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Insertion of Pex5p into the Peroxisomal Membrane Is Cargo Protein-dependent*

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It is now generally accepted that Pex5p, the receptor for most peroxisomal matrix proteins, cycles between the cytosol and the peroxisomal compartment. According to current models of peroxisomal biogenesis, this intracellular trafficking of Pex5p is coupled to the transport of newly synthesized peroxisomal proteins into the organelle matrix. However, direct evidence supporting this hypothesis was never provided. Here, using an *in vitro* peroxisomal import system, we show that insertion of Pex5p into the peroxisomal membrane requires the presence of cargo proteins. Strikingly the peroxisomal docking/translocation machinery is also able to catalyze the membrane insertion of a Pex5p truncated molecule lacking any known cargo-binding domain. These results suggest that the cytosol/peroxisomal cycle in which Pex5p is involved is directly or indirectly regulated by Pex5p itself and not by the peroxisomal docking/translocation machinery.

Peroxisomal matrix proteins are synthesized on free ribosomes and post-translationally imported into the organelle (for review, see Refs. 1 and 2). The vast majority of proteins destined to this compartment are recognized by Pex5p, the peroxisomal targeting signal 1 (PTS1)[1] receptor (3–5). Structurally Pex5p can be divided into two domains. The first half of the protein has been shown to be involved in an intricate network of protein-protein interactions (6–10). The peroxisomal targeting information of Pex5p resides in this domain (7). The C-terminal half of Pex5p is involved in binding the so-called PTS1 sequence, a degenerated tripeptide present at the C terminus of practically all peroxisomal matrix proteins (11–14).

The observation that Pex5p has a dual subcellular distribution soon led several authors to propose that Pex5p could be a cycling receptor (5, 15, 16). According to this model, newly synthesized PTS1-containing proteins would be recognized by cytosolic Pex5p. The cargo protein-Pex5p complex would then dock at the peroxisomal membrane, triggering translocation of the cargo protein across the organelle membrane. Somewhere during this process, Pex5p would be released from the membrane back to the cytosol to catalyze further transport cycles (for review, see Refs. 1 and 2).

Experimental data compatible with this model were first provided by Dodt and Gould (17); by manipulating temperature and ATP levels in cultured mammalian cells the intracellular distribution of Pex5p could be altered. More recently Dammai and Subramani (18) have shown that Pex5p goes through multiple rounds of cycling between the cytosol and the peroxisome. Thus, the two intracellular pools of Pex5p are interchangeable.

The fact that Pex5p cycles between the cytosol and the peroxisome has been considered the landmark observation in favor of the cycling receptor model. However, direct evidence showing that the intracellular movement of Pex5p is coupled to the transport of PTS1-containing proteins was never provided. Furthermore, by proposing that these two events are coupled, the model implicitly assumes the existence of regulatory mechanisms enabling cargo-loaded Pex5p molecules to be inserted into the peroxisomal membrane and excluding free Pex5p molecules from the peroxisomal docking/translocation machinery; otherwise a futile energy-requiring cycle would be possible. This constraint is difficult to conciliate with recent observations showing that Pex5p molecules lacking any known cargo-binding domain are specifically targeted to the peroxisome *in vivo* (7). Obviously several hypotheses can be forwarded to explain this phenomenon even in the light of the cycling receptor model. However, such a hypothesis will only be valid, and probably of utmost importance in understanding the mechanism of peroxisomal docking of Pex5p, when definite proof for a cargo-induced peroxisomal targeting of Pex5p is provided.

Recently we described a peroxisomal *in vitro* import system particularly suited to study Pex5p trafficking (19). In this work, using the same experimental approach, we present data strongly suggesting that insertion of Pex5p into the peroxisomal membrane is PTS1-dependent. Strikingly a truncated Pex5p molecule lacking any known cargo-binding domain is also a substrate for the machinery that drives insertion of Pex5p into the peroxisomal membrane. These results suggest that no crucial protein-protein interactions occur between the peroxisomal docking/translocation machinery (20–22) on one side and the cargo proteins or the Pex5p C-terminal receptor domain on the other. The implications of these observations on the mechanism regulating the docking/insertion of Pex5p into the peroxisomal membrane are discussed.

EXPERIMENTAL PROCEDURES

In vitro import experiments using rat liver PNS fractions were performed in import buffer (0.25 M sucrose, 50 mM KCl, 5 mM MOPS-KOH, pH 7.2, 3 mM $MgCl_2$, 1 mM EDTA-NaOH, pH 7.2, 0.2% (w/v) lipid-free bovine serum albumin, and 20 μM methionine) exactly as described previously (19). The synthesis of ^{35}S-labeled Pex5p (the large isoform) has already been described (19). cDNAs encoding ΔC1Pex5p and ΔC2Pex5p preceded by the T7 RNA polymerase promotor were obtained

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[1] The abbreviations used are: PTS, peroxisomal targeting sequence; GST, glutathione *S*-transferase; PNS, rat liver postnuclear supernatant; TPRs, Pex5p domain (amino acid residues 312–639) comprising its tetratricopeptide repeats; MOPS, 4-morpholinepropanesulfonic acid.

by PCR amplification of pGEM4-Pex5 (19) using the forward primer 5′-AGTCAGTGAGCGAGGAAGCGGAAGAGC-3′ and the reverse primers 5′-CGCGCTCTTTCATTAGTACCCCTTATCATA-3′ (for ΔC1Pex5p) or 5′-GTCCCGCTATTACGTGTGCTGCAGATCCTC-3′ (for ΔC2Pex5p). These cDNA fragments were then subjected to *in vitro* transcription/translation as described previously (19).

A recombinant protein containing GST fused to amino acid residues 312–639 of Pex5p (GST-TPRs) was obtained as follows. Plasmid pGEM4-Pex5p was subjected to PCR using the primers 5′-GCGAGAATTCATGGATGACCTTACGTCAGCTACCTATGA-3′ and 5′-GGGTCTAGAGCGGCCGCGTCGACCTGTCACTGGGGCAGGCC-3′. The amplified fragment was inserted into the *Eco*RI and *Not*I sites of pGEX-5X-1 plasmid (Amersham Biosciences). GST-TPRs and GST were expressed in the XL1-Blue strain of *Escherichia coli*, purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Biosciences), and dialyzed against the following solution: 0.25 M sucrose, 5 mM MOPS-KOH, pH 7.2, and 1 mM EDTA-NaOH, pH 7.2.

For the production of the fusion proteins GST-SKL and GST-LKS, the primers 5′-GATCCCCACAATTCCCAGGTCGATCCAAGCTTTGAGC-3′ and 5′-GGCCGCTCAAAGCTTGGATCGACCTGGGAATTGTGGG-3′ (for GST-SKL) and 5′-GATCCCCACAATTCCCAGGTCGACTTAAGTCCTAAGC-3′ and 5′-GGCCGCTTAGGACTTAAGTCGACCTGGGAATTGTGGG-3′ (for GST-LKS) were annealed. The DNA dimers were purified by non-denaturing PAGE, eluted from the gel, and ligated to pGEX-4T-3 (Amersham Biosciences) previously digested with the *Bam*HI and *Not*I restriction enzymes. The fusion proteins were expressed and purified as described above. The peptides CRYHLKPLQSKL (Pep-SKL) and CRYHLKPLQLKS (Pep-LKS) were synthesized by Sigma Genosys.

RESULTS AND DISCUSSION

Insertion of Pex5p into the Peroxisomal Membrane Is PTS1-dependent—In a recent work, we described a cell-free *in vitro* import system to study Pex5p association with and release from the peroxisomal compartment (19). In this work, we have used this experimental system to address a crucial issue in the field of peroxisomal biogenesis: is the intracellular cycling of Pex5p coupled to the transport of PTS1-containing proteins across the peroxisomal membrane?

As an attempt to solve this question, we first tried to determine whether or not import of Pex5p into the peroxisome could be stimulated by supplementing the import reaction with a PTS1-containing recombinant protein, GST-SKL. The concentration of GST-SKL in the import assays was 8 μM, a value more than 100-fold the dissociation constant reported for the Pex5p-PTS1 complex (14). Considering that Pex5p is a low abundance protein in rat liver (0.008% of total liver protein; calculated from the data in Ref. 20), this concentration of GST-SKL should ensure an almost complete saturation of Pex5p. As a negative control, we used GST-LKS, a glutathione *S*-transferase molecule containing at its C terminus a non-functional PTS1-like sequence (23). The results of this experiment are shown in Fig. 1*A*; no stimulation on the import of *in vitro* synthesized Pex5p could be observed.

We repeated this experiment but this time using a PTS1-containing peptide (Pep-SKL). The biological activity of this peptide is well documented (23, 24). Although the concentration of the peptide used in this experiment was 350-fold the dissociation constant of the Pex5p-PTS1 complex, again no stimulation on the peroxisomal import of Pex5p could be detected (see Fig. 1*B*).

There are two possibilities to explain this negative result: either insertion of Pex5p into the peroxisomal membrane is not coupled to the transport of cargo proteins or the cytosolic phase of the PNS fractions used in these import assays already has a sufficient amount of PTS1-containing proteins. Considering that 1) rat liver peroxisomes are particularly prone to disruption during tissue homogenization and represent 2% of total liver protein (25) and 2) peroxisomal proteins are not processed after import, *i.e.* they retain their peroxisomal targeting information (26), we reasoned that the second possibility should be tested.

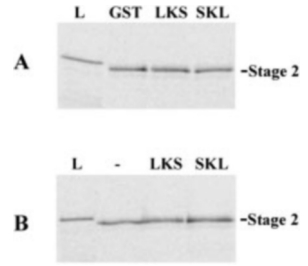

FIG. 1. **The basal level of peroxisomal *in vitro* import of Pex5p is not increased by PTS1-containing peptides/proteins.** *A*, PNS fractions were incubated with ^{35}S-labeled Pex5p in import buffer containing 5 mM ATP in the presence of 8 μM GST (*lane GST*), GST-LKS (*lane LKS*), or GST-SKL (*lane SKL*) fusion proteins for 30 min at 26 °C. Samples were then treated with proteinase K to degrade non-imported ^{35}S-labeled Pex5p, and the organelles were pelleted and subjected to SDS-PAGE. An autoradiograph of the gel is shown. *B*, PNS fractions were incubated with ^{35}S-labeled Pex5p in import buffer containing 5 mM ATP either in the absence (*lane* −) or in the presence of synthetic peptides (25 μM final concentration) CRYHLKPLQSKL (*lane SKL*) and CRYHLKPLQLKS (*lane LKS*). Samples were processed as described above. *Stage 2*, a peroxisomal population of Pex5p partially accessible to proteinase K (19). *Lane L*, ^{35}S-labeled Pex5p (10% of the input in each import reaction).

If insertion of Pex5p into the peroxisomal membrane occurs only when the PTS1 receptor is in a complex with cargo proteins, then sequestering these cargo proteins should inhibit the peroxisomal import of ^{35}S-labeled Pex5p. For this purpose a GST recombinant protein containing amino acid residues 312–639 of Pex5p (GST-TPRs) was produced. This domain of Pex5p contains the PTS1 binding activity of Pex5p but lacks its peroxisomal targeting information (7, 10).

As shown in Fig. 2, when *in vitro* synthesized Pex5p is subjected to an import reaction in the presence of 0.17 μM GST-TPRs, no protease-resistant Pex5p can be detected (Fig. 2, *A* and *B*, *lanes 6*). Adding the same amount of GST to the import reaction has no effect on the import of Pex5p into the organelle (Fig. 2, *lanes 3*; see also Fig. 3*B*). When these import assays are performed in the presence of a GST fusion protein (GST-LKS) or a peptide (Pep-LKS), both containing a non-functional PTS1-like sequence, the inhibitory properties of GST-TPRs on the *in vitro* import of Pex5p remain unchanged (Fig. 2, *lanes 5*). In sharp contrast, when GST-SKL or Pep-SKL are used under the same conditions this inhibition is partially reverted (Fig. 2, *lanes 4*; see legend to Fig. 2). The reason why a complete reversion is not observed is not known at the moment. It is possible that addition of GST-TPRs to PNS fractions not only titrates PTS1-containing proteins but also some other(s) component(s) necessary for the efficient targeting of Pex5p to the peroxisomal compartment. Although additional work will be necessary to clarify this matter the data presented here are clear in one point: insertion of Pex5p into the peroxisomal membrane is PTS1-dependent.

C-terminal Truncated Versions of Pex5p Are Substrates for the Peroxisomal Docking/Insertion Machinery—Recently Dodt *et al.* (7) have mapped the region of Pex5p responsible for its peroxisomal targeting. After transfection of human fibroblasts with plasmids encoding epitope-tagged N-terminal fragments of Pex5p the authors were able to show a peroxisomal location for a recombinant protein containing just the first 214 amino acid residues of Pex5p. Although the exact peroxisomal location of this recombinant protein was not defined in that study (*i.e.* no distinction between Pex5p molecules inserted into the peroxisomal membrane or just adsorbed at the surface of the organelle was made), the fact that this domain of Pex5p lacks any cargo protein-interacting domain and yet is correctly tar-

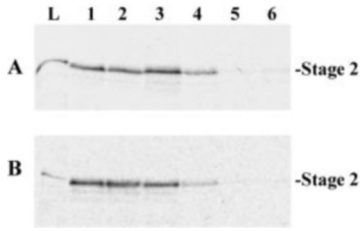

FIG. 2. **Insertion of Pex5p into the peroxisomal membrane is PTS1-dependent.** *In vitro* synthesized Pex5p was incubated with PNS fractions in the presence of 0.17 μM GST (*lanes 1–3*) or GST-TPRs (*lanes 4–6*). In *A*, GST-SKL (*lanes 1* and *4*) or GST-LKS (*lanes 2* and *5*) fusion proteins were also included in the import reactions (8 μM final concentration). In *B*, import reactions received Pep-SKL (*lanes 1* and *4*), Pep-LKS (*lanes 2* and *5*), or no peptide (*lanes 3* and *6*). Peptides were used at 25 μM final concentration. All import reactions were performed in the presence of 5 mM ATP. After 30 min at 26 °C, protein samples were processed as described in the legend to Fig. 1. The ratio of stage 2 Pex5p in *lanes 4* to *lanes 1* is 0.64 ± 0.11 (n = 4) and 0.31 ± 0.08 (n = 4) in *A* and *B*, respectively. *Stage 2* and *Lane L*, see the legend to Fig. 1.

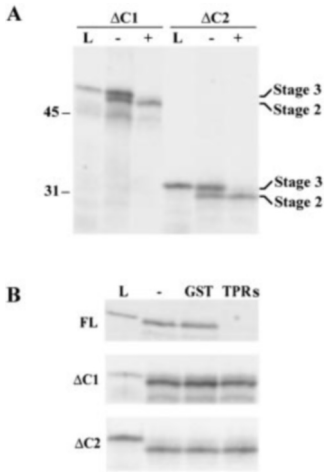

FIG. 3. **C-terminal truncated Pex5p molecules are substrates for the peroxisomal docking/insertion machinery.** *A*, *in vitro* synthesized ΔC1Pex5p and ΔC2Pex5p were subjected to import reactions in the presence (*lanes +*) or absence (*lanes −*) of exogenous ATP. After 30 min at 26 °C, protein samples were processed as described in the legend to Fig. 1. *Stage 3*, a peroxisomal population of Pex5p completely resistant to the action of proteinase K (19); *Stage 2*, see the legend to Fig. 1. *Lanes L*, ^{35}S-labeled ΔC1Pex5p or ΔC2Pex5p (1% of the input in each import reaction). The *numbers* at the *left* indicate the molecular masses of the applied standards in kDa. *B*, insertion of ΔC1Pex5p and ΔC2Pex5p into the peroxisomal membrane is not inhibited by GST-TPRs. ^{35}S-Labeled full-length Pex5p (*FL*) or the truncated versions ΔC1Pex5p (Δ*C1*) or ΔC2Pex5p (Δ*C2*) were incubated with PNS fractions in the absence (*lanes −*) or in the presence of GST (*lane GST*) or GST-TPRs (*lane TPRs*) fusion proteins. Import reactions were performed in the presence of 5 mM ATP. After proteinase K treatment, the organelles were isolated and subjected to SDS-PAGE. *Lanes L*, reticulocyte lysates containing ^{35}S-labeled full-length Pex5p (*panel FL*) or the ΔC1Pex5p and ΔC2Pex5p truncated versions (*panels* Δ*C1* and Δ*C2*, respectively).

geted to the peroxisome led us to investigate this phenomenon in more detail.

Thus, we tried to determine whether or not C-terminal truncated versions of Pex5p still retain the capacity of being inserted into the peroxisomal membrane. Two different ^{35}S-labeled proteins were synthesized: ΔC1Pex5p, amino acid residues 1–324 of Pex5p, and ΔC2Pex5p, amino acid residues 1–197 of Pex5p. Import reactions were performed in the presence or absence of exogenous ATP as described previously (19). The results of this experiment are shown in Fig. 3*A*. In both cases, protease-resistant species corresponding to stage 2 Pex5p (*lanes* + ATP) and stage 2 plus stage 3 Pex5p (*lanes* − ATP) can be easily detected (see legends to Figs. 1 and 3 and Ref. 19). This result could suggest that amino acid residues 1–197 of Pex5p are sufficient to drive insertion of Pex5p into the peroxisomal membrane. There is, however, another possibility. In our experimental system import of these truncated forms of Pex5p is performed in the presence of endogenous rat liver Pex5p. Recently it was suggested that Pex5p may form a homodimer or a homotetramer and that this polymeric Pex5p is probably the active form of the PTS1 receptor (27, 28). Thus, in principle, the ^{35}S-labeled proteins used here could interact with endogenous Pex5p, which in turn would drive insertion of ΔC1Pex5p and ΔC2Pex5p into the peroxisomal membrane. One easily testable prediction of such a hypothesis is that if import of full-length Pex5p is inhibited (*e.g.* by exploring the properties of GST-TPRs), then import of ΔC1Pex5p and ΔC2Pex5p should also be inhibited.

The results of this experiment are shown in Fig. 3*B*. GST-TPRs has no effect on the peroxisomal import of both ΔC1Pex5p and ΔC2Pex5p. Thus, our data confirm and extend the observations made by Dodt *et al.* (7): the N-terminal domain of Pex5p can be targeted to and inserted into the peroxisomal membrane. This observation may have major implications on the mechanism regulating the process of docking/insertion of Pex5p into the peroxisomal membrane. Indeed, the fact that ΔC2Pex5p is a substrate for the docking/insertion machinery suggests that no crucial protein-protein interactions occur between this machinery on one side and the receptor domain of Pex5p or the cargo proteins on the other. If this proves to be the case, then how is cycling of Pex5p regulated? We can think of only one possibility: Pex5p itself directly or indirectly regulates this cycle. Many different hypotheses can be envisaged to explain such a mechanism. For instance, binding of cargo proteins to the receptor domain of Pex5p could induce conformational alterations on the PTS1 receptor, activating (*e.g.* exposing) its peroxisomal targeting domain; this regulatory mechanism would have been lost in both ΔC1Pex5p and ΔC2Pex5p (*i.e.* the peroxisomal targeting domain in these truncated molecules would be constitutively active). Data suggesting that Pex5p N-terminal and C-terminal domains interact with each other are already available and could support this hypothesis (27). Alternatively cytosolic Pex5p is kept away from the peroxisomal compartment due to an interaction with some (still unknown) protein. Such a putative factor would only bind to cargo-unloaded Pex5p molecules through an interaction requiring the receptor domain of Pex5p (which is not present in both ΔC1Pex5p and ΔC2Pex5p).

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