidomethylated NH₂-terminal fragment is labeled with an asterisk. B, peptide sequence of the 1968.9 MH⁻ fragment indicated in A. Fragments representing b and y ions are shown. Ac, acetyl group. CAM, carbamidomethyl group. amu, atomic mass units.
None of the cysteine point mutants were able to complement the growth of a pex5/H9004 strain on oleate (Fig. 4C, lower panel and data not shown), a phenotype that was caused by the inability of the mutant proteins to import PTS1 proteins (supplemental materials Fig. S1). Immunoblot analysis of total lysates showed that the pattern of ubiquitination of the C6R mutant was similar to that of wild type Pex5p in a pex4Δ mutant (Fig. 4B). These data suggest that although the C6R mutant does not import PTS1 proteins, it still associates with the peroxisomal membrane and is ubiquitinated in an Ubc4p-dependent manner. The introduction of the cysteine mutation into Pex5p lacking lysines 18 and 24 (C6R K18R/K24R, not shown) or all NH2-terminal lysines (C6R/K0N) resulted in a significant reduction of the ubiquitinated Pex5p bands (Fig. 4B, compare C6R with C6R/K0N). However, these mutants were unable to rescue the pex5/H9004 phenotype (Fig. 4C and supplemental materials Fig. S1) indicating that the presence of the cysteine residue is essential for the function of Pex5p and that the growth phenotype of the C6R mutant was not caused by Ubc4p-dependent ubiquitination of the protein.

To directly assess the role of the conserved cysteine residue in Ubc4p-independent ubiquitination, we immunoprecipitated different Pex5p mutants from cells expressing Myc-tagged ubiquitin and analyzed ubiquitination by anti-Myc immunoblotting (Fig. 4C). Whereas the lysine-less Pex5p mutant (K0N) was still modified, this modification was no longer visible when the conserved cysteine was mutated to arginine (C6R/K0N). The ubiquitinated species seen in the C6R mutant most likely represents Ubc4p-dependent modification on lysines. Together, the results suggest that the conserved cysteine residue plays a crucial role in the Ubc4p-independent ubiquitination of Pex5p.

The NH2-terminal Domain of Human Pex5p Can Functionally Replace That of S. cerevisiae Pex5p and Ubiquitination of the Chimeric Protein Requires the Conserved Cysteine—The sequence conservation of the NH2-terminal 35–40 amino acids in the Pex5p proteins, including the strictly conserved cysteine residue (Fig. 4A), suggests that this part of the protein serves an important function and may be sufficient for Ubc4p-independent ubiquitination of Pex5p. We therefore replaced the first 42 amino acids of

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**Figure 4.** A conserved cysteine residue in the NH2-terminal region of Pex5p is crucial for Ubc4p-independent ubiquitination and function. A, sequence alignment showing the NH2-terminal 42 amino acids of a number of Pex5p (upper panel) and Pex18/20p (lower panel) proteins from different species. * indicates the conserved cysteine residue. Arrowheads indicate lysine residues 18 and 24 in S. cerevisiae Pex5p. Sc, S. cerevisiae; Pp, P. pastoris; Hp, H. polymorpha; Hs, Homo sapiens; Mm, Mus musculus; Yp, Yarrowia lipolytica. B, pex5Δ, pex4Δpex5Δ, or pex5ΔpexΔ cells bearing a plasmid expressing Myc-tagged ubiquitin (+) or a control vector (−) and co-expressing wild type Pex5p (WT), Pex5p cysteine mutant (C6R), or Pex5p C6R without NH2-terminal lysines (C6R K0N) were lysed and cell extracts were analyzed by SDS-PAGE and anti-Pex5p immunoblotting (IB). The open arrowhead indicates a band that cross-reacts with the anti-Pex5p antibody. Asterisk indicates the Myc-ubiquitin-conjugated Pex5p species in the C6R mutant protein. C, pex5Δ cells co-expressing Myc-tagged ubiquitin and wild type Pex5p (WT), Pex5p lacking NH2-terminal lysines (K0N), Pex5p with the conserved cysteine residue mutated to an arginine (C6R), or a similar construct without NH2-terminal lysines (C6R K0N) were lysed and subjected to immunoprecipitation (IP) and immunoblotting, as described in the legend to Fig. 1 (upper panels) or spotted onto plates containing oleate as the sole carbon source and grown for 7 days at 28 °C (lower panel).
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FIGURE 5. The NH₂-terminal domain of human Pex5p can functionally replace that of S. cerevisiae Pex5p and is ubiquitinated in a cysteine-dependent manner. pex5Δ cells co-expressing Myc-tagged ubiquitin and either wild type S. cerevisiae Pex5p (ScPex5p), a chimeric Pex5p constructs containing the first 41 amino acids from human Pex5p (Hs/Sc) or the same chimera with cysteine 11 mutated to arginine (Hs C11R/Sc), the chimera lacking the NH₂-terminal lysines (HsK,N/Sc) or lacking both the NH₂-terminal lysines and cysteine 11 (HsC₁₁,R K₅,N/Sc), were lysed and subjected to immunoprecipitation (IP) and immunoblotting (IB) as described in the legend to Fig. 1 (upper panels), or spotted onto plates containing olate as the sole carbon source and grown 7 days at 28 °C (lower panel).

S. cerevisiae (Sc) Pex5p with the first 41 amino acids from human Pex5p (Hs/Sc), in which the conserved cysteine residue is present at position 11, and analyzed both the ubiquitination status and the functionality of the chimeric protein. Remarkably, the chimeric Pex5p was modified at a level comparable with that of ScPex5p, and could fully complement the pex5Δ phenotype (Fig. 5). Again, mutation of all lysines in the Pex5p chimera (Hs K₅,N/Sc) did not block ubiquitination and the mutant protein could restore growth of the pex5Δ strain on olete, albeit incompletely. The reduced amounts of the mutant protein present in the cell may account for this phenotype (Fig. 5). Mutation of the cysteine residue either in the Pex5p chimera (Hs C₁₁R/Sc) or in the lysine-less chimera (Hs C₁₁R K₅,N/Sc) resulted in an almost complete inhibition of ubiquitination and rendered the protein non-functional. These data re-emphasize the fact that the conserved cysteine residue is critical for both Ubc4p-independent ubiquitination and function of Pex5p.

The Ubiquitinated K₅,N Form of Pex5p Is Susceptible to β-Mercaptoethanol—The results obtained so far imply that the conserved cysteine residue in the NH₂-terminal domain of Pex5p can function as a site for ubiquitin attachment. Conjugation of ubiquitin to a cysteine residue would result in the formation of a thioester bond between the COOH group of the terminal glycine residue in ubiquitin and the SH group of the cysteine residue. This bond, which is also the type of linkage E1 and E2 enzymes form with ubiquitin, can be broken by the reducing agent β-mercaptoethanol, whereas a ubiquitin-lysine linkage (isopeptide or amide bond) is not susceptible to β-mercaptoethanol (9). We compared the effect of β-mercaptoethanol on the two different forms of ubiquitinated Pex5p. Treatment of immunoprecipitates of the K₅,N form of Pex5p with β-mercaptoethanol drastically reduced the amount of ubiquitinated Pex5p (Fig. 6A). In contrast, the levels of lysine-linked ubiquitinated Pex5p, isolated from the pex4Δ strain, were unaffected by this treatment (Fig. 6B). The data clearly show that the ubiquitin linkage in the K₅,N form of Pex5p behaves as a thioester bond and not as an amide bond, adding strong support for the conserved cysteine being the conjugation site for Ubc4p-independent ubiquitination.

Ubc4p-independent Ubiquitination of Pex5p Requires Pex4p—Previously, it has been shown that ubiquitination of Pex5p in the deletion strains pex4Δ, pex22Δ, pex1Δ, pex6Δ, and pex15Δ is dependent on the E2 enzyme Ubc4p (20–22). The E2 responsible for the ubiquitination of Pex5p in wild type cells, however, has not yet been identified although Pex4p (Ubc10p) is a very likely candidate (13, 14, 35). Our observation that Ubc4p-dependent ubiquitination of Pex5p could be efficiently blocked by mutation of the lysine residues present in the NH₂-terminal region (Fig. 2), allows a direct test of the involvement of Pex4p in Ubc4p-independent ubiquitination. To this end, the ubiquitination patterns of the Pex5p K₅,N mutant were compared with those of wild type Pex5p (WT), both in a pex4Δpex5Δ and a pex5Δpex6Δ strain, using immunoprecipitation analysis (Fig. 7). The results show that the lysine-less Pex5p mutant is only ubiquitinated in the pex5Δpex6Δ strain and not in the pex4Δpex5Δ strain (Fig. 7, K₅,N), conditions in which wild type Pex5p displayed the expected ubiquitination patterns (Fig. 7, WT). These data demonstrate that Ubc4p-independent ubiqui-
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Ubc4p and Ubc5p, two redundant E2 enzymes, resulted in slow growth and a PTS1 import defect. The identification of the two lysines (Lys\textsuperscript{18} and Lys\textsuperscript{24}) in Pex5p that are used as targets of Ubc4p-dependent ubiquitination (Fig. 2) allowed us to address the function of this modification in wild type cells. Our data show that replacement of these lysines by arginine residues does not affect the function of the protein (Fig. 2), which suggests that Ubc4p-dependent ubiquitination of Pex5p does not play an essential role in the import of PTS1 proteins. This is in line with previous work in other organisms, where lysine residues present in the NH\textsubscript{2}-terminal region of H. polymorpha Pex5p (33) and P. pastoris Pex20p (24) at positions 21 and 19, respectively, were shown to be essential for Ubc4p-dependent ubiquitination, but their mutation had no effect on protein function. Interestingly, sequence alignments of the NH\textsubscript{2}-terminal region of different Pex5p and Pex20p family members (Fig. 4) shows that, in all cases, a lysine residue is present in the first 25 amino acids. It appears, therefore, that Ubc4p-dependent ubiquitination of the (co)-receptors Pex5p and Pex20p on lysines is a conserved, but non-essential, process that is activated in certain mutants blocked in a step at which these receptors are recycled. Whether the protein is degraded by the proteasome seems to depend on the organism.

**Ubc4p-independent Ubiquitination**—We have shown that Ubc4p-independent ubiquitination of Pex5p also occurs in the NH\textsubscript{2}-terminal 308 amino acids of the protein but does not require lysine residues (Fig. 2). This raised the question as to which other residue(s) could potentially act as an attachment site for ubiquitin. Our data point toward a well conserved cysteine residue that is essential for both Ubc4p-independent ubiquitination and function of Pex5p. First, mutation of the cysteine (Cys\textsuperscript{6}) in the lysine-less K0N mutant blocked Ubc4p-independent ubiquitination and resulted in a protein no longer able to rescue the pex5\Delta phenotype (Fig. 4). Second, by swapping the NH\textsubscript{2}-terminal region of ScPex5p with that of HsPex5p, we again showed that the presence of a cysteine residue, cysteine 11 in human Pex5p, is essential for both the Ubc4p-independent ubiquitination and receptor function (Fig. 5). Finally, and most significantly, biochemical studies showed that the ubiquitin linkage in the lysine-less form of Pex5p is susceptible to the reducing agent β-mercaptoethanol, whereas ubiquitin-lysine bonds are not (Fig. 6). The theoretical possibility remains

\[\text{FIGURE 7. Ubc4p-independent ubiquitination of Pex5p requires Pex4p, pex5\Delta, pex4\Delta pex5\Delta, and pex5\Delta pex6\Delta cells co-expressing Myc-tagged ubiquitin and either wild type (WT) or mutant forms of Pex5p were subjected to immunoprecipitation (IP) and immunoblotting (IB) as described in the legend to Fig. 1.}\]
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that the cysteine does not act as the final conjugation site but is involved in the transfer of ubiquitin to a side chain of another residue in Pex5p, analogous to the transfer of ubiquitin from the active site cysteine of an E2 enzyme to the substrate. Transfer to a lysine seems unlikely because none of the lysine mutants had a phenotype, whereas mutation of the cysteine alone resulted in a non-functional protein. If the residue involved in ubiquitin transfer is essential, one might expect the residue(s) that receive the ubiquitin to be, likewise, essential. In the absence of any lysines, in Pex5p K0N, other potential targets would include the α-NH2 group or serine, threonine, and tyrosine residues that could form ester bonds with ubiquitin through their hydroxyl groups. The α-NH2 group is a highly unlikely conjugation site, as we have shown that it is blocked by acetylation (Fig. 3), which is a co-translational modification (39). Even if a small portion of Pex5p remains un-acetylated, the susceptibility of the ubiquitinated Pex5p K0N to β-mercaptoethanol indicates that the linkage between Pex5p and ubiquitin is not an amide bond, the type of bond formed between NH2 groups and ubiquitin. The same argument applies to an ester bond that would be formed between ubiquitin and a serine, threonine, or tyrosine residue as this type of linkage would also not be susceptible to β-mercaptoethanol. We attempted to isolate the ubiquitinated forms of Pex5p for mass spectrometric analysis to confirm the role of the cysteine residue as the conjugation site, but we were unable to isolate sufficient amounts of modified Pex5p. This may be due to the weak nature of the thioester bond between ubiquitin and Pex5p.

Our data strongly suggest that the conserved cysteine residue in Pex5p represents the conjugation site for ubiquitin. So far, ubiquitination on a cysteine has only been suggested for the major histocompatibility complex class I heavy chain, a reaction that is catalyzed by a viral E3 ligase (9). Our data would represent the first example of cysteine ubiquitination performed by the cellular ubiquitination machinery. Why a cysteine, rather than a more standard lysine residue, is the preferred conjugation site remains to be investigated. We speculate that the timely removal of the ubiquitin from Pex5p may represent an important step in the import cycle, a process that may occur at a faster rate when ubiquitin is linked to a cysteine, as thioester bonds are more labile than isopeptide linkages. The small amounts of ubiquitinated Pex5p that are present at any one time in the cell are in line with this suggestion, although it cannot be ruled out that the liability of the thioester bond to reduction may also hamper the detection.

Which step in the import cycle is regulated by Ubc4p-independent ubiquitination of Pex5p remains to be addressed, although two recent observations suggest a role in the recycling of Pex5p from the peroxisomal membrane to the cytosol. First, Costa-Rodrigues and co-workers (40) showed that the extreme NH2-terminal 17 amino acids of human Pex5p containing the conserved cysteine are essential for the release of the receptor from the peroxisome membrane. Second, the group of Subramani (41) suggested that the conserved cysteine residue near the NH2 terminus of P. pastoris Pex20p (cysteine 8) is required for cytosolic relocation of peroxisomal Pex20p. Although modification of the cysteine residue in Pex20p was not addressed in this article, it is conceivable that this cysteine is also a target for ubiquitination.

Using subcellular fractionation, we have, thus far, been unable to show an accumulation of the C6R/K0N or the C6R mutants at the peroxisomal membrane, suggesting that cysteine ubiquitination of Pex5p may be required for another important step in the import cycle, for example, PTS1 cargo release/delivery. The severe PTS1 protein import defect observed in the C6R and C6R/K0N mutants (supplementary materials Fig. S1) is in line with this suggestion, whereas a deficiency in Pex5p recycling would be expected to result in a milder PTS1 protein import defect (16). We are currently investigating these possibilities further. Irrespective of its possible function, the highly conserved nature of the cysteine residue in both Pex5p and the Pex20p families implies that cysteine ubiquitination is not restricted to yeast but may also occur in other organisms.

**Pex4p: The E2 Required for Ubc4p-independent Ubiquitination**—Our finding that the E2 enzyme Pex4p (Ubc10p) is required for the Ubc4p-independent ubiquitination of Pex5p resolves a long-standing debate about the possible substrates of this ubiquitin-conjugating enzyme. Following its identification in the early 1990s as a genuine E2 enzyme, ubiquitinated peroxins were not identified until nearly 10 years later (20, 23). All potential Pex4p substrates belong to the two families of cycling (co)-receptors involved in either the PTS1 (Pex5p) or PTS2 (Pex20/Pex18p) protein import pathways. However, the notion that in the absence of Pex4p, Pex5p and Pex20p are still ubiquitinated by another E2 (Ubc4p) has cast serious doubts on the role of Pex4p in ubiquitination of these proteins (20, 24, 33). By blocking ubiquitination on lysines, through mutation of the target residues, we now show that Pex4p is required for the Ubc4p-independent ubiquitination of Pex5p (Fig. 7).

In a pex6Δ strain, Pex4p-dependent ubiquitination of Pex5p is undisturbed (Fig. 7), an observation that explains why in this strain and, by inference, in the pex1Δ and pex15Δ strains, larger ubiquitinated species (3–4 ubiquitins) are seen than those found in a pex4Δ cell (1–2 ubiquitins). In the absence of Pex6p, Pex1p, or Pex15p, both Pex4p and Ubc4p modify Pex5p, whereas in the absence of Pex4p, only Ubc4p-mediated ubiquitination occurs. The similarity in the ubiquitination pattern observed with the C6R mutant of Pex5p in wild type cells and wild type Pex5p in pex4Δ cells (Fig. 4) supports the notion that both mutants have a deficiency in the same process, i.e. Pex4p-dependent ubiquitination of Pex5p.

Perspectives—Until recently, it was believed that the attachment of the first ubiquitin moiety to a substrate protein invariably occurs on an NH2 group present on either an internal lysine or the NH2-terminal residue. However, the recent finding that ubiquitin may also be conjugated to a cysteine residue has added a further level of complexity to ubiquitin-regulated processes (9). Our observation that ubiquitination on a cysteine is likely to occur on proteins involved in peroxisome biogenesis indicates that this alternate form of ubiquitination may be more widespread in nature than previously thought. Its recent discovery may be explained by the relative instability of a thioester bond (cysteine-ubiquitin) compared with an amide linkage (lysine/αNH2-ubiquitin). The identification of more proteins...
that are ubiquitinated on a cysteine will help to unravel the potential function(s) of this novel form of ubiquitination.

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