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A PEX7-centered perspective on the peroxisomal targeting signal type 2-mediated protein import pathway.

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SUMMARY

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and transported to the organelle by shuttling receptors. Matrix proteins containing a type 1 signal are carried to the peroxisome by PEX5, whereas those harboring a type 2 signal are transported by a PEX5-PEX7 complex. The pathway followed by PEX5 during the protein transport cycle has been characterized in detail. In contrast, not much is known regarding PEX7. In this work we show that PEX7 is targeted to the peroxisome in a PEX5- and cargo-dependent manner where it becomes resistant to exogenously added proteases. Entry of PEX7 and its cargo into the peroxisome occurs upstream of the first cytosolic ATP-dependent step of the PEX5-mediated import pathway, *i.e.*, before monoubiquitination of PEX5. PEX7 passing through the peroxisome becomes partially, if not completely, exposed to the peroxisome matrix milieu suggesting that cargo release occurs at the trans side of the peroxisomal membrane. Finally, we found that export of peroxisomal PEX7 back into the cytosol requires export of PEX5 but, strikingly, the two export events are not strictly coupled indicating that the two proteins leave the peroxisome separately.

Keywords: Peroxisomes, PEX7, PEX5, PTS2-containing protein, cargo protein translocation, import pathway

INTRODUCTION

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and post-translationally targeted to the organelle via one of two peroxisomal targeting sequences (PTSs): 1) the PTS type 1 (PTS1), a small peptide frequently ending with the sequence SKL located at the C termini of the vast majority of matrix proteins (1, 2) and 2) the PTS2, a degenerated nonapeptide present at the amino termini of a few matrix proteins (3-5). In contrast to the PTS1, the PTS2 is generally cleaved when the protein reaches the organelle matrix (5-7). In mammals and many other organisms both PTS1 and PTS2 proteins are transported to the organelle by PEX5, the peroxisomal shuttling receptor (8-11). The interaction of PEX5 with PTS1 proteins is direct (12-16) whereas the interaction between PEX5 and PTS2 proteins requires the adaptor protein PEX7 (17-19). Interestingly, not all PEX5 proteins in a mammalian cell are capable of binding PEX7. This is due to alternative splicing of the *PEX5* transcript which yields two major isoforms of the receptor, PEX5S and PEX5L. In contrast to PEX5L, PEX5S is not able to bind PEX7 because it lacks an internal 37 amino acid domain (8, 10). The situation in yeasts is different. While these organisms also use PEX5 to target PTS1 proteins to the peroxisome, import of PTS2 proteins is promoted by PEX7 and a species-specific member of the so-called PEX20 family (19-23), a group of proteins that have no mammalian counterpart but that display functional similarities with the N-terminal half of PEX5L (17, 19, 24).

The pathway followed by PEX5 during the protein transport process is reasonably known (25-28). After binding a cargo protein in the cytosol, PEX5 interacts with the peroxisomal docking/translocation machinery (DTM) (29), a peroxisomal membrane protein complex comprising PEX13, PEX14 and the RING peroxins PEX2, PEX10 and PEX12 (30-32). Following this docking event PEX5 gets inserted into the DTM acquiring a transmembrane topology (33, 34), a step that results in the translocation of the cargo protein across the organelle membrane and its release into the peroxisomal lumen (35, 36). Interestingly, none of these steps require cytosolic

ATP (35-37). PEX5 is then extracted from the DTM through a two-step mechanism. First, PEX5 is monoubiquitinated at a conserved cysteine (Cys 11 in human PEX5) (38, 39); this monoubiquitinated PEX5 species is subsequently dislocated from the DTM in an ATP-dependent manner by the receptor export module (REM), a protein complex comprising the two mechanoenzymes PEX1 and PEX6 (37, 40-42). Finally, ubiquitin is removed from PEX5 probably by a combination of enzymatic and non-enzymatic mechanisms (43-45).

In contrast to all the data available for PEX5, our knowledge on the pathway followed by PEX7 during the PTS2 protein import is still incomplete. Actually, for mammalian PEX7, besides a recent report showing that the protein associates with peroxisomes and acquires a protease-protected status in a cytosolic ATP-independent manner (46), not much else is presently known. Data on PEX7 from yeasts are somewhat more abundant (reviewed in (4)). For instance, it has been suggested that yeast PEX7 interacts first with the PTS2 cargo protein and subsequently with a member of the PEX20 family; this cytosolic trimeric complex then interacts with the DTM, leading to the translocation of the cargo protein into the matrix of the organelle (47). Such pathway would suggest that PEX7 reaches the peroxisome in a cargo-dependent manner, as is in fact the case for mammalian PEX5 working in the PTS1 protein import pathway (29). Intriguingly, however, PEX7 can also be found in peroxisomes in strains lacking PEX20 and that, therefore, do not import PTS2 proteins (48).

There are also some data on the intraperoxisomal pathway followed by yeast PEX7. According to Lazarow and co-workers this protein is completely translocated across the peroxisomal membrane during its normal protein transport cycle (49). However, as stated above, these organisms use a member of the PEX20 family, and not PEX5, to transport PEX7-PTS2 cargo protein complexes to the peroxisome. This fact together with the idea that PEX20 itself may accompany PEX7 during its journey through the peroxisome matrix (48, 50) raises doubts on whether or not the yeast data can be extrapolated to the mammalian system (see also Discussion).

92 In this work we have optimized a previously established peroxisomal in vitro import system to
93 study the pathway followed by mammalian PEX7 during the PTS2 protein import cycle. We found
94 that PEX7 reaches the peroxisome in a PEX5L- and PTS2-dependent manner where it acquires a
95 protease-protected status. Acquisition of this status occurs upstream of the first cytosolic ATP-
96 dependent step, *i.e.*, before ubiquitination of PEX5L. This in vitro import system also allowed us
97 to characterize the export step of PEX7. Our results show that whenever export of PEX5L is
98 inhibited that of PEX7 is also blocked. This suggests that PEX7 exits the organelle through the
99 DTM site occupied by PEX5L. Importantly, in vitro imported PEX5L and PEX7 display different
100 export kinetics suggesting that their export is uncoupled. Finally, we provide evidence indicating
101 that PEX7 travelling through the peroxisome becomes partially, if not completely, exposed to the
102 peroxisome matrix milieu.
103

MATERIAL AND METHODS

Plasmids

The cDNA coding for the full-length human PEX7 (pGEM4-PEX7) was obtained by PCR amplification using the plasmid SC119985 (OriGene) as template and the primers 5'-GCCTCTAGAGCCACCATGAGTGCGGTGTGCGGTGGA-3' and 5'-GCGCGGTACCTCAAGCAGGAATAGTAAGAC-3'. The amplified fragment was cloned into the *Xba*I and the *Kpn*I sites of pGEM4® (Promega). A plasmid encoding PEX7 possessing a tryptophan instead of a leucine at position 70 (PEX7(L70W)) was obtained with the QuikChange® site-directed mutagenesis kit (Stratagene) using pGEM4-PEX7 as template and the primers 5'-GGAATGATGGTTGGTTTGATGTGACTTGG -3' and 5'-CCAAGTCACATCAAACCAACCATCATTCC -3'. A plasmid encoding preL4R-PEX7, a PEX7 protein possessing at its N terminus the peptide MAQRRQVVLGHLRGPADSGWMPQAAPCLSGASR was constructed as follows. Plasmid SC119985 was used as template in a PCR reaction with the primers 5'-GCCTCTAGAATGAGTGCGGTGTGCGGTGGA-3' and 5'-GCGCGGTACCTCAAGCAGGAATAGTAAGAC-3' and the obtained DNA fragment was inserted into *Xba*I/*Kpn*I-digested pGEM4® (Promega). This plasmid was then digested with *Sph*I and *Xba*I and ligated to the pre-annealed primers 5'-CCACCATGGCGCAGAGGCGGCAGGTAGTGCTGGGCCACCTGAGGGGTCCGGCCGATT CCGGCTGGATGCCGCAGGCCGCGCCTTGCCTGAGCGGTGCCT-3' and 5'-CTAGAGGCACCGCTCAGGCAAGGCGCGGCCTGCGGCATCCAGCCGGAATCGGCCGGA CCCCTCAGGTGGCCCAGCACTACCTGCCGCCTCTGCGCCATGGTGGCATG-3'. The peptide preceding PEX7 in the preL4R-PEX7 fusion protein contains amino acid residues 2-30 of human pre-thiolase in which leucine 4 was replaced by an arginine (numbering of full-length human pre-thiolase (51)). This peptide still contains the cleavage site for the matrix processing

peptidase, but the L4R mutation abolishes its PTS2 function (52). The plasmid encoding full-length human thiolase precursor (pGEM4-pre-Thiolase) was described elsewhere (35). A plasmid coding for pre-thiolase possessing the L4R mutation (pGEM4-preL4R-Thiolase) was obtained with the QuickChange® site-directed Mutagenesis Kit (Stratagene), using pGEM4-pre-Thiolase as template and the primers 5'- ATGCAGAGGCGGCAGGTAGTGCTGGG -3' and 5'- CCCAGCACTACCTGCCGCCTCTGCAT -3'. The plasmid pGEM4-PEX5L, encoding the large isoform of human PEX5, was described before (34). The plasmid encoding amino acid residues 1-324 of PEX5L possessing an alanine at position 11 (ΔC1PEX5L(C11A)) was obtained with the QuikChange® site-directed mutagenesis kit (Stratagene), using pET28-ΔC1PEX5L as template (53) and primers described elsewhere (44). The cDNA coding for histidine-tagged PEX7 was obtained by PCR amplification using the plasmid SC119985 (OriGene) as template and the primers 5'-GTATGAGCCATATGAGTGCGGTGTGCGGTGGAG-3' and 5'-GGCCGCGGAATTCTCAAGCAGGAATAGTAAGAC-3'. The amplified fragment was cloned into the *Nde*I and the *EcoR*I sites of pET-28a (Novagen). The cDNA encoding the mature form Phytanoyl-CoA hydroxylase (m-PHYH) was obtained by PCR amplification of the plasmid described in (54) using the primers 5'- GGCGCGGTACCATCAGGGACTATTTCCTCTGCC -3' and 5'- GGCGCAAGCTTTCAAAGATTGGTTCTTTCTCC -3' and cloned into the *Kpn*I and *Hind*III sites of pQE31 (Qiagen).

Recombinant Proteins

The recombinant large isoform of human PEX5 (PEX5L) (55), PEX5L containing the missense mutation N526K (PEX5L(N526K)) (56), proteins comprising amino acid residues 1-324 or 315-639 of PEX5L (ΔC1PEX5L and TPRs, respectively) and ΔC1PEX5L containing the missense mutation C11A (ΔC1PEX5L(C11A)) (53, 57), a protein comprising the first 287 amino acid residues of the small isoform of human PEX5 (ΔC1PEX5S) (35), the GST-ubiquitin fusion protein

(GST-Ub) (38), the precursor of human Phytanoyl-CoA hydroxylase (p-PHYH) and its mature form (m-PHYH) (54), human PEX19 (58) and a protein comprising the first 80 amino acid residues of human PEX14 (NDPEX14) (57), were obtained as described previously. Histidine-tagged PEX7 was expressed in the BL21(DE3) strain of *Escherichia coli* and obtained as inclusion bodies. The fusion protein was purified by HIS-Select™ nickel affinity gel (Sigma) under denaturing conditions (6 M guanidine hydrochloride) and concentrated by trichloroacetic acid precipitation.

In Vitro Import/Export Reactions

Liver post-nuclear supernatants (PNS) from rat or PEX7 knockout mouse were prepared as described before (34). In a typical import reaction (100 µl final volume), ^{35}S-labeled proteins (1-2 µl of the rabbit reticulocyte lysates; see below) were diluted to 10 µl with 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 *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, 2 mM reduced glutathione, final concentrations) and added to 500 µg of liver PNS that had been primed for import (5 min incubation at 37 ºC in import buffer containing 0.3 mM ATP; see (35, 37) for details). Reactions were incubated for 30 min at 37 °C, unless otherwise stated. ATP or AMP-PNP were used at 3 mM, final concentration. NTP depletion from both PNS and reticulocyte lysates using apyrase (Grade VII, Sigma) was done exactly as described (36). Where indicated, import reactions were supplemented with recombinant PEX5 proteins (PEX5L, PEX5L(N526K), ΔC1PEX5L, ΔC1PEX5L(C11A) or ΔC1PEX5S; 30 nM final concentrations), GST-Ub or bovine ubiquitin (10 µM), and recombinant p-PHYH or m-PHYH (140 nM, final concentration). After import, reactions were treated with pronase (500 µg/ml final concentration) for 45 min on ice and processed for SDS-PAGE/autoradiography exactly as described before (35). In some experiments, organelles

were resuspended in import buffer and subjected to pronase digestion in the presence or absence of 1% (w/v) Triton X-100.

In the in vitro export assays, radiolabeled proteins were first subjected to an import assay for 15 min. Further import was then stopped either by adding recombinant NDPEX14 to the reaction (30 µM final concentration), or by isolating the organelles by centrifugation and resuspending them in import buffer. In earlier experiments, cytosolic proteins derived from 500 µg of liver PNS were also added. The organelle suspensions were then incubated at 37 ºC in the presence of either 5 mM ATP or AMP-PNP.

For the PTS2-only in vitro import/export experiments, PNS were pre-incubated with 1 µM recombinant TPRs for 10 min on ice, before starting the import assays. This recombinant protein, corresponding to the C-terminal half of PEX5, comprises its PTS1-binding domain and is used here to sequester endogenous PTS1-containing proteins (13, 29, 56). Also, the reticulocyte lysates containing ^{35}S-PEX7 and ^{35}S-PEX5L (2 µl each) were pre-incubated with recombinant p-PHYH (20 min at 23 ºC in 10 µl of import buffer) to favor formation of the trimeric PEX5L-PEX7-PTS2 complex. The export incubation was carried out as described above, but in the presence of 1 µM TPRs and 10 µM NDPEX14.

Subcellular fractionation

Pronase-treated organelles from an import reaction or rat liver purified peroxisomes were resuspended in 20 mM MOPS-KOH, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 2 mM DTT, 0.1 mg/ml phenylmethanesulfonylfluoride, 1:500 (v/v) mammalian protease inhibitors mixture (Sigma) and disrupted by sonication using a SONOPULS HD2200-BANDELIN equipped with a MS 73 microtip. The sonication conditions used (40% duty cycle, 10% output power for just 25 s) were established as the mildest ones resulting in a quantitative extraction of catalase from

peroxisomes. Membrane and matrix components were separated by centrifugation at 100,000 g for 60 min.

Miscellaneous

All ^{35}S-labeled proteins were synthesized using the TNT® T7 quick coupled transcription/translation kit (Promega) in the presence of [^{35}S]methionine (specific activity >1000 Ci/mmol; PerkinElmer Life Sciences). Although no attempts were made to quantify the amounts of radiolabeled proteins in our reactions, note that, according to the manufacturer, 1 µl of reticulocyte lysate typically produces 2-6 ng of radiolabeled protein. For ^{35}S-PEX7 this corresponds to a final concentration of 0.6-3.4 nM in the import assays. An antibody directed to human PEX7 was produced in rabbits using recombinant histidine-tagged PEX7. The antibody directed to PEX13 was described elsewhere (59) and the one against catalase was purchased from Research Diagnostics, Inc. (catalogue number RDI-CATALASEabr). All antibodies were detected using goat alkaline phosphatase-conjugated anti-rabbit antibodies (A9919; Sigma).

Densitometric analyses of autoradiography films were performed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011).

RESULTS

PEX7 reaches the peroxisome in a PEX5L- and PTS2-dependent manner. We have previously described an improved in vitro system to characterize the peroxisomal import mechanism of pre-thiolase, a PTS2-containing protein (35). The system relies on a rat liver post-nuclear supernatant as a source of peroxisomes and cytosolic components, supplemented with either recombinant ΔC1PEX5L (amino acid residues 1-324 of PEX5L) or PEX5L(N526K) (PEX5L possessing a lysine at position 526 instead of an asparagine; (56, 60)). These two PEX5 proteins contain an intact PEX7-binding domain as well as all the other elements required for a productive interaction with the peroxisomal protein import machinery, and thus they are still competent in the PTS2-mediated import pathway. However, they do not bind efficiently PTS1 proteins (8, 60, 61), an advantage when studying the PTS2-mediated import pathway (see below). In this work we used this improved system to analyze PEX7.

Figure 1A shows the results of in vitro import assays performed with both ^{35}S-labeled PEX7 and pre-thiolase. In the absence of ΔC1PEX5L, or in the presence of ΔC1PEX5S (a protein almost identical to ΔC1PEX5L that lacks the PEX7-binding domain; see Introduction), only a small fraction of protease-protected (imported) thiolase was observed in organelle pellets, as expected (35), and the same is true for ^{35}S-PEX7 (lanes 1 and 3). A 5-fold increase in the amounts of both radiolabeled proteins was observed when the import assays were supplemented with either recombinant ΔC1PEX5L or PEX5L(N526K) (lanes 2 and 5). Recombinant PEX5L also improves the import efficiencies of both pre-thiolase and PEX7 but only by a factor of ≈2.5 (compare lanes 1 and 4). The weaker stimulatory effect obtained with PEX5L is probably due to the fact that this protein also interacts with endogenous PTS1-containing proteins present in the PNS, creating a competition problem at the peroxisomal DTM (see also (35)). Importantly, the in vitro import yields of ^{35}S-PEX7 obtained in the presence of ΔC1PEX5L can be further improved by a factor of 2 when a recombinant PTS2 protein, pre-phytanoyl-CoA 2-hydroxylase (p-PHYH), is added to the

assay (Fig. 1B, compare lanes 3 and 4). The stimulatory effect of p-PHYH on PEX7 import contrasts with its inhibitory effect on pre-thiolase import (Fig. 1B, compare lanes 3 and 4). Recombinant phytanoyl-CoA 2-hydroxylase lacking the PTS2 (m-PHYH) has no such effects (Fig. 1C, compare lanes 2 and 3). These findings strongly indicate that the ^{35}S-PEX7 protein used in these experiments is truly functioning in the PTS2-mediated protein import pathway. Further data corroborating this conclusion were obtained when a PNS from PEX7 knockout mice (62) was used in import assays. As shown in Fig. 1D (lane 1), an assay using PNS from these mice supplemented with ΔC1PEX5L (and 2 µl of a mock-translated reticulocyte lysate) failed to reveal import of pre-thiolase. In contrast, addition of just 2 µl of the lysate containing ^{35}S-PEX7 was sufficient to promote import and partial processing of pre-thiolase (Fig. 1D, lane 2). A non-functional PEX7 protein harboring a mutation previously described in a patient with Rhizomelic Chondrodysplasia Punctata Type 1 (PEX7(L70W); (63)), was not competent in this assay, as expected (Fig. 1D, lane 3). Note that PEX7 alone is readily degraded by the protease used in these assays (Fig. 1E, lane 2) and that the resistance it acquires during in vitro import vanishes in the presence Triton X-100, a mild detergent that solubilizes biological membranes (Fig. 1F, lane 2). Taken together, the experiments described above strongly indicate that in vitro synthesized PEX7 reaches the peroxisome in a PEX5L- and PTS2-dependent manner where it acquires a protease-protected status.

The energetics of PEX7 import. We have previously shown that: 1) PEX5L becomes inserted into the DTM in a cytosolic ATP-independent process (37, 40, 64) and 2) translocation of pre-thiolase across the DTM into the peroxisomal matrix occurs upstream of the first cytosolic ATP-dependent step, *i.e.*, before monoubiquitination of PEX5L (35). Not surprisingly, we found that the energetic requirements of PEX7 import are identical, as was in fact also reported by others (46). As shown in Fig. 2A neither supplementation of import reactions with AMP-PNP (a non-hydrolyzable ATP analog; (65)), nor pre-treatment of the ^{35}S-PEX7 protein and PNS with apyrase

(an enzyme that hydrolyzes ATP and other NTPs; (66)) blocked PEX7 import (compare lane 1 with lanes 2 and 3, respectively).

Interestingly, although export of peroxisomal PEX7 is ATP-dependent (as it will be shown below), the levels of peroxisomal PEX7 observed under the different energetic conditions are identical. This finding suggests that export of PEX7 from the peroxisome becomes a rate-limiting step in this optimized in vitro import system.

The data in Fig. 2A showing that PEX7 import is not blocked in assays containing apyrase, a condition previously shown to block PEX5L monoubiquitination (35, 36), also suggests that import of PEX7, like import of pre-thiolase, occurs upstream of PEX5L monoubiquitination. Additional data supporting this conclusion are presented in Fig. 2B. Identical amounts of protease-protected ^{35}S-PEX7 and thiolase were obtained in import reactions supplemented with either ΔC1PEX5L or ΔC1PEX5L(C11A), a mutant protein that possesses an alanine at position 11. The substitution of cysteine 11 by an alanine results in a PEX5 protein that can still enter the DTM but that is no longer monoubiquitinated (44).

The N terminus of peroxisomal PEX7 is exposed into the organelle matrix. The fact that peroxisomal ^{35}S-PEX7 is resistant to exogenously added proteases suggests that PEX7 exposes no major domains into the cytosol but provides no clues on how deep in peroxisomes it reaches. To address this issue we adapted a strategy previously used by others to show that a portion of the polypeptide chain of peroxisomal PEX5L reaches the peroxisomal matrix (52). Specifically, we synthesized a PEX7 protein having at its N terminus a cleavable, but otherwise non-functional, mutant version of thiolase pre-sequence and asked whether this PEX7 protein (hereafter referred to as preL4R-PEX7) could be cleaved in our in vitro import assays. A control experiment with a pre-thiolase carrying the same mutation (L4R) confirmed that this mutant pre-sequence is not functional in our in vitro assays (Fig. 3A). As shown in Fig. 3B, preL4R-PEX7 subjected to in vitro import assays not only acquired a protease-resistant status in a PEX5L- and PTS2-dependent

manner but was also converted into a 2-3 kDa shorter protein. Furthermore, preL4R-PEX7, like PEX7, is also able to restore import of pre-thiolase in PNS from the PEX7 knockout mice (Fig. 3C). Processing of preL4R-PEX7 requires its passage through the peroxisome because nearly no processed PEX7 could be detected when the import assays were performed in the presence of NDPEX14, a recombinant protein comprising the PEX5-binding domain of PEX14 ((67); Fig. 3D, compare lanes 1 and 5 with lanes 3 and 7, respectively). As shown before, this recombinant protein completely blocks the PEX5-mediated protein import pathway (36). Interestingly, when the protease treatment was omitted, cleaved PEX7 was also detected in the supernatant of the import assays but only under import-permissive conditions (Fig. 3D, compare lanes 2 and 4) suggesting that our in vitro system can also be used to study PEX7 export. Finally, and in agreement with the data shown in Fig. 2B, cleavage of preL4R-PEX7 was also observed when its import was promoted by ΔC1PEX5L(C11A) (Fig. 3E). Interestingly, when this export-incompetent PEX5L species is used in these assays, almost no cleaved PEX7 is recovered in the supernatant fraction (Fig. 3E, compare lanes 3 and 4) suggesting that export of cleaved PEX7 is somehow dependent on PEX5L ubiquitination/export (see also below). In summary, these results indicate that at least the N terminus of PEX7 reaches a location where it can be cleaved by the protease that processes PTS2 proteins, *i.e.*, the peroxisomal matrix.

Export of PEX7 from the peroxisome requires export of PEX5L, but the two events are not strictly coupled. PEX7 functions as a shuttling receptor, meaning that peroxisomal PEX7 is eventually exported back to the cytosol (4). Aiming at characterizing in detail this process we developed a two-step protocol in which ^{35}S-PEX7 is first subjected to an import assay, and after blocking further import (see Material and Methods for details), the organelle suspension is then subjected to a second incubation step, the export assay. The results of one these assays performed under standard conditions show that the amount of organelle-associated protease-protected ^{35}S-PEX7 decreases over time with the concomitant appearance of ^{35}S-PEX7 in the supernatant (Fig.

4A). Interestingly, experimental conditions that inhibit export of peroxisomal PEX5L back into the cytosol, also block export of PEX7. As shown in Fig. 4B (*top*) almost no export of PEX7 was detected in assays made in the presence AMP-PNP (see also Fig. 4C). This non-hydrolyzable ATP analogue still allows PEX5L monoubiquitination at the DTM but blocks the receptor export module (45). A similar inhibition was observed when both the import and export incubations were made in the presence of a GST-ubiquitin fusion protein (Fig. 4B, *middle* and Fig. 4C). As shown before, ubiquitination of DTM-embedded PEX5L with this ubiquitin analogue results in a species that is no longer export-competent (38). Note that we have been unable to detect any ubiquitination of PEX7 in our in vitro assays (even under non-reducing conditions; data not shown) suggesting that the effect of GST-Ub on PEX7 export occurs via PEX5L. In agreement with this interpretation, and with the data shown in Fig. 3E, when ^{35}S-PEX7 was imported in the presence of ΔC1PEX5L(C11A) no significant export of ^{35}S-PEX7 was detected (Fig. 4B, *bottom* and Fig. 4C). Thus, peroxisomal PEX7 is exported back into the cytosol only when PEX5L is also exported.

Several hypotheses could explain this phenomenon. An obvious one would be to assume that export of PEX7 is coupled to that of PEX5L. Alternatively, it might be that PEX5L arrested at the DTM simply blocks the site used by PEX7 to exit the organelle. In an attempt to clarify this issue we determined the export kinetics of both proteins. Obviously, such an experiment would only be informative if we could find conditions where PEX5L reaches the peroxisome in a PTS2-only mode. With this in mind, we performed in vitro assays in the presence of a recombinant protein comprising the PTS1-binding domain of PEX5 (referred to as TPRs), a strategy previously shown to efficiently block the PTS1-dependent targeting of PEX5L to the peroxisome (29, 56), and asked whether peroxisomal targeting of PEX5L could be recovered by adding ^{35}S-PEX7 and recombinant p-PHYH to the import assays. As shown in Fig. 5A this strategy turned out to be feasible. Using these experimental conditions we then employed the two-step import-export protocol described above to compare the export kinetics of ^{35}S-PEX7 and ^{35}S-PEX5L. Briefly,

after an import step performed in presence of AMP-PNP, the organelles were isolated by centrifugation, resuspended in import buffer and subjected to an export assay. Aliquots were then withdrawn at various time points, and protease-treated organelles were analyzed by SDS-PAGE/autoradiography. As shown in Fig. 5B, two populations of ^{35}S-PEX5L displaying different protease susceptibilities were detected in this experiment, as expected (34, 38, 64). The most abundant at time zero of the export step is the so-called stage 3 PEX5L, a DTM-embedded monoubiquitinated species that leaves the peroxisome very rapidly in the presence of ATP (Fig. 5B, compare lanes 0′ and 2′; see also (38, 64) and legend to Fig. 5B for additional details regarding the properties of peroxisomal PEX5L). The other population is stage 2 PEX5L (the precursor of stage 3 PEX5L), a non-ubiquitinated species that is cleaved at its N terminus by the protease used in these assays yielding a 2-kDa shorter protein. Due to the fact that the buffer used in the export step lacked ubiquitin and components of the ubiquitin-conjugating cascade, the majority of stage 2 PEX5L was not converted into stage 3 PEX5L and therefore remained in the organelles. Densitometric analyses of autoradiographs revealed that about 70% of total peroxisomal ^{35}S-PEX5L left the organelle in the first 2 min of the export incubation (Fig. 5B; lower panel). Importantly, the export kinetics of ^{35}S-PEX7 is considerably slower, a difference particularly evident at the 2-min time point of the export assay. Apparently, when PEX5L is exported from the peroxisome it leaves behind a fraction of PEX7, a finding strongly suggesting that export of the two proteins is not coupled. In summary, the data in Fig. 4 and 5 suggest that at least a fraction of PEX7 and PEX5L leave the peroxisome separately but through the same site; the finding that no peroxisomal PEX7 is exported whenever PEX5L is arrested at the DTM suggests that DTM-embedded PEX5L behaves as a plug blocking the release of peroxisomal PEX7 into the cytosol (see also Discussion).

Peroxisomal PEX5L engaged in the PTS2 import pathway remains tightly bound to the organelle membrane. All the presently available data suggest that PEX5L shuttles between the

cytosol and the peroxisomal DTM where it acquires a transmembrane topology, without ever entering completely into the organelle matrix (29, 34, 37, 38, 68). However, it is important to note all those data were obtained with experimental systems in which PEX5L is mostly involved in the PTS1-mediated protein import pathway. Considering a previously proposed hypothesis that PEX20, the yeast functional counterpart of PEX5L, may enter completely into the organelle matrix together with PEX7 (50), it might be possible that mammalian PEX5L functioning in the PTS2 import pathway also follows a similar route. To address this possibility we used the PTS2-dependent import assay described above and tried to determine whether ^{35}S-PEX5L co-fractionates with either membrane or matrix peroxisomal proteins. Briefly, protease-treated organelles from ATP- or AMP-PNP-supplemented import assays were disrupted by sonication and subjected to ultracentrifugation to separate membrane from soluble proteins. The efficiency of this procedure was assessed by monitoring the behavior of catalase, a peroxisomal matrix protein (69, 70) and PEX13, an intrinsic peroxisomal membrane protein and a component of the DTM (71). As shown in Fig. 6A, ^{35}S-PEX5L quantitatively co-fractionated with the membrane marker PEX13. This result strongly indicates that, similarly to the situation in the PTS1-mediated import pathway, peroxisomal PEX5L engaged in the PTS2 protein import pathway remains tightly bound to the peroxisomal membrane. A different behavior was observed for PEX7. Indeed, although a major fraction of ^{35}S-PEX7 was found in the membrane pellet some protein was also detected in the soluble fraction. A similar distribution was observed for endogenous rat liver PEX7 present in highly pure peroxisome preparations (Fig. 6B). The detection of a soluble population of PEX7 in these experiments could well support the idea that PEX7 is completely released into the matrix of the organelle during the PTS2 import pathway, although further data are necessary to corroborate this possibility (see also Discussion).

DISCUSSION

In this work we show that mammalian PEX7 is targeted to the peroxisome in a PEX5L- and PTS2-dependent manner where it acquires resistance to exogenously added proteases. Importantly, both PEX7 and pre-thiolase, a PTS2 protein, reach this protease-protected location in a cytosolic ATP-independent manner ((35, 46), and this work), implying that the PEX7-PTS2 protein complex enters the peroxisome upstream of the first ATP-dependent step of the PEX5L-mediated protein import pathway, *i.e.*, prior to monoubiquitination of DTM-embedded PEX5L. Additional data presented in this work corroborate this conclusion. As shown in Fig. 2B and 3E, a mutant version of PEX5L that cannot be monoubiquitinated at the DTM is as functional as the normal protein in promoting peroxisomal import of both PEX7 and pre-thiolase. Clearly, the PEX5L-mediated entry of both PEX7 and its cargo into the peroxisome is not linked to monoubiquitination of PEX5L at the DTM. Interestingly, this conclusion is in contrast to the so-called "export-driven import model", a hypothetical mechanism recently proposed for the yeast PEX18/PEX7 system (72, 73). According to this idea, monoubiquitination/export of PEX18, a member of the PEX20 family and a functional counterpart of PEX5L in the PTS2 protein import pathway, is mechanically linked to the translocation of PEX7, and presumably its cargo, across the peroxisomal membrane. Seemingly, the different architectures of the PTS2 protein import machineries in these organisms translate into at least some significant mechanistic differences.

One of the aims of this work was to characterize the intraperoxisomal pathway followed by mammalian PEX7 during the PTS2 protein transport cycle. Up till now, there was only one study addressing this problem in a systematic manner. This is a work by Lazarow and colleagues describing the properties of a yeast PEX7-green-fluorescent-protein (GFP) fusion protein, a protein that although unable to complement the phenotype of a ΔPEX7 strain, accumulates massively in the peroxisomal matrix (49). As shown by those authors, cleavage of the fusion protein at the PEX7-GFP junction yielded a PEX7 protein that could now exit the organelle and rescue the

phenotype of the ΔPEX7 strain. Apparently, there is a way out of the peroxisome for a PEX7 protein that was artificially accumulated in the matrix of the organelle. Based on those findings it was proposed that PEX7 follows an "extended cycling mechanism", *i.e.*, that PEX7 enters completely into the peroxisome matrix during the PTS2 protein transport cycle (49). The results described here for preL4R-PEX7 strongly suggest that at least the N terminus of mammalian PEX7 enters sufficiently deep into the peroxisome matrix milieu to become accessible to the peroxisomal protease that cleaves the engineered pre-sequence. Furthermore, fractionation of organelles by sonication did reveal the existence of a PEX7 pool displaying the properties expected for a peroxisomal matrix protein. Thus, the data presented here for mammalian PEX7 are surely compatible with the "extended cycling mechanism" proposed by Lazarow and colleagues (see Fig. 7, pathway A). However, we must note that proteins weakly associated with a biological membrane may also be extracted into the soluble fraction by sonication. Therefore, we cannot formally exclude a scenario in which PEX7, like PEX5L, is retained at the DTM during all the steps occurring at the peroxisome, exiting the DTM only after PEX5L export (Fig. 7, pathway B). It is important to note that this second possibility would still be compatible with the data on yeast PEX7. Indeed, if we assumed that yeast PEX20 family members are retained at the DTM during their passage through the peroxisome exposing their PEX7-binding domain into the organelle matrix, as it is likely the case for mammalian PEX5L, then it would be also reasonable to assume that any functional PEX7 generated de novo in the peroxisomal matrix could interact with a DTM-embedded PEX20 protein, thus returning to its normal pathway.

Many important aspects of the PTS2-mediated protein import pathway remain unclear. One directly related to this work regards the molecular details of PEX7 export. Our data suggest that PEX7 leaves the peroxisomal compartment through the DTM site occupied by PEX5L and that peroxisomal PEX5L and PEX7 probably exit the organelle separately. However, the implications of these findings on the molecular mechanism of PEX7 export are largely dependent on whether or

not PEX7 enters completely into the organelle matrix. In pathway A (Fig. 7), the DTM would have the capacity to interact with a matrix PEX7 protein and somehow promote its export in a PEX5L-independent manner, while retaining all resident peroxisomal proteins in the matrix of the organelle. Pathway B, on the other hand, obviates the need for such a selectivity filter at the matrix side of the DTM and suggests that the ATP-dependent extraction of PEX5L from the DTM could also be coupled to the disruption of the interaction between PEX5L and PEX7, thus preparing PEX7 for a new PTS2 recognition event. Regardless of the pathway followed by PEX7, it is clear from our data that its export from the peroxisome requires PEX5-free DTMs, and therefore the action of the mechanoenzymes PEX1 and PEX6. Thus, these ATP-dependent enzymes surely influence PEX7 export but whether this functional connection is merely indirect (i.e., via PEX5 export) or direct remains to be determined.

Another issue that warrants future studies regards the protein transport capacity of PEX5L. Can a single molecule of PEX5L simultaneously transport a PTS1 and a PTS2 protein to the peroxisome, or are these mutually exclusive events? Clearly, further work is necessary to understand these complex details of the peroxisomal protein import machinery.

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686 **FIGURE LEGENDS**

687 **Fig. 1- ^{35}S-PEX7 acquires a protease-protected and organelle-associated status in in vitro**
688 **import reactions in a PEX5L- and PTS2-dependent manner.**

689 **A-C,** In vitro import assays of ^{35}S-PEX7 and ^{35}S-pre-thiolase in the absence or presence of the
690 indicated recombinant proteins. PEX5L(N526K) is indicated by "PEX5NK". Lanes I, 10% (A and
691 B) or 5% (C) of the reticulocyte lysates containing ^{35}S-PEX7 and ^{35}S-pre-thiolase used in each
692 reaction. Lane I in C, 5% of the reticulocyte lysates containing ^{35}S-PEX7 and ^{35}S-pre-thiolase used
693 in each reaction. The asterisk in A and B marks a radiolabeled band occasionally produced by the
694 in vitro translation kit in an unspecific manner. **D**, PEX7, but not PEX7(L70W), promotes import
695 of ^{35}S-pre-thiolase to peroxisomes from PEX7 knockout mice. PNS from PEX7 knockout mouse
696 liver was used in import assays with ^{35}S-pre-thiolase in the presence of a mock-translated
697 reticulocyte lysate (lane 1) or lysates containing ^{35}S-PEX7 (lane 2) or ^{35}S-PEX7(L70W) (lane 3).
698 Lanes I_1, I_2 and I_3, 5% of the reticulocyte lysates containing ^{35}S-pre-thiolase, ^{35}S-PEX7 and ^{35}S-
699 PEX7(L70W) used in the reactions, respectively. pre-Thiol and m-Thiol, precursor and mature
700 forms of thiolase, respectively. **E**, Soluble ^{35}S-PEX7 is completely susceptible to pronase in the
701 absence of Triton X-100. **F**, Organelles from an import assay made in the presence of recombinant
702 ΔC1PEX5L and p-PHYH were isolated by centrifugation, resuspended in import buffer and
703 subjected to pronase digestion in the absence (lane 1) or presence (lane 2) of 1% (w/v) Triton X-
704 100. Lane I, 5% of the reticulocyte lysate containing ^{35}S-PEX7. In A-D and F, pronase-treated
705 organelles were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane.
706 Autoradiographs (upper panels) and the corresponding Ponceau S-stained membranes (lower
707 panels) are shown.
708
709

Fig. 2- The energetics of PEX7 import.

A, A primed rat liver PNS fraction (see Material and Methods) was incubated with ^{35}S-PEX7 for 7 min in import buffer containing ΔC1PEX5L and p-PHYH in the presence of either ATP (lane 1), or AMP-PNP (lane 2). An identical assay but using apyrase-treated PNS and ^{35}S-PEX7 was also performed (lane 3). Lanes I_1 and I_2, 5% of the reticulocyte lysates containing ^{35}S-PEX7 used in lanes 1 and 2 (- apyrase), and lane 3 (+ apyrase), respectively. **B**, A non-monoubiquitinatable form of PEX5 (ΔC1PEX5L(C11A)) is as efficient as ΔC1PEX5L in targeting PEX7 and pre-thiolase to the peroxisome. Import assays with ^{35}S-PEX7 and ^{35}S-pre-thiolase were made in import buffer containing ATP and GST-Ub, in the absence (lane 1) or presence of recombinant ΔC1PEX5L (lane 2) or ΔC1PEX5L(C11A) (lane 3). Note that ubiquitination of ΔC1PEX5L with GST-Ub results in a species that is no longer export-competent (38, 53). Lane I, 5% of the reticulocyte lysates containing ^{35}S-PEX7 and ^{35}S-pre-thiolase were mixed and loaded together in the same lane. Pronase-treated organelles were analyzed as described in Fig. 1. Autoradiographs (upper panels) and the corresponding Ponceau S-stained membranes (lower panels) are shown.

Fig. 3- PEX7 becomes transiently exposed to the organelle matrix during the PTS2-mediated protein import pathway.

A, ^{35}S-pre-thiolase containing an arginine instead of a leucine at position 4 (preL4R-Thiol; lane 2), in contrast to ^{35}S-pre-thiolase (pre-Thiol; lane 1), is not imported in vitro. Lanes I_1, I_2 and I_3, 5% of the reticulocyte lysates containing ^{35}S-PEX7, ^{35}S-pre-thiolase and ^{35}S-preL4R-thiolase, respectively. **B**, ^{35}S-preL4R-PEX7 was subjected to import assays in the absence (lane 1) or presence of the indicated recombinant proteins (lanes 2-4). Pronase-treated organelles were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. clv-PEX7, cleaved ^{35}S-preL4R-PEX7. **C**, ^{35}S-preL4R-PEX7 promotes import of ^{35}S-pre-thiolase to peroxisomes from PEX7 knockout mice. PNS from PEX7 knockout mice was used in import assays with ^{35}S-pre-

thiolase in the presence of either a mock-translated reticulocyte lysate (lane 1) or a lysate containing ^{35}S-preL4R-PEX7 (lane 2). Import and processing of ^{35}S-pre-thiolase is best seen in import assays using unlabeled/cold preL4R-PEX7 (lane 3) due to the fact that mature thiolase co-migrates with uncleaved preL4R-PEX7 (lane 2, asterisk). Lanes I_1 and I_2, 5% of the reticulocyte lysates containing ^{35}S-preL4R-PEX7 and ^{35}S-pre-thiolase used. **D**, Control experiments showing that processing of ^{35}S-preL4R-PEX7 in import assays occurs only under import-permissive conditions. ^{35}S-preL4R-PEX7 was subjected to import assays in the presence of the indicated recombinant proteins. At the end of the incubation the samples were halved and treated or not with pronase, as indicated. The import reactions were then centrifuged to obtain organelle pellets (P) and supernatants (S). Total pellets (derived from 500 µg of PNS) and ¼ of the corresponding supernatants were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. The asterisk indicates a soluble minor preL4R-PEX7-derived fragment displaying some resistance to pronase. PEX19, a protein involved in another aspect of peroxisome biogenesis (74), was used in these assays as a negative control for NDPEX14. **E**, Import assays with ^{35}S-preL4R-PEX7 were performed in the presence of ΔC1PEX5L (lanes 1 and 3) or ΔC1PEX5L(C11A) (lanes 2 and 4). Pronase-treated organelles (lanes P) and untreated supernatants (lanes S) were analyzed as in D. Autoradiographs (upper panels) and the Ponceau S-stained membranes (lower panels) are shown. Lanes I, 5% of the reticulocyte lysate containing ^{35}S-preL4R-PEX7 used in each reaction.

Fig. 4- PEX7 is exported back to the cytosol in a PEX5L export-dependent manner.

A, ^{35}S-PEX7 was imported for 15 min in the presence of p-PHYH, ΔC1PEX5L, ubiquitin and ATP. The reaction mix was then diluted with ice-cold import buffer and the organelles were isolated by centrifugation and subjected to an export assay in the presence of ATP (see Material and Methods for details). Aliquots were collected at the indicated time points, and one half was treated with pronase while the other was left untreated. Equivalent amounts of organelles from the

pronase-treated aliquots and supernatants from the untreated aliquots (derived from 125 µg of PNS) were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. **B**, PEX7 export assays. In "standard reactions", the ΔC1PEX5L- and p-PHYH-mediated import of ^{35}S-PEX7 was allowed to occur at 37 ºC for 15 min in the presence of ubiquitin and ATP. At this point, import was inhibited by the addition of NDPEX14 (30 µM) and the reaction further incubated. Aliquots were taken at the indicated time points. Pronase-treated organelles were subjected to SDS-PAGE analysis and blotted onto a nitrocellulose membrane. PEX7 export was inhibited when ATP was replaced by AMP-PNP (*top*). Likewise, replacing ubiquitin by GST-Ub in the import step inhibits subsequent export of PEX7 (*middle*). The same inhibition was observed when recombinant ΔC1PEX5L was replaced by ΔC1PEX5L(C11A) (*bottom*). Lanes I, 5% of the reticulocyte lysates containing ^{35}S-PEX7. Autoradiograph (upper panels) and the corresponding Ponceau S-stained membrane (lower panels) are shown. **C**, The bar graph shows the average percentage of PEX7 export after 20 min under the conditions described in B. Standard deviations ($n \geq 3$) are also presented.

Fig. 5- Peroxisomal PEX5L and PEX7 display different export kinetics.

A, Targeting of PEX5L to the peroxisome in a PTS2-only in vitro import system. A reticulocyte lysate containing ^{35}S-PEX5L was pre-incubated with either a mock-translated lysate (lane 1) or a lysate containing ^{35}S-PEX7 plus 0.5 µg of p-PHYH (lane 2). Each mixture was then subjected to import assays using PNS supplemented with ATP and 1 µM recombinant TPRs, the PTS1-binding domain of PEX5. After pronase treatment, organelles were subjected to SDS-PAGE analysis and blotted onto a nitrocellulose membrane. Lanes I_1 and I_2, 5% of the reticulocyte lysates containing ^{35}S-PEX7 and ^{35}S-PEX5L used in the assays, respectively. **B**, A mixture of ^{35}S-PEX7 and ^{35}S-PEX5L pre-incubated with recombinant p-PHYH was subjected to a 15 min import assay using TPRs-treated PNS in the presence of AMP-PNP. The reaction was diluted with ice-cold import

buffer, and the organelles were isolated by centrifugation, resuspended in import buffer and subjected to an export assay in the presence of ATP, TPRs and NDPEX14. Aliquots were collected at the indicated time points. Pronase-treated organelles were analyzed as in A. Lanes I_1 and I_2, 2% of the reticulocyte lysates containing ^{35}S-PEX7 and ^{35}S-PEX5L used in the assays, respectively. The bar graph shows averages and standard deviations (n=3) of the amounts of peroxisomal ^{35}S-PEX7, stage 2 ^{35}S-PEX5L (PEX5 stg2) and stage 3 ^{35}S-PEX5L (PEX5 stg3) at each time point. Stage 2 and stage 3 PEX5 are two DTM-embedded transmembrane PEX5 populations (38, 44). Stage 2 PEX5 is converted into stage 3 PEX5 by monoubiquitination at its cysteine 11. The two populations display different susceptibility to proteases: stage 2 PEX5 is cleaved near the N terminus yielding a 2-kDa shorter protein, whereas stage 3 PEX5 is completely resistant because the N-terminal domain is protected by the covalently attached ubiquitin moiety. Note that stage 3 PEX5L runs exactly as unmodified full-length PEX5L upon SDS-PAGE under reducing conditions because the PEX5-ubiquitin thiolester linkage is destroyed by DTT. The open arrow head indicates an export-incompetent N-terminally truncated PEX5L species produced in the in vitro transcription/translation reactions (see also (56)). This species also serves as an internal negative control in the export assay.

Fig. 6- Peroxisomal PEX5L remains tightly bound to the peroxisomal membrane while a fraction of PEX7 behaves as a matrix protein.

A, A mixture of ^{35}S-PEX7 and ^{35}S-PEX5L pre-incubated with p-PHYH was subjected to an import assay using TPR-treated PNS in the presence of ATP (left panel) or AMP-PNP (right panel), as indicated. After pronase treatment, organelles were disrupted by sonication. Half of the suspension was left on ice (lanes T) while the other half was subjected to ultracentrifugation to obtain membrane (P) and soluble (S) fractions. Samples were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. After autoradiography to detect ^{35}S-PEX7 and ^{35}S-PEX5L, the

membrane was probed with antibodies against Catalase (α-CATALASE) and PEX13 (α-PEX13). PEX5 stg2 and PEX5 stg3, stage 2 and stage 3 ^{35}S-PEX5L, respectively. Note that PEX13 is converted into a 28-30 kDa fragments after protease treatment (33). **B**, An identical sonication experiment was done using rat liver purified peroxisomes. The nitrocellulose membrane was also probed with antibodies against PEX7 (α-PEX7).

Fig. 7- Working model for the PEX5L-PEX7-mediated import pathway.

After its assembly in the cytosol, the trimeric PEX5L-PEX7-PTS2 protein complex docks at the docking/translocation machinery (DTM) [arrow 1]. This receptor-cargo complex then becomes inserted into the DTM [arrow 2]. This step culminates with the PTS2 cargo protein being delivered to the organelle matrix (where the PTS2 is cleaved) and PEX5L displaying a transmembrane topology (*i.e.*, stage 2 PEX5L). At this stage, PEX7 is completely protected from exogenous proteases exposing at least its N terminus to the peroxisome matrix. PEX7 may be completely released from the DTM into the matrix milieu (pathway A) or may be retained at the DTM until the export step (pathway B). Following insertion into the DTM, PEX5L is monoubiquitinated at the conserved cysteine 11 residue [arrow 3], yielding stage 3 PEX5L. Monoubiquitination of PEX5L allows its ATP-dependent extraction from the DTM [arrow 4], and the subsequent export of PEX7 [arrow 5]. After deubiquitination of PEX5L [arrow 6], the protein transport cycle restarts.