A Cargo-centered Perspective on the PEX5 Receptor-mediated Peroxisomal Protein Import Pathway*

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Tânia Francisco, Tony A. Rodrigues, Marta O. Freitas, Cláudia P. Grou, Andreia F. Carvalho, Clara Sá-Miranda, Manuel P. Pinto, and Jorge E. Azevedo

From the Organelle Biogenesis and Function Group and the Lysosome and Peroxisome Biology Unit, Instituto de Biologia Celular e Molecular (IBMC), Universidade do Porto, R. do Campo Alegre, 823, 4150-180 Porto and the Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, R. de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

Background: How the soluble receptor PEX5 delivers its cargoes to the peroxisome remains largely unknown.

Results: Cargo translocation occurs after docking of the receptor at the peroxisome and before any ATP-dependent step.

Conclusion: Translocation is concomitant with PEX5 insertion into the docking/translocation machinery.

Significance: These results support a model in which cargoes are pushed across the peroxisomal membrane by PEX5.

Peroxisomal matrix proteins are encoded by nuclear genes and synthesized on free ribosomes in the cytosol (1). Their sorting to the organelle relies on one of two types of peroxisomal targeting signals (PTS).5 The PTS type 1 (PTS1) consists of a conserved tripeptide, usually with the sequence S-K-L, present at the C terminus of the vast majority of peroxisomal matrix proteins (2, 3). The PTS2 is a degenerated nonapeptide located at the N terminus of only a small number of proteins (4–6). Targeting of newly synthesized matrix proteins to the organelle requires a complex machinery comprising both cytosolic and peroxisomal membrane proteins. A central component of this machinery is PEX5, a monomeric 70-kDa protein rich in intrinsically disordered domains (7–9), which interacts directly with PTS1 proteins. This interaction is mediated mainly by the PTS1 of the cargo protein on one side and the tetratricopeptide repeats (TPRs) present in the C-terminal half of PEX5 on the other, but other regions of the cargo protein and other domains of PEX5 are also involved (11–16). Interestingly, recent data suggest that PEX5 may also act as a chaperone at least for some PTS1 proteins (11). In mammals, plants, and many other organisms, PEX5 is also in charge of transporting PTS2 proteins to the peroxisome (17–20). In this case, however, the interaction is not direct but rather mediated by the adaptor protein PEX7 (5, 21, 22).

According to current models (23–26), after binding newly synthesized matrix proteins in the cytosol, PEX5 interacts with the peroxisomal docking/translocation module (DTM), a multisubunit protein assembly comprising five core components: PEX13 and PEX14 and the RING finger peroxins PEX2, PEX10, and PEX12 (27–29). This interaction results in the insertion of PEX5 into the DTM, with PEX5 acquiring a transmembrane topology (Refs. 30 and 31; see also “Discussion”). PEX5 is then monoubiquitinated at a conserved cysteine residue (32, 33), a modification required for the next step of the pathway, the ATP-dependent extraction of monoubiquitinated PEX5 back across the peroxisomal membrane.
The Import Mechanism of a Peroxisomal PTS1 Protein

![A diagram showing the import mechanism of a peroxisomal PTS1 protein.](image)

FIGURE 1. \[^{35}S\]SCPx is specifically imported into peroxisomes in vitro. A, preincubation of \[^{35}S\]SCPx with recombinant PEX5 improves its in vitro import efficiency. Two chemically identical import reactions were assembled, differing solely in the step of the protocol where recombinant PEX5 was added. In one reaction (lane 3), \[^{35}S\]SCPx was preincubated in the absence of PEX5 and added to a PNS in ATP-containing import buffer. After the addition of recombinant PEX5, the reaction was incubated for 15 min at 37 °C. In the other reaction (lane 4), \[^{35}S\]SCPx was preincubated with recombinant PEX5, added to a PNS in the same buffer, and incubated under the same conditions. Proteinase K-treated organelles were then subjected to SDS-PAGE/Western blotting/autoradiography. Lanes 1 and 2 contain 5% of the preincubated \[^{35}S\]SCPx proteins used in the assays shown in lanes 3 and 4, respectively. The autoradiograph (upper panel) and the Ponceau S-stained membrane (lower panel) are shown. B, in vitro synthesized \[^{35}S\]SCPx interacts with PEX5. \[^{35}S\]SCPx was preincubated for 30 min at room temperature in the absence or presence of 1 μM recombinant PEX5, as indicated. After adding a mixture of protein standards, the samples were subjected to sucrose gradient centrifugation. After fractionation, equivalent aliquots were subjected to SDS-PAGE/Western blotting. Note that the sedimentation coefficient of \[^{35}S\]SCPx increases in the presence of PEX5, indicating that the two proteins interact. Autoradiographs are shown. Protein standards used were: ovalbumin (Ova; 45 kDa), bovine serum albumin (BSA; 66 kDa), and aldolase (Ald; 140 kDa). C, organelles from an in vitro import reaction were resuspended in import buffer and treated with proteinase K (PK) in the absence (lane 2) or presence of Triton X-100 (TX-100; lane 3). Samples were analyzed as in A. The behaviors of endogenous SCPx and catalase (Cat) are shown. D, \[^{35}S\]SCPx preincubated with recombinant PEX5 was subjected to a standard import assay. Aliquots of the reaction were withdrawn at the indicated time points, treated with proteinase K, and processed for SDS-PAGE/autoradiography (upper panel). The Ponceau S-stained membrane is also shown (lower panel). E, \[^{35}S\]SCPx was preincubated in the absence (−) or presence (+) of the indicated recombinant proteins. Protein mixtures were then subjected to standard import assays and analyzed as described above. Note that PEX19 is involved in a different aspect of peroxisomal biogenesis (59) and was used here just as a negative control. F, a protease-treated import reaction was subjected to Nycodenz gradient centrifugation. The behaviors of \[^{35}S\]SCPx, endogenous SCPx, catalase (Cat), cytochrome c (Cyt c; a mitochondrial marker), and two endoplasmic reticulum proteins (KDEL; recognizes GRP72 and GRP98) are shown. The fraction of catalase detected at the top of the gradient represents mostly catalase that has leaked from peroxisomes during PNS preparation. Unlike soluble SCPx, soluble mouse catalase is quite resistant to proteinase K (see C). Lanes 1 in C, D, and E, 5% of the \[^{35}S\]SCPx protein used in each reaction. Numbers to the left indicate the molecular masses of protein standards in kDa.
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**EXPERIMENTAL PROCEDURES**

**Proteins and Plasmids**—The recombinant large isoform of human PEX5 (PEX5 (8, 17, 43)), proteins comprising amino acid residues 1–324 and 315–639 of PEX5 (ΔC1PEX5 and TPRs, respectively (7)), PEX5 containing the missense mutation N526K (PEX5(N526K) (44–46)), TPRs with the missense mutation N526K (TPRs(N526K), numbering of full-length PEX5 (44)), a protein comprising the first 80 amino acid residues of human PEX14 (NDPEX14 (7)), PEX19 (47), and a glutathione S-transferase-ubiquitin fusion protein (GST-Ub (32)) were obtained as described previously. PEX5 possessing an alanine at position 11 was obtained with the QuikChange® site-directed mutagenesis kit (Stratagene), using pQE30−PEX5 as the template (8, 38). pGEM4 (Promega)-based plasmids encoding PEX5 possessing a lysine (PEX5(C11K)) or an alanine residue (PEX5(C11A)) at position 11 were described before (38). The cDNA encoding SCPx (clone MmCD00313611, PlasmID, Dana Farber/Harvard Cancer Center; (41)) was amplified by PCR using the primers 5′-GCGCGGCTAGGACACGTGCCTTCTGTTGTTT-3′ and 5′-GCGCGGCTGATCTACAAGGTTGCCC-3′ and cloned into XbaI/KpnI-digested pGEM4 vector (Promega). The cDNA encoding human 2,4-dienoyl-CoA reductase (DECR2) was obtained from the plasmid pKDN36 (48) (a kind gift of Dr. Marc Fransen from KU Leuven, Belgium) by PCR using the primers 5′-GATATTCTAGAGCCACATGCGCCAGCCCAGCCGC-3′ and 5′-CGCCGCTGATCTACGATCTAGGTGGAGAGAAGAGA-3′. The DNA fragment was digested with XbaI and Kpnl and cloned into the XbaI/Kpnl-digested pGEM4 vector (Promega). 35S-labeled proteins were synthesized in vitro as described before (40).

**In Vitro Import Experiments**—Mouse liver postnuclear supernatant (PNS) was prepared as described before (49). The reticulocyte lysate containing 35S-labeled SCPx ([35S]SCPx) was diluted 1:10 in import buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 3 mM MgCl2, 0.16 M methylamine, 2 μg/ml N-(trans-epoxysuccinyl)-l-leucine 4-guanidinobutylamide) and incubated for 15 min at 37 °C in the absence or presence of one or more of the following recombinant proteins: PEX5 or PEX5(N526K) or PEX5(C11A) (30 nM final concentration), TPRs or TPRs(N526K) (10 μM final concentration), and NDPEX14 or TPRs (20 μM final concentration). In a standard import reaction (100 μl final volume), 10 μl of the

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**FIGURE 2. Import of a PTS1 protein into peroxisomes does not require cytosolic ATP.** A, [35S]SCPx was preincubated in the presence of either recombinant PEX5 (lanes 1 and 2) or PEX5(C11A) (lane 3). An aliquot of the PEX5-containing [35S]SCPx was further treated with apyrase (lane 2). These samples were then subjected to import assays, as follows: lane 4, assay containing ATP and [35S]SCPx preincubated with PEX5; lane 5, the same as in lane 4 but in the presence of AMP-PNP instead of ATP; lane 6, assay containing [35S]SCPx preincubated with PEX5 and PNS, both pretreated with apyrase; lane 7, import assay containing ATP and [35S]SCPx preincubated with PEX5(C11A); lane 8, the same as in lane 4 but also containing recombinant NDPEX14. Samples were processed as described in the legend for Fig. 1A. Lanes 1–3, 5% of the [35S]SCPx samples used in the assays. B, [35S]-labeled PEX5(C11K) was subjected to in vitro import reactions containing ubiquitin aldehyde and either ATP (lanes 2 and 3) or AMP-PNP (lanes 4 and 5). After 7 min at 37 °C, the import reactions were centrifuged to obtain an organelle pellet (P) and a supernatant (S), and both fractions were analyzed by SDS-PAGE/Western blotting. Note that AMP-PNP allows PEX5(C11K) ubiquitination but not its export into the soluble phase of the import reaction (39). C, [35S]-labeled PEX5(C11K) was incubated in the absence (−) or presence (+) of apyrase (lanes 1 and 2, respectively). The first of these samples (minus apyrase) was subjected to an in vitro import assay in the presence of ATP (lane 3); the apyrase-treated [35S]-labeled PEX5(C11K) was subjected to an import assay using apyrase-treated PNS (lane 4). Both reactions also contained 15 μM GST-Ub. The organelles were then isolated by centrifugation and analyzed by SDS-PAGE/Western blotting/autoradiography. Note that GST-Ub is efficiently used by the machinery that monoubiquitinates PEX5 but, in contrast to ubiquitin, results in a PEX5 species that is not exported from the DTM (32, 38). Also, monoubiquitinated PEX5(C11K) is more stable than monoubiquitinated PEX5 upon SDS-PAGE. This property increases the sensitivity of the ubiquitination assays (38). D, [35S]DECR2 was subjected to import assays exactly as described in A for [35S]SCPx. The asterisk indicates an unspecific radiolabeled band produced during the in vitro translation reaction. Lane 1 in B and lanes 1 and 2 in C, 5% of the [35S]-labeled PEX5(C11K) used in the assays.
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FIGURE 3. In vitro imported \[^{35}S\]SCPx behaves as endogenous SCPx upon fractionation of peroxisomes. \[^{35}S\]SCPx preincubicated with the indicated PEX5 proteins was subjected to in vitro import assays under different energetic conditions, as specified. At the end of the import reaction, the organelles were treated with proteinase K, isolated by centrifugation, and sonicated. One-half of these samples was kept on ice ("K treatment (see also Ref. 62).

Results

Our laboratory has been using an in vitro import strategy to dissect the mechanism of protein translocation across the mammalian peroxisomal membrane. This strategy comprises three steps: 1) in vitro synthesis of a \[^{35}S\]S-labeled reporter protein; 2) incubation of the reporter protein with a PNS, a source of peroxisomes and cytosolic components; and 3) treatment of the organelle suspension with a large amount of a protease (such as proteinase K) to degrade nonimported (accessible) reporter protein while preserving the fraction that was imported. This strategy works particularly well when the reporter protein is PEX5 itself (38, 49). In contrast, the import yields obtained using peroxisomal matrix proteins as reporters are in general rather poor. As explained in detail elsewhere (40), a major problem stems from the fact that PNS contains large amounts of soluble PTS1 proteins that have leaked from peroxisomes during tissue homogenization. These soluble proteins compete with the \[^{35}S\]S-labeled reporter protein for PEX5 binding, thus resulting in low import yields.

We found that the amounts of protease-protected \[^{35}S\]SCPx in in vitro import assays can be dramatically improved by preincubating this PTS1 protein with recombinant PEX5 before proceeding with the import reaction (Fig. 1A, compare lanes 3 and 4). Possibly, this step allows the reporter protein to form a complex with PEX5 (Fig. 1B) with no competition from the PTS1 proteins present in the PNS. We note that \[^{35}S\]SCPx is extremely susceptible to proteinase K, a property that is not altered by the precubincation step with PEX5 nor by the simple presence of PNS during the protease treatment (e.g. see the temperature dependence experiments below). Actually, the protease-resistant status of \[^{35}S\]SCPx subjected to import assays vanishes when the protease treatment is made in the presence of the mild detergent Triton X-100 (Fig. 1C, upper panel), thus suggesting that its protease resistance reflects a

\[\text{diluted lysate were added to 400 \, \mu g} \text{ of PNS protein that had been primed for import (incubation for 5 min at 37 °C in import buffer containing 0.3 mM ATP; see Refs. 40 and 50). Import assays also contained 2 mM glutathione and, as indicated, ATP (3 mM), AMP-PNP (3 mM), bovine ubiquitin (15 \mu M), GST-Ub (15 \mu M), or ubiquitin aldehyde (3 \mu M). In the apyrase experiments, both the diluted lysate and the PNS in import buffer were incubated at 37 °C with apyrase (20 units/ml, Grade VII, Sigma) for 5 and 2 min, respectively, before starting the import assay. When comparing import efficiencies of SCPx under different energetic conditions, import assays were performed for just 7 min to minimize differences induced by the time-dependent occupation of the DTM by PEX5 (40). After import, samples were subjected to proteinase K digestion (400 \, \mu g/ml, 40 min on ice). Processing of organelles for SDS-PAGE/autoradiography was done as described before (40). In some experiments, organelles were isolated by centrifugation, resuspended in import buffer, and subjected to proteinase K digestion (50 \, \mu g/ml) in the absence or presence of 1% (w/v) Triton X-100. Sonication and fractionation of organelles were done exactly as described (40).}

\[\text{Nycodenz and Centrifugation of Sucrose Gradients—Nycodenz gradient centrifugation of protease-treated import reactions was done as described (47) except that a Nycodenz gradient comprising 1.5 ml of 45% (w/v), 7 ml of 28% (w/v), 2 ml of 25% (w/v), and 2 ml of 20% (w/v) Nycodenz in 5 mM MOPS-KOH, pH 7.2, and 1 mM EDTA-NaOH, pH 8.0, was used and the centrifugation conditions were 59,000 \times g for 3 h at 4 °C in a vertical rotor (STEPSAVER\textsuperscript{TM} 65V13, Sorvall\textsuperscript{®}). For the sucrose gradient centrifugation analyses, 10 \mu l of SCPx lysate in 200 \mu l of a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA-NaOH, pH 8.0, and 1 mM DTT, supplemented or not with 25 \mu g of recombinant PEX5, were loaded onto the top of a continuous 5–20% (w/v) sucrose gradient in the same buffer. After centrifugation at 247,000 \times g for 29 h at 4 °C in an SW41 rotor (Beckman), 13 aliquots were collected from the bottom of the tube. Ovalbumin (3.6 S), bovine serum albumin (4.3 S), and aldolase (7.4 S), were used as internal sedimentation coefficient standards.}

\[\text{Antibodies—The antibodies directed to SCPx (19182-1-AP; ProteinTech\textsuperscript{TM}), catalase (C0979; Sigma), KDEL (ab12223; Abcam), and cytochrome c (556433; BD Pharmingen\textsuperscript{TM}) were purchased. The antibody directed to PEX14 was described before (29). Rabbit and mouse antibodies were detected using goat alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibodies (A9919 and A2429, respectively; Sigma) or goat anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology).}

\[\text{RESULTS}

\[\text{diluted lysate were added to 400 \, \mu g} \text{ of PNS protein that had been primed for import (incubation for 5 min at 37 °C in import buffer containing 0.3 mM ATP; see Refs. 40 and 50). Import assays also contained 2 mM glutathione and, as indicated, ATP (3 mM), AMP-PNP (3 mM), bovine ubiquitin (15 \mu M), GST-Ub (15 \mu M), or ubiquitin aldehyde (3 \mu M). In the apyrase experiments, both the diluted lysate and the PNS in import buffer were incubated at 37 °C with apyrase (20 units/ml, Grade VII, Sigma) for 5 and 2 min, respectively, before starting the import assay. When comparing import efficiencies of SCPx under different energetic conditions, import assays were performed for just 7 min to minimize differences induced by the time-dependent occupation of the DTM by PEX5 (40). After import, samples were subjected to proteinase K digestion (400 \, \mu g/ml, 40 min on ice). Processing of organelles for SDS-PAGE/autoradiography was done as described before (40). In some experiments, organelles were isolated by centrifugation, resuspended in import buffer, and subjected to proteinase K digestion (50 \, \mu g/ml) in the absence or presence of 1% (w/v) Triton X-100. Sonication and fractionation of organelles were done exactly as described (40).

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Several experiments were performed to test the specificity of this in vitro import system. Acquisition of a protease-protected status by \(^{35}\text{S}\)SCPx is time- (Fig. 1D) and temperature-dependent (see below). Furthermore, no protease-protected \(^{35}\text{S}\)SCPx was obtained when PEX5 was replaced by a PEX5 mutant protein that is unable to bind PTS1 proteins efficiently (PEX5(N526K) (45, 46)) (Fig. 1E, lane 4). The same result was obtained when \(^{35}\text{S}\)SCPx was preincubated with PEX5 plus a molar excess of a protein comprising the PTS1-binding domain of PEX5 (TPRs; Fig. 1E, lane 5). The latter protein can still bind PTS1 proteins efficiently but lacks the N-terminal domain of PEX5 required for a productive interaction with the peroxisomal DTM (51). A mutant version of TPRs carrying the N526K mutation (TPRs(N526K)) has no such effect (lane 6). Finally, no protease-resistant SCPx was observed when the reporter protein was preincubated with PEX5 plus a recombinant protein comprising the N-terminal domain of PEX14, a component of the DTM (lane 7). This domain of PEX14 binds with high affinity to the so-called diaromatic motifs present in the N-terminal half of PEX5 (52), which are essential for PEX5 function (53).

To confirm that the membrane-bound organelle to which PEX5 targets SCPx is in fact the peroxisome, a protease-treated import reaction was subjected to Nycodenz gradient centrifugation (54). As shown in Fig. 1F, most in vitro imported \(^{35}\text{S}\)SCPx was found in fractions 1–3, the region of the gradient containing intact peroxisomes. Taken together, these data show that \(^{35}\text{S}\)SCPx can be efficiently imported into peroxisomes in vitro.

Having established the robustness and specificity of this in vitro import system, we then asked whether import of SCPx requires hydrolysis of cytosolic ATP. Two different strategies were used to address this question. In the first, a PNS that had been primed for import in the presence of 0.3 mM ATP (see “Experimental Procedures” for details) was used in an import reaction containing 3 mM AMP-PNP. AMP-PNP is a potent inhibitor of ATPases cleaving the bond between the \(\beta\)- and \(\gamma\)-phosphate groups of ATP. Note that ubiquitination of PEX5 at the DTM still occurs in the presence of AMP-PNP because the ubiquitin-activating enzyme uses this ATP analog quite efficiently (39, 55). However, export of monoubiquitinated PEX5 from the DTM to the cytosol, a process catalyzed by the ATPases PEX1/PEX6, is completely blocked by AMP-PNP (39, 55). However, export of monoubiquitinated PEX5 from the DTM to the cytosol, a process catalyzed by the ATPases PEX1/PEX6, is completely blocked by AMP-PNP (39).

As shown in Fig. 2A, the import efficiencies of \(^{35}\text{S}\)SCPx in reactions supplemented with either ATP (lane 4) or AMP-PNP (lane 5) are essentially the same. Thus, a 10-fold molar excess of AMP-PNP over ATP does not result in an inhibition of SCPx import, although export of monoubiquitinated PEX5 is blocked under these conditions (Fig. 2B), as expected (39).

In the second strategy, both \(^{35}\text{S}\)SCPx and a primed PNS were treated with apyrase, an enzyme that hydrolyzes ATP and other NTPs (56), before the import reaction. As shown in Fig. 2A, the import efficiency of SCPx was not decreased by the apyrase treatment (compare lanes 4 and 6). A control experiment shows that the apyrase treatment efficiently depletes ATP from the reactions because ubiquitination of PEX5 was no longer observed under these conditions (Fig. 2C), as described before (40). Taken together, these results strongly suggest that import of SCPx into peroxisomes requires neither monoubiquitination of PEX5 nor even hydrolysis of cytosolic ATP. Additional evidence supporting the first of these conclusions was obtained when \(^{35}\text{S}\)SCPx was preincubated with recombinant PEX5(C11A) instead of PEX5. PEX5(C11A) possesses an alanine at position 11, a non-ubiquitatable amino acid residue. Therefore, this mutant PEX5 is not monoubiquitinated at the DTM and, consequently, it is not exported back to the cytosol.

**FIGURE 4. Temperature dependence of the docking/insertion of PEX5 into the DTM and SCPx import.** A, \(^{35}\text{S}\)SCPx preincubated with a mixture of recombinant and \(^{35}\text{S}\)-labeled PEX5(C11A) was subjected to import assays at different temperatures. After 15 min, the samples were halved and treated (lanes +) or not (lanes −) with proteinase K (PK). The organelles were analyzed by SDS-PAGE/autoradiography (upper panel). The Ponceau S-stained membrane is also shown (lower panel). B, \(^{35}\text{S}\)SCPx preincubated as described above was subjected to import assays at 0 or 37 °C in the presence of either recombinant ΔC1PEX5 or PEX19 (5 μM each), as indicated. Protease-treated and untreated organelles were then analyzed as above. C, organelles from an import assay performed at 0 °C (lane 2) were resuspended in import buffer containing either recombinant ΔC1PEX5 or PEX19, incubated for 15 min, and reisolated by centrifugation. Organelle pellets (P) (lanes 3 and 5) and the corresponding supernatants (S) (lanes 4 and 6) were analyzed as above. Lanes 1 in A, B, and C, 5% of the radiolabeled proteins used in the assays.
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FIGURE 5. Working model for the PEX5-mediated protein import pathway. After binding a PTS1 protein in the cytosol, PEX5 docks at the DTM in a reversible manner. PEX5 then becomes inserted into the DTM, pushing the cargo protein across the organelle membrane. In contrast to PEX5 recycling, which includes monoubiquitination and PEX1/PEX6-catalyzed extraction of the receptor from the DTM, the docking and translocation steps do not require cytosolic ATP.

(38). As shown in Fig. 2A, PEX5(C11A) is as efficient as PEX5 in these assays (compare lanes 4 and 7).

The ability to be imported into peroxisomes in a PEX5 monoubiquitination- and cytosolic ATP-independent manner is not a particularity of SCPx. Indeed, exactly the same results were obtained when [35S]DECR2, another peroxisomal matrix PTS1 protein, was used in these assays (Fig. 2D).

The experiments above indicate that [35S]SCPx acquires a protease-protected peroxisomal location in a process that requires PEX5 but not hydrolysis of cytosolic ATP. However, it remained unclear whether the protease-protected [35S]SCPx detected in those experiments represents a protein that was already completely translocated into the peroxisomal matrix or a species that is still associated with the DTM. To address this issue, [35S]SCPx preincubated with either PEX5 or PEX5(C11A) was subjected to import assays in the presence of ATP or AMP-PNP or apyrase, as specified in Fig. 3. After protease treatment, the organelles were isolated by centrifugation, disrupted by sonication, and ultracentrifuged to obtain membrane and soluble fractions. The efficiency of the fractionation procedure was assessed by Western blotting using antibodies directed to PEX14 (a peroxisomal intrinsic membrane protein), cytochrome c (a peripheral membrane mitochondrial protein), and catalase (a soluble peroxisomal matrix protein). As shown in Fig. 3, a major fraction of endogenous SCPx was found in the soluble fraction, although some protein was also recovered in the membrane pellet. The dual behavior of SCPx might be related to the fact that the C-terminal domain of SCPx binds membrane lipids (57). Importantly, and regardless of the experimental conditions used in the import assays, in vitro imported [35S]SCPx displayed exactly the behavior of endogenous SCPx, thus suggesting that it represents a species that was already translocated into the matrix of the organelle.

The data presented above suggest that translocation of SCPx across the peroxisomal membrane occurs upstream of PEX5 monoubiquitination step. According to current models, there are only two events occurring at the peroxisome before this step: 1) docking of the PEX5-cargo protein complex at the DTM and 2) insertion of PEX5 into this machinery. We note, however, that although there is abundant experimental evidence supporting the concept that PEX5 becomes inserted into the DTM (30, 31, 35, 50), data regarding the docking step itself are still very limited. Actually, it is presently unknown whether such a step really exists in a mechanistic sense or whether docking and insertion of PEX5 into the DTM are simply the beginning and the end of a single step. To clarify this issue, we explored the fact that insertion of PEX5 into the DTM is inhibited at low temperatures (58) and asked whether docking of the PEX5-cargo protein complex can still occur under those conditions. Fig. 4 shows in vitro import assays where [35S]SCPx was preincubated with a mixture of recombinant PEX5(C11A) and 35S-labeled PEX5(C11A), thus allowing us to monitor the behavior of the cargo and the receptor simultaneously. As expected, insertion of PEX5(C11A) into the DTM (as assessed by the acquisition of a protease-resistant status (49)) does not occur at low temperatures (Fig. 4A, compare lanes 2 and 3 with lanes 4 – 6). Importantly, import of SCPx displays the same temperature dependence profile. When the protease treatment was omitted, both 35S-labeled PEX5(C11A) and 35S-labeled SCPx were found in the organelle fractions even in import reactions performed at low temperatures (Fig. 4A, lanes 7 and 8). A considerable fraction of these proteins is specifically adsorbed to the peroxisome because their amounts in assays containing recombinant ΔC1PEX5, a PEX5 protein that lacks the PTS1-binding domain but that is still competent in entering the DTM (30, 39), are smaller than those observed in the presence of PEX19, a protein involved in another aspect of peroxisome biogenesis (59) and used here as a negative control (Fig. 4B, compare lanes 2 and 4 with 6 and 8, respectively). A similar competition phenomenon was observed when organelles isolated from an import assay performed at 0 °C were washed in buffer containing ΔC1PEX5 (Fig. 4C, compare lanes 3 and 4 with lanes 5 and 6, respectively).
respectively). Apparently, both $^{35}$S-labeled PEX5(C11A) and $^{35}$S-labeled SCPx can interact in a specific and reversible manner with peroxisomes. Taken together, these results provide evidence for the existence of a mechanistically distinct docking step of the PEX5-cargo protein at the DTM and suggest that import of SCPx occurs concomitantly with insertion of PEX5 into the DTM.

**DISCUSSION**

We have previously shown that cytosolic PEX5 becomes transiently inserted into the peroxisomal membrane DTM in a process that is cargo protein-dependent but independent of cytosolic ATP (30, 31, 50). Those findings together with data suggesting that DTM-embedded PEX5 adopts a transmembrane topology exposing a major fraction of its polypeptide chain into the lumen of the organelle (31, 49) led us to propose that the PEX5-mediated translocation of cargo proteins across the peroxisomal membrane is an ATP-independent event that occurs concomitantly with insertion of PEX5 into the DTM (50, 60). According to this model, the driving force for the protein translocation step resides in the strong protein-protein interactions that are established between PEX5 on one side and components of the DTM on the other; ATP hydrolysis is necessary only at later steps, to extract PEX5 from the DTM, thus resetting the protein transport system (see Ref. 23 and references cited therein). Although essentially all the data on PEX5 collected since then using several experimental systems are compatible with such a model (35, 36), the fact remained that there was no direct evidence showing ATP-independent import of a peroxisomal matrix protein was available for many years. This gap in experimental evidence was partially filled in by recent findings showing that translocation of pre-thiolase (a PTS2 protein) across the peroxisomal membrane occurs before monoubiquitination of PEX5 at the DTM, in a cytosolic ATP-independent manner (40). However, considering that PTS2 proteins are targeted to the peroxisome by a PEX5-PEX7 protein complex and that there are some data suggesting that PEX7 may actually enter the organelle matrix together with PTS2 proteins (61), it remained unclear whether the mechanistic data gathered for PTS2 proteins were also valid for PTS1 proteins.

The data presented here indicate that neither a nonhydrolyzable ATP analog nor the depletion of ATP from import reactions affects the PEX5-mediated peroxisomal import of PTS1 proteins. These findings, besides indicating that import of PTS1 proteins does not require cytosolic ATP, also imply that the import process occurs before PEX5 monoubiquitination. Indeed, the import efficiencies and behaviors of $^{35}$S-labeled SCPx upon the peroxisome fractionation experiments were the same when using recombinant PEX5 or PEX5(C11A). Thus, contrary to previous hypotheses (23, 33), neither ubiquitination of PEX5 at the DTM nor the ATP-dependent extraction of monoubiquitinated PEX5 from the DTM plays a role in the cargo protein translocation steps.

The conclusion that import of a PTS1 protein occurs upstream of PEX5 monoubiquitination immediately indicates that translocation of the cargo protein across the organelle membrane occurs during the docking/insertion of PEX5 at/into the DTM. By performing import reactions at several temperatures, it was possible to resolve these two steps. We found that docking of both PEX5 and SCPx at the organelle surface can still occur at 0 °C; insertion of PEX5 into the DTM and import of SCPx into peroxisomes, however, were only detected at higher temperatures.

In summary, the results presented here suggest that translocation of a PTS1 protein across the organelle membrane occurs downstream of the docking step and upstream of PEX5 ubiquitination, concomitantly with the insertion of the receptor into the DTM. These findings provide a hitherto missing cargo-centered perspective to support a model in which PEX5, besides working as a soluble receptor, also functions as a translocator pushing cargo proteins across the peroxisomal membrane as it gets inserted into the peroxisomal docking/translocation machinery (Fig. 5).

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