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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 glycine 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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[4] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; DTM, docking/translocation machinery; Ub-Pex5p, monoubiquitinated Pex5p species; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PTS1, peroxisomal targeting signal type 1; CHO, Chinese hamster ovary; PNS, postnuclear supernatant; MOPS, 4-morpholinepropanesulfonic acid; NEM, *N*-ethylmaleimide; Ubal, ubiquitin aldehyde; DUB, deubiquitinating enzyme; ATPγS, adenosine 5-*O*-(thiotriphosphate); GST-Ub, glutathione *S*-transferase-ubiquitin; GST-SKL, glutathione *S*-transferase fusion protein containing a PTS1 signal at the C terminus; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein.

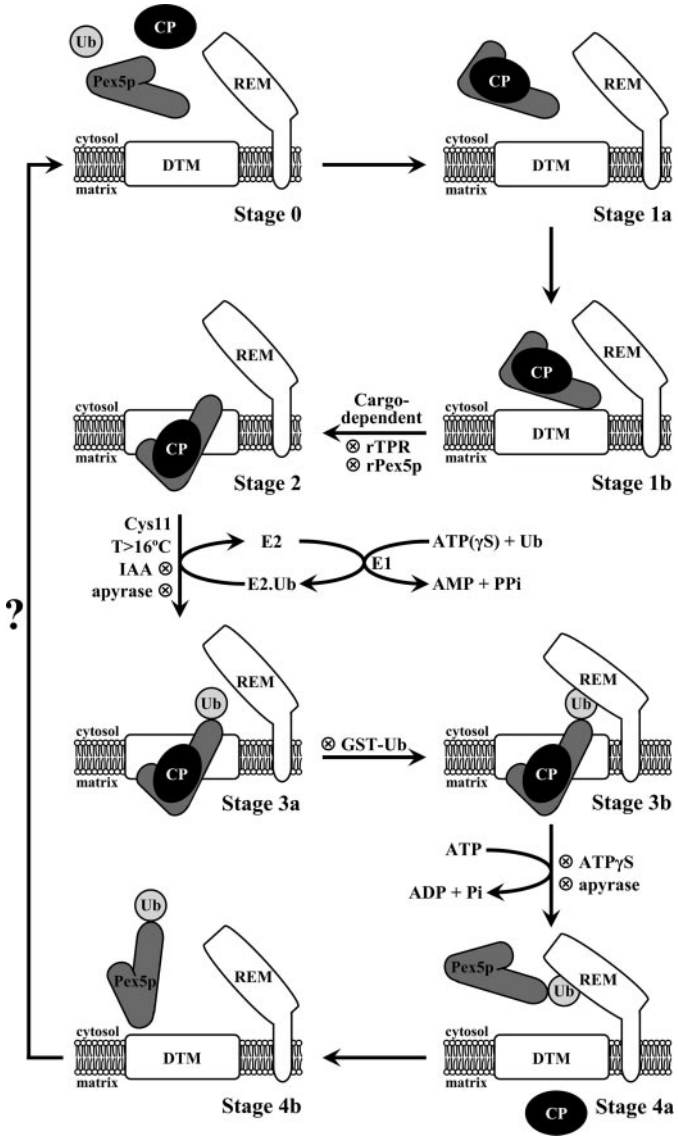

FIGURE 1. **Model of the Pex5p-mediated peroxisomal protein import pathway.** There are five major stages in this protein sorting pathway (numbered 0–4). Substages (*a* and *b*) are mostly of conceptual nature. The different stages have been characterized with the *in vitro* system used here, applying several strategies that block (⊗) 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.[5] Monoubiquitination of stage 2 Pex5p yielding stage 3 Pex5p occurs at Cys^{11} of Pex5p. The reaction requires ATP or ATPγS (*ATP(γS)*) 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.

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 *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.

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′-GGTGGAGGCCGAAGCCGGGGGTGCCAAC-3′ and 5′-GTTGGCACCCCCGGCTTCGGCCTCCACC-3′ for the Pex5(C11A)p-encoding plasmid; 5′-GGTGGAGGCCGAACGCGGGGGTGCCAAC-3′ and 5′-GTTGGCACCCCCGCGTTCGGCCTCCACC-3′ for the Pex5(C11R)p-encoding plasmid; and 5′-GGTGGAGGCCGAAAAGGGGGGTGCCAAC-3′ and 5′-GTTGGCACCCCCCTTTTCGGCCTCCACC-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′-GCCCGTCGACCTGTCACTGGGGCAGGCCAAAC-3′) and cloning the BglII/SalI-digested PCR products into the multiple cloning site of pIRES-EGFP-PTS1 (34) digested with the same restriction enzymes. All of the constructs were verified by DNA sequencing (Agowa).

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 CellM imaging station (Olympus) equipped with U-MNUA2, U-MNIBA3, and U-MWIY2 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 before (26). For the steady-state analysis of rat liver Pex5p, an SEM buffer supplemented with 20 mM *N*-ethylmaleimide (NEM), 1:300 (v/v) protease inhibitor mixture and 10 μg/ml phenylmethylsulfonyl fluoride (Sigma) was used.

In Vitro Assays—*In vitro* import reactions (100 μl final volume) containing 400 μg of PNS protein and 1 μl of the relevant ^{35}S-labeled protein (see below) in import buffer (0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH, pH 7.2, 3 mM $MgCl_2$, 20 μM methionine, and 2 μg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide) were incubated for 30 min at 37 °C in the presence of 5 mM ATP and 10 μM bovine ubiquitin, unless otherwise indicated. Ubiquitin aldehyde (Ubal; Biomol), an inhibitor of deubiquitinating enzymes (DUBs), was used at a final concentration of 3 μM. Proteasome inhibitors MG132 and clastolactacystin β-lactone (Biomol) each at 10 μM final concentration were included in some of the initial experiments, but similar results were obtained in their absence. When specified, 20 units/ml apyrase (grade VII, Sigma) and 2 μg of recombinant Pex5p (38) were added to the reactions. NEM treatment of import reactions was done on ice for 5 min by adding 25 μl of a freshly made 0.1 M solution/100 μl *in vitro* reaction. To separate organelles from soluble proteins, the *in vitro* reactions were diluted with SEM buffer (typically 900 μl) and centrifuged at 13,000 rpm for 20 min at 4 °C using a Biofuge pico centrifuge (Heraeus). To determine the lability of the soluble Ub-Pex5p thiol ester conjugate (stage 4) in the presence of GSH, a standard *in vitro* reaction containing bovine ubiquitin, Ubal, and proteasome inhibitors was performed. At the end of the 37 °C incubation, the reaction was diluted with 400 μl of SEM buffer supplemented with the Ubal and proteasome inhibitors (but lacking NEM) and centrifuged as above. The supernatant was then incubated for the indicated time periods at 37 °C, pH 7.2, with 5 mM GSH (added from a freshly prepared 0.1 M solution in 20 mM MOPS-KOH, 1 mM EDTA, pH 7.2 adjusted at 37 °C). After addition of NEM (final concentration, 20 mM) and incubation on ice for 5 min, the samples were subjected to trichloroacetic acid precipitation and processed for SDS-PAGE (7). To determine the half-life of the membrane-bound Ub-Pex5p conjugate (stage 3) in the presence of GSH, a total 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 ATPγS 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 ^{35}S-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 ^{35}S-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 μg 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 SW41 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—^{35}S-Labeled Pex5p proteins were synthesized in rabbit reticulocyte lysates using the TnT® quick coupled transcription/translation system (Promega) in the presence of EasyTag™ L-[^{35}S]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 UN-SCAN-IT automated system. Pex5p was detected on Western blots using an antibody directed to human Pex5p (40) or by blot overlay using human ^{35}S-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 Cys^{11} 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 in our *in vitro* system when using this mutated Pex5p species so that we could directly compare the properties of the Ub-Pex5p thiol ester conjugate with those of an almost identical molecule differing only in the covalent bond linking ubiquitin to the peroxin. Obviously, there was a risk that this mutant protein would no longer be recognized by the peroxisomal E3 ligase(s) catalyzing this ubiquitination event. However, and surpassing our expectations, this turned out not to be the case, as described below.

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 Cys^{11} 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 Cys^{11} for the dislocation of Pex5p back into the cytosol.

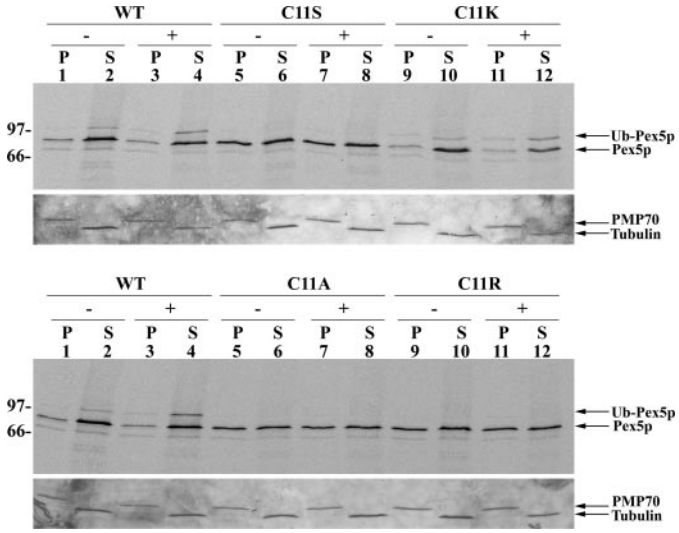

FIGURE 2. **Pex5(C11K)p is a substrate for the peroxisomal import and export machinery.** Import reactions containing 400 μg of PNS protein were programmed with ^{35}S-labeled Pex5p (*WT*) or with mutant versions in which Cys11 was replaced by a serine (*C11S*), a lysine (*C11K*), an alanine (*C11A*), or an arginine (*C11R*). The samples were incubated for 30 min at 37 °C in the absence (−) or presence (+) of Ubal. After treatment with NEM, the samples were separated into an organelle pellet (*P*) and a supernatant (*S*) by centrifugation, subjected to SDS-PAGE under nonreducing conditions, and blotted onto a nitrocellulose. The membrane was exposed to an x-ray film and afterward probed sequentially with antibodies against PMP70 and α-tubulin. Note that there is always a large fraction of ^{35}S-labeled Pex5p that never passes through the DTM. Competition with endogenous rat liver Pex5p surely contributes to the low yields of Ub-Pex5p in these assays. The *numbers* to the *left* indicate the molecular masses of reduced protein standards in kDa.

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 DUBs (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 DUBs) 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

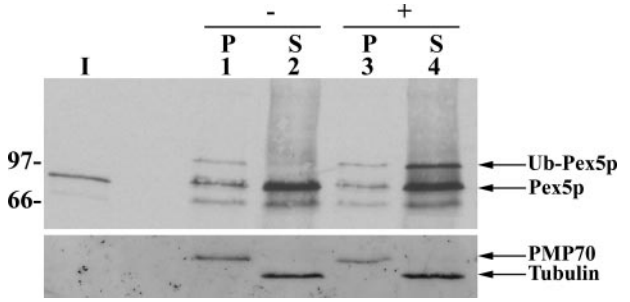

FIGURE 3. **Stage 3 and stage 4 Pex5p display a different susceptibility to DUBs.** ^{35}S-labeled Pex5p was subjected to *in vitro* import reactions containing 800 μg of PNS in 100 μl, in the absence (−) or presence (+) of Ubal. After 20 min at 37 °C, apyrase was added, and the reactions were incubated for 3 min and supplemented with recombinant Pex5p. Incubation proceeded for an additional 20 min. After NEM treatment, the reactions were separated into organelle pellets (*P*) and supernatants (*S*), subjected to SDS-PAGE under nonreducing conditions, and blotted onto a nitrocellulose membrane. The membrane was exposed to an x-ray film and afterward probed sequentially with antibodies against PMP70 and α-tubulin. *Lane I*, 10% of the ^{35}S-labeled Pex5p reticulocyte lysate used in each lane. The *numbers* to the *left* indicate the molecular masses of reduced protein standards in kDa.

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. 4*A*, 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. 4*B*, *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. 4*C*, 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*).

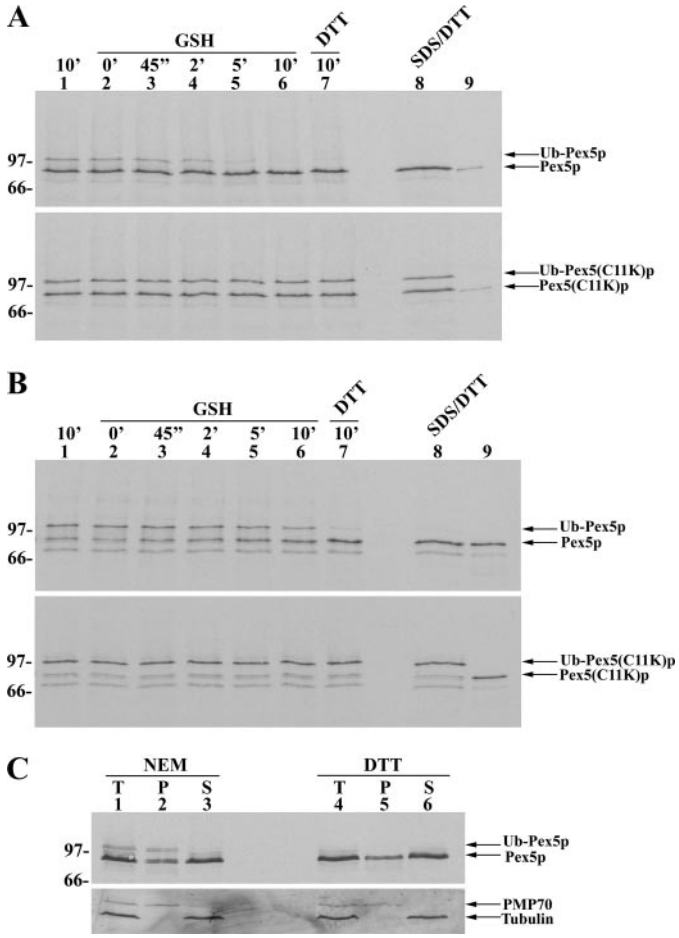

FIGURE 4. **The susceptibility of the Ub-Pex5p thiol ester bond to GSH is context-dependent.** *A*, soluble Ub-Pex5p (*upper panel*) and Ub-Pex5(C11K)p (*lower panel*) were generated as described under "Experimental Procedures," incubated with 5 mM GSH (*lanes 3–6*) or 5 mM DTT (*lane 7*) for the indicated periods of time, and treated with 20 mM NEM for 5 min on ice. The samples in *lanes 1* and *2* received the same amount of NEM-neutralized GSH after 10 and 0 min of incubation at 37 °C, respectively. All of the samples were subjected to trichloroacetic acid precipitation and analyzed by SDS-PAGE under nonreducing (*lanes 1–7*) or reducing conditions (*lanes 8*; 100 mM DTT, 2% (w/v) SDS, 15 min at 65 °C). *Lanes 9*, rabbit reticulocyte lysates containing the ^{35}S-labeled Pex5p and Pex5(C11K)p proteins. *B*, the same as in *A*) with the exception that membrane bound Ub-Pex5p (*upper panel*) and Ub-Pex5(C11K)p (*lower panel*) were used. Autoradiographs are shown. *C*, a rat liver PNS prepared in the presence of NEM (*T*) was separated into an organelle pellet (*P*) and supernatant (*S*) by centrifugation. Equivalents to 80 μg of PNS protein were analyzed by SDS-PAGE under nonreducing (*NEM*; *lanes 1–3*) or reducing conditions (*DTT*; *lanes 4–6*). Endogenous rat Pex5p, Ub-Pex5p, PMP70, and α-tubulin are indicated. The *numbers* to the *left* indicate the molecular masses of reduced protein standards in kDa.

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-

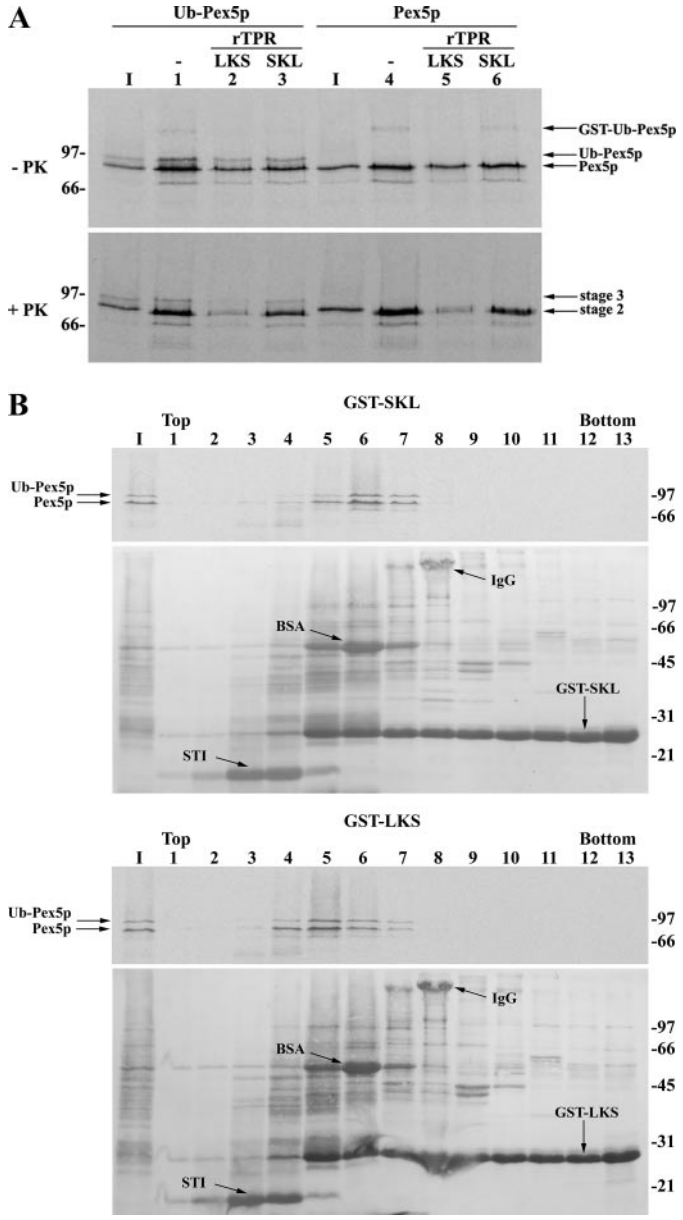

FIGURE 5. **Stage 4 Pex5p interacts with the DTM in a cargo-dependent manner.** *A*, the supernatant of an *in vitro* reaction containing a mixture of ^{35}S-labeled Pex5p and Ub-Pex5p or a similar supernatant containing only the ^{35}S-labeled Pex5p were treated with iodoacetamide and subjected to an *in vitro* reaction at 16 °C for 30 min (see "Experimental Procedures" for details). A vast excess of GST-Ub was added to the reaction medium to assess the inhibition of *de novo* ubiquitination. The reactions in *lanes 2* and *5* also contained a recombinant protein comprising the PTS1-binding domain of Pex5p (r*TPR*) plus a control peptide (*LKS*). The reactions in *lanes 3* and *6* contained this inhibitory recombinant protein neutralized with a vast molar excess of PTS1-containing peptide (*SKL*). At the end of the incubation half of each sample was subjected to proteinase K treatment. Protease-treated (+*PK*) and untreated (−*PK*) halves were incubated on ice with 20 mM NEM, diluted with SEM buffer, and subjected to centrifugation. Organelle pellets were analyzed under nonreducing conditions by SDS-PAGE/autoradiography. Note the near absence of *de novo* ubiquitination of Pex5p (*GST-Ub-Pex5p*) in these reactions. *Lanes I*, 2.5% of the iodoacetamide-treated Ub-Pex5p and Pex5p radiolabeled proteins used in each lane. The *numbers* to the *left* indicate the molecular masses of reduced protein standards in kDa. *B*, sucrose gradient sedimentation analysis of stage 4 Pex5p. A mixture of soluble iodoacetamide-treated ^{35}S-labeled Pex5p and Ub-Pex5p was preincubated with either GST-SKL or negative control protein, GST-LKS. A mixture of proteins with known sedimentation coefficients was added to the samples immediately before loading onto the top of sucrose gradients containing either GST-SKL or GST-LKS, respectively. After centrifugation, the fractions were collected from the bottom of the tube, 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 I*, 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.

pendent extraction from a membrane-embedded protein complex (the DTM) again exposed and sensitive to GSH and DUBs.

Deubiquitination of Pex5p Is Not a Mandatory Step in the Pex5p-mediated Import Pathway—The experiment presented in Fig. 2 revealed yet another interesting aspect of the Pex5p-mediated protein import pathway. Indeed, the relative amounts of unconjugated and ubiquitinated Pex5p proteins detected in the organelle pellets seem to parallel the ones found in the supernatants. Considering that the organelle pellets are not significantly contaminated with soluble proteins (as assessed by the distribution of tubulin), this finding suggested to us that Ub-Pex5p and Ub-Pex5(C11K)p may have the capacity of re-entering the DTM. To test this idea we produced soluble Ub-Pex5p in an *in vitro* reaction, treated the mixture of Ub-Pex5p and Pex5p with iodoacetamide, and performed a second *in vitro* assay at 16 °C to inhibit *de novo* ubiquitination (Fig. 1). A vast excess of GST-Ub was also included in this second *in vitro* reaction, allowing us to assess the degree of inhibition of *de novo* ubiquitination. As shown in Fig. 5*A*, Ub-Pex5p does acquire a protease-resistant status when incubated with a PNS fraction, suggesting that it entered the DTM. Importantly, as observed for Pex5p (*lanes 4–6*), insertion of Ub-Pex5p into the DTM is cargo protein-dependent (compare *lanes 1* and *2*; see also Fig. 1). Thus, deubiquitination of Pex5p is not a mandatory step in the Pex5p-mediated import pathway.

One implication of this finding is that ubiquitination of Pex5p does not change significantly its cargo binding capacity. The experiment presented in Fig. 5*B* corroborates this interpretation. Here, a mixture of soluble Pex5p and Ub-Pex5p was subjected to sucrose gradient centrifugation in the presence or absence of a PTS1-containing recombinant protein (GST-SKL). As shown before (38), in the absence of GST-SKL, the majority of Pex5p sediments as a monomeric protein above bovine serum albumin (Fig. 5*B*, *lower panel*, *lane 5*). The abnormal sedimentation behavior of Pex5p is due to the natively unfolded nature of a major part of its polypeptide chain (39). When GST-SKL is included in the gradient solution, a complex comprising one molecule of Pex5p and one of GST-SKL is formed (38), and this complex now sediments slightly below bovine serum albumin (*upper panel, lane 6*; note that although both Pex5p and albumin are more abundant in *lane 6*, there is more albumin in *lane 5* than in *lane 7*, whereas similar amounts of Pex5p are found in these two lanes). Importantly, the distributions of Ub-Pex5p in these gradients parallel those of Pex5p. Taken together, these observations suggest that Pex5p and Ub-Pex5p have similar cargo binding capacities.

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 Pex5p in our *in vitro* system. To determine whether the same is true *in vivo*, fibroblasts from Pex5-KO mice

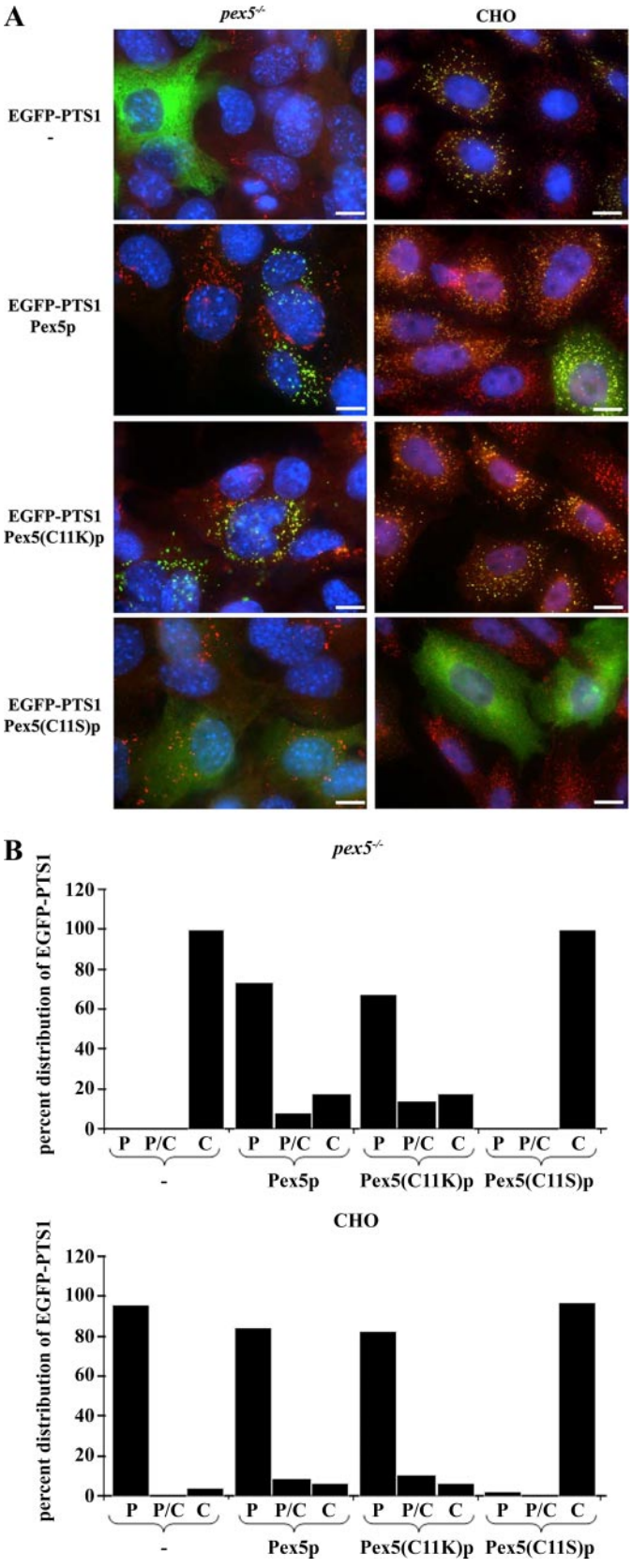

FIGURE 6. **Localization of EGFP-PTS1 in *pex5*$^{-/-}$ and CHO cells upon co-expression with Pex5p, Pex5(C11K)p, or Pex5(C11S)p.** *A*, Pex5p-deficient mouse fibroblasts (*pex5*$^{-/-}$) or CHO cells were transiently transfected with a bicistronic plasmid encoding EGFP-PTS1 and no other protein (−) or one of the indicated Pex5p variants. After 48 h, the cells were fixed and processed for fluorescence microscopy by using anti-Pex14p antibodies (*red*). The nuclei were counterstained with 4′,6′-diamino-2-phenylindole (*blue*). *Scale bar*, 10 μm. *B*, percentage of distribution of EGFP-PTS1 localizations in *pex5*$^{-/-}$ and CHO cells upon co-expression with Pex5p, Pex5(C11K)p, or Pex5(C11S)p. The subcellular localization of EGFP-PTS1 was determined by its punctate (peroxisomal, *P*), diffuse (cytosolic, *C*), or bimodal (peroxisomal/cytosolic, *P/C*) staining pattern in at least 200 cells, and the results were quantified.

(35) 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

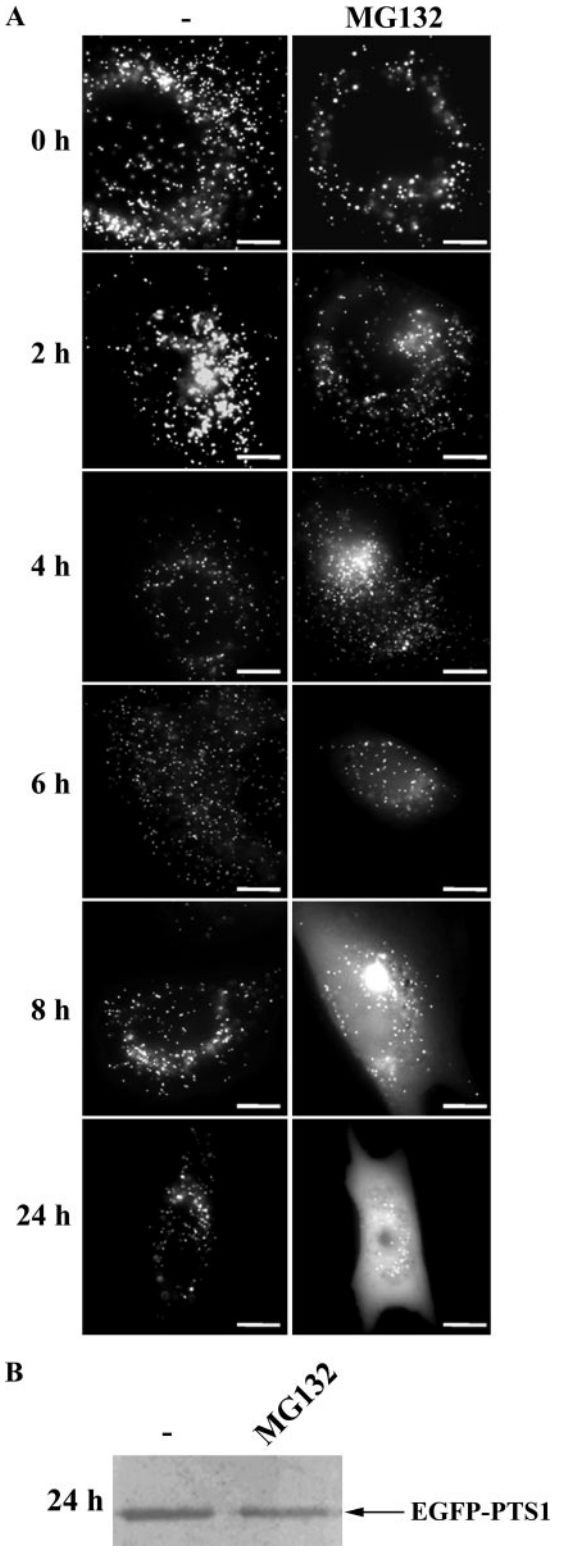

FIGURE 7. **The proteasome inhibitor MG132 blocks the PTS1 import pathway *in vivo*.** *A*, CHO cells were transfected with a plasmid coding for EGFP-PTS1, and the transfected cells were enriched by drug selection (G418) for 3 weeks. Next, the cells were grown in the absence (−) or presence of 5 μM MG132. At the indicated time points, the cells were fixed and processed for direct fluorescence analysis. *Scale bar*, 10 μm. The images shown for each time point are representative for each condition. *B*, the levels of EGFP-PTS1 in cells treated or not with MG132 for 24 h were monitored by Western blotting using an anti-EGFP antibody.

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 Cys^{11} 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,

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,[5] 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 factor 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.

[5] C. P. Grou, A. F. Carvalho, M. P. Pinto, S. J. Huybrechts, C. Sá-Miranda, M. Fransen, and J. E. Azevedo, unpublished results.

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, $pK_a = \sim 9.0$ (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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