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The N Terminus of the Peroxisomal Cycling Receptor, Pex5p, Is Required for Redirecting the Peroxisome-associated Peroxin Back to the Cytosol*

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Most newly synthesized peroxisomal matrix proteins are transported to the organelle by Pex5p, a remarkable multidomain protein involved in an intricate network of transient protein-protein interactions. Presently, our knowledge regarding the structure/function of amino acid residues 118 to the very last residue of mammalian Pex5p is quite vast. Indeed, the cargo-protein receptor domain as well as the binding sites for several peroxins have all been mapped to this region of Pex5p. In contrast, structural/functional data regarding the first 117 amino acid residues of Pex5p are still scarce. Here we show that a truncated Pex5p lacking the first 110 amino acid residues (ΔN110-Pex5p) displays exactly the peroxisomal import properties of the full-length peroxin implying that this N-terminal domain is involved neither in cargo-protein binding nor in the docking/translocation step of the Pex5p-cargo protein complex at the peroxisomal membrane. However, the ATP-dependent export step of ΔN110-Pex5p from the peroxisomal membrane is completely blocked, a phenomenon that was also observed for a Pex5p version lacking just the first 17 amino acid residues but not for a truncated protein comprising amino acid residues 1–324 of Pex5p. By exploring the unique properties of ΔN110-Pex5p, the effect of temperature on the import/export kinetics of Pex5p was characterized. Our data indicate that the export step of Pex5p from the peroxisomal compartment (in contrast with its insertion into the organelle membrane) is highly dependent on the temperature.

All peroxisomal matrix proteins are synthesized in the cytosol and post-translationally imported into the organelle. Specific targeting of these proteins to the peroxisome is promoted by one of two receptor proteins, the peroxins Pex5p and Pex7p (reviewed in Refs. 1–4). By far the vast majority of peroxisomal matrix proteins use Pex5p as their import receptor. Proteins belonging to this family possess a peroxisomal targeting signal type 1 (PTS1),[1] a C-terminal tripeptide with the sequence SKL, or a conserved variant (5–7). A very small group of peroxisomal matrix proteins is targeted to the organelle by Pex7p. This peroxin recognizes a degenerated nona-peptide complying to the consensus sequence (R/K)(L/I/V)X_5(H/Q)(L/A/F), the so-called peroxisomal targeting signal type 2 (PTS2) (8–10). In mammals and plants, but not in lower eukaryotes, targeting of these PTS2-containing proteins seems to require Pex5p, suggesting that the two import pathways actually converge at the level of the PTS1 receptor (11–21).

The Pex5p-mediated protein import process can be divided into four major steps (reviewed in Refs. 1–4). In the first step, newly synthesized peroxisomal proteins interact with their receptor while still in the cytosol. The molecular basis for this interaction is now well defined. Indeed, both structural and biochemical studies have shown that the seven tetratricopeptide repeats present in the C-terminal half of Pex5p provide the binding site for the PTS1 sequence (7, 22–27). In the second step, these soluble cargo protein-Pex5p complexes are recognized by a complex protein machinery present at the peroxisomal membrane. Although the exact composition and architecture of this machinery are still ill defined, recent protein purification studies suggest that Pex2p, Pex10p, Pex12p, Pex13p (see "Discussion"), and Pex14p are the core components of this protein complex (28–30). Somewhere during or immediately after this recognition event, cargo proteins are translocated across the membrane of the peroxisome (step 3). There is still some controversy regarding the fate of Pex5p during this step. Indeed, some authors have proposed that Pex5p is completely translocated across the organelle membrane together with the cargo proteins (31, 32). However, data suggesting that Pex5p stays in contact with the peroxisomal membrane during this step are also available (see below and Ref. 33). Finally, Pex5p is recycled (exported) back to the cytosol in order to catalyze further rounds of transportation (step 4). Besides the fact that ATP hydrolysis is required at this stage (34), not much is known regarding this step.

Thus, although the data supporting this general model are now abundant (35–37), it is clear that we are still far from understanding the mechanistic details of this process. Much of what we know regarding this issue has been inferred from steady-state level analysis of peroxisomal Pex5p in several

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[1] The abbreviations used are: PTS, peroxisomal targeting sequence; PNS, post-nuclear supernatant; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione *S*-transferase; TPRs, Pex5p domain (amino acid residues 312–639) comprising its tetratricopeptide repeats; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

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mutant cell lines (35, 36). It is true that this type of studies has been quite valuable in assigning general functions to components involved in protein translocation across the peroxisomal membrane. However, it is also evident that different experimental strategies have to be used in order to characterize in detail the mechanism of this process.

We have recently described an *in vitro* system particularly suited to study the process of association (import)/dissociation (export) of Pex5p with and from the peroxisomal compartment. It consists of incubating ^{35}S-labeled Pex5p with a post-nuclear supernatant from rat liver, and it explores the fact that Pex5p acquires a protease-resistant status when inserted into the peroxisomal membrane (38, 39). By using this experimental strategy, it was shown that insertion of Pex5p into the peroxisomal membrane requires the presence of PTS1-containing proteins in the import medium and depends on available Pex14p, an intrinsic membrane component of the peroxisomal docking/translocation machinery (39, 40). Unexpectedly, insertion of Pex5p into the organelle membrane does not require ATP, suggesting that the driving force for protein translocation across the peroxisomal membrane resides on protein-protein interactions. In contrast, export (recycling) of Pex5p from the peroxisomal compartment is ATP-dependent (34). At the peroxisomal membrane level, two different populations of Pex5p could be identified and characterized using protease-protection assays (39). In the presence of ATP, conditions in which Pex5p continuously enters and exits the peroxisomal membrane, peroxisomal Pex5p behaves as a transmembrane protein exposing the majority of its mass into the peroxisomal lumen with only a small N-terminal fragment of ~2 kDa accessible to the protease from the cytosolic side of the membrane. This is the so-called stage 2 Pex5p. Under ATP-limiting conditions or in the presence of nonhydrolyzable ATP analogues, a second membrane population of Pex5p is observed. This population of Pex5p, referred to as stage 3 Pex5p, is completely resistant to the protease as long as the organelles are kept intact. It was also shown that stage 2 is the precursor of stage 3 Pex5p and that this population of Pex5p leaves the peroxisomal membrane rapidly in the presence of ATP. Most interestingly, both stage 2 and stage 3 Pex5p can be immunoprecipitated by anti-Pex14p antibodies. These observations led to the proposal that Pex14p remains in contact with Pex5p during most of the steps (if not all) occurring at the peroxisomal membrane, a conclusion that is in perfect agreement with the protein purification studies cited above (28–30).

Here we have used this *in vitro* system to characterize the role of the first 110 amino acid residues of Pex5p. By using several criteria, it is shown that a truncated version of Pex5p lacking this domain displays exactly the peroxisomal import properties of the full-length version of the peroxin, implying that this domain is necessary neither for cargo-protein binding nor for insertion into the peroxisomal membrane. However, in contrast to full-length Pex5p, N-terminal truncated Pex5p is unable to exit the peroxisomal compartment. By using the unique properties of this truncated version of Pex5p we have characterized the import/export kinetics of full-length Pex5p.

MATERIALS AND METHODS

Rat liver post-nuclear supernatants (PNS) and peroxisomes were prepared as described before (34) in SEM buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.4, 1 mM EDTA-NaOH, pH 7.4) supplemented with 2 μg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide. *In vitro* import experiments using PNS or purified peroxisomes were performed in import buffer (0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH, pH 7.4, 3 mM $MgCl_2$, 0.2% (w/v) lipid-free bovine serum albumin, 20 μM methionine, and 2 μg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide). Import reactions were performed in a 100-μl final volume using 150 μg of PNS protein, unless indicated otherwise. Where specified, an ATP-regenerating system (10 mM creatine phosphate and creatine phosphokinase 5 units/ml, final concentrations) was included in the import reactions. With this modification the Pex5p export activity of peroxisomes is maintained for longer periods (~45 min; data not shown). Proteinase K treatment of import reactions and processing of protein samples for SDS-PAGE and autoradiography were done exactly as described (39).

The cDNAs encoding full-length human Pex5p (the large isoform; Ref. 11) or the C-terminal truncated version comprising amino acid residues 1–324 of Pex5p (ΔC1-Pex5p) preceded by the T7 RNA polymerase promotor were obtained as described previously (39, 40). The cDNAs encoding the N-terminal truncated versions of Pex5p lacking the first 17 or the first 110 amino acid residues (ΔN17-Pex5p and ΔN110-Pex5p, respectively) were obtained by using an expression PCR strategy (41). In the first PCR, the pGEM4-Pex5p plasmid (39) was amplified by using the forward primers 5′-GAGCCACCATGAAGCTC-GCCGGGCACT-3′ (for ΔN17-Pex5p) or 5′-GACCCACCATGGACTTG-GCCTTGTCTGAGAACTGG-3′ (for ΔN110-Pex5p) and the reverse primer 5′-GAGACTGTCACTGGGGCAGGCCAAACATAG-3′. In the second PCRs, this reverse primer was used together with the forward primer 5′-GAATTCTAATACGACTCACTATAGGGAGAGCCACCATG-3′. cDNAs were subjected to *in vitro* transcription using T7 RNA polymerase (Roche Applied Science). ^{35}S-Labeled proteins were synthesized using the translation kit Retic Lysate IVT™ (Ambion) in the presence of Redivue™ L-[^{35}S]methionine (specific activity >1000 Ci/mmol) following the manufacturer's instructions. In antibody inhibition experiments, 150 μg of rat liver PNS or 80 μg of purified peroxisomes were preincubated with 30 μg of nonimmune or anti-Pex14p immunoglobulins (29) or 10 μg of nonimmune or anti-Pex13p (42) Fab fragments in 100 μl of import buffer for 20 min on ice, before starting the import reaction by adding the radiolabeled proteins.

The synthesis and purification of glutathione *S*-transferase (GST), a recombinant protein comprising GST fused to amino acid residues 312–639 of Pex5p (GST-TPRs; Ref. 40), GST-Pex5p (39), GST-SKL (GST containing a PTS1 signal at the C terminus; Ref. 40), and GST-LKS (GST ending with a nonfunctional PTS1-like sequence; Ref. 40) were described before. These recombinant proteins were used in import reactions at final concentrations of 0.17 μM (GST and GST-TPRs), 0.85 μM (GST-Pex5p), and 8 μM (GST-SKL and GST-LKS).

IgGs were purified from rabbit sera using protein A-Sepharose beads according to the manufacturer (Amersham Biosciences). Preparation of Fab fragments using immobilized papain was carried out using the ImmunoPure Fab preparation kit (Pierce). Densitometric analysis of x-ray films was performed using the UNSCAN-IT automated digitizing system.

RESULTS

Pex5p Lacking the First 110 Amino Acid Residues Is Efficiently Targeted to the Peroxisomal Compartment—The large isoform of mammalian Pex5p (hereafter referred to as Pex5p; Ref. 11) is a remarkable multidomain protein of 639 amino acid residues involved in a complex network of transient protein-protein interactions. Presently, our knowledge on the structure/function of amino acid residues 118–639 of mammalian Pex5p is quite vast. Indeed, a variety of biochemical and structural studies have shown that this region of the protein contains the binding site for PTS1-containing cargo proteins (7, 22–27), seven Pex14p-binding domains (43–45), and binding sites for Pex7p (11–14), Pex12p (46), and Pex13p (45). In sharp contrast, not much is known regarding the role of the first 117 amino acid residues of Pex5p. That this domain is important for the function of Pex5p *in vivo* was recently shown (45). However, the reason behind this observation remains unclear. In order to address this issue, we have used an *in vitro* import system to compare the properties of a truncated version of Pex5p lacking the first 110 amino acid residues (ΔN110-Pex5p) with the ones displayed by the full-length peroxin.

When ^{35}S-labeled full-length Pex5p is subjected to an import reaction in the absence of exogenous nucleotides, two different peroxisomal membrane populations of the peroxin are detected upon protease treatment of the import reactions (Fig. 1*A*, *lane 1*). These correspond to stage 2 Pex5p (the faster migrating band) and stage 3 Pex5p (see Introduction). In the presence of

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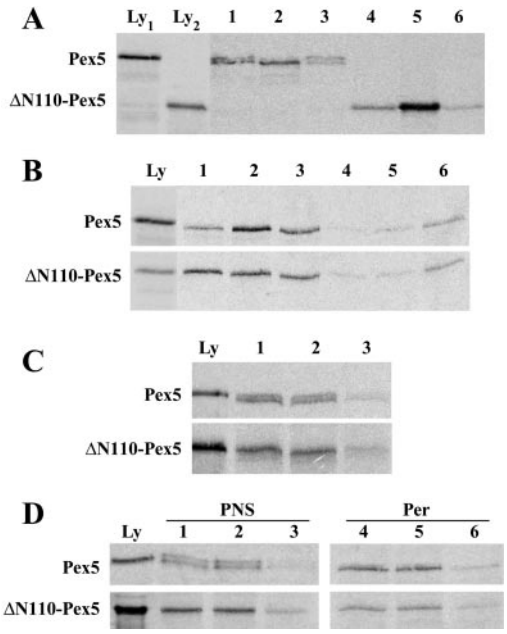

FIG. 1. **Pex5p lacking the first 110 amino acid residues is correctly targeted to the peroxisomal membrane.** *A*, PNS fractions were incubated for 45 min with ^{35}S-labeled Pex5p (*lanes 1–3*) or ^{35}S-labeled ΔN110-Pex5p (*lanes 4–6*) in import buffer containing no exogenous nucleotides (*lane 1* and *4*), 5 mM ATP (*lane 2* and *5*), or 5 mM ATPγS (*lane 3* and *6*). *Lanes Ly1* and *Ly2*, ^{35}S-labeled Pex5p and ^{35}S-labeled ΔN110-Pex5p, respectively, used in the import reactions (~5% of input). *B*, *in vitro* synthesized Pex5p or ΔN110-Pex5p were incubated with PNS fractions in import buffer containing 5 mM ATP in the presence of 0.17 μM GST (*lanes 1–3*) or GST-TPRs (*lanes 4–6*). GST-LKS (*lanes 2* and *5*) or GST-SKL (*lanes 3* and *6*) fusion proteins were also included in the import reactions (8 μM final concentrations). *C*, PNS fractions were incubated for 20 min on ice with nonimmune IgGs (*lane 2*), anti-Pex14p IgGs (*lane 3*), or in the absence of IgGs (*lane 1*) and subjected to an import reaction with ^{35}S-labeled Pex5p or ΔN110-Pex5p in the absence of exogenous nucleotides. *D*, PNS fractions or purified peroxisomes were preincubated 20 min on ice with nonimmune Fab fragments (*lane 2* and *5*), anti-Pex13p Fab fragments (*lane 3* and *6*), or in the absence of Fab fragments (*lane 1* and *4*) and were subjected to an import reaction with ^{35}S-labeled Pex5p or ^{35}S-labeled ΔN110-Pex5p in the absence of exogenous nucleotides. *Per*, purified peroxisomes. *Lanes Ly*, ^{35}S-labeled Pex5p (*upper panels*) and ^{35}S-labeled ΔN110-Pex5p (*lower panels*) used in the *in vitro* reactions (~5% of input). All the *in vitro* reactions were performed at 26 °C, treated with proteinase K and analyzed by SDS-PAGE autoradiography.

exogenous ATP, stage 2 Pex5p is by far the most prominent population (Fig. 1*A*, *lane 2*). In the presence of ATPγS, the two membrane populations of Pex5p are again detected (Fig. 1*A*, *lane 3*). However, the levels of these populations are decreased when compared with those detected in the absence of exogenous ATP. It should be noted that this phenomenon does not reflect the need for ATP during the import step of Pex5p. It was recently shown that this step is ATP-independent (34). This difference is related to the fact that import reactions performed in the absence of exogenous ATP do contain some ATP (from both the PNS fraction and reticulocyte lysate). This endogenous ATP promotes some degree of export of rat liver Pex5p from the docking/translocation sites thus freeing these sites for the ^{35}S-labeled Pex5p. When the results obtained with ΔN110-Pex5p are analyzed, two major differences are evident. The first is that in the absence of exogenous ATP (*lane 4*) or in the presence of ATPγS (Fig. 1*A*, *lane 6*), it is no longer possible to detect two protease-resistant populations. Please note that the difference between stage 2 and stage 3 Pex5p is that only the former can be cleaved by proteinase K, a cleavage that occurs around amino acid residues 15–20 (39). Obviously, in the absence of this domain, as it is the case for ΔN110-Pex5p, no cleavage can occur. Thus, this result is in perfect agreement with the proposed membrane topology for stage 2 Pex5p (39). The second difference regards the relative amount of ΔN110-Pex5p detected in the absence (*lane 4*) and presence (*lane 5*) of exogenous ATP. When this ratio is compared with the one obtained for the full-length peroxin, it is clear that more protease-resistant ΔN110-Pex5p than full-length Pex5p can be obtained in an import reaction containing exogenous ATP. The reason behind this observation is explained below.

As described previously, insertion of full-length Pex5p into the peroxisomal membrane requires the existence of PTS1-containing proteins in the import medium. If these PTS1 cargo proteins are sequestered from the medium by adding an excess of a recombinant protein comprising the receptor domain of Pex5p but not its peroxisomal targeting domain (GST-TPRs), then insertion of Pex5p into the organelle membrane is no longer observed (40). We used this strategy to characterize further the import properties of ΔN110-Pex5p. As shown in Fig. 1*B*, insertion of ΔN110-Pex5p into the peroxisomal membrane is blocked when GST-TPRs is added to the import reaction (*lanes 4* and *5*). However, this inhibitory effect is suppressed in the presence of an excess of a recombinant PTS1-containing protein (Fig. 1*B*, *lane 6*). Thus, insertion of ΔN110-Pex5p into the peroxisomal membrane is cargo protein-dependent.

Another criterion currently used to demonstrate the specificity of this *in vitro* system relies on the use of an antibody directed to Pex14p. Preincubation of PNS fractions with this antibody decreases the amount of Pex5p that is inserted into peroxisomal membrane (39). This observation corroborates previous findings showing that the import of a PTS1-containing protein is also blocked in the presence of anti-Pex14p IgGs (47). Thus, we tested the inhibitory effect of anti-Pex14p IgGs on the membrane insertion process of ΔN110-Pex5p. As shown in Fig. 1*C*, the amount of protease-resistant ΔN110-Pex5p decreases when the PNS fraction is preincubated with the anti-Pex14p antibody (Fig. 1*C*, compare *lanes 1* and *2* with *lane 3*).

Exactly the same inhibition can be observed when IgGs (data not shown) or Fab fragments directed to Pex13p are used in these *in vitro* experiments. The insertion of either Pex5p or ΔN110-Pex5p into the peroxisomal membrane is highly inhibited (Fig. 1*D*, compare *lanes 1* and *2* with *lane 3*). Most importantly, this inhibitory effect of anti-Pex13p Fab fragments is still observed when these two radiolabeled proteins are subject to import reactions using purified rat liver peroxisomes (Fig. 1*D*, compare *lanes 4* and *5* with *lane 6*). As described recently, isolated peroxisomes are not functional in catalyzing the export of Pex5p back to the cytosolic compartment (34). Thus, this result indicates that most (if not all) active docking/translocation sites of the peroxisomal membrane contain Pex13p (see "Discussion").

Taken together, these data indicate that insertion of ΔN110-Pex5p into the peroxisomal membrane is as specific as the insertion of the full-length peroxin. In addition, these data allow us to conclude that the first 110 amino acid residues of Pex5p do not play any crucial role in either cargo-protein binding or in the docking/translocation step of the Pex5p-cargo protein complex at the peroxisomal membrane.

Peroxisomal ΔN110-Pex5p Is Not a Substrate for the Peroxisomal Recycling Machinery—As noted above, the amount of protease-protected ΔN110-Pex5p obtained upon *in vitro* import experiments in the presence of exogenous ATP is remarkably higher than the one observed for the full-length peroxin. Considering that under these conditions full-length Pex5p enters and exits the peroxisomal membrane (34), this observation immediately suggested to us that the export step of ΔN110-Pex5p from the peroxisomal membrane could somehow be abnormal. To test this hypothesis, an *in vitro* pulse-

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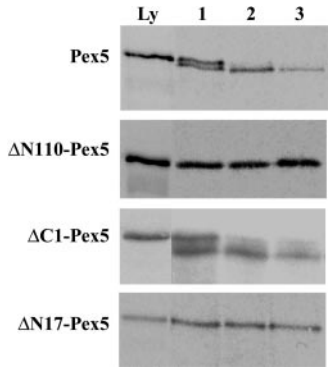

FIG. 2. **Peroxisomal export competencies of truncated versions of Pex5p.** *In vitro* synthesized Pex5p, ΔN110-Pex5p, ΔC1-Pex5p, and ΔN17-Pex5p were incubated with the PNS fraction (450 μg of protein in a 300-μl final volume) in the absence of exogenous ATP for 15 min at 26 °C. A vast excess of recombinant GST-Pex5p was then added to the reactions to block further insertion of the Pex5p proteins into the peroxisomal membrane, and 7 min later, an aliquot corresponding to 150 μg of PNS protein was removed (*lane 1*). ATP (5 mM final concentration) was then added to the import reactions. Aliquots of the import reactions (150 μg of PNS protein) were taken 8 and 23 min later (*lanes 2* and *3*, respectively). All the samples were treated with proteinase K and analyzed by SDS-PAGE autoradiography. *Lanes Ly*, radiolabeled proteins used in the *in vitro* reactions.

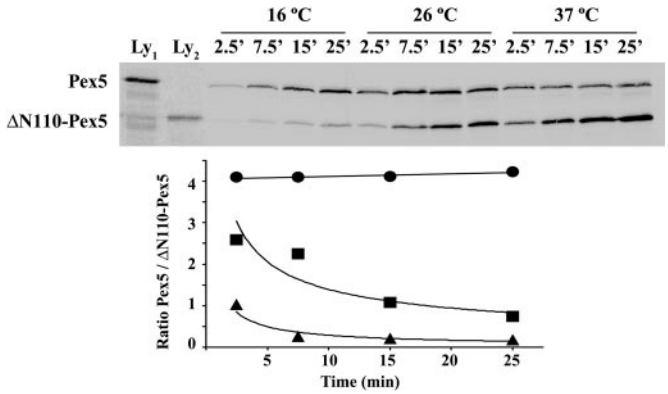

FIG. 3. **Export of Pex5p from the peroxisomal compartment, but not its insertion into the peroxisomal membrane, is blocked at low temperatures.** A PNS fraction (5.4 mg of protein) was incubated at 26 °C for 10 min in 600 μl of import buffer containing 5 mM ATP to release endogenous Pex5p from peroxisomal docking/translocation sites (34). Equal portions of this PNS fraction (1.8 mg of protein) were then added to tubes containing a mixture of ^{35}S-labeled Pex5p and ΔN110-Pex5p in 200 μl of import buffer supplemented with 5 mM ATP and an ATP-regenerating system, pre-equilibrated at 16, 26, or 37 °C. Aliquots of the three import reactions (corresponding to 450 μg of PNS protein) were removed at the indicated time points, subjected to proteinase K treatment, and analyzed by SDS-PAGE autoradiography. *Lanes Ly1* and *Ly2*, ^{35}S-labeled Pex5p and ^{35}S-labeled ΔN110-Pex5p, respectively, used in the import reactions. A densitometric analysis of protease-resistant Pex5p and ΔN110-Pex5p is also presented. The Pex5p/ΔN110-Pex5p ratios obtained at 16 (*circles*), 26 (*squares*), and 37 °C (*triangles*) are plotted against time.

chase import experiment was performed (39). Besides full-length Pex5p and ΔN110-Pex5p, a C-terminal truncated protein comprising amino acid residues 1–324 of Pex5p (ΔC1-Pex5p) was also included in this analysis. The import (but not the export) properties of ΔC1-Pex5p were described before (40). ^{35}S-Labeled Pex5p, ΔN110-Pex5p, or ΔC1-Pex5p were separately incubated with a PNS fraction for 15 min in the absence of exogenous ATP. At this time, a vast excess of recombinant GST-Pex5p was added to the import reactions to block further import of the ^{35}S-labeled proteins. Seven min later, an aliquot of each import reaction was taken (Fig. 2, *lane 1*), and immediately, ATP (5 mM) was added to the reactions. Aliquots were removed 8 (Fig. 2, *lane 2*) and 23 min later (*lanes 3*). As expected, the amount of peroxisomal ^{35}S-labeled Pex5p decreases over time indicating that Pex5p is capable of exiting the peroxisomal membrane (39). A similar result was obtained for ΔC1-Pex5p. In contrast, the amount of protease-protected ΔN110-Pex5p remains constant during the complete incubation time indicating that, indeed, ΔN110-Pex5p is unable to exit the peroxisomal compartment.

We have tried to define more precisely the amino acid residues of Pex5p required for the export step. This proved to be quite a difficult task. Indeed, several attempts to express *in vitro* several N-terminal truncated forms slightly longer than ΔN110-Pex5p were unsuccessful. Translation of these mRNAs resulted in either no protein synthesis or in the synthesis of truncated Pex5p products migrating faster than ΔN110-Pex5p upon SDS-PAGE (data not shown). Only in the last part of this work did we manage to obtain a correct, although weak, *in vitro* expression when using an mRNA coding for a truncated form of Pex5p lacking just the first 17 amino acid residues (ΔN17-Pex5p). The export competence of this protein was then assessed by using the pulse-chase strategy described above. The results of this analysis are also shown in Fig. 2. The amount of protease-resistant ΔN17-Pex5p remains constant during the complete incubation time as observed for ΔN110-Pex5p.

Two conclusions can be drawn from these results. First, amino acid residues 325–639 (the second half of Pex5p) are not required for the export step suggesting that this portion of the PTS1 receptor does not establish crucial interactions with the peroxisomal export machinery. Second, amino acid residues 18–110 *per se* are not sufficient to enable peroxisomal Pex5p to be exported back to the cytosolic fraction.

Import/Export of Pex5p into/from the Peroxisomal Membrane, Temperature Dependence—We are still far from being able to describe the Pex5p-mediated process of protein import into peroxisomes by using defined chemical equations. The complexity of the process is huge, and problems are encountered in the simple effort of defining the stoichiometry of the Pex5p-cargo protein interaction (48). Even more obscure is the nature of the interaction of the Pex5p-cargo protein complex with the docking/translocation machinery, virtually nothing is known in the chemical sense (*i.e.* number of steps, rate constants, etc.). Our knowledge of the export step of Pex5p from the peroxisomal membrane is not better. It has been shown that ATP hydrolysis is required at this step (34), but the nature of the ATPases involved or the need for additional factors is unknown. Surely one strategy to start addressing all these issues is to obtain some basic data regarding the kinetics of these processes. Here the effects of temperature on the peroxisomal import/export processes of Pex5p are described. Two observations prompted us to perform this analysis. The first is related to the finding that in our *in vitro* system the amount of protease-protected ^{35}S-labeled Pex5p is maximal at 26 °C, not at 37 °C (39), a more physiological value. The second observation regards the fact that Pex5p can be accumulated at the peroxisomal compartment *in vivo* just by decreasing the temperature of the cell cultures (35).

A PNS fraction was incubated at 26 °C for 10 min in the presence of 5 mM ATP in order to release endogenous Pex5p from the peroxisomal docking/translocation sites. Equal portions of this PNS fraction were then added to mixtures containing ^{35}S-labeled Pex5p and ΔN110-Pex5p in import buffer pre-equilibrated at 16, 26, or 37 °C. The amounts of protease-protected radiolabeled proteins as function of time in each of these three reactions were quantified. The results of this experiment are shown in Fig. 3. At 16 °C, a steady increase on the amounts of protease-protected Pex5p and ΔN110-Pex5p is observed. Most importantly, a graphical representation of the ratio of Pex5p to ΔN110-Pex5p as function of time yields a

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straight line with a slope close to 0. Considering that ΔN110-Pex5p is unable to exit the peroxisomal compartment, this result implies that full-length Pex5p is not exported from the peroxisomal membrane at this temperature. Quite different results are obtained at 26 or 37 °C. Under these conditions, a steady increase in the amounts of protease-protected ΔN110-Pex5p is still observed, as expected. However, the amounts of protease-protected Pex5p converge to steady-state values very rapidly, particularly at 37 °C. Accordingly, graphical representations of these data result in concave curves tending to a zero value. Exactly the same kinetic behavior was observed for both Pex5p and ΔN110-Pex5p when these experiments were performed using the radiolabeled proteins separately (data not shown).

Taken together these data provide the mechanistic explanation for the two observations cited above. By decreasing the temperature in the *in vitro* or the *in vivo* assays, one is preferentially inhibiting the peroxisomal export step of Pex5p. Obviously, this leads to the accumulation of Pex5p at the peroxisomal compartment.

The results presented in Fig. 3 reveal two other interesting features of the Pex5p cycling pathway. The first is related to the export rate of Pex5p from the peroxisomal membrane. Considering that a constant value for the amount of protease-protected Pex5p is reached within the first 2.5 min of incubation at 37 °C, implying that the export flux of Pex5p equals its import flux, then the half-life of the peroxisomal pool of Pex5p must be smaller than 2.5 min. The second finding derives from the results obtained at 16 °C. Although Pex5p is unable to exit the peroxisomal compartment at this temperature, no significant amounts of stage 3 Pex5p are detected under these conditions. This observation suggests that the transition of Pex5p from stage 2 to stage 3 is temperature-dependent. In order to confirm this property, the following experiment was performed. ^{35}S-Labeled Pex5p and ΔN110-Pex5p (used here as an internal control) were incubated with a PNS fraction for 15 min at 26 °C in the presence of 1 mM ATP. After blocking further import of the radiolabeled proteins by adding a vast excess of recombinant GST-Pex5p, equal portions of the reaction were transferred to tubes containing import buffer and ATPγS (10 mM final concentration) pre-equilibrated at 16, 26, or 37 °C. Aliquots of the import reactions were removed 8 and 23 min later and processed for SDS-PAGE. As shown in Fig. 4, incubation of PNS fractions containing stage 2 Pex5p at 16 °C in the presence of ATPγS does not result in the appearance of stage 3 Pex5p. In contrast, large amounts of stage 3 Pex5p are obtained at 26 and 37 °C. We conclude that conversion of stage 2 into stage 3 Pex5p is temperature-dependent.

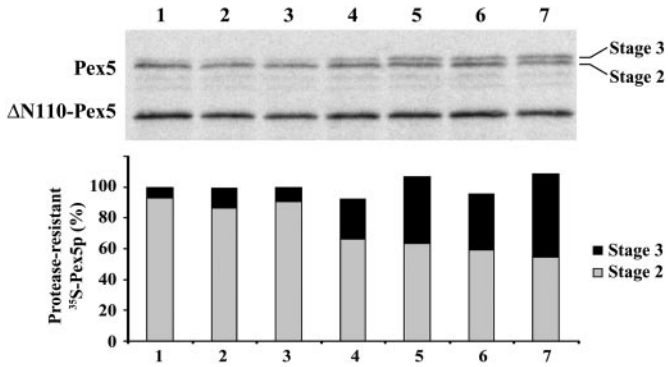

FIG. 4. **Transition of stage 2 Pex5p into stage 3 Pex5p is temperature-dependent.** A mixture of ^{35}S-labeled Pex5p and ^{35}S-labeled ΔN110-Pex5p was incubated with a PNS fraction in import buffer containing 1 mM ATP for 15 min at 26 °C. Recombinant GST-Pex5p was then added to the reaction, and 7 min later, an aliquot was removed (*lane 1*). Equal portions of this reaction were then transferred to tubes containing import buffer supplemented with ATPγS (10 mM final concentration) pre-equilibrated at 16 (*lanes 2* and *3*), 26 (*lanes 4* and *5*), or 37 °C (*lanes 6* and *7*). Aliquots of the import reactions were taken 8 (*lanes 2*, *4*, and *6*) and 23 min later (*lanes 3*, *5*, and *7*). All the samples were treated with proteinase K and analyzed by SDS-PAGE autoradiography. A densitometric analysis of protease-resistant Pex5p is presented in the graphic. The amounts of protease-resistant Pex5p (stage 2 plus stage 3 Pex5p) were normalized for the amount of ΔN110-Pex5p present in each lane. The amount of protease-resistant Pex5p in *lane 1* was set to 100%. The *gray portions* of the *bars* represent stage 2 Pex5p; *black portions* represent stage 3 Pex5p. *Numbers* in the *x* axis refer to the lanes of the autoradiography.

DISCUSSION

In this work we present mechanistic data regarding the Pex5p-mediated peroxisomal protein import pathway with emphasis on its last step, the ATP-dependent recycling step of Pex5p. It is shown that the first 110 amino acid residues of Pex5p are involved neither in cargo-protein binding nor in the docking/translocation step that occurs at the peroxisomal membrane. From the four experimental criteria used to support this conclusion (see Fig. 1), one is presented here for the first time and deserves some comments. This regards the inhibitory effect of anti-Pex13p antibodies on the peroxisomal import step of Pex5p. Pex13p is an intrinsic membrane component of the peroxisomal membrane probably involved in the recycling step of Pex5p back to the cytosolic compartment (45, 49). *In vitro* binding assays and yeast and bacterial two-hybrid experiments have shown that this peroxin is capable of interacting directly with Pex14p and Pex5p (50). Although there are no doubts that *in vivo* the interaction of Pex13p with Pex5p is transient, the same cannot be said regarding the Pex13p-Pex14p interaction. Indeed, when peroxisomal membranes from both rat and yeast are solubilized with the mild detergent digitonin only substoichiometric amounts of Pex13p can be found in association with Pex14p (29, 30). Two hypotheses were formulated to explain these observations: either the Pex13p-Pex14p interaction is too weak to resist the solubilization procedure or there is in fact a Pex13p protein complex (lacking Pex14p and other known peroxins) playing some still undefined role on the process of protein translocation across the peroxisomal membrane (30). The finding that anti-Pex13p Fab fragments block almost completely the insertion of Pex5p into the peroxisomal membrane implies that most (if not all) active docking/translocation sites of the peroxisomal membrane possess Pex13p and, thus, strongly support the first hypothesis.

Considering all the Pex5p protein interaction data published until now, the observation that the first 110 amino acid residues of Pex5p are involved neither in cargo-protein binding nor in the docking/translocation step is not surprising. Indeed, ΔN110-Pex5p still contains the cargo protein-binding site as well as all the binding sites for Pex7p, Pex12p, Pex13p and Pex14p (reviewed in Refs. 3 and 4). Unexpected, however, is the observation that ΔN110-Pex5p is not a substrate for the peroxisomal export machinery catalyzing the export of Pex5p back to the cytosol. Two major conclusions can be drawn from this result. The first regards the insertion step of Pex5p into the peroxisomal membrane. The fact that the amount of protease-protected ΔN110-Pex5p remains constant in our pulse-chase experiments (see Fig. 2) suggests that this step is an irreversible event. The second conclusion regards the role of Pex5p itself during the export step. *A priori*, two different mechanisms leading to the release of Pex5p from the docking/translocation machinery could be hypothesized. Pex5p could be a passive entity in this step, *i.e.* the ATPase promoting the export of Pex5p from the peroxisomal membrane could simply disassemble the docking/translocation machinery sequestering its Pex5p-interacting peroxins. Alternatively, Pex5p could play an active role in this process, *e.g.* Pex5p could be pulled from the

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docking/translocation machinery by the ATPase. By showing that a Pex5p domain with no role in the docking/translocation step is required for the export of Pex5p from the peroxisomal membrane, our data strongly favor this last possibility.

Experiments aiming at refining the Pex5p residues required for the export step indicate that residues 18–110 *per se* are not sufficient to sustain export of Pex5p from the peroxisomal membrane. This observation suggests that the first 17 amino acid residues of Pex5p play a major role in this process. In this context, it may be relevant to note that, although the N-terminal half of Pex5p is poorly conserved among different species, the sequence Cys-X_n-Asn-Pro-Leu (where X_n is any 3–9 amino acid residues) is found at the N termini of most Pex5p proteins (data not shown). Also interesting is the observation that similar motifs (Cys-X_n-Asn-(Ala/Gly)-(Leu/Ala)) are found at the N termini of *Neurospora crassa* and *Yarrowia lipolytica* Pex20p (19, 20). This peroxin is thought to escort the PTS2 receptor to the peroxisomal membrane, a role that in mammals and plants is performed by Pex5p (19–21). Whether this N-terminal domain of Pex20p is also crucial for the export of this peroxin from the docking/translocation machinery remains to be determined.

The reason why these residues of Pex5p are so important for its function remains unknown. However, it must be noted that there are now three independent observations pointing to the existence of a strong correlation between ATP, an active ATPase, and the export of Pex5p on one side and the first 15–20 amino acid residues of Pex5p on the other side. The first, as described here, is the fact that ΔN17-Pex5p is not a substrate for the export machinery. The second regards the observation that in our *in vitro* system, stage 3 Pex5p can only be detected in the absence of exogenous ATP or in the presence of nonhydrolyzable ATP analogues. As suggested recently (39), the difference between stage 2 and stage 3 Pex5p may reside simply in a protection effect performed by the ATPase catalyzing the export step of Pex5p over its first 20 amino acid residues. The third is that no stage 3 Pex5p can be detected in an incomplete *in vitro* system in which full-length Pex5p is not exported from the peroxisomal membrane. It was proposed that the export machinery in this *in vitro* system is either absent or inactive (34). Thus, all these observations converge to the same possibility, namely that the N terminus of Pex5p provides the binding site for the ATPase catalyzing its export.

The central role of the N terminus of Pex5p in the export step is also emphasized by the results obtained with ΔC1-Pex5p. Although this truncated protein contains only amino acid residues 1–324 of Pex5p, our data show that ΔC1-Pex5p is also a substrate for the export machinery. This observation implies that the second half of Pex5p, a region that contains the cargo-protein binding domain, is not involved in crucial interactions with the export machinery. Thus, the triggering signal for the export machinery does not reside in conformational alterations occurring at the C-terminal half of Pex5p, when the receptor releases its cargo on the luminal side of the peroxisomal membrane. Instead, such signal emerges from the N terminus of Pex5p. It is possible that at the end of the translocation step some of the membrane peroxins in contact with the N terminus of Pex5p suffer some conformational alterations. These structural rearrangements could, on the one hand, decrease the affinity of Pex5p to its cargo and, on the other hand, activate the ATPase-promoting Pex5p export. Possible candidates to carry these functions are already available. Pex13p in mammals and Pex13p and/or Pex8p in yeast are components of the docking/translocation machinery that are probably involved in late steps of the transport cycle (45, 49, 51). However, a more direct mechanism is also feasible. It is possible that only at the end of the translocation step Pex5p presents the stereochemical properties required by the ATPase.

The kinetics of the export step of Pex5p from the peroxisomal compartment was also characterized. It is shown that this step is highly dependent on temperature. A maximum of export activity was detected at 37 °C, whereas no activity could be detected at 16 °C. In contrast, insertion of Pex5p into the peroxisomal membrane could be detected even at this low temperature. These data provide the mechanistic explanation for the observation that Pex5p can be accumulated at the peroxisomal compartment *in vivo* just by decreasing the temperature of the cell cultures (35). Accumulation of Pex5p at the peroxisomal compartment does not result from a direct inhibition of the translocation step, as suggested earlier, but rather from a block in the export step of Pex5p. In our *in vitro* experiments, and possibly in the *in vivo* situation, this inhibition occurs at the step where stage 2 Pex5p is converted into stage 3 Pex5p. Finally, the results presented here suggest that the export step of Pex5p from the peroxisomal compartment is quite fast at 37 °C, with the peroxisomal pool of Pex5p displaying a half-life smaller than 2.5 min.

To conclude, we emphasize that the results and experimental tools described here pave the way to many new experiments. By exploring the unique properties of ΔN110-Pex5p, it is now possible to measure simultaneously the peroxisomal import and export fluxes of Pex5p. The specific effects of many other experimental variables on each of these processes can now be addressed.

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