

The Human Peroxisomal Targeting Signal Receptor, Pex5p, Is Translocated into the Peroxisomal Matrix and Recycled to the Cytosol

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Summary

Peroxisomal targeting signals (PTSs) are recognized by predominantly cytosolic receptors, Pex5p and Pex7p. The fate of these PTS receptors following their interactions on the peroxisomal membrane with components of docking and putative translocation complexes is unknown. Using both novel and multiple experimental approaches, we show that human Pex5p does not just bind cargo and deliver it to the peroxisome membrane, but participates in multiple rounds of entry into the peroxisome matrix and export to the cytosol independent of the PTS2 import pathway. This unusual shuttling mechanism for the PTS1 receptor distinguishes protein import into peroxisomes from that into most other organelles, with the exception of the nucleus.

Introduction

Proteins imported into the peroxisome matrix use either C-terminal tripeptide (PTS1) or N-terminal nonapeptide (PTS2) peroxisome targeting signals that are recognized by cognate receptors, Pex5p and Pex7p, respectively (Rachubinski and Subramani, 1995). In several eukaryotes, including mammals, these receptors are predominantly cytosolic where they bind to the PTS-containing cargo and subsequently ferry them to the peroxisome membrane (Dodt et al., 1995; Wiemer et al., 1995; Dodt and Gould, 1996).

Pex5p and Pex7p dock at the peroxisomal membrane via a complex of proteins consisting of Pex3p, Pex13p, Pex14p, and Pex17p (Subramani et al., 2000; Johnson et al., 2001). Additionally, Pex5p interacts with Pex10p and Pex12p (Chang et al., 1999; Okumoto et al., 2000), which, along with Pex2p, are zinc binding, ring-finger proteins. These zinc binding proteins act downstream of the docking complex and some or all of these proteins are postulated to constitute the translocation complex involved in peroxisomal matrix protein import.

Peroxisomal import has no strict parallels to the transport of proteins across membranes of the endoplasmic reticulum, mitochondrion, or the chloroplast. A striking difference between peroxisomal protein import and that into other organelles is that peroxisomes allow the entry of folded, oligomerized proteins, or even the “piggy back” import of non PTS-containing cargo into the or-

ganelle matrix (Glover et al., 1994; McNew and Goodman, 1994; Walton et al., 1995).

The predominantly cytosolic location of the PTS receptors in many organisms has raised questions regarding the dynamics of the PTS receptors during the import cycle. Do Pex5p and Pex7p shuttle their PTS-containing cargo from the cytosol to the surface of the peroxisome, and then return to the cytosol for another round of import (simple shuttle model), or do the PTS receptors enter and exit the peroxisome during each round of import (extended shuttle model) (Rachubinski and Subramani, 1995)? We demonstrate that Pex5p is translocated into the peroxisomal matrix and is capable of returning to the cytosol during the import cycle, as predicted by the extended shuttle model.

Results

Detection of Peroxisomal Entry and Exit of GFP and Pex5p Fusions

A peroxisome-specific, posttranslational modification was used to show that Pex5p enters the peroxisome matrix. From earlier studies on the protease responsible for the maturation of precursor thiolase (prethiolase) in rat liver, we knew that its activity is peroxisome specific, and that a minimal prethiolase processing site (PPS) (cleavage following the amino acid sequence AAPC) is functional, irrespective of its location, when transplanted to other peroxisomal matrix proteins. We therefore designed fusion proteins of the short form of human Pex5p (Pex5pS) containing the PPS, and used multiple experimental approaches to assess whether the proteins had entered the peroxisome matrix and recycled back to the cytosol.

Fusion constructs, engineered to allow detection of peroxisomal import and export, were stably integrated into immortalized cells derived from an individual with no peroxisome import defects (HeLa) and from patients with a Pex5p (Ala-T) or Pex7p deficiency (Bro-T). The defined peroxisomal matrix protein import deficiencies in Ala-T and Bro-T cells allowed us to study the shuttling phenomenon with greater clarity.

The fusions (Figure 1A) contained, from the N terminus, the following features: a wild-type (PTS2wt) or mutant PTS2 (PTS2mut) sequence, the prethiolase processing site (arrow), the FLAG epitope (sequence DYKDDDDK) for detection of the unprocessed and processed forms, and a reporter (either EGFP or Pex5p). The processing events at the PPS were observed using either M2 or M1 antibodies, which recognize the FLAG epitope in specific contexts within a protein. The M2 antibody recognizes the FLAG epitope in any context and should, therefore, detect both unprocessed and processed forms of the fusion proteins. The M1 antibody, however, recognizes the FLAG epitope only when it is present at the extreme N terminus of the protein because it requires an N-terminal Asp, which is exposed only upon correct proteolytic processing at the PPS. Therefore, it is diagnostic of proper cleavage at this site by the prethiolase protease.

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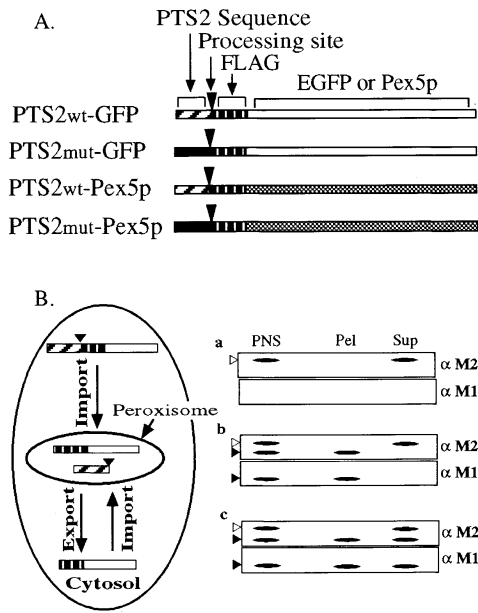


Figure 1. Constructs and Strategy used to Demonstrate Pex5p Shuttling

(A) Fusion constructs of EGFP and Pex5p stably integrated into HeLa, Ala-T, and Bro-T cells. Relevant components and epitopes, and their order within the fusion proteins are indicated.

(B) Strategy used to detect peroxisomal entry and exit of the fusion proteins using subcellular fractionations and immunoblotting with M1 and M2 antibodies. The diagram on the left represents the import into, and export out of, peroxisomes for the fusion proteins, while panels a, b, and c (right) show schematic gels of the expected subcellular distributions of the proteins in immunoblotting with M1 and M2 antibodies. Details are in the Results section.

Unimported, and therefore unprocessed, fusion proteins would be cytosolic and be recognized only by the M2, but not by the M1, antibody. In subcellular fractions obtained by differential centrifugation (Experimental Procedures), the unprocessed form would be detected by the M2 antibody in PNS and Sup fractions, and not in the Pel fraction, if processing by the prethiolase protease was efficient (Figure 1B, panel a). If the substrate was imported into peroxisomes (either via the PTS2 sequence or an unidentified PTS within the reporter protein), it would be cleaved at the PPS by the peroxisome-specific, prethiolase protease. This would generate a smaller, processed protein, which would be detected by the M1 antibody. Assuming incomplete import of the substrate into the peroxisomes and no export, the M2 antibody would recognize both the unprocessed and processed forms in PNS and Sup fractions and only the processed form in the Pel fraction, whereas the M1 antibody would detect the processed form in the PNS and Pel fractions (Figure 1B, panel b). Finally, if the substrate was imported into peroxisomes and exported back into the cytosol, the processed form would be recognized by both antibodies in the PNS, Pel, and Sup fractions (Figure 1B, panel c). It should be noted that the smaller size of the processed form of the reporter fusion, its recognition by the M1 and M2 antibodies, and its localization in the Pel fraction, together, demonstrate peroxisomal import, whereas its smaller size, recogni-

tion by M1 and M2 antibodies, and location in the Sup fraction show export from the peroxisome matrix to the cytosol.

The use of a substrate without an export signal (GFP) showed that artifactual export into the cytosol did not occur as a result of leaky peroxisomes. Rigorous controls established that the prethiolase protease is peroxisome specific, and does not leak out in a functional form into the cytosol (see below).

Peroxisomal Targeting of GFP Fusions Is Dependent on the PTS2 Pathway

Using fluorescence microscopy in HeLa cells, GFP-SKL, as well as the PTS2wt-GFP fusion, were found to be targeted to peroxisomes via the PTS1 and PTS2 import pathways, respectively. In contrast, the PTS2mut-GFP fusion remained cytosolic in HeLa, Ala-T, and Bro-T cells, showing that peroxisomal targeting of the PTS2-GFP fusions requires the PTS2 pathway (data not shown).

In Ala-T cells, both GFP-SKL and PTS2mut-GFP were cytosolic, but PTS2wt-GFP was peroxisomal, indicating an intact PTS2, but a defective PTS1, import pathway. In Bro-T cells lacking a functional PTS2 receptor, both PTS2-GFP fusions were cytosolic while GFP-SKL was peroxisomal, indicating a functional PTS1, but a defective PTS2, pathway (data not shown).

The behavior of the GFP fusions was also analyzed independently by immunoblot analysis (Figure 2). The PTS1-dependent peroxisomal marker, catalase, was in the Pel fraction of HeLa (Figure 2A) and Bro-T cells (Figure 2C), but in the Sup fraction of Ala-T cells (Figure 2B). The PTS2wt-GFP fusion was in the organelle pellet in HeLa (Figure 2A, left panel) and Ala-T cells (Figure 2B, left panel), indicative of its targeting to peroxisomes via the PTS2 pathway. In contrast, PTS2mut-GFP was not associated with the peroxisomes and was found in the Sup fraction of HeLa (Figures 2A and 2B, right panels), and Bro-T cells (Figure 2C, right panel). Note that catalase import into peroxisomes was normal, as expected in Bro-T cells, but PTS2wt-GFP was cytosolic and unprocessed (Figure 2C, left panel). The absolute requirement for both a functional PTS2 sequence and Pex7p for peroxisomal localization of the PTS2wt-GFP fusion, and the correlation between pelletable GFP fusions and their peroxisomal localization, show that these proteins detected in the pellet are not in aggregates or on other membranes.

Peroxisome-Specific Processing of PTS2wt-GFP Requires the PTS1 Import Pathway

We asked whether entry into the peroxisomal matrix could be detected using cleavage by the prethiolase protease and simultaneously sought evidence for the peroxisome-specific action of this protease. Subcellular fractions from HeLa cells stably expressing the PTS2-GFP fusions were immunoblotted with M2 and anti-catalase antibodies. Untransfected cells did not express any GFP fusion (Figures 2A–2C). Both unprocessed and processed forms were detected in HeLa cells expressing PTS2wt-GFP (Figure 2A, left panel) and colocalized in the organelle pellet with peroxisomal catalase.

In HeLa cells, the absence of any processed

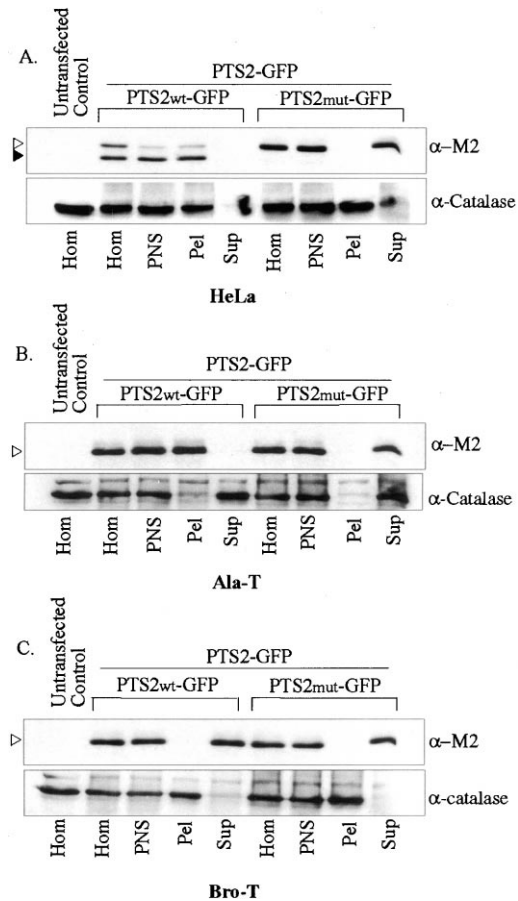


Figure 2. Processing and Subcellular Locations of PTS2-GFP Fusions

Untransfected cells or (A) HeLa, (B) Ala-T, or (C) Bro-T cells, expressing either PTS2wt-GFP or PTS2mut-GFP fusions, were subjected to differential centrifugation, and immunoblotted with M2 and anti-catalase antibodies. Open and solid arrows denote the unprocessed and processed forms of the fusion protein, respectively. Hom, cell homogenate.

PTS2mut-GFP shows that no processing of the fusion protein can occur at the PPS while the protein is cytosolic either during its synthesis or prior to its transit to the peroxisome matrix (Figure 2A, right panel). It is also evident that the prethiolase protease did not leak out in a functional form from the peroxisomal matrix. The peroxisomal marker catalase was primarily in the organelle pellet showing that peroxisomes were intact. These conclusions are supported by additional experiments in Ala-T and Bro-T cells where PTS2mut-GFP was cytosolic, but unprocessed (Figure 2B and 2C, right panels).

Expression of PTS2-GFP fusions in Ala-T cells provided insight into the import pathway used by the prethiolase protease. In Ala-T cells, even when the PTS2wt-GFP fusion was localized to peroxisomes, it remained unprocessed, suggesting the absence of the prethiolase protease in the peroxisome matrix. In contrast, in Pex7p-deficient Bro-T cells (Figure 2C), both the PTS2-GFP fusions were cytosolic and unprocessed. These results strongly imply that the protease depends on a functional PTS1 pathway for entry into peroxisomes.

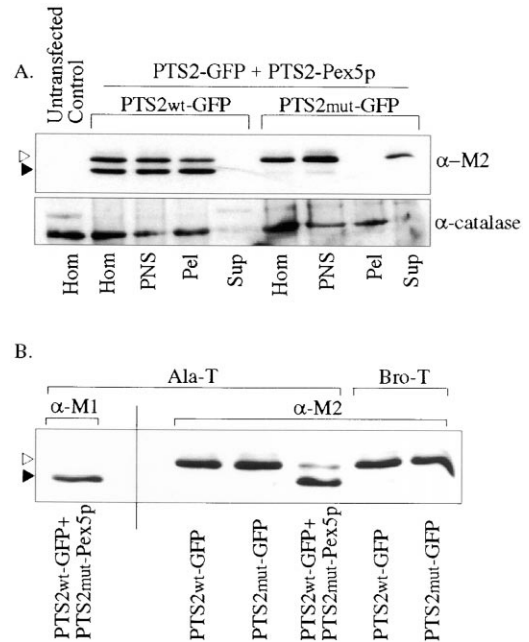


Figure 3. Peroxisomal Entry of PTS2wt-GFP and Specificity of the Prethiolase Protease

(A) Complementation of the PTS1 import defect in Ala-T cells coexpressing the PTS2-GFP and PTS2mut-Pex5p fusions. Differential centrifugation fractions were immunoblotted with M2 or anti-catalase antibodies.

(B) Peroxisome-specific cleavage of the PTS2wt-GFP fusion by the prethiolase protease. Cell lysates expressing the constructs were immunoblotted with either M1 or M2 antibodies, as shown. Symbols are as in Figure 2.

To further test this possibility and to document peroxisomal entry of the fusion protein, we coexpressed PTS2mut-Pex5p in Ala-T cells producing PTS2-GFP fusions. The coexpression of PTS2mut-Pex5p in Ala-T cells made no difference to either the processing or subcellular location of PTS2mut-GFP, as expected, but it did indeed restore the proper targeting of catalase to the Pel fraction (Figure 3A, right panel). However, upon coexpression of the Pex5p fusion with PTS2wt-GFP, this fusion protein was processed, and its cleavage coincided with the restoration of the PTS1 import pathway, as shown by the presence of catalase in the Pel fraction (Figure 3A, left panel). This is additional evidence that the prethiolase protease uses the PTS1 pathway for peroxisomal entry.

The absence of cytosolic prethiolase protease activity, when this enzyme is presumably mislocalized to the cytosol in Ala-T cells (Figure 2B, right panel), shows that this protease functions exclusively inside the peroxisomes. The peroxisome-specific cleavage of the reporter is therefore a diagnostic hallmark of entry into the peroxisomal matrix.

The Peroxisome-Specific Prethiolase Protease Activity Is Sequence Dependent

The protease activity was sequence specific because, in Ala-T cells coexpressing PTS2mut-Pex5p, the processed form of PTS2wt-GFP was detected by the M1

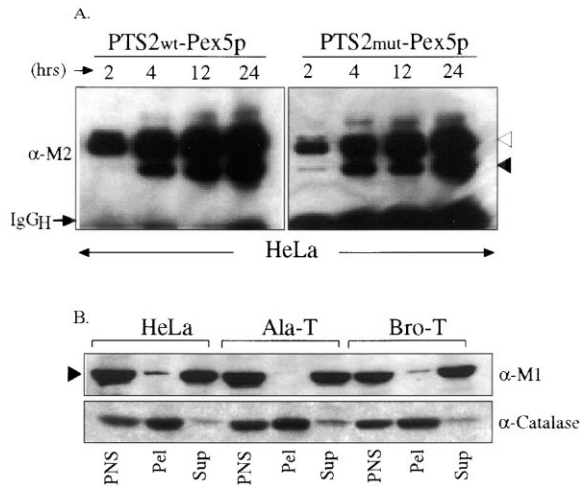


Figure 4. Pex5p Enters and Exits the Peroxisomal Matrix Independent of a Functional PTS2 Import Pathway

Arrow symbols are as in Figure 2. (A) Expression levels and processing at the PPS of PTS2-Pex5p fusions in HeLa cells. Cell lysates at the indicated time points were immunoprecipitated with anti-Pex5p antibody and blotted with M2 antibody. (B) Subcellular fractions of HeLa, Ala-T, or Bro-T cells expressing PTS2mut-Pex5p were immunoblotted with M1 and anti-catalase antibodies.

antibody (Figure 3B). Several experiments (Figures 2B, 2C, and 3B) in which only the unprocessed form of the PTS2-GFP fusions (expressed in either Ala-T or Bro-T) accumulated indicated that the uncleaved form could only be detected by M2, and not by M1, antibody. Both the processed and unprocessed forms were detected by the M2 antibody, as expected (Figure 3A, left panel). The appearance of the processed form was accompanied by restoration of the PTS1 import pathway in Ala-T cells (Figure 3A, left panel), and the detection of the processed form by the M1 antibody (Figure 3B, lane 1). This is evidence that the lower molecular weight species is the processed PTS2wt-GFP and that the processing occurs specifically at the PPS. These data also prove that the processing is due to the prethiolase protease because it occurs only at the known cleavage site for this enzyme.

The PTS2wt-GFP Fusions Remain in the Peroxisome and Fail to Exit into the Cytosol

Analysis of subcellular fractions by immunoblotting with M2 antibody indicated that when peroxisomes were intact, no cytosolic processed form of PTS2wt-GFP was detected (Figure 3A, left panel). Therefore, the fusion protein enters the peroxisome matrix using its PTS2 sequence and is cleaved there by the protease, but cannot return to the cytosol.

The PTS2-Pex5p Fusions Enter the Peroxisome Matrix in a PTS2-Independent Manner

We then addressed whether Pex5p could enter and exit the peroxisomal matrix. Examination of the expression pattern of PTS2-Pex5p in HeLa cells, following induction with doxycyclin, allowed us to choose the 24 hr time point for most experiments (Figure 4A). In these cells, the PTS2-Pex5p fusions were processed, indicative of

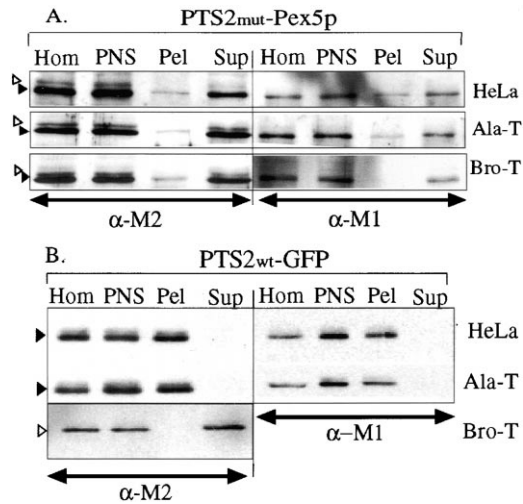


Figure 5. The PTS2mut-Pex5p and PTS2wt-GFP Fusions Enter the Peroxisome Matrix, but Only the PTS2mut-Pex5p Fusion Recycles back to the Cytosol

The fusion proteins, (A) PTS2mut-Pex5p and (B) PTS2wt-GFP, were coexpressed in HeLa, Ala-T, or Bro-T cells and the subcellular distributions of the processed and unprocessed forms of these fusion proteins were analyzed by immunoblotting of the same samples with M1 and M2 antibodies.

entry into the peroxisome matrix. Additionally, the processing of these fusion proteins was independent of a functional PTS2 sequence since both PTS2wt-Pex5p and PTS2mut-Pex5p exhibited equivalent amounts of the unprocessed and processed forms using the M2 antibody (Figure 4A).

Pex5p Cycles between the Peroxisome Matrix and the Cytosol

In cells stably expressing PTS2mut-Pex5p, we tested whether the processed Pex5p in the peroxisomal matrix could recycle back to the cytosol. Analysis of subcellular fractions, from HeLa, Ala-T, and Bro-T cells expressing PTS2mut-Pex5p, indicated unequivocally that the processed form was detected specifically by M1 antibody and that it was predominantly cytosolic (Sup fraction) (Figure 4B). Catalase was peroxisomal (Figure 4B), confirming the integrity of the peroxisomes. The observed subcellular distribution of catalase was similar in all three cell lines because while HeLa and Bro-T have a functional PTS1 pathway, the Ala-T cells were complemented by the PTS2mut-Pex5p fusion for PTS1 import. The combined results demonstrate that the Pex5p fusion enters the peroxisome matrix and recycles back into the cytosol.

Additional experiments, employing HeLa, Ala-T, and Bro-T cells coexpressing PTS2mut-Pex5p and PTS2wt-GFP fusions, confirmed that Pex5p enters and exits the peroxisome matrix while PTS2wt-GFP only enters the organelle. In these experiments, PTS2wt-GFP was used to demonstrate the integrity of peroxisomes isolated from the same cells expressing the Pex5p fusions.

Three important observations were made in these experiments (Figure 5A). First, the PTS2mut-Pex5p fusion was processed, indicating entry into the peroxisome

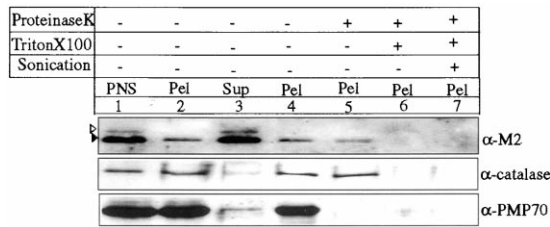


Figure 6. Protease Protection Assay Showing That a Portion of Peroxisome-Associated PTS2mut-Pex5p Is inside Peroxisomes
Lanes 1–4 show the distribution, in subcellular fractions of HeLa cells, of peroxisomal matrix (catalase) and membrane markers (PMP-70) and of the unprocessed and processed forms of PTS2mut-Pex5p. Proteins in the pellet fractions were treated with Proteinase-K, with or without sonication and detergent (Triton X-100). Samples were subjected to SDS-PAGE and immunoblotting with appropriate antibodies.

matrix. Second, at similar induction times, the amounts of the processed forms of PTS2mut-Pex5p were comparable in all three cell lines, showing that the entry of Pex5p into peroxisomes is independent of the PTS2 pathway. Finally, most of the processed forms were cytosolic, as detected by both M2 and M1 antibodies, and only a minor fraction was associated with the Pel fraction, demonstrating export from the peroxisome to the cytosol. In the same cells, the PTS2wt-GFP fusion was completely processed in HeLa and Ala-T cells, but not in Bro-T cells where it remained in the cytosol. More importantly, the processed PTS2wt-GFP was found solely in the Pel, and not in the Sup, fraction (Figure 5B, see results with M2 and M1), and so was catalase (data not shown), indicating that peroxisomes were intact and that the observed predominantly cytosolic localization of PTS2mut-Pex5p was not due to its leakage from peroxisomes. This is strong evidence that Pex5p enters and exits the peroxisome, even when catalase and the processed PTS2wt-GFP are strictly peroxisomal.

The Processed Pex5p Associated with Peroxisomes Is Partially Protease Resistant

Protease protection is a reliable method for investigating whether a given protein, present in the peroxisomal fraction, is inside the matrix or associated on the surface of the peroxisomal membrane. The protease protection assay (Figure 6) demonstrated that the peroxisome-associated, processed PTS2mut-Pex5p was partially resistant to protease. The complete disappearance of cytosolically exposed PMP70 and the total protection of catalase, under the same conditions, indicated that the conditions used did not rupture the peroxisomes. The slight decrease in the amount of Pex5p and the complete protease resistance of catalase (Figure 6, compare lanes 4 and 5) suggested that a fraction of the processed form of Pex5p is present on the outside of the peroxisomal membrane. This implies that the previously cycled Pex5p (processed form) can undergo another round of peroxisomal docking. The complete degradation of Pex5p and catalase, upon sonication or addition of detergent, strongly suggests that the Proteinase K-resistant fraction of Pex5p was indeed in the peroxisomal matrix.

Bimodal Distribution of the PTS2-Pex5p Fusion between Peroxisomes and Cytosol

Indirect immunofluorescence with M1 and M2 antibodies provided additional evidence of Pex5p cycling. We used defined concentrations of either digitonin alone, followed by fixation, to permeabilize just the plasma membrane but not the peroxisomal membrane, or with Triton X-100 to permeabilize both membranes before fixation and incubation with appropriate antibodies. The efficacy of these permeabilization conditions was demonstrated using HeLa cells (Figure 7A). Since PMP70 is on the cytosolic face of the peroxisomal membrane, both permeabilization conditions allowed its detection on punctate peroxisomes using anti-PMP70 antibody. In contrast, catalase, a peroxisomal matrix protein, was not detected upon permeabilization with digitonin alone, but was in punctate peroxisomes using Triton X-100 (Figure 7A).

Upon permeabilization of both plasma and peroxisomal membranes with Triton X-100, both M1 and M2 antibodies revealed a predominantly cytosolic, as well as punctate signals, for the Pex5p fusion suggestive of peroxisomal localization (Figure 7B). The punctate signals corresponding to the exposed M1 epitope did colocalize with PMP70 in many cells (Figure 7B), showing that some of the processed Pex5p was peroxisome associated.

Upon selective permeabilization of only the plasma membrane with digitonin, the cytosol could be washed out. The cells were then fixed and the peroxisomal membrane was permeabilized with Triton X-100. This procedure reduces the predominantly cytosolic fluorescence observed with M1 and M2 antibodies (Figure 7B). Under these conditions, a pronounced punctate signal was observed in all the cells, with both M1 and M2 antibodies (Figure 7C). Both these signals colocalized with PMP70, showing that the punctate signals correspond to peroxisomes. These experiments support the biochemical experiments and prove that processed Pex5p associates with peroxisomes. However, because weak punctate signals were also seen with both M1 and M2 antibodies using digitonin alone (data not shown), we conclude that a detectable amount of processed Pex5p was docked on the cytosolic face of the peroxisome, suggestive of successive rounds of peroxisomal entry and exit.

Kinetics of Cycling of Pex5p Fusions into and out of Peroxisomes

The kinetics of shuttling of Pex5p were determined by pulse-chase experiments. HeLa cells expressing PTS2mut-Pex5p were induced with doxycyclin and simultaneously metabolically labeled for 3 hr at 37°C. At various chase times, the cells were subjected to differential centrifugation, and the subcellular fractions were immunoprecipitated with M2 antibodies.

The autoradiogram of samples analyzed by SDS-PAGE revealed a decrease in the unprocessed form over the chase period and a corresponding increase in the processed forms in the Pel and Sup fractions (Figure 8A). The control, catalase, was exclusively in the peroxisome fraction (data not shown), indicating that peroxisomes were intact. The sustained increase in the processed form of Pex5p fusion indicates that, in normal cells, the

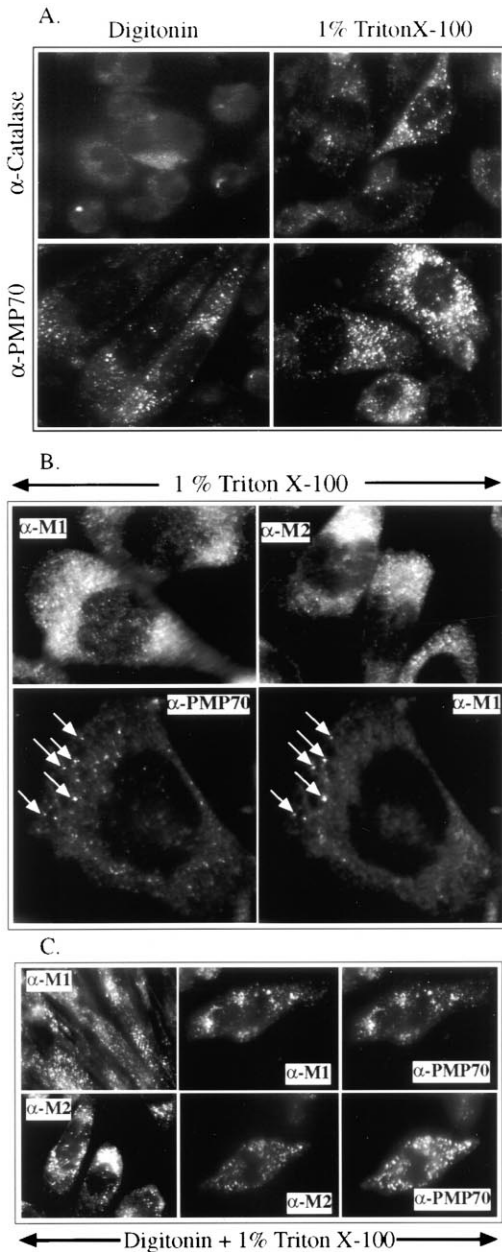


Figure 7. Colocalization of the Processed Form of PTS2mut-Pex5p with Peroxisomes in HeLa Cells

(A) Cells expressing the PTS2mut-Pex5p fusion were treated either with digitonin (25 μ g/ml) alone (Experimental Procedures), to permeabilize only the plasma membrane (left panels), or with Triton X-100 (1%) to permeabilize both plasma and peroxisomal membranes (right panels). Indirect immunofluorescence was performed with antibodies against the peroxisomal matrix and membrane markers, catalase (left panels) and PMP-70 (right panels), respectively.

(B) Indirect immunofluorescence with M1 (top left and bottom right panel), M2 (top right panel), and anti-PMP70 antibodies (bottom left panel) and colocalization of the processed form of PTS2mut-Pex5p with PMP70 (lower left and right panels) in cells permeabilized with Triton X-100.

(C) Detection of the intraperoxisomal processed form of PTS2mut-Pex5p employing sequential permeabilization of the plasma membrane with digitonin (25 μ g/ml), followed by washes to remove the cytosol, and subsequent permeabilization of the peroxisomal mem-

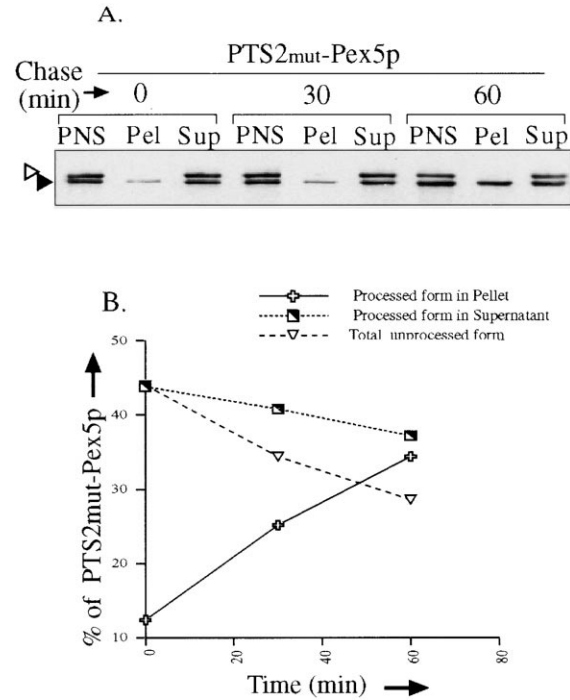


Figure 8. Pulse-Chase Analysis of Peroxisomal Import and Export of the PTS2mut-Pex5p Fusion Protein in HeLa Cells

Conditions for the pulse-chase are in Experimental procedures. Subcellular fractions obtained at each time point were immunoprecipitated using M2 antibodies, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography. (A) Autoradiogram showing the amount of processed and unprocessed forms of PTS2mut-Pex5p associated with subcellular fractions over the chase period. (B) Densitometric analysis of the pulse-chase results.

peroxisomal entry of Pex5p is a continuous processes, strengthening the argument that Pex5p enters the peroxisomal lumen as part of its normal functional cycle. After the 60 min chase, not all the unprocessed form had entered the peroxisomes and undergone processing, probably because of competition between the unprocessed Pex5p fusion and the processed and endogenous forms of Pex5p for reentry into peroxisomes.

Densitometric analysis of the pulse-chase data revealed certain interesting aspects about shuttling. Because the Pex5p fusion is synthesized during the 3 hr pulse, and some of it shuttles into and out of peroxisomes during this time, substantial processing of the Pex5p fusion was seen at 0 min of chase. The total unprocessed form decreased exponentially over the entire chase period with a half-time of about 90 min, accompanied by a corresponding increase in the total processed form (Figure 8B). Furthermore, the increment in the processed form in Pel fraction between the time points (Figure 8B, compare amounts in Pel at 0, 30, and 60 min) could be accounted by the combined decrease from the Sup fraction of the unprocessed and processed

brane with Triton X-100 (1%). Indirect immunofluorescence was performed with M1, M2, and anti-PMP70 antibodies. The central and right panels show colocalization in the same cells.

forms. The reduction of the processed form from the Sup, and its appearance in the Pel fraction, show that the processed form of Pex5p can undergo additional rounds of cycling through the peroxisome matrix. Furthermore, it supports the idea that Pex5p is not destined for degradation, as a dead-end intermediate might be, either in the peroxisome matrix or the cytosol.

Discussion

The PTS receptors, Pex5p and Pex7p, have been reported to be localized either predominantly in the cytosol (Dodt et al., 1995; Wiemer et al., 1995; Elgersma et al., 1996; Gould et al., 1996) or inside the peroxisomes (Szilard et al., 1995; Zhang and Lazarow, 1996) in different organisms. The varying subcellular locations of these receptors have influenced models regarding their role in peroxisomal protein import. The intraperoxisomal Pex5p or Pex7p observed in some organisms has been interpreted to mean that these receptors pull PTS-containing cargo across the peroxisomal membrane, in a manner analogous to the action of heat shock proteins as chaperones within the mitochondrial matrix to facilitate protein import (Szilard et al., 1995; Zhang and Lazarow, 1996). This intraperoxisomal chaperone model is very different from the more prevalent view that in *S. cerevisiae*, *P. pastoris*, CHO, and human cells, Pex5p is primarily cytosolic and acts by delivering PTS-containing cargo from the cytosol to the peroxisome. There are no experimental data that address how this shuttling might occur, although the “simple shuttle” and “extended shuttle” models represent hypothetical possibilities. We present conclusive evidence that, in the context of the three models for Pex5p function (intraperoxisomal chaperone, simple shuttle, and extended shuttle), Pex5p operates via the extended shuttle mechanism.

Features of the Strategy Used to Show Pex5p Shuttling in Human Cells

There are three important elements in the method we used to demonstrate Pex5p shuttling in human cells—(1) we were able to successfully drive the import of the PTS2wt-GFP fusion into the peroxisome matrix by the use of a PTS2 sequence from thiolase, (2) we show that the prethiolase protease exhibits peroxisome-specific cleavage of the reporter fusions at the PPS, and (3) the PTS2wt-GFP fusion can enter, but not exit, peroxisomes. The import of the PTS2-GFP fusions into the peroxisomes is strictly dependent on the presence of a wild-type PTS2 sequence and on the presence of a functional PTS2 receptor, Pex7p. This is proved by (a) the targeting of the PTS2wt-GFP, but not the PTS2mut-GFP, fusion to peroxisomes (Figures 2A and 2B) in HeLa and Ala-T cells, (b) the cleavage of the PTS2wt-GFP, and not the PTS2mut-GFP, fusion in peroxisomes in HeLa cells (Figure 2A, compare left and right panels), and (c) the lack of peroxisomal targeting of both PTS2-GFP constructs in Pex7p-deficient Bro-T cells (Figure 2C). The strict peroxisome specificity of the prethiolase protease is supported by the absence of any cleavage of cytosolically localized PTS2mut-GFP in HeLa cells (Figure 2A, right panel), or of PTS2wt-GFP and PTS2mut-GFP fusions in Bro-T cells (Figure 2C). Furthermore, in

view of the fact that the peroxisomal targeting of the prethiolase protease is dependent on a functional PTS1 pathway (Figure 2B, left panel), it is remarkable that in Ala-T cells, there is no cleavage of the cytosolic PTS2mut-GFP fusion, even when the protease is also likely to be cytosolic. Finally, as shown in Figure 3B, the cleavage by the prethiolase protease exposes the M1 epitope on the reporter fusions, proving that the protease acts only at the PPS and nowhere else on the reporter fusions. The inability of the PTS2wt-GFP fusion to be exported out of peroxisomes after its cleavage is shown by the absence of any processed fusion protein in the Sup fraction (Figures 2A and 3A, left panels).

Evidence for the Extended Shuttle Model for Pex5p Function

The shuttle mechanism requires that Pex5p enter and exit the peroxisome in a manner that represents a functional cycle. Our demonstration of Pex5p entry into the peroxisome matrix was based on multiple experimental approaches that include the peroxisome-specific cleavage of Pex5p fusion proteins, detection of the cleaved form of the Pex5p fusion by M1 antibody, presence of the processed Pex5p fusion in the peroxisome pellet fraction in subcellular fractionation experiments, protease resistance of the Pex5p fusion cleaved in the peroxisome, indirect immunofluorescence, and finally pulse-chase experiments.

The cleavage of the PTS2wt-Pex5p fusion in HeLa cells and the recognition of the processed form by both M1 and M2 antibodies (Figures 5A and 5B), in combination with the peroxisome specificity of the prethiolase protease, show that the fusion enters the peroxisome matrix. We were unable to detect any of the unprocessed forms of Pex5p fusion in the organelle pellet fractions in differential centrifugation experiments, suggesting that the prethiolase protease acts fast relative to the kinetics of entry of the reporter into the peroxisomes, and that all the pellet-associated Pex5p must be peroxisomal. The processed, peroxisomally localized PTS2mut-Pex5p fusion is inside the peroxisomes as shown by the protease resistance of much of the processed protein associated with the Pel fraction, under conditions where the peroxisomal markers, cytosolically exposed PMP70, and matrix-associated catalase behave as expected (Figure 6). The differential and sequential permeabilization data also show that some of the processed form of the PTS2mut-Pex5p fusion, recognized by both M1 and M2 antibodies, is peroxisome associated (Figure 7B) and inside the matrix (Figure 7C). Finally, the kinetics of Pex5p recycling clearly show a precursor-product relationship between the disappearance of the unprocessed form of the Pex5p fusion from the cytosol and the accumulation of the processed form of the Pex5p fusion in the Pel fraction (peroxisomes) (Figure 8B). These data provide definitive evidence that the Pex5p fusion enters the peroxisome matrix, where it is processed by the prethiolase protease.

Likewise, several results support the export of the processed Pex5p fusion from the peroxisomes back to the cytosol. If the Pex5p fusion was able to enter peroxisomes, but not exit from the matrix back to the cytosol, as in the case of the PTS2wt-GFP fusion (Figure

3A, left panel), one would expect to find the processed Pex5p only in the Pel, and not the Sup, fraction. However, in all experiments (Figures 4B, 5A, 7B, and 8), the majority of the processed form was in the Sup or cytosol. The cytosolic location of the processed Pex5p fusion was not due to leakage from peroxisomes because the marker catalase was predominantly in the Pel fraction (Figure 4B) and inside peroxisomes (Figures 6 and 7A). Additionally, the PTS2wt-GFP fusion was exclusively in the Pel fraction (Figure 5B) in the same cells. These studies show clearly that the Pex5p fusion, after entry into the peroxisome matrix and proteolytic processing at the PPS, is exported back into the cytosol.

Evidence for Multiple Rounds of Pex5p Recycling

Three lines of evidence support the notion that Pex5p is involved in multiple rounds of cycling through the peroxisome matrix. The kinetic data (Figure 8B) show that the net increase in the processed Pex5p associated with the Pel fraction can be accounted for by the sum of the decrease, from the Sup fraction, of processed and unprocessed Pex5p. In the absence of random proteolytic degradation of Pex5p in our experiments, we conclude that the processed form of Pex5p disappearing from the Sup reenters the peroxisome matrix in a subsequent round and therefore appears in the Pel fraction. Additionally, the immunofluorescence data (Figure 7C and data not shown) show that upon permeabilization of HeLa cells expressing PTS2mut-Pex5p with digitonin alone, some M1- and M2-reactive Pex5p is on the outside of the peroxisomes, suggestive of docking of processed Pex5p as a prelude to another round of peroxisomal import. Finally, consistent with this docking, some of the processed Pex5p fusion in the Pel fraction is protease sensitive (Figure 6).

Pex5p Shuttling in Relation to Modes of Action of other Signal Sequence Receptors

For organelles such as mitochondria and chloroplasts, the relevant receptors (Tom20, Tom22, Tom37, Tom70 for mitochondria; Toc159 for chloroplasts) reside in the outer membrane and no receptor shuttling is involved (Chen et al., 2000; Lithgow, 2000). The signal recognition particle (SRP) involved in the insertion of secretory pathway proteins across the ER membrane resides in the cytosol and shuttles between the cytosol and the ER membrane (Potter and Nicchitta, 2000). Only the nuclear import receptors of the importin family recognize cargo in the cytosol, shuttle them into the organelle, and then recycle to the cytosol (Gorlich and Kutay, 1999). Our model for the shuttling of Pex5p is most similar in design to nuclear import, a point that should be noted in considerations of the evolutionary origin of peroxisomes. Both peroxisomal and nuclear import of proteins represent posttranslational mechanisms, and both allow transport of folded and oligomeric proteins.

Experimental Procedures

Oligonucleotides and Generation of Duplex DNA

The complementary oligonucleotides PTS2C and PTS2R coded for the minimal rat PTS2 sequence (MHRLQVVLGHL) (Swinkels et al., 1991). A degenerate nucleotide was used to generate either the wild-type or mutant PTS2 coding sequences (L4R). The second

oligonucleotide set (PPFLC and PPFLR) encoded the PPS (Hijikata et al., 1987) contiguous with the FLAG epitope. The oligonucleotides also contained overhangs on both ends to enable cloning into a NcoI site. The complementary oligonucleotides were denatured separately at 80°C, chilled on ice for 5 min, mixed at equal concentrations, and duplex formation was allowed to occur at room temperature for 6 hr. The resulting duplexes (PTS2 and PPFL) were checked by 12% native polyacrylamide gel electrophoresis (PAGE) and appropriate bands were eluted for cloning. Primer sequences are available on request.

Plasmids

The Tet-On system (Clontech, Palo Alto, CA) was chosen for tight regulation of gene expression. All the fusion constructs were finally made in pTRE-2 for expression in human cell lines. The NcoI site at the translation initiation site of the human *PEX5* cDNA in pJ7-PTS1R (Wiemer et al., 1995) was used to sequentially insert the PPFL and then the PTS2 duplex DNAs. The entire fusion constructs were then PCR amplified using oligonucleotides (PFL5F and PFL5R) containing SacII and BglIII sites at 5' and 3' ends, respectively, and subcloned between the SacII and BamHI sites of pTRE-2 to yield PTS2mut-Pex5p and PTS2wt-Pex5p.

The PTS2-GFP fusions with wild-type and mutated versions of the PTS2 sequence were made by digesting the PTS2mut-Pex5p and PTS2wt-Pex5p constructs with NsiI-NotI and replacing Pex5p with an in-frame EGFP fragment from pEGFP (Clontech) digested with PstI-NotI. This resulted in PTS2mut-GFP and PTS2wt-GFP, respectively. pEGFP-SKL was made by using a reverse primer containing sequences coding for SKL-STOP and PCR amplified (primers FEGFP and REGFP) using as template the EGFP portion from pEGFP. The PCR product was digested with BamHI-NotI and cloned between the BamHI-NotI sites of pTRE-2.

Stable Cell Lines, Cell Growth, and Induction

HeLa cells were used as a control cell line representing the phenotype of wild-type cells with intact PTS1 and PTS2 pathways. Ala-T cells are defective in the PTS1 pathway due to a point mutation (N489K), and thereby mislocalize PTS1-containing proteins but properly target PTS2-containing proteins. Bro-T cells are defective in the PTS2 pathway and mislocalize the PTS2-, but not the PTS1-containing, proteins due to a nonfunctional PTS2 receptor, Pex7p (L292ter). Tet-On stable cell lines expressing the fusion proteins in HeLa, Ala-T, and Bro-T were generated according to manufacturer's instructions. The clones finally selected exhibited very low background and high level expression of the fusion proteins upon induction with doxycyclin.

The growth and maintenance of cell lines has been described earlier (Wiemer et al., 1995). Expression of the various fusion proteins was induced using doxycyclin (Clontech) at 1 μ g/ml concentration generally for 24 hr.

Subcellular Fractionations and Protease Protection Assay

Subcellular fractionations were performed on ice essentially as described (Wilcke et al., 1995; Otera et al., 2000) with modifications. About 4×10^7 cells were detached from culture dishes, washed three times with PBS, and resuspended in ice-cold STE (0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA) homogenization buffer containing the recommended concentrations of mammalian protease inhibitors (Sigma Chemical Co., St. Louis, MO). The cells were subjected to Dounce homogenization using 25 strokes by hand in a 2 ml homogenizer (Kontes, Vineland, NJ). The nuclei were separated by centrifugation at $773 \times g$ (2500 rpm). The supernatant was then subjected to centrifugation at $3726 \times g$ (5500 rpm) for 20 min to separate large organelles. The supernatant was carefully removed and part of it was saved as a cleared post-nuclear fraction (PNS). The rest of the PNS was subjected to centrifugation at $27,717 \times g$ (15,000 rpm) (for protease protection experiments) or directly centrifuged at $100,000 \times g$ (45,000 rpm) for 30 min to obtain a peroxisome-enriched pellet (Pel) and the cytosolic supernatant (Sup) fractions. The peroxisome-enriched pellet fraction was resuspended in the same volume as the supernatant.

Protease protection was performed essentially as described (Gouveia et al., 2000). The peroxisomal pellet, recovered after cen-

trifugation at $27,717 \times g$, was carefully resuspended in ST buffer (250 mM Sucrose, 50 mM Tris-HCl, pH 7.4) and equally distributed into several tubes containing freshly prepared Proteinase K (Sigma; prepared in 10 mM Tris-HCl, 1 mM calcium acetate, pH 7.5) at a final concentration of 100 $\mu\text{g}/\text{ml}$. As a control, 2 mg whole-cell lysate (prepared in ST buffer) was used to monitor protease digestion over the period of incubation. Samples were then incubated on ice for 20 to 60 min, and immediately processed for SDS-PAGE analysis. Staining of gels of control digests indicated complete digestion within 20 min.

Pulse-Chase Experiments

About 10^7 HeLa cells stably expressing the PTS2mut-Pex5p fusion were serum starved for 6 hr in methionine- and cysteine-free DMEM (ICN, Irvine, CA) prior to pulse-chase. The cells were induced with doxycyclin at 1 $\mu\text{g}/\text{ml}$ concentration and simultaneously labeled with 1 mCi of ^{35}S -(methionine and cysteine) mixture (Translabel™, ICN) for 3 hr. Post labeling, the cells were quickly washed in the cold, twice with PBS and twice with complete medium, and chased with enriched complete medium (DMEM, 20% FBS, prewarmed at 37°C) for 60 min at 37°C, 5% CO_2 . Subcellular fractions were prepared at the indicated time points and samples were then processed for immunoprecipitation.

Antibodies, Immunoblotting, and Immunoprecipitation

Monoclonal mouse M1 and M2 antibodies (Sigma) recognize the FLAG epitope in specific contexts (see Results). The peroxidase-conjugated, goat anti-mouse secondary antibody (Biorad, Hercules, CA) was used at a dilution of 1:2500. Rabbit polyclonal anti-catalase antibody (Rockland Immunochemicals, Gilbertsville, PA) coupled to peroxidase was used at 1:5000 dilution. Rabbit polyclonal anti-PMP70 primary antibody was used at 1:2000 dilution. Rabbit polyclonal anti-Pex5p antibody (lab stock) was used at 1:1000 dilution for immunoprecipitations. The goat anti-rabbit secondary antibody conjugated to peroxidase (Biorad) was used at 1:2500 dilution.

Immunoblotting was performed under standard conditions. The membranes containing the transferred proteins were blocked for 1 hr at room temperature with 5% nonfat milk in TBSC (50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl_2 , pH 7.4) for M1 and M2 antibodies (as recommended by manufacturer). Following incubation with primary and secondary antibodies, the membranes were developed with the ECL chemiluminescence detection system. For detection of catalase or PMP70, TBST (TBS, 0.1% Tween20) was used instead of TBSC in the procedure.

Immunoprecipitations were performed using M2 antibody linked to agarose (Sigma) or with Pex5p antibodies as described earlier (Rieder and Emr, 1997).

Semipermeabilization and Sequential Permeabilization of Cell Membranes and Immunofluorescence

Anti-rabbit antibody coupled to an Alexa dye (Alexa488, Molecular Probes Eugene, OR), excitable at 488 nm, was used to detect either PMP70 or catalase, while anti-mouse antibodies coupled to Alexa546, excitable at 546 nm, allowed detection of the PTS2-Pex5p fusions.

Cells were grown on poly-lysine-coated coverslips to 80% confluency for immunofluorescence experiments. For selective permeabilization of only the plasma membrane but not the peroxisomal membrane (Dodt et al., 1995), the coverslips were placed in PBS containing digitonin (25 $\mu\text{g}/\text{ml}$) exactly for 90 s and washed extensively with PBS to remove cytosol. The coverslips were fixed in formaldehyde (3.7% in PBS), washed, and incubated exactly for 30 min with 1:300 dilution of rabbit anti-PMP70 antibodies, 1:1000 dilution of rabbit anti-catalase antibody, or 1:300 dilution of mouse monoclonal M1 and M2 antibodies. The coverslips were extensively washed and incubated with 1:200 dilution of the corresponding Alexa dye-conjugated secondary antibodies for 30 min. The coverslips were finally washed and mounted in PBS containing 20% glycerol. Permeabilization of plasma and peroxisomal membranes, without digitonin treatment, was achieved by fixing the cells and incubating the coverslips in PBS containing 1% Triton X-100 for 5 min, followed by incubation with primary and secondary antibodies as mentioned above. For sequential permeabilization of the peroxi-

somal membrane, after semipermeabilization with digitonin, the fixed coverslips were treated with 1% Triton X-100 in PBS for 5 min and the rest of the procedure was essentially as described above.

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