Properties of the Ubiquitin-Pex5p Thiol Ester Conjugate*

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Pex5p, the peroxisomal protein cycling receptor, binds newly synthesized peroxisomal matrix proteins in the cytosol and promotes their translocation across the organelle membrane. During its transient passage through the membrane, Pex5p is monoubiquitinated at a conserved cysteine residue, a requisite for its subsequent ATP-dependent export back into the cytosol. Here we describe the properties of the soluble and membrane-bound monoubiquitinated Pex5p species (Ub-Pex5p). Our data suggest that 1) Ub-Pex5p is deubiquitinated by a combination of context-dependent enzymatic and nonenzymatic mechanisms; 2) soluble Ub-Pex5p retains the capacity to interact with the peroxisomal import machinery in a cargo-dependent manner; and 3) substitution of the conserved cysteine residue of Pex5p by a lysine results in a quite functional protein both in vitro and in vivo. Additionally, we show that MG132, a proteasome inhibitor, blocks the import of a peroxisomal reporter protein in vivo.

Since the discovery of the ubiquitin-conjugating cascade nearly 30 years ago, thousands of proteins have been shown to be modified by ubiquitin (1, 2). In many cases ubiquitination of a protein is linked to its proteasomal degradation (3), whereas in a growing number of examples, ubiquitination of a protein is used as a transient modification to modulate its biological properties (for a review see Ref. 4). Regardless of the final outcome, it is generally assumed and in many cases demonstrated that ubiquitin is covalently attached through an amide bond involving the carboxyl group of the last glycin of ubiquitin on one hand, and an amino group of the targeted protein on the other (5). Recent findings from several laboratories, however, suggest that this rule is not always valid, and proteins ubiquitinated at serines and threonines (yielding oxyesters) or even cysteines (forming thiol esters) have been identified (6–10).

Protein ubiquitination at cysteine residues is a particularly puzzling phenomenon for two reasons. First, on a thermodynamic basis it is the least favorable event (the approximate free energy changes for acyl shifts from a thiol ester to a thiol, alcohol, and amine are 0, −2.4, and −11 kcal/mol, respectively (11, 12)). Second, although data on the half-lives of ubiquitin-protein thiol ester conjugates under physiologically relevant conditions are scarce, it is known that ubiquitin thiol esters are easily disrupted by nucleophiles such as GSH (13), raising the possibility that, to some degree, proteins subjected to this kind of conjugation may undergo futile ubiquitination/deubiquitination cycles. Thus, a thiol ester bond appears not to be the most efficient way to link ubiquitin to a protein, unless, of course, the aim is to create an activated (easily transferable) form of ubiquitin, as is in fact the case with ubiquitin-activating enzymes (E1s),4 ubiquitin-conjugating enzymes (E2s), and some ubiquitin ligases (E3s) (2).

In the last years we have been characterizing Pex5p, one of the three presently known proteins claimed to be ubiquitinated at a cysteine residue (6–10). Pex5p is a central component of the peroxisomal protein import machinery functioning as a shuttle receptor in the transport of newly synthesized peroxisomal matrix proteins from the cytosol into the peroxisome (14–17). According to current models, the Pex5p-mediated protein import pathway can be divided into five major stages (numbered 0–4; see Fig. 1 and Ref. 18 for a recent review). First, free/soluble Pex5p (stage 0) binds peroxisomal matrix proteins in the cytosol. The Pex5p-cargo protein complex (stage 1) then docks at the peroxisomal docking/translocation machinery (DTM), a membrane-embedded protein assembly comprising the core subunits Pex13p, Pex14p and the three RING (really interesting new gene) finger peroxins Pex2p, Pex10p, and Pex12p (19–21). The interaction of the Pex5p-cargo protein complex with the DTM

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ultimately results in the insertion of Pex5p into this machinery (stage 2) with the concomitant translocation of the cargo protein across the peroxisomal membrane. Next, Pex5p is monoubiquitinated at a conserved cysteine residue present near the N terminus of the peroxin (7, 10). This monoubiquitinated Pex5p (Ub-Pex5p; stage 3) is then dislocated in an ATP-dependent process by Pex1p and Pex6p, two members of the AAA (ATPases associated with various cellular activities) family, yielding a soluble Ub-Pex5p species (stage 4) (7, 22, 23). Finally, it is assumed, although not yet demonstrated, that the ubiquitin moiety is removed from stage 4 Pex5p, thus regenerating stage 0 Pex5p.

In this work we used an established in vitro system (7, 24–29) to characterize the properties of monoubiquitinated Pex5p. We provide evidence suggesting that the stage 4-to-stage 0 transition can occur by two independent mechanisms, one of enzymatic nature and the other involving a simple nucleophilic attack of the thiol ester bond by GSH. Interestingly, the soluble Ub-Pex5p thiol ester conjugate (stage 4) is still a substrate for the DTM, suggesting that ubiquitination of Pex5p does not change its cargo protein binding properties. Notably, the conserved cysteine residue of Pex5p can be substituted by a lysine with no detectable loss of functionality both in vitro and in vivo assays. Finally, our data suggest that treatment of cell cultures with MG132, a proteasome inhibitor (30), leads to a block of the peroxisomal import machinery.

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**FIGURE 1. Model of the Pex5p-mediated peroxisomal protein import pathway.** There are five major stages in this protein sorting pathway (numbered 0–4). Substages (a and b) are mostly of conceptual nature. The different stages have been characterized with the in vitro system used here, applying several strategies that block (22) the pathway at different steps: Stage 0, cytosolic cargo-free Pex5p (protease accessible). Stage 1, cytosolic Pex5p-cargo protein complex (protease accessible). Stage 2, Pex5p embedded in the peroxisomal docking/translocation machinery (DTM) (only ~2 kDa of Pex5p N terminus are accessible to exogenously added proteinase K). Stage 3, DTM-embedded monoubiquitinated Pex5p (Pex5p is completely resistant to proteinase K most likely because the ubiquitin moiety of this conjugate protects the peroxin). Stage 4, protease accessible monoubiquitinated Pex5p (in our experimental conditions the majority of stage 4 Pex5p is a soluble protein, i.e., Stage 4b). Insertion of Pex5p into the DTM is cargo protein-dependent. The addition of a vast excess of a recombinant protein comprising the PTS1-binding domain of Pex5p (rTPR) to in vitro reactions sequesters endogenous cargo proteins, thus blocking the insertion of Pex5p into the DTM. Recombinant full-length Pex5p (rPex5p) has the same effect but, in addition, also competes with radiolabeled Pex5p for the DTM. Monoubiquitination of stage 2 Pex5p yielding stage 3 Pex5p occurs at Cys11 of Pex5p. The reaction requires ATP or ATP-γS (ATPγS) and is temperature-dependent (T > 16°C). Treatment of Pex5p with iodoacetamide (IAA) blocks this cysteine and thus its ubiquitination. The ubiquitin analogue GST-Ub is also used efficiently by the ubiquitin-conjugating cascade acting on Pex5p. However, this stage 3 species is no longer a substrate for the receptor export module (REM), presumably because of the bulkiness of GST-Ub. Apyrase hydrolyzes ATP and thus blocks Pex5p both at stage 2 and 3b levels. The mechanism of the stage 4-to-stage 0 transition (question mark) is addressed in this work. CP, cargo protein; Ub, ubiquitin. See Ref. 18 and references cited therein for details.

**EXPERIMENTAL PROCEDURES**

*Plasmids—*cDNAs encoding the large isoform of human Pex5p (31, 32) possessing an alanine (Pex5(C11A)p), an arginine (Pex5(C11R)p), or a lysine (Pex5(C11K)p) at position 11 were obtained with the QuikChange® site-directed mutagenesis kit (Stratagene), using pGEM4-Pex5 (26) as the template. The primers used were: 5′-GGTGAGGGCCAGCGGGGGGTGCCAAC-3′ and 5′-GTTGGCCACCCCGGCTCCGCC-TCCACC-3′ for the Pex5(C11A)p-encoding plasmid; 5′-GGTGAGGGCCAGCGGGGGGTGCCAAAC-3′ and 5′-GTTGGCCACCCCGGCTCCGCC-TCCACC-3′ for the Pex5(C11R)p-encoding plasmid; and 5′-GTTGGCCACCCCGGCTCCGCC-TCCACC-3′ for the Pex5(C11K)p-encoding plasmid. The pGEM4-Pex5(C11S)p has been previously described (24). The monocistronic mammalian expression plasmid encoding a EGFP fusion protein containing at its C terminus a peroxisomal targeting signal type 1 (EGFP-PTS1) has been described elsewhere (33). The bicistronic mammalian expression vectors coding for EGFP-PTS1 and the nontagged long isoforms of Pex5p, Pex5(C11S)p, or Pex5(C11K)p were constructed by amplifying the corresponding cDNAs by PCR (primers, 5′-GGGAGATCTACCATGGCAATGCGGGAGCTG-3′ and 5′-GGAGGCCGAACGCGGGGGTGCCAAC-3′ and 5′-GTTGGCCACCCCGGCTCCGCC-TCCACC-3′ for the Pex5(C11A)p-encoding plasmid; and 5′-GTTGGCCACCCCGGCTCCGCC-TCCACC-3′ for the Pex5(C11K)p-encoding plasmid).

*Cell Culture, Transfections, and (Immuno)fluorescence microscopy—*Chinese hamster ovary (CHO) cells and immortalized mouse Pex5p-deficient fibroblasts (35) were cultured as
described elsewhere (36). The cells were transfected by using Lipofectamine Plus (Invitrogen). In some experiments, the transfected cells were first enriched on G418 (300 μg/ml) for at least 3 weeks. The proteasome inhibitor MG132 was initially dissolved in dimethyl sulfoxide, diluted with α-minimal essential medium complete medium (Lonza), and added to the G418-enriched cells. Control cells received the same amount of dimethyl sulfoxide. The peroxisomal localization of EGFP-PTS1 was assessed by co-localization studies with Pex14p (37). Fluorescence was evaluated on a CellIM imaging station (Olympus) equipped with U-MNUA2, U-MNBIA3, and U-MWY2 fluorescence mirror units.

Preparation of Postnuclear Supernatants (PNS) from Rat Liver—Rat liver PNS for in vitro assays were prepared in SEM buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, 1 mM EDTA-NaOH, pH 7.2) supplemented with 2 μg/ml N-(trans-epoxysuccinyl)-l-leucine 4-guanidinobutylamide, as described (26). For the steady-state analysis of rat liver Pex5p, an organelle pellet enriched in stage 3 Pex5p was prepared as follows. An in vitro reaction containing bovine ubiquitin, Ubal and proteasome inhibitors was first incubated for 5 min at 37 °C in the presence of 0.5 mM ATP (to release the DTM from the endogenous Pex5p (29)) followed by a second 25-min incubation in the presence of 7 mM ATP·γS to accumulate stage 3 Pex5p. A total organelle pellet was obtained by centrifugation and resuspended in 500 μL of SEM buffer supplemented with Ubal and proteasome inhibitors. Treatment with 5 mM GSH and processing of the samples for SDS-PAGE was done as above. Cargo protein-dependent import experiments using iodoacetamide-treated substrates were done as follows. Two reactions (tubes A and B, respectively) containing 1 mg of PNS protein each in 250 μL of import buffer supplemented with ATP, 1 μM bovine ubiquitin, and Ubal were assembled on ice. Twelve microliters of 35S-labeled Pex5p were then added to reaction A, and both tubes were incubated at 37 °C for 30 min. At the end of the incubation, the tubes were centrifuged (see above), the supernatants were recovered and placed on ice, and 12 μL of 35S-labeled Pex5p were then added to supernatant B. Both supernatants were treated with iodoacetamide (final concentration, 12 mM) on ice for 15 min. In parallel, a tube containing 4.8 mg of PNS protein in 720 μL of import buffer supplemented with 1 mM ATP and Ubal was incubated at 37 °C for 3 min (to release the DTM from endogenous Pex5p). This suspension was put at 16 °C and supplemented with 12 μM GST-Ub, and 120-μL aliquots were pipetted into two sets of three tubes (tubes 1–3). Tubes 1 contained no addition; tubes 2 contained a recombinant protein comprising the PTS1-binding domain of Pex5p (referred to as TPR-Pex5p in Ref. 39) plus a PTS1-containing peptide (CRYHLKPLQSKL) (27); and tubes 3 contained this recombinant protein plus a control peptide (CRYHLKPLQLKS). The final concentration of the recombinant protein and peptides were 1.7 and 33 μM, respectively. Each set of tubes was then programmed with 80 μL/tube of supernatant A or B, and incubation was continued at 16 °C for 30 min. At the end of the incubation one half of each reaction was treated with NEM, and the organelles were isolated by centrifugation. The other half was treated with proteinase K as described before (7) but using a lower protease concentration (250 μg/ml) to minimize degradation of the ubiquitin moiety in the stage 3 Pex5p species.

Sucrose Gradient Centrifugation—Eighty microliters of a supernatant from an in vitro reaction performed in the presence of Ubal were treated with iodoacetamide as described above and incubated on ice for 5 min with 3 μM of recombinant Pex5p. The solution was brought to 400 μL with buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA-NaOH) and halved. One half received GST-SKL, a GST containing a PTS1 signal at the C terminus; the other received GST-LKS, a GST ending with a nonfunctional PTS1-like sequence (27), both at 2 μM final concentration. After 10 min at room temperature, 30 μg each of bovine IgGs (6.9 S), bovine serum albumin (4.3 S), and soybean trypsin inhibitor (2.3 S) were added to the samples as internal standards (the numbers in parentheses represent the sedimentation coefficients). These mixtures were then applied onto the top of continuous 5–30% (w/v) sucrose gradient in buffer A supplemented with 0.25 μM of the corresponding GST fusion protein, GST-SKL or GST-LKS, respectively. After centrifugation at 39,000 rpm for 29 h at 4 °C in a SW27 rotor (Beck-
man), 13 fractions of 0.8 ml were collected from the bottom of the tube, immediately precipitated with trichloroacetic acid, and analyzed by SDS-PAGE under nonreducing conditions. All of the recombinant proteins and sedimentation standards were pretreated with iodoacetamide (10 mM) before use.

**Miscellaneous**—[^25S]-Labeled Pex5p proteins were synthesized in rabbit reticulocyte lysates using the TNT® quick coupled transcription/translation system (Promega) in the presence of EasyTag™ L-[^35S]methionine (specific activity, >1000 Ci/mmol; PerkinElmer Life Sciences) following the manufacturer’s instructions. SDS-PAGE (performed at 4°C) and autoradiography were done as described (7). Densitometric analysis of autoradiographed gels was performed using the UNSCAN-IT automated system. Pex5p was detected on Western blots using an antibody directed to human Pex5p (40) or by blot overlay using human[^35S]-labeled Pex14p, as described before (41). Rabbit polyclonal antisera against HsPex14p and EGFp have been described elsewhere (36). The anti-PMP70 antibody (42) was kindly provided by Dr. Wilhelm J. Just (University of Heidelberg, Heidelberg, Germany). The anti-α-tubulin was purchased from Sigma. Rabbit and mouse antibodies were detected on Western blots using alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgGs (Sigma), respectively.

**RESULTS**

**Substitution of Cys11 of Pex5p by a Lysine Results in a Peroxisomal Import/Export-competent Protein**—To characterize the properties of the Ub-Pex5p thiol ester conjugate, we started our work by generating a mutant Pex5p protein, Pex5(C11K)p, in which the conserved cysteine residue at position 11 was replaced by a lysine. Our aim was to detect some ubiquitination event. However, and surpassing our expectations, this turned out not to be the case, as described before.

In addition to Pex5(C11K)p, we included in some experiments three other mutant versions, Pex5(C11S)p, Pex5(C11A)p and Pex5(C11R)p, all lacking the conserved Cys11 residue and possessing at this position a serine, alanine, or arginine residue, respectively. The properties of Pex5(C11S)p have been described before (7, 24). Because Pex5(C11S)p is a very poor substrate for peroxisome-dependent monoubiquitination, it accumulates at the peroxisomal membrane. The data in Fig. 2 (upper panel) show precisely this behavior. When Pex5(C11S)p is incubated with a PNS fraction in the presence of ATP, conditions under which wild-type Pex5p continuously enters and exits the peroxisomal DTM (25, 26, 29), a significant fraction (45%) co-sediments with the organelles (lane 5). A much smaller fraction of the wild-type peroxin (15%) is found in the organelle pellet because as the protein gets inserted into the DTM, it is also pumped out back into the cytosol (lane 1). Large fractions of protein in the organelle pellets were also found for Pex5(C11A)p and Pex5(C11R)p (compare lane 1 with lanes 5 and 9 in lower panel), emphasizing the importance of Cys11 for the dislocation of Pex5p back into the cytosol.

Remarkably, Pex5(C11K)p behaves as the wild-type peroxin (15% in the organelle pellet; compare lanes 1 and 9 in upper panel), suggesting that this variant enters the DTM and is actively dislocated into the cytosol by the peroxisomal export machinery. The presence of monoubiquitinated forms of both Pex5p and Pex5(C11K)p in the supernatant fractions (lanes 2 and 10) indicates that a fraction of both proteins present in the soluble phase of these reactions has indeed passed through the peroxisomal DTM, because only Pex5p proteins that have inserted into the DTM are monoubiquitinated in this in vitro system (28). Thus, Pex5(C11K)p is as functional as Pex5p in this assay.

**The Stage 4-to-Stage 0 Pex5p Transition Can Occur by Both Enzymatic and Nonenzymatic Mechanisms**—Interestingly, the amounts of Ub-Pex5p and Ub-Pex5(C11K)p that can be detected in these reactions increase when Ubal, an inhibitor of DubUs (43, 44), is included in the import buffer (Fig. 2, compare lanes 2 and 10 with 4 and 12, respectively). The fraction of ubiquitinated species undergoing this Ubal-sensitive deubiquitinating reaction in our standard conditions is, however, relatively modest (about 30%). A clearer result was obtained when the concentration of the PNS fraction in the assays (and so the concentration of DubUs) was increased by a factor of two and deubiquitination of Ub-Pex5p was allowed to proceed for 20 min in the absence of de novo ubiquitination. For this purpose, Pex5p was first incubated for 20 min with the PNS fraction in import buffer containing ATP to generate stage 3 and stage 4
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Pex5p. Apyrase and a vast excess of recombinant Pex5p were then added to the reaction (Fig. 1). As shown in Fig. 3, when this procedure is performed in the presence of UbAl both stage 3 and stage 4 Pex5p can be easily detected in the assay (lanes 3 and 4, respectively). In contrast, in the absence of the DUB inhibitor only stage 3 Ub-Pex5p is detected (compare lanes 1 and 2). This observation suggests that stage 3, unlike stage 4 Pex5p, is inaccessible to DUBs.

DUBs are not the only cellular components that are diluted in our assays. For the problem being addressed here, i.e. the lability of the Ub-Pex5p thiol ester conjugate, another potentially important variable is the GSH concentration. GSH is a major nucleophile in vivo (45), and as stated previously, it can disrupt ubiquitin-protein thiol ester conjugates quite efficiently (13). Thus, we determined the stability of both stage 3 and stage 4 Pex5p in the presence of 5 mM GSH, the physiological concentration in rat liver (46). As shown in Fig. 4A, stage 4 Pex5p is quite sensitive to GSH (half-life, 2.3 min; upper panel, lanes 2–6). As expected, Ub-Pex5(C11K)p is destroyed neither by GSH (lower panel, lanes 2–6) nor by dithiothreitol (DTT; lane 7). The Ub-Pex5(C11K)p conjugate is also not destroyed by heat treatment in the presence of 100 mM DTT and 2% SDS (lane 8) in agreement with the fact that in this conjugate an amide bond links Ub to Pex5(C11K)p. Interestingly, although stage 3 Pex5p still displays some sensitivity to GSH, its stability in the presence of this nucleophile is much higher than the one observed for stage 4 Pex5p (half-life, 10 min; Fig. 4B, upper panel, lanes 2–6). Incubation of stage 3 Pex5p in the presence of the smaller and stronger nucleophile DTT leads, nevertheless, to the disruption of the thiol ester bond (lane 7).

To further characterize the properties of stage 3 and stage 4 Pex5p, we have analyzed the steady-state levels of rat liver Pex5p and Ub-Pex5p in a PNS freshly prepared in the presence of 20 mM NEM. This alkylating agent inhibits DUBs and also blocks the thiol group of endogenous GSH. As shown in Fig. 4C, a small fraction of total rat liver Pex5p can be detected as a 100-kDa species that, based on its molecular mass and DTT sensitivity, corresponds to Ub-Pex5p (compare lanes 1 and 4). Importantly, virtually all rat liver Ub-Pex5p is found in the organelle pellet after centrifugation of the PNS (lane 2). Its absence in the supernatant fraction of this centrifugation (lane 3) suggests that stage 4 Pex5p is rapidly deubiquitinated in vivo.

In conclusion, these data indicate that stage 3 Pex5p is much more resistant to the action of DUBs and GSH than stage 4 Pex5p. From another perspective, these results illustrate how a cysteine residue in one protein can be, at one time, sufficiently exposed to the cytosolic milieu allowing its derivatization with an 8.5-kDa protein (i.e. ubiquitin) becoming, immediately after, protected from both nucleophilic attack by a 0.3-kDa molecule (i.e. GSH) and the action of DUBs and, finally, after ATP-de-
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The in Vivo Properties of Pex5(C11K)p and Pex5(C11S)p—The data presented above suggest that Pex5(C11K)p is as functional as Pex5 in our in vitro system. To determine whether the same is true in vivo, fibroblasts from Pex5-KO mice

subjected to SDS-PAGE under nonreducing conditions, and blotted onto nitrocellulose membranes. Autoradiographs and the corresponding Ponceau S-stained membranes are shown. The internal sedimentation standards (soybean trypsin inhibitor (STI), bovine serum albumin (BSA), and immunoglobulins (IgG)) as well as GST-SKL and GST-LKS are indicated. Lanes 1, 25% of the iodoacetamide-treated Ub-Pex5p and Pex5p radiolabeled proteins used in each lane. The numbers to the right indicate the molecular masses of reduced protein standards in kDa.
were transiently transfected with bicistronic plasmids encoding EGFP-PTS1 (a protein efficiently targeted to the peroxisome in a Pex5p-dependent manner (47)) and Pex5p, Pex5(C11S)p, or Pex5(C11K)p. Forty-eight hours post-transfection, the cells were analyzed by (immuno)fluorescence microscopy. As shown in Fig. 6, EGFP-PTS1 displays a peroxisomal localization in most cells transfected with the Pex5p- or Pex5(C11K)p-encoding plasmids, as assessed by its co-localization with Pex14p, a peroxisomal membrane protein (37, 48). In contrast, no peroxisomal EGFP-PTS1 was detected when using the Pex5(C11S)p-encoding plasmid in these experiments (Fig. 6). Our in vitro observations showing that this mutant protein is not a substrate for the peroxisomal ubiquitination machinery probably explain this result. Indeed, considering that peroxisomes contain very low amounts of DTM components compared with peroxisomal matrix proteins (21), i.e. peroxins are used in a catalytical manner, it is possible that Pex5(C11S)p undergoes one protein transport event becoming trapped at the DTM and thus blocking it.

In vivo data favoring this interpretation were obtained when CHO cells were transiently transfected with the above mentioned bicistronic plasmids (Fig. 6). Whereas a peroxisomal localization for the EGFP-PTS1 reporter protein was obtained with the Pex5p- and Pex5(C11K)p-encoding constructs, this reporter protein was mainly detected in the cytosol of cells expressing Pex5(C11S)p. Thus, substitution of the conserved cysteine of Pex5p by a serine, but not by a lysine, results in a dominant negative protein, as expected.

The Proteasome Inhibitor MG132 Blocks the PTS1 Import Pathway in Vivo—The reason for the unconventional ubiquitination of Pex5p could be related to the chemical lability of stage 4 Pex5p. As proposed recently (18) (see also “Discussion”), the fact that the stage 4-to-stage 0 transition may occur by both an enzymatic and nonenzymatic mechanism could decrease the half-life of soluble Ub-Pex5p and thus its probability of being recognized by some component of the ubiquitin-proteasome system. If correct, this would imply that Pex5(C11K)p is less stable than Pex5p in vivo, a phenomenon that might be observed by comparing the levels of these proteins in cell cultures grown in the absence or presence of proteasome inhibitors. Unfortunately, despite several attempts using CHO cell lines stably expressing EGFP-PTS1 and Pex5p or Pex5(C11K)p, we have been unable to confirm this possibility (data not shown; see also “Discussion”). Nevertheless, an interesting finding was made during the course of that work: MG132, a proteasome inhibitor, interferes with the peroxisomal targeting of EGFP-PTS1. This phenomenon is illustrated in Fig. 7 showing a CHO cell line stably expressing this peroxisomal reporter protein and grown in the presence or absence of MG132 for different periods of time.

Inhibition of the proteasome could lead to the accumulation of polyubiquitinated Pex5p molecules, which might block the
peroxisomal DTM. Alternatively, the decrease in the cellular ubiquitin levels induced by MG132 (49) could simply impede export of Pex5p from the DTM. Presently, we favor this last possibility because no ubiquitinated Pex5p protein could be detected in total protein extracts obtained from MG132-treated cell cultures (data not shown).

**DISCUSSION**

One of the aims of this work was to characterize the last step of the Pex5p-mediated import pathway, the deubiquitination of soluble Ub-Pex5p (stage 4 Pex5p). Our data suggest that stage 4 Pex5p is indeed converted into stage 0 Pex5p and that this deubiquitination process may occur by a combination of enzymatic and nonenzymatic mechanisms. Importantly, both mechanisms are context-dependent with the membrane-bound Ub-Pex5p (stage 3) displaying a higher resistance to both. The inaccessibility of stage 3 Pex5p to DUBs and GSH should avoid futile ubiquitination-deubiquitination cycles at the DTM.

The exact fractions of stage 4 Pex5p following each of these deubiquitinating pathways cannot be determined from our experiments. This would require knowing the identity and the in vivo concentration of the DUB(s) involved in this process. It seems clear, however, that the nonenzymatic pathway is not a crucial feature of the Pex5p-mediated import pathway because substitution of the conserved cysteine of Pex5p by a lysine results in a functional protein. This does not necessarily mean that the nonenzymatic deubiquitinating pathway has no importance at all, as discussed below.

Interestingly, deubiquitination of stage 4 Pex5p seems not to be a mandatory step in the Pex5p-mediated import pathway. Indeed, soluble Ub-Pex5p can still acquire a protease-resistant status when incubated with organelles in the presence of cargo proteins. We note that a bypass of the stage 4-to-stage 0 transition probably does not occur in vivo because a steady-state level analysis of endogenous ubiquitinated Pex5p in PNS fractions revealed that stage 4 Pex5p is below the detection limit of our assays. Similar steady-state data have been reported for yeast Pex5p (10). Thus, in vivo the stage 4-to-stage 0 transition is probably much faster than the rate at which stage 4 is generated. Nevertheless, the in vitro assays and the sedimentation analyses reported here do suggest that ubiquitination of Pex5p does not alter its cargo protein binding capacity, a property that, if extrapolated to the membrane-bound Pex5p species, could imply that ubiquitination of stage 2 Pex5p is not linked to the cargo protein release step. Furthermore, the finding that stage 4 Pex5p can return to the DTM in a cargo-dependent manner together with its sedimentation behavior also supports the idea that Pex5p is released from the Pex1p/Pex6p protein complex still in its ubiquitinated form.

The most striking observations made in this work concern the properties of Pex5(C11K)p. The fact that this mutant protein enters the DTM, acquires a monoubiquitin, and is exported from the DTM as efficiently as the wild-type Pex5p protein suggests that neither Cys11 nor the thiol ester bond in the Ub-Pex5p conjugate play a key role in any of the steps occurring at the DTM. The same conclusion can be drawn from our in vivo data showing that Pex5(C11K)p, but not Pex5(C11S)p, is capable of rescuing the phenotype of Pex5-KO cells. Thus,
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although sufficiently important to have been conserved throughout the evolution, it seems that the cysteine residue of Pex5p or the thiol ester bond in which it is involved has just an ancillary function in this protein sorting pathway. Alternatively, the reason why a cysteine and not a lysine is used in this ubiquitination event could be related to some regulatory pathway acting on Pex5p through its conserved cysteine rather than to the actual mechanism of protein translocation across the peroxisomal membrane. The exact nature of this ancillary function/regulatory role, however, remains unknown (but see below). Nevertheless, the results presented here do allow us to exclude some possibilities. For instance, our data argue strongly against the possibility of Pex5p being a HECT (homologous to E6AP C-terminus)-like E3 ubiquitin ligase (50) because the stability of the isopeptide bond in the Ub-Pex5(C11K)p conjugate would make any Pex5(C11K)p-promoted ubiquitination extremely difficult. This same reasoning also suggests that the ubiquitination step of Pex5p at the DTM can be made irreversible without any deleterious effects. In fact, and in agreement with this conclusion, we have been unable to detect such putative reversibility when using the wild-type peroxin in in vitro assays, suggesting that a kinetic barrier is raised immediately after the ubiquitination process. Our in vitro data also seem to exclude the possibility that the cysteine residue provides some advantage over a lysine residue in the stage 2-to-stage 3 Pex5p transition. The thiol group of cysteine is a better nucleophile than the ε-amine of lysine, a property that, in principle, could result in a faster ubiquitination reaction of Pex5p at the DTM. However, ubiquitination of stage 2 Pex5p is a rate-limiting step in our in vitro system (see Ref. 7), and so any difference in the ubiquitination rates of Pex5p and Pex5(C11K)p would have been easily detected.

If Pex5p is not an HECT-like E3 protein, if there is no mechanistic need for a reversible ubiquitination step at the DTM, and if a cysteine residue presents no advantage over a lysine residue in the stage 2-to-stage 3 transition, why is Pex5p ubiquitinated at a cysteine residue? Two different hypotheses can be raised, one centered on the chemical lability of the thiol ester bond of stage 4 Pex5p, and the other related to the biological properties of cysteine residues. In the first, one could assume that, although this protein import pathway can work with a lysine-based ubiquitination of Pex5p, there are advantages in disrupting the soluble Ub-Pex5p conjugate as fast as possible using for this purpose a nonenzymatic cleavage in addition to the DUB-mediated one. As discussed recently (18), these advantages could include a decreased half-life of stage 4 Pex5p, which might decrease its probability of being recognized by some component of the ubiquitin-proteasome system, like an E4 enzyme (ubiquitin-chain elongation factor) (51) or the proteasome itself (52). Considering the unavoidably high dilution of the ubiquitin-proteasome system in our in vitro assays and the high nonphysiological levels of Pex5p or Pex5(C11K)p attained in the transfection experiments, differences in the stabilities of the two proteins could have been easily missed in this work.

In the second hypothesis the biological properties of cysteine residues, and not the lability of the thiol ester bond in which the conserved cysteine of Pex5p is involved, is the important feature. Cysteine residues can be modified in numerous ways (e.g., oxidation, glutathionylation, nitrosylation, acylation, etc.) (53, 54), often resulting in the modulation of protein activity. In the case of Pex5p, modification of its conserved cysteine would have a dramatic effect because it would lead to a block of the peroxisomal DTM with the concomitant mislocalization of newly synthesized peroxisomal proteins into the cytosol. Coupled with an appropriate transcription regulation of peroxisomal protein-encoding genes, such a hypothetical mechanism could result in an advantageous mislocalization of some peroxisomal enzymes into the cytosol (e.g. catalase in an oxidative stress situation). Clearly, further data are necessary to understand why the peroxisomal protein import machinery uses this peculiar type of ubiquitination.

In this work we describe some of the properties of an ubiquitin-protein thiol ester conjugate. In agreement with previous data on the sensitivity of the ubiquitin-E1 thiol ester conjugate to GSH (half-life of 1.4 min in 5 mM GSH, pH 7.2; estimated from the data in Ref. 13 and assuming that the glutathiolate anion, pKₐ = −9.0 (55), is the attacking species in this reaction), we show that stage 4 Pex5p is also easily disrupted by GSH (half-life of 2.3 min in 5 mM GSH, pH 7.2). However, the results obtained with stage 3 Pex5p also suggest that thiol ester bonds in ubiquitin-protein conjugates may exist in environments inaccessible to GSH. Thus, although ubiquitination of proteins at cysteine residues seems particularly suited for transient events, as is the case in the Pex5p-mediated import pathway, it also has the potential to be as efficient and durable as the classical lysine-based ubiquitination, provided that some entity (e.g. an interacting protein) protects the thiol ester bond from nucleophile attack. Considering that most current protocols aiming at identifying/characterizing ubiquitinated proteins include at least one reduction step with DTT/β-mercaptoethanol (56), reagents that also cleave thiol esters, it is thus possible that many more proteins ubiquitinated at cysteine residues will be found in the near future.

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