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Peroxisomal monoubiquitinated PEX5 interacts with the AAA ATPases PEX1 and PEX6 and is unfolded during its dislocation into the cytosol

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PEX1 and PEX6 are two members of the ATPases associated with diverse cellular activities (AAA) family and the core components of the receptor export module of the peroxisomal matrix protein import machinery. Their role is to extract monoubiquitinated PEX5, the peroxisomal protein-shuttling receptor, from the peroxisomal membrane docking/translocation module (DTM), so that a new cycle of protein transportation can start. Recent data have shown that PEX1 and PEX6 form a heterohexameric complex that unfolds substrates by processive threading. However, whether the natural substrate of the PEX1–PEX6 complex is monoubiquitinated PEX5 (Ub-PEX5) itself or some Ub-PEX5–interacting component(s) of the DTM remains unknown. In this work, we used an established cell-free *in vitro* system coupled with photoaffinity cross-linking and protein PEGylation assays to address this problem. We provide evidence suggesting that DTM-embedded Ub-PEX5 interacts directly with both PEX1 and PEX6 through its ubiquitin moiety and that the PEX5 polypeptide chain is globally unfolded during the ATP-dependent extraction event. These findings strongly suggest that DTM-embedded Ub-PEX5 is a *bona fide* substrate of the PEX1–PEX6 complex.

Peroxisomes are cytoplasmic organelles delimited by a single membrane found in almost all eukaryotes (1). In mammals, they harbor a set of ~100 different proteins and are involved in several metabolic pathways, such as β-oxidation of fatty acids, synthesis of plasmalogens and bile acids, and detoxification of glyoxylate (2–6). Despite their simplicity, peroxisomes play important roles in human health and development, as demonstrated by a group of inherited metabolic disorders in which peroxisomes are partially or even completely defective, the peroxisomal biogenesis disorders (7). These diseases are caused by mutations in genes encoding proteins mechanistically involved in several aspects of peroxisome biogenesis, the so-called peroxins or PEX proteins (6–8). In mammals, 16 peroxins are presently known, 10 of which are components of the machinery that sorts newly synthesized proteins to the organelle matrix (reviewed in Ref. 9).

Proteins destined for the peroxisomal matrix are synthesized in the cytosol and transported to the organelle membrane by PEX5, the peroxisomal matrix protein–shuttling receptor (10–20). There, cargo-loaded PEX5 interacts with the docking/translocation module (DTM),[4] a multisubunit transmembrane complex comprising the core components PEX2, PEX10, PEX12, PEX13, and PEX14 (21, 22). This interaction culminates with the insertion of PEX5 into the DTM with the concomitant translocation and release of the cargo protein into the organelle matrix (23–26). No ATP hydrolysis or membrane potential is needed for these steps; the driving force for the complete cargo transport process resides in the strong protein–protein interactions that are established between PEX5 and DTM components (23, 25, 27).

After releasing its cargo, PEX5 has to be extracted from the DTM so that a new protein transport cycle can be initiated. Recycling of PEX5 involves three steps. First, DTM-embedded PEX5 is monoubiquitinated at a conserved cysteine residue (Cys-11 in the human protein) (28, 29). Then monoubiquitinated PEX5 (Ub-PEX5) is extracted into the cytosol in an ATP hydrolysis–dependent manner by the so-called receptor export module (REM) (27, 30, 31). This is a protein complex compris-

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[4] The abbreviations used are: DTM, docking/translocation module; AAA, ATPases associated with diverse cellular activities; DHFR, dihydrofolate reductase; EGFP, enhanced GFP; HA, influenza hemagglutinin; MTX, methotrexate; *p*Bpa, *p*-benzoyl-L-phenylalanine; PNS, postnuclear supernatant; REM, receptor export module; Ub-PEX5, monoubiquitinated PEX5; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; ATPγS, adenosine 5′-*O*-(thiotriphosphate); Ub, ubiquitin; RIPA, radioimmune precipitation assay.

ing PEX1 and PEX6, two members of the AAA ATPases family, plus a poorly conserved peroxisomal membrane protein (PEX26 in mammals, APEM9 in plants, or PEX15 in yeasts/fungi), which anchors the ATPases to the organelle membrane (32–34). Finally, Ub-PEX5 is deubiquitinated in the cytosol probably by a combination of enzymatic and nonenzymatic events (35–37).

Although the general properties of the PEX5-mediated protein import pathway are known, the mechanistic details of each of its steps remain mostly unclear. In this work, we focused on the mechanism used by the REM to extract Ub-PEX5 from the DTM.

An abundance of structural/functional data was recently reported for the yeast REM. These include EM structures of the PEX1–PEX6 complex, an X-ray structure of the cytosolic domain of PEX15, and the structure of a trimeric complex containing these three proteins (reviewed in Ref. 38) (39–42). These studies revealed that PEX1 and PEX6 assemble into a ring-shaped heterohexameric complex, best described as a trimer of PEX1–PEX6 heterodimers, displaying a relatively large pore at its center. These data, together with mutational analyses of PEX1 and PEX6 showing that the conserved hydrophobic residues of the so-called pore loops are important for PEX1–PEX6 function, might suggest that the PEX1–PEX6 complex uses its pore to handle substrates (39, 40, 42). Robust data showing that the PEX1–PEX6 complex indeed unfolds substrates in a pore loop–dependent manner using a processive threading mechanism were reported very recently (42). Surprisingly, the unfolding/threading activity of the PEX1–PEX6 complex was detected using a soluble fragment of PEX15 as substrate, but whether or not PEX15 is a natural substrate for these ATPases remains unknown, as thoroughly discussed by the authors of that work (42) (also see "Discussion").

Here, we asked whether or not DTM-embedded Ub-PEX5 displays properties of a *bona fide* REM substrate. Our data show that DTM-embedded Ub-PEX5 interacts directly with PEX1–PEX6 and is globally unfolded during the ATP-dependent extraction step. This suggests that Ub-PEX5 is indeed a natural substrate of the REM.

Results

The results reported below were obtained with a cell-free *in vitro* system that recapitulates all the steps of the PEX5-mediated protein import pathway. This experimental system was thoroughly described recently (43), but a brief explanation is provided here for clarity. The core of the system is a rat liver post-nuclear supernatant (PNS; the source of peroxisomes and cytosolic proteins), which is incubated with an *in vitro* synthesized radiolabeled PEX5 protein under appropriate conditions. During this incubation, a fraction of the radiolabeled PEX5 protein becomes inserted into the peroxisomal DTM, where it is subsequently monoubiquitinated at cysteine 11 and rapidly exported into the cytosol by the REM in an ATP-dependent manner. A number of strategies can be used to block this pathway (43). For instance, AMP-PNP and ATPγS, two ATP analogs used in this work, are excellent substrates for the ubiquitin-conjugating cascade (36, 44) but potent inhibitors of the REM (36). Thus, *in vitro* assays containing one of these nucleotides will reveal an accumulation of Ub-PEX5 at the peroxisomal DTM, particularly so if the PNS was previously "primed" (*i.e.* preincubated with a small amount of ATP to release endogenous PEX5 from the DTMs) (25). Two types of *in vitro* assays are used here: the so-called single-step and two-step import/export assays. The first involves a single incubation of the reaction mixture. If such an assay is performed in the presence of ATP, then all of the steps of the pathway will occur (*i.e.* radiolabeled PEX5 will continuously interact with the DTM, where it is monoubiquitinated and subsequently exported into the soluble phase of the assay). Thus, if at the end of the incubation period, the PNS is centrifuged to separate organelles from soluble proteins, most monoubiquitinated PEX5 will be detected in the soluble fraction. In the two-step import/export assay, a radiolabeled PEX5 protein is first accumulated at the DTM in the presence of AMP-PNP or ATPγS (the first step of the assay); the organelles are then isolated by centrifugation, resuspended, and incubated in buffer containing ATP (the second step) to allow export of the Ub-PEX5 protein into the soluble phase of the assay (36). The two-step assay is particularly suited to characterize events downstream of the monoubiquitination step. For practical reasons related to the lability of the thiol ester bond linking ubiquitin to cysteine 11 of PEX5 (29), all of the PEX5 proteins used in this work contain instead a lysine at position 11. These C11K mutants are fully functional both *in vitro* and *in vivo* (35), but the corresponding monoubiquitinated forms are much more resistant upon SDS-PAGE, which can be performed under reducing conditions. Also, many of the experiments described here were performed with a truncated protein comprising amino acid residues 1–324 of PEX5 and derivatives of it. This protein lacks the globular tetratricopeptide repeat domain of PEX5 and thus is unable to bind efficiently PTS1-containing proteins. However, all of the other functions of the receptor are preserved in this C-terminal truncated species (45). Finally, two readouts are possible in these assays. One, a protease-protection assay, explores the fact that PEX5, or truncated versions of it, are extremely sensitive to proteases, such as proteinase K, unless they are inserted into the DTM. In the other, no protease treatment is performed; one just monitors the distribution of monoubiquitinated PEX5 species between the organelle and soluble fractions of the assay by SDS-PAGE/autoradiography. It is important to note that in the latter case, large amounts of nonubiquitinated PEX5 species will be detected in the organelle pellets. Although a fraction of this material represents nonubiquitinated PEX5 specifically interacting with the DTM, the majority may represent PEX5 protein nonspecifically adsorbed to the organelles, some of which may even appear in the supernatant fraction of a two-step assay. Thus, no conclusions are drawn from the behavior of these nonubiquitinated PEX5 species.

Neither the N nor the C terminus of DTM-embedded Ub-PEX5 is important for the export step

Many members of the AAA family of mechanoenzymes exploit the presence of extended N or C termini in their substrates to unfold or pull them out of protein complexes (46, 47). For instance, katanin, a member of the AAA microtubule–severing family, extracts tubulin from microtubules by grabbing and pulling its disordered C-terminal tail (48, 49). As a first step to understand how Ub-PEX5 is dislocated from the DTM, we asked whether a similar mechanism might be valid for the peroxisomal REM.

We know from previous work that no particular C-terminal domain of PEX5 is important for the dislocation process because truncated proteins comprising amino acid residues 1–324, 1–197, or 1–125 of PEX5 are all functional in the dislocation step (45, 50). Also, we have recently shown that a PEX5 protein possessing an enhanced GFP (EGFP) moiety at its C terminus is still a substrate for the dislocation machinery (51). Indeed, although that PEX5-EGFP fusion protein was not efficiently released from the DTM, protease protection assays revealed that most of the protein that accumulated at the peroxisome in the presence of ATP was already largely exposed into the cytosol, indicating that the C-terminal EGFP moiety interferes with the dislocation machinery only at late stages of the extraction process. Additional data indicating that the presence of an unrelated protein domain at the C terminus of a PEX5 protein does not interfere with the dislocation process were obtained when a ^{35}S-labeled fusion protein comprising the first 324 amino acid residues of PEX5 fused to ubiquitin lacking its last two glycine residues (PEX5(1–324;C11K)-Ub(1–74); see Fig. 1 for a schematic representation of the PEX5 proteins used in this work) was used in a cell-free *in vitro* two-step import/export assay (36). Similarly to ^{35}S-labeled PEX5(1–324; C11K), used here as a positive control (Fig. 2*A*, *two bottom panels*) (36), monoubiquitinated PEX5(1–324;C11K)-Ub(1–74) was efficiently dislocated into the soluble phase of the reaction when ATP (but not AMP-PNP) was used in the second step of the assay. Identical results were obtained with a similar PEX5 protein in which mouse dehydrofolate reductase (DHFR) was substituted for Ub(1–74) (see below). Thus, we next focused on the N terminus of PEX5.

DTM-embedded PEX5 exposes a 2 to 3-kDa domain from its N terminus into the cytosol (45, 52). This small domain includes cysteine 11, the residue that has to be monoubiquitinated so that PEX5 can be extracted by the REM. Thus, the REM might extract Ub-PEX5 from the DTM by interacting with its first 10 amino acid residues. To test this, we produced a protein comprising amino acid residues 10–324 of PEX5 (PEX5(10–324;C11K)) and assessed the capacity of this protein to enter the DTM, be monoubiquitinated, and be extracted back into the cytosol using a single-step *in vitro* import/export assay. As shown in Fig. 2*B*, the amounts of monoubiquitinated PEX5(10–324;C11K) and PEX5(1–324;C11K) detected in the supernatants of the reactions made in the presence of ATP are similar. Taken together, these data suggest that if the REM recognizes DTM-embedded Ub-PEX5 through a direct interaction, this interaction does not involve a free disordered N- or C-terminal end on PEX5.

DTM-embedded Ub-PEX5 can be cross-linked to both PEX1 and PEX6 through its ubiquitin moiety

DTM-embedded PEX5 can only be exported back into the cytosol after monoubiquitination at its Cys-11 residue. Thus, in principle, the ubiquitin moiety attached to PEX5 might interact with the REM. However, experimental evidence supporting this possibility is still lacking. We note that there are some data suggesting that both nonubiquitinated and monoubiquitinated PEX5 interact with AWP1, a cytosolic protein reported to bind PEX6 and seemingly necessary for the export step (53). However, AWP1 does not interact with the PEX1–PEX6 protein complex, the active form of the REM (53), and thus the exact role of AWP1 in the dislocation process remains undefined.

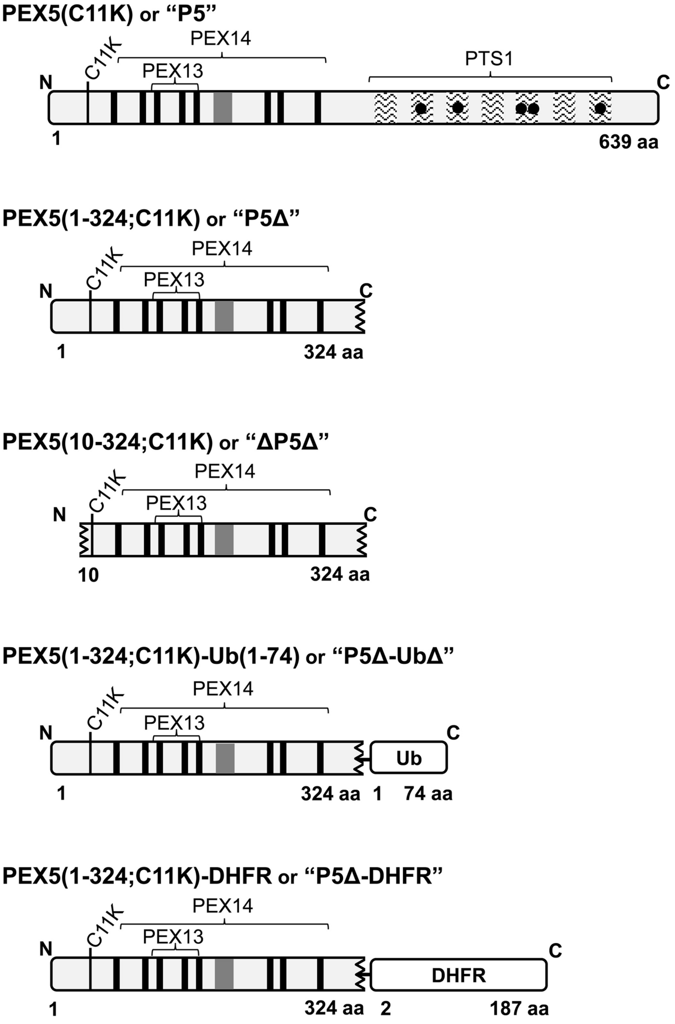

Figure 1. *Schematic representation* of PEX5 species used in this work. The C11K substitution, the PEX13- and PEX14-binding pentapeptide motifs (*black bars* (74, 75)), the PEX7-binding domain (*gray box* (76)), the PTS1-binding tetratricopeptide repeats (*zigzag boxes* (65)), and the 5 cysteine residues present in PEX5(C11K) (*black dots*) are indicated. Abbreviated names of the different PEX5 species, as used throughout the figures, are shown in *quotation marks*.

Aiming at better understanding the role of ubiquitin in PEX5 dislocation, we used a photocross-linking approach (54, 55) to identify the molecular neighbors of the ubiquitin moiety in the DTM-embedded Ub-PEX5 species. For this purpose, four recombinant ubiquitin molecules, each harboring a photocross-linking *p*-benzoyl-L-phenylalanine (*p*Bpa) residue at a different position (see Fig. 3*A*), were produced and used in cell-free *in vitro* assays programmed with ^{35}S-labeled PEX5(1–324; C11K) and performed in the presence of AMP-PNP. Organelle suspensions from these assays were then exposed to UV light to elicit photocross-linking and subjected to SDS-PAGE/autoradiography analyses. The results of these experiments are shown in Fig. 3*B*. In all cases, the most prominent UV-induced radio-

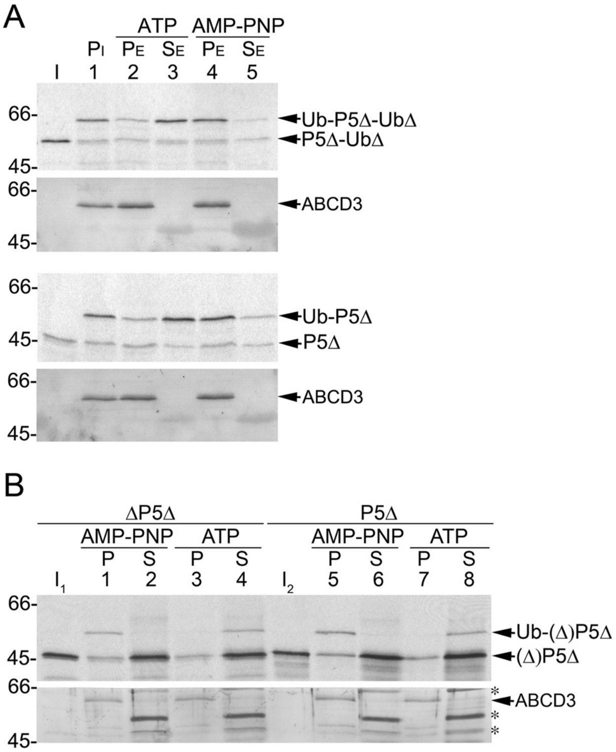

Figure 2. Neither the N nor the C terminus of DTM-embedded Ub-PEX5 is important for the export step. *A*, PEX5(1–324;C11K) containing a ubiquitin moiety at its C terminus is extracted by the REM. Organelles from a PEX5(1–324;C11K)-Ub(1–74) (*P5Δ-UbΔ*) or PEX5(1–324;C11K) (*P5Δ*) import reaction made in the presence of AMP-PNP (*PI*; *lane 1*) were subjected to a second incubation in the presence of either ATP, to promote export (*lanes 2* and *3*), or AMP-PNP, to maintain the REM blocked (*lanes 4* and *5*). Organelle (*PE*; *lanes 2* and *4*) and soluble fractions (*SE*; *lanes 3* and *5*) were then analyzed by SDS-PAGE/Western blotting/autoradiography. A representative experiment ($n = 3$) is shown. The distribution of the peroxisomal membrane protein ABCD3 is shown. *Lane I*, 5% of the indicated radiolabeled protein used in the assay. *B*, the first nine amino acid residues of PEX5 are not required for its export. Radiolabeled PEX5(10–324;C11K) (*ΔP5Δ*) or PEX5(1–324;C11K) (*P5Δ*) were subjected to a single-step assay in the presence of either AMP-PNP or ATP, as indicated. Organelle pellets (*P*) and supernatants (*S*) were then analyzed by SDS-PAGE/Western blotting/autoradiography. A representative experiment ($n = 3$) is shown. Immunoblot against ABCD3 is shown. *, nonspecific proteins occasionally recognized by the ABCD3 antibody. *Lanes* I_1 and I_2, 10% of the indicated radiolabeled protein used in the assay. In *A* and *B*, *numbers* to the *left* indicate the molecular masses of protein standards in kDa.

labeled bands were detected in the high-molecular weight region of the SDS-gel, suggesting that Ub-PEX5(1–324;C11K) (molecular mass of 45 kDa) was cross-linked to some large proteins, probably PEX1 and/or PEX6 (note that PEX1 and PEX6 are by far the two largest components of the peroxisomal protein import machinery, displaying molecular masses of 141 and 104 kDa, respectively (56–58)).

To determine whether or not PEX1 and/or PEX6 are components of these cross-linked products, photocross-linked organelles were solubilized in a denaturing SDS-containing buffer and subjected to immunoprecipitation using either control IgGs or antibodies directed to PEX1 or PEX6. As shown in Fig. 3*C*, we were able to immunoprecipitate significant amounts of PEX1 without precipitating also PEX6, and vice versa (see the *two bottom panels*), showing that protein complexes were efficiently disrupted by the solubilization procedure. Autoradiography analyses of immunoprecipitates revealed that the main photocross-linked products obtained with Ub(A28*p*Bpa), Ub(D39*p*Bpa), and Ub(Q49*p*Bpa) comprise PEX6, whereas those obtained with Ub(Q62*p*Bpa) contain PEX1. Clearly, the ubiquitin moiety of DTM-embedded Ub-PEX5(1–324;C11K) is very close to both PEX1 and PEX6, probably not more than a few Å apart (59). Furthermore, the fact that Ub(Q62*p*Bpa)-PEX5(1–324;C11K) reacts mainly with PEX1, whereas PEX5(1–324;C11K) modified with any of the other three *p*Bpa-modified ubiquitins reacts mainly with PEX6, suggests that the ubiquitin moiety in the DTM-embedded Ub-PEX5 species does not wobble freely, but rather exists in a movement-restricted location (*i.e.* in a binding site).

Extraction of Ub-PEX5 from the DTM involves/requires global unfolding of its polypeptide chain

As stated above, recent data suggest that both nonubiquitinated and monoubiquitinated PEX5 somehow interact with REM components (53, 60). However, the mechanistic meaning of these interactions remains unknown. Given the recent demonstration that the yeast PEX1–PEX6 complex uses a threading mechanism to unfold a substrate (42), we reasoned that assessing whether or not DTM-embedded Ub-PEX5 is globally unfolded during the extraction step would shed light on this issue.

Two strategies were used for this purpose. In the first, we used the classical approach of fusing DHFR to a reporter protein that is used as the substrate in a process requiring unfolding of its polypeptide chain (61). This approach explores the fact that DHFR is a relatively pliable protein that can be unfolded by several molecular machineries, including ATPases of the AAA family (62, 63). However, this property changes completely in the presence of methotrexate (MTX), a folate analog that binds to DHFR, greatly stabilizing its structure (64). We produced a fusion protein comprising the first 324 amino acid residues of PEX5(C11K) followed by mouse DHFR (PEX5(1–324;C11K)-DHFR) and used this protein, as well as PEX5(1–324;C11K), in two-step *in vitro* import/export assays, which were done either in the presence or in the absence of MTX (see "Experimental procedures" for details). As expected, MTX did not block export of monoubiquitinated PEX5(1–324;C11K); as shown in Fig. 4*A* (*III*), a considerable fraction of Ub-PEX5(1–324;C11K) was found in the soluble phase of the MTX-containing assay after incubation in the presence of ATP but not AMP-PNP (compare *lanes 4* and *6*; see also Fig. 2*A*). A similar behavior was observed for Ub-PEX5(1–324;C11K)-DHFR in the assay lacking MTX (Fig. 4*A* (*I*)), although the export efficiency of this species seems to be already compromised (see legend to Fig. 4 for a quantification of export efficiencies). Importantly, in the presence of MTX and ATP, export of Ub-PEX5(1–324;C11K)-DHFR was strongly inhibited (Fig. 4*A* (*II*), compare *lanes 4* and *6*). Furthermore, under these conditions, organelle-associated monoubiquitinated PEX5(1–324;C11K)-DHFR was mostly accessible to exogenously added proteinase K, indicating that the export step was blocked at a late stage (Fig. 4*B*). Thus, as previously described for a PEX5-EGFP fusion protein (51), the ATP-dependent dislocation of this species by the REM can be initiated but not terminated efficiently. Besides confirming that the identity of PEX5 C terminus is irrelevant for the initiation of the extraction step, these findings indicate that an irrelevant

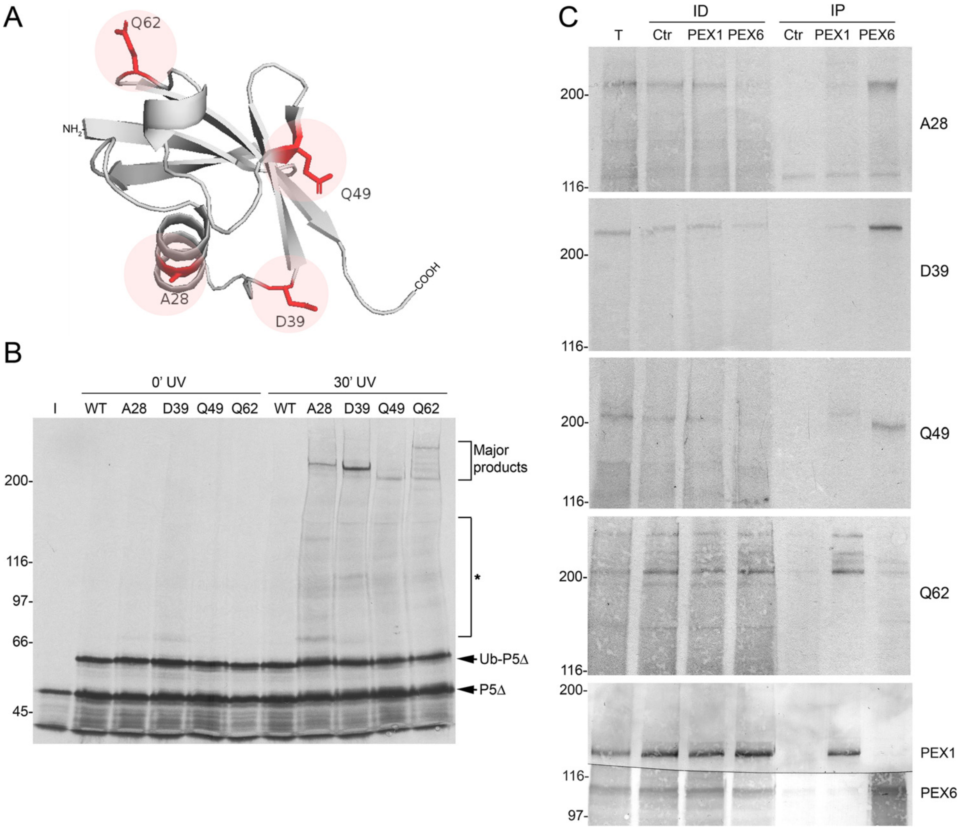

Figure 3. Monoubiquitinated PEX5(1–324;C11K) can be specifically photocross-linked to PEX1 and PEX6 through its ubiquitin moiety. *A*, ubiquitin structure (Protein Data Bank code 1UBQ (77)) highlighting the relative positions of the amino acid residues replaced by a photoreactive *p*Bpa. *B*, radiolabeled PEX5(1–324;C11K) (*P5Δ*) was used in import assays containing AMP-PNP and either HA-tagged WT ubiquitin or one of four ubiquitin mutants possessing a single *p*Bpa residue at the indicated position (*A28*, *D39*, *Q49*, or *Q62*). Import reactions were halved and were irradiated (*30′ UV*) or not (*0′ UV*) with UV light while kept on ice. Organelles were isolated by centrifugation and analyzed by SDS-PAGE/Western blotting/autoradiography. *Lane I*, 5% of the radiolabeled protein used in each reaction. Major cross-linking products are indicated. *, some minor ^{35}S-labeled cross-linked species were also detected. One of four experiments with similar results is shown. *C*, organelle fractions of UV-irradiated import reactions (as shown in *B*) were solubilized and immunoprecipitated with anti-PEX1 (PEX1), anti-PEX6 (PEX6), or a rabbit control serum (control (*Ctrl*)) in RIPA buffer. Total protein (*T*), immunoprecipitated protein (*IP*), and corresponding immunodepleted fractions (*ID*) were analyzed by SDS-PAGE/Western blotting/autoradiography. Each immunoprecipitation was performed at least twice. Following film exposure, the nitrocellulose membranes were cut slightly above the 116-kDa marker, and the upper and lower strips were probed with anti-PEX1 and anti-PEX6 antibodies, respectively (the membrane from the Ub(Q62*p*Bpa) experiment is shown here). In *B* and *C*, *numbers* to the *left* indicate the molecular masses of protein standards in kDa.

protein domain appended to the C terminus of the receptor inhibits its release by the REM, particularly if that domain is tightly folded. In the second approach, we adapted an elegant assay recently developed for the yeast PEX1–PEX6 complex, which monitors global unfolding of a protein substrate by determining the accessibility of its cysteine residues to maleimide-containing reagents (42). Full-length PEX5(C11K), which possesses five relatively unexposed cysteines at its C-terminal TPR domain (65) (see also Fig. 1), and PEG-maleimide of 5 kDa were used in these experiments. Again, a two-step import/export protocol was used. Briefly, an organelle pellet containing DTM-embedded Ub-PEX5 was resuspended in import buffer and divided into three aliquots. One aliquot received ATPγS, to maintain the REM blocked (29), plus PEG-maleimide and was incubated for 3 min at 37 °C. The second aliquot received PEG-maleimide plus a 2:1 ATP/ATPγS mixture and was also incubated for 3 min at 37 °C (note that ATP alone also works in this experiment (data not shown), but the PEGylation yields are slightly better with this mixture, presumably because it decreases the export rate); the third aliquot received the 2:1 ATP/ATPγS mixture and, after a 10-min incubation at 37 °C (to release Ub-PEX5 into the soluble phase of the assay), was treated for an additional 3 min at 37 °C with PEG-maleimide. After quenching unreacted PEG-maleimide with an excess of DTT, organelles and soluble proteins were then separated by centrifugation, and both were analyzed by SDS-PAGE/autoradiography. As shown in Fig. 5, Ub-PEX5 arrested at the DTM (*lane 2*) was not PEGylated, whereas only a small fraction of already released (soluble) Ub-PEX5 (*lane 7*) was PEGylated during the 3-min incubation. In contrast, the presence of PEG-maleimide during the 3-min export step led to an almost quantitative PEGylation of Ub-PEX5 (*lane 5*). Altogether, these data strongly suggest that extraction of Ub-PEX5 from the peroxisomal DTM involves unfolding of its polypeptide chain.

Discussion

It has long been known that extraction of Ub-PEX5 from the peroxisomal DTM is an ATP-dependent process that requires the AAA ATPases PEX1 and PEX6 (27, 30, 31), but how exactly this occurs has remained mysterious. *A priori*, and as discussed before (50), there are two possibilities: 1) the REM could interact with Ub-PEX5 and pull it from the DTM, releasing it into

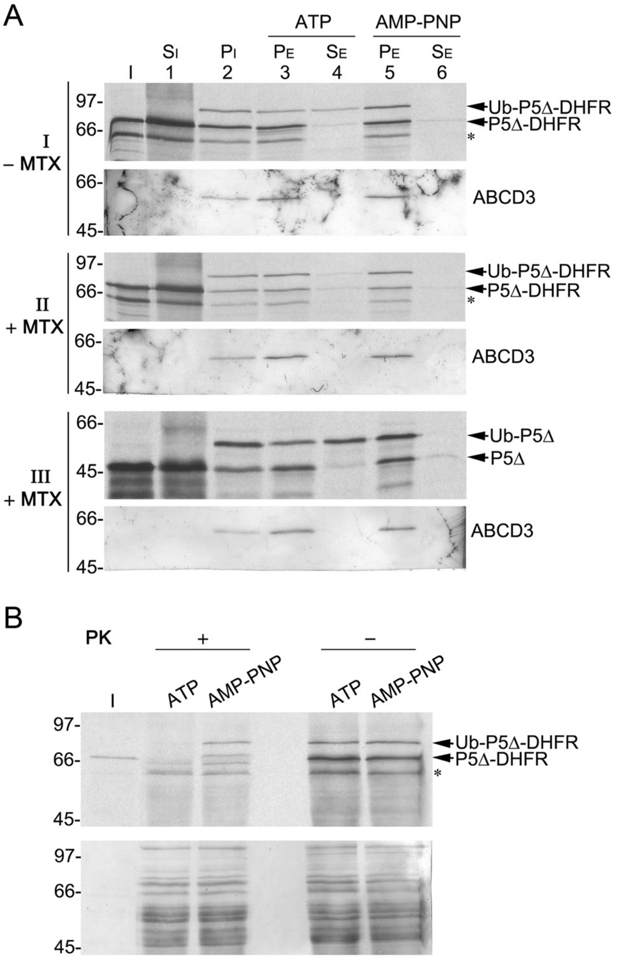

Figure 4. A tightly folded domain appended to the C terminus of PEX5(1–324;C11K) inhibits its export. *A*, radiolabeled PEX5(1–324;C11K)-DHFR (P5Δ-DHFR) and PEX5(1–324;C11K) (P5Δ) were used in two-step import/export assays carried out in the absence (*I*; −*MTX*) or presence of 2 μM methotrexate (*II* and *III*; +*MTX*). After the import step, organelles (*PI*; *lane 2*) were separated from the cytosolic fraction (*SI*; lane 1) and subjected to the second step of the assay in the presence of either ATP (*lanes 3* and *4*) or AMP-PNP (*lanes 5* and *6*). Organelles (*PE*; *lanes 3* and *5*) and soluble fractions (*SE*; *lanes 4* and *6*) were analyzed by SDS-PAGE/Western blotting/autoradiography. Autoradiographs and ABCD3 immunoblots are shown. *SI*, equivalent to 50 μg of PNS; *PI*, *PE*, and *SE*, equivalent to 250 μg of PNS. *Lane I*, 25% of the radiolabeled protein used in the reactions. *, an N-terminally truncated product of PEX5(1–324;C11K)-DHFR that can be imported but not exported (78). The percentages of exported species (defined as the ratio of ubiquitinated species in *SE* to the sum of ubiquitinated species in *SE* and *PE*) were determined by densitometry analyses of three independent experiments. Values of 40% (range 26–47%), 10% (range 7–13%), and 64% (range 53–82%) were obtained for Ub-P5Δ-DHFR minus MTX, Ub-P5Δ-DHFR plus MTX, and Ub-P5Δ plus MTX, respectively. *B*, radiolabeled PEX5(1–324;C11K)-DHFR (P5Δ-DHFR) was subjected to single-step assays in the presence of MTX and either ATP or AMP-PNP. After incubation, samples were halved and treated (*lanes* +) or not (*lanes* −) with proteinase K (*PK*). Organelles were isolated and analyzed by SDS-PAGE/Western blotting/autoradiography. *I*, 5% of the radiolabeled protein used in the assay. The autoradiograph (*top*) and the corresponding Ponceau S–stained membrane (*bottom*) of a representative experiment (*n* = 3) are shown. *, as in *A*. *Numbers* to the *left* indicate the molecular masses of protein standards in kDa.

the cytosol, or 2) the REM could bind and unfold/sequester DTM components that interact with Ub-PEX5, thus disrupting all of the interactions that maintain the receptor at the peroxisomal membrane. Although the first possibility is conceptually more straightforward, recent data on yeast and mammalian PEX1 and PEX6 proteins could actually support the second mechanism. We are referring to the fact that *in vitro*, the yeast PEX1–PEX6 complex can bind and unfold a soluble fragment of PEX15, the yeast orthologue of mammalian PEX26, but not a linear ubiquitin-PEX5 fusion protein, and to the findings that both mammalian and yeast PEX1–PEX6 complexes interact with the PEX5–PEX14 complex via PEX26 and PEX15, respectively (42, 60). Together, these data might suggest that the interaction between PEX5 and PEX14, the main PEX5-binding component of the DTM, is disrupted by the PEX1–PEX6 complex via PEX15/PEX26 (42). Although such a mechanism is plausible, the data supporting it can actually be interpreted differently. Indeed, as recently discussed by Gardner *et al.* (42), the interaction between PEX15/PEX26 and the PEX5–PEX14 complex may simply reflect a role of PEX15/PEX26 in recruiting Ub-PEX5 to the PEX1–PEX6 complex, and the fact that the yeast PEX1–PEX6 complex does not unfold a soluble linear ubiquitin-PEX5 fusion protein *in vitro* could mean that the AAA complex recognizes only correctly monoubiquitinated PEX5 (*i.e.* PEX5 ubiquitinated at its residue 11) or that the interaction between the PEX1–PEX6 complex and PEX5 requires other peroxins, which were not present in those *in vitro* assays. Finally, the fact that yeast PEX1–PEX6 unfolds a C-terminally truncated PEX15 protein *in vitro* does not necessarily mean that it does so with full-length PEX15 *in vivo*, particularly when we consider that the free disordered C-terminal end of the recombinant PEX15 fragment engaged by the PEX1–PEX6 complex in those *in vitro* assays corresponds to an internal domain in the membrane-embedded full-length PEX15 protein. Clearly, additional work was necessary to clarify this issue.

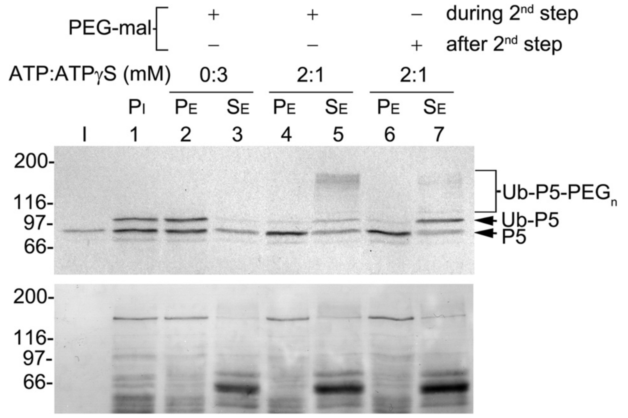

Figure 5. Buried cysteine residues present in the C-terminal half of PEX5 become exposed during the export step. Full-length PEX5(C11K) (*P5*) was subjected to import reactions in the presence of ATPγS. Isolated organelles (*PI*) were resuspended and used in three different export assays, performed in the presence of ATPγS (export blocked) or a 2:1 ATP/ATPγS mixture (export induced). PEG-maleimide 5,000 Da (*PEG-mal*) was added either immediately before or after Ub-PEX5(C11K) (*Ub-P5*) export. PEGylation was done for 3 min at 37 °C. Organelle (*PE*) and supernatant (*SE*) fractions were processed for SDS-PAGE/Western blotting/autoradiography. The autoradiograph (*top*) and the corresponding Ponceau S–stained membrane (*bottom*) of a representative experiment (*n* = 3) are shown. *Lane I*, 2% of the radiolabeled protein used in the assay. *Ub-P5-PEGn*, PEGylated Ub-P5 species. *Numbers* to the *left* indicate the molecular masses of protein standards in kDa.

Here, we used an established cell-free *in vitro* system to address this problem. We provide evidence strongly supporting the view that mammalian Ub-PEX5 is a *bona fide* substrate of the REM. Indeed, we found that DTM-embedded Ub-PEX5 can be photocross-linked to both PEX1 and PEX6. This suggests that Ub-PEX5 interacts directly with the AAA ATPases of the REM, an expected property for an authentic substrate. More importantly, we have shown that 1) PEX5 cysteine residues located dozens/hundreds residues apart from the pentapeptide motifs that mediate the interaction of PEX5 with the DTM (see Fig. 1) become largely exposed to a maleimide reagent during the ATP-dependent dislocation process, and 2) fusing the N-terminal half of PEX5 (a domain fully functional in both the import and export steps (50)) to mouse DHFR results in a protein that arrests at the export step particularly when the stability of DHFR is increased by MTX. Thus, together, these results suggest that DTM-embedded Ub-PEX5 undergoes global unfolding during the ATP-dependent extraction process and that its unfolding is mandatory for a complete extraction from the organelle surface.

Although it is still unclear how DTM-embedded Ub-PEX5 is recruited to the translocation pore of the PEX1–PEX6 hexameric complex, the data presented here, together with previous findings, allow us to propose two hypothetical models. Both take into account that the minimal information necessary to engage the REM resides in the ubiquitin moiety itself plus the region comprising residues 10–125 of PEX5 (45) (this work). In one model, a protein loop in the 10–125 region of PEX5 would enter the PEX1–PEX6 pore through its cis side (*i.e.* the membrane-facing side of the PEX1–PEX6 complex). ATP hydrolysis would then lead to the translocation of the remaining Ub-PEX5 domains through the REM pore, ultimately releasing the complete substrate into the cytosol, at the trans side of the PEX1–PEX6 complex. The rather large pore of the PEX1–PEX6 complex, which may widen even more during substrate translocation, as proposed recently for CDC48 (66), together with the finding that PEX5(1–324;C11K)-Ub(1–74) displays no detectable export problems (see Fig. 2*A*) might support this possibility.

In the second model, the PEX1–PEX6 ring would assemble around the short exposed segment of PEX5 that follows residue 11 (45). This would place this short segment of PEX5 inside the pore and the ubiquitin moiety of Ub-PEX5 already at the trans side of the PEX1–PEX6 complex. ATP hydrolysis would then lead to the complete dislocation of the PEX5 polypeptide chain into the cytosol. Discriminating between these two possibilities will be a challenging task, but the experimental tools and strategies developed in this work will surely help us to understand this and other still unclear aspects of the peroxisomal matrix protein import machinery.

Experimental procedures

DNA constructs

Plasmids encoding the large isoform of human PEX5 possessing a lysine instead of a cysteine residue at position 11 (pGEM4-PEX5(C11K) (35)), a C-terminally truncated form containing residues 1–324 (pET28-PEX5(1–324) (67)), an N-terminally truncated form containing residues 315–639 (pQE30-PEX5(315–639) (68)), human UCHL3 (pET28a-UCHL3 (69)), and influenza hemagglutinin (HA)-tagged human ubiquitin (pET28a-HA-Ub (36)) were described previously. The plasmid pEVOL-*p*BpF, which encodes the tRNA/tRNA synthetase pair necessary for the *in vivo* incorporation of the photocross-linker *p*Bpa, in response to an amber codon, was a gift from Peter Schultz (54) (Addgene plasmid 31190).

The PEX5(1–324;C11K) cDNA, encoding amino acid residues 1–324 of PEX5(C11K) was obtained by PCR amplification of the plasmid pGEM4-PEX5(C11K) using the primers 5′-GCCCAATACGCAAACCGCCTCTCC-3′ and 5′-GCGCGGATCCTCATTAGTACCCCTTATCATAGGTAGCTG-3′. The purified product was used directly in TNT® quick-coupled transcription/translation reactions (see “Miscellaneous methods”).

pGEM4-PEX5(10–324;C11K), a plasmid encoding residues 10–324 of PEX5(C11K), was obtained by PCR amplification of pGEM4-PEX5(C11K) with primers 5′-GGAATAAGTCGACATGGAAAAGGGGGGTGC-3′ and 5′-CGGGCAGGTCTAGATCAGTACCCCTTATCATAGGTAGC-3′. The purified product was digested and cloned into the SalI and XbaI sites of pGEM4 (Promega).

A plasmid encoding residues 1–324 of PEX5(C11K) fused to the N terminus of mouse DHFR was obtained as follows. First, the DHFR coding region was amplified by PCR using the plasmid pYES2-cytb2(1–107)DHFR (70) (a kind gift of Dr. Dejana Mokranjac, Ludwig-Maximilians Universität, Munich, Germany) and the primers 5′-GCGCCGTCTAGAGGATCTGGGGTTCGACCATTGAACTGCATC-3′ and 5′-GCGCGCGGTACCTTAGTCTTTCTTCTCGTAGACTTCAAAC-3′. The purified product was digested and cloned into the XbaI and KpnI sites of pGEM4 (Promega), originating pGEM4-DHFR. Then a DNA fragment encoding residues 1–324 of PEX5(C11K) was amplified from pGEM4-PEX5(C11K) by PCR with the primers 5′- GTCTGCCGGTCGACGCCACCATGGCAATGCGGGAGCTG-3′ and 5′-GCGCCGTCTAGATCCACTTCCGTACCCCTTATCATAGGTAGCTGACG-3′. The purified fragment was digested and cloned into the SalI/XbaI-digest of pGEM4-DHFR, originating pGEM4-PEX5(1–324;C11K)-DHFR.

To obtain a plasmid encoding residues 1–324 of PEX5(C11K) fused to human ubiquitin lacking the two C-terminal Gly residues, a DNA fragment encoding residues 1–74 of ubiquitin was amplified from pET28a-HA-Ub using the primers 5′-GCGTACTCTAGAAGCGGCATGCAGATCTTCGTGAAGAC-3′ and 5′-GCTCGCGGTACCTCATCTGAGACGGAGGACCAG-3′. The purified product was digested and cloned into the XbaI/KpnI digest of pGEM4-PEX5(1–324;C11K)-DHFR, originating pGEM4-PEX5(1–324;C11K)-Ub(1–74).

To generate a plasmid encoding HA-tagged ubiquitin with a C-terminal His$_6$ tag (pET28a-HA-Ub-His), the STOP codon of pET28a-HA-Ub was mutated by site-directed mutagenesis (QuikChange II site-directed mutagenesis kit, Agilent Technologies) using the primers 5′-GTCTCAGAGGTGGTTCAGCGAATTCGAGC-3′ and 5′-GCTCGAATTCGCTGAACCACCTCTGAGAC-3′.

Plasmids encoding HA-Ub-His harboring single amber codon mutations (A28X, D39X, Q49X, or Q62X) were obtained by site-directed mutagenesis of pET28a-HA-Ub-His with the following primers: 5′-GAGAATGTCAAGTAGAAGATCCAAGACAAGGAAGGC-3′ and 5′-GCCTTCCTTGTCTTGGATCTTCTACTTGACATTCTC-3′ for the A28X mutation; 5′-GCATCCCTCCTTAGCAGCAGAGGTTGATC-3′ and 5′-GATCAACCTCTGCTGCTAAGGAGGGATGC-3′ for the

D39X mutation; 5′-CTTTGCTGGGAAATAGCTGGAAGATGGACGC-3′ and 5′-GCGTCCATCTTCCAGCTATTTCCCAGCAAAG-3′ for the Q49X mutation; and 5′-CTACAACATCTAGAAAGAGTCCACCCTGC-3′ and 5′-GCAGGGTGGACTCTTTCTAGATGTTGTAG-3′ for the Q62X mutation.

To obtain a plasmid encoding histidine-tagged residues 1–421 of human PEX6, the plasmid PTY03 (71) was digested with NcoI, and the relevant fragment was cloned into the NcoI site of pET9dNHis$_6$ (a kind gift of G. Stier, EMBL, Heidelberg, Germany) (72). All generated plasmids were sequence-verified.

Expression and purification of recombinant proteins

The histidine-tagged proteins, PEX5(1–324), PEX5(315–639) (hereafter referred to as TPRs), and UCHL3 were purified as described previously (68, 69). All recombinant proteins were stored in Buffer A (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT).

HA-Ub-His was expressed in *Escherichia coli* BL21(DE3) strain by induction with 1 mM isopropyl 1-thio-D-galactopyranoside for 3 h at 37 °C in LB medium. Pelleted cells were resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 50 μg/ml phenylmethylsulfonyl fluoride, 1:500 (v/v) protease inhibitor mixture (catalog no. P8340, Sigma-Aldrich), and 200 μg/ml lysozyme), incubated on ice for 30 min, and disrupted by sonication. Histidine-tagged proteins were purified by Ni Sepharose 6 Fast Flow affinity chromatography (GE Healthcare) according to the manufacturer's instructions. Proteins were concentrated, and the buffer was exchanged to Buffer A by repeated cycles of centrifugation and dilution using a Vivaspin PES concentrator (molecular weight cut-off 3,000; Sartorious).

For incorporation of *p*Bpa, HA-Ub-His amber mutants were expressed in BL21(DE3) cells carrying the plasmid pEVOL-*p*BpF. Induction was carried out for 3 h at 37 °C with 1 mM isopropyl 1-thio-D-galactopyranoside and 0.02% arabinose in 2xYT medium supplemented with 0.2 mM *p*Bpa (catalog no. F-2800; Bachem). Purification was carried out as above. Histidine tags were removed from HA-Ub-His fusion proteins by digestion with the deubiquitinating enzyme UCHL3 (10 ng of UCHL3 per μg of HA-Ub-His protein; 1 h at 37 °C). His-PEX6(1–421) was expressed in *E. coli* BL21(DE3) and purified under denaturing conditions (8 M urea) by nickel-nitrilotriacetic acid chromatography (Qiagen) with refolding on the column following the manufacturer's instructions.

In vitro peroxisomal import/export assays

In vitro import/export assays of radiolabeled PEX5 proteins were performed as described previously (43). Briefly, 1–3 μl of ^{35}S-labeled PEX5 proteins were used in reactions containing 0.6–1 mg of a rat liver PNS primed for import (*i.e.* incubated for 5 min at 37 °C in the presence of 0.4 mM ATP to free DTMs of endogenous PEX5 (43)) in import buffer (20 mM MOPS-KOH, pH 7.4, 0.25 M sucrose, 50 mM KCl, 3 mM MgCl$_2$, 20 μM methionine, 2 μg/ml E-64, 2 mM reduced GSH, 10 μM bovine ubiquitin, final concentration). Reactions were incubated at 37 °C for 20 min in the presence of either 3 mM ATP or AMP-PNP. Reactions were then diluted with SEMK (20 mM MOPS-KOH, pH 7.4, 0.25 M sucrose, 1 mM EDTA-NaOH, pH 8.0, 80 mM KCl), and supernatant and organelle fractions were isolated by centrifugation at ~16,000 × *g* for 15 min at 4 °C. Following protein precipitation with 10% (w/v) TCA, both fractions were analyzed by SDS-PAGE/Western blotting/autoradiography. Where indicated, import reactions were treated with proteinase K exactly as described before (43).

For the two-step assays, radiolabeled PEX5 proteins were first imported in the presence of 3 mM AMP-PNP (first step), and organelles were isolated as above. Organelle pellets were carefully resuspended in ice-cold import buffer (which in some experiments also contained 0.5 μM BSA and 1 μM recombinant PEX5(1–324)), supplemented with either 3 mM ATP or AMP-PNP, and subjected to a second incubation (second step) for 3 min at 37 °C. Soluble and organelle fractions were isolated and analyzed by SDS-PAGE/Western blotting/autoradiography, as described above.

As required, import/export reactions were supplemented with deubiquitinating enzyme inhibitor ubiquitin-vinyl methyl ester (2 μM (36)) to detect Ub-PEX5 in supernatants; recombinant TPRs (1 μM) to inhibit the import of rat endogenous PEX5 during import of C-terminally truncated [^{35}S]PEX5 species; and HA-Ub (WT and *p*Bpa-containing mutants (10 μM) in substitution of bovine ubiquitin) for photocross-linking experiments. MTX, used to impair the export of DHFR fusion PEX5, was added to reticulocyte lysates (preincubated for 10 min at 25 °C) and to import reactions at 2 μM.

Photocross-linking and immunoprecipitation experiments

For photocross-linking experiments, import reactions of [^{35}S]PEX5(1–324;C11K) were performed in the presence of 3 mM AMP-PNP and either HA-tagged WT or mutant ubiquitins containing a photoreactive *p*Bpa residue. Following import (37 °C for 30 min), reactions were halved and put on ice. One half was exposed to UV light (30 min on ice, Blak-ray™ B-100AP 100-watt lamp, ~7-cm distance). Organelles from both halves were then isolated by centrifugation and analyzed by SDS-PAGE/Western blotting/autoradiography, as described previously.

To immunoprecipitate cross-linked products, organelle fractions of UV-irradiated import reactions were solubilized in 50 mM Tris, pH 8.0, 1 mM DTT, 1% SDS for 10 min at 65 °C and later diluted to RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, final concentration) supplemented with 1:200 (v/v) protease inhibitor mixture. The lysate was clarified by centrifugation through a cellulose acetate membrane (Corning® Costar® Spin-X® 0.22 μm) and incubated (3 h at 4 °C) with 50 μl (bed volume) of G protein–coupled Sepharose preloaded with either anti-PEX1, anti-PEX6, or control rabbit Igs. Beads were then washed three times with 1 ml of RIPA buffer and once with 1 ml of TBS (50 mM Tris, pH 7.4, 150 mM NaCl). Bound proteins were eluted with Laemmli sample buffer. Total, unbound, and 5 eq of bound protein fractions were subjected to SDS-PAGE/Western blotting and autoradiography.

PEGylation susceptibility assays

For the PEGylation assays, organelles derived from [^{35}S]PEX5-(C11K) import reactions made in the presence of 3 mM ATPγS were resuspended in import buffer containing either 3 mM

ATPγS (where export remains blocked) or a mixture of 2 mM ATP plus 1 mM ATPγS (where export can occur). Methoxy-PEG-maleimide 5,000 Da (catalog no. 63187, Sigma-Aldrich) was added at 4 mM (final concentration) either immediately before incubating at 37 °C for 3 min or after Ub-PEX5(C11K) was exported into the soluble fraction by a 10-min incubation at 37 °C. PEG-maleimide was allowed to react only for 3 min before quenching with 100 mM DTT (final concentration) for 3 min at 37 °C. Reactions were diluted with ice-cold SEMK, centrifuged to isolate organelle and supernatant fractions, and processed for SDS-PAGE/Western blotting/autoradiography.

Miscellaneous methods

^{35}S-Labeled 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.

The antibody against ABCD3 (73) was kindly provided by Dr. Wilhelm W. Just (University of Heidelberg). The anti-PEX6 antibody was raised in rabbit against human His-PEX6-(1–421). Rabbit polyclonal anti-PEX1 antibody (NBP1-80577; Novus Biologicals) was purchased. All primary antibodies were detected with goat alkaline phosphatase–conjugated anti-rabbit antibodies (A9919; Sigma-Aldrich).

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