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Peroxin Puzzles and Folded Freight: Peroxisomal Protein Import in Review

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Peroxisomes are organelles that perform a variety of functions, including the metabolism of hydrogen peroxide and the oxidation of fatty acids. Peroxisomes do not possess organellar DNA; all peroxisomal matrix proteins are posttranslationally translocated into the organelle. The mechanism of peroxisomal protein translocation has been the subject of vigorous research in the past decade. Many of the proteins (peroxins, abbreviated Pex) that play critical roles in peroxisome biogenesis have been identified through functional complementation of yeast strains and of Chinese hamster ovary cell lines that are defective in peroxisome biogenesis. Researchers are now turning towards biochemical and genetic analyses of these peroxins to define their roles in peroxisome biogenesis and to discover interacting protein partners. Evidence suggests that some of the interacting partners include molecular chaperones. Several current models for peroxisomal protein import are presented.

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Introduction

Peroxisomes are ubiquitous organelles that participate in hydrogen peroxide metabolism, β-oxidation of fatty acids, and other important metabolic pathways (Olsen and Harada 1995; van den Bosch et al. 1992). Peroxisomes usually measure 0.5–1.0 μm in diameter and have a simple architecture; a single membrane encloses an electron-dense matrix that often appears granular or contains a crystalline core. Peroxisomes do not possess an organellar genome (Douglass et al. 1973; Kamiryo et al. 1982). All peroxisomal matrix proteins are encoded by nuclear genes, translated on free cytosolic ribosomes, and posttranslationally transported into the organelle (reviewed in Lazarow 1995; Olsen 1998; Rachubinski and Subramani 1995; Subramani 1993).

The fact that peroxisomal matrix proteins are synthesized in the cytoplasm implies the need for peroxisome-specific mechanisms to target and translocate these proteins across the peroxisomal membrane. Several predictions about how this system functions may be made from research in other posttranslational protein import systems, especially mitochondrial and chloroplastic protein import (Haucke and Schatz 1997; Rassow and Pfanner 1995; Schatz and Dobberstein 1996). The first prediction is that peroxisomal proteins possess specific targeting signals. Second, these targeting signals interact with peroxisomal import receptors. Third, if protein conformation is essential for the protein's import competence, chaperone proteins may also interact with the targeted protein. Fourth, the targeted protein or its receptor interacts with components of the translocation apparatus. Fifth, the protein is translocated across the membrane through this apparatus.

These predictions seem straightforward; the overall model appears simple. Research in the past decade,

however, has led to several unexpectedly complicated models for peroxisome biogenesis. This review focuses on three areas of peroxisome biogenesis research: peroxisomal targeting signals (PTSs); proteins which participate in peroxisome biogenesis, including chaperones; and the import competence of various assembly states of the targeted peroxisomal protein.

Peroxisomal targeting signals

Peroxisomal proteins are directed to the organelles by PTSs. Two types of signals have been identified for proteins destined for the peroxisomal matrix. The first, designated PTS1, is a carboxyl-terminal tripeptide that is necessary and sufficient to target proteins to peroxisomes (reviewed in Erdmann et al. 1997; Olsen 1998; Subramani 1993; Subramani 1996). A PTS1 consists of the amino acids serine-lysine-leucine, or conserved substitutions of these residues. The position of the signal at the extreme carboxyl terminus of the protein is important for proper targeting; addition of amino acids to the carboxyl terminus beyond the tripeptide abolishes targeting of proteins to peroxisomes (Gould et al. 1989; Miyazawa et al. 1989). In addition, placement of a PTS1 at the amino terminus or at internal positions in a passenger protein does not target the passenger protein to peroxisomes (Subramani 1993). The majority of peroxisomal matrix proteins identified possess a PTS1 (de Hoop and AB 1992; Erdmann et al. 1997; Olsen and Harada 1991).

The second type of peroxisomal targeting signal (PTS2) contains a nine amino acid consensus sequence; the PTS2 nonapeptide is usually found within 20–30 amino acids of the amino terminus (reviewed in de Hoop and AB 1992; Erdmann et al. 1997; Gietl 1996; McNew and Goodman 1996; Olsen 1998; Roggenkamp 1992; Subramani 1993). In plants and mammals PTS2 sequences are cleaved in the organelle matrix, but cleavage is not required for import. In some yeasts, such as *Saccharomyces cerevisiae*, the signal remains uncleaved after import. Compared to the number of peroxisomal proteins that have PTS1 signals, few PTS2 proteins have been characterized. The most well-characterized PTS2 protein is thiolase, a fatty-acid β-oxidation enzyme found in plant, animal and yeast peroxisomes. Currently, three PTS2 proteins have been identified in mammals: thiolase (Bout et al. 1988; Hijikata et al. 1990; Osumi et al. 1991; Swinkels et al. 1991; Tsukamoto et al. 1994a), alkyl-dihydroxyacetonephosphate synthase (de Vet et al. 1998), and phytanoyl-CoA hydroxylase (Jansen et al. 1997). Citrate synthase and malate dehydrogenase are PTS2 proteins in plants (Gietl 1990; Kato et al. 1996); amine oxidase and Pex8p are PTS2 proteins in yeasts (Bruinenberg et al. 1989; Faber et al. 1995; Waterham et al. 1994). Interestingly, Pex8p contains both PTS1 and PTS2 signal sequences (Waterham et al. 1994).

Compared to matrix protein targeting mechanisms, the targeting of peroxisomal membrane proteins is not well understood, primarily because few peroxisomal membrane proteins have been characterized. What is clear is that membrane proteins are targeted by a mechanism distinct from PTS1- and PTS2-protein targeting. Yeast strains defective in both PTS1- and PTS2-protein import often possess an intact peroxisomal membrane, with membrane proteins, but they lack matrix proteins (Lazarow 1993; Purdue and Lazarow 1995; Subramani 1993; Subramani 1997; Van der Leij et al. 1992). The occurrence of these "peroxisomal membrane ghosts" indicates that peroxisomal membrane proteins are still targeted and inserted into the membrane and that they utilize a different pathway than the peroxisomal matrix proteins.

The targeting signals of four peroxisomal membrane proteins from yeasts have been studied. *Candida boidinii* Pmp47 is an integral membrane protein with six putative membrane-spanning domains (Dyer et al. 1996; McCammon et al. 1994). A 20 amino acid, matrixfacing, hydrophilic loop between the fourth and fifth transmembrane segments is both necessary and sufficient to target Pmp47 to the peroxisomal membrane. In contrast, *Pichia pastoris* Pex3p and *Hansenula polymorpha* Pex3p integral membrane proteins are targeted by 40 residues and 16 residues at the amino terminus, respectively (Baerends et al. 1996; Wiemer et al. 1996). Finally, the targeting signal for *S. cerevisiae* Pex15p is found within 82 amino acids of the carboxyl terminus, in the matrix-localized tail of the protein (Elgersma et al. 1997; Erdmann et al. 1997). This signal is necessary, but not sufficient for targeting Pex15p to peroxisomal membranes (Elgersma et al. 1997).

Interestingly, both *H. polymorpha* Pex3p and *S. cerevisiae* Pex15p can also be targeted to the endoplasmic reticulum (ER). Deletion of the Pex3p and Pex15p membrane targeting signals causes the proteins to accumulate in the ER (Elgersma et al. 1997). However, deletion of the Pmp47 targeting signal causes mislocalization to the cytosol (Dyer et al. 1996). Tabak and his colleagues (Elgersma et al. 1997) suggest that a subset of peroxisomal membrane proteins are targeted to peroxisomes through the ER (i.e., cotranslationally), while others rely on

posttranslational transport from the cytosol. Thus, there may be two types of peroxisomal membrane targeting signals. The dual localization of Pex3p and Pex15p, and other evidence, has led some authors to propose that peroxisomes arise from ER-derived vesicles. Because the role of the ER in peroxisome biogenesis is beyond the scope of this review, interested readers should consult other discussions on this topic (e.g., Elgersma et al. 1997; Erdmann et al. 1997; Kunau and Erdmann 1998).

Peroxins

Significant progress in the field of peroxisomal protein import has come from the identification of the proteins responsible for peroxisome biogenesis; these proteins have been termed peroxins (pex). Peroxins are involved in matrix protein import, membrane biogenesis, peroxisome proliferation, and organelle inheritance. They are assigned numbers in accordance with a recently unified nomenclature system (Distel et al. 1996). Due in large part to the power of yeast genetics and to research on human peroxisome biogenesis disorders, over 20 peroxins have been identified and characterized (reviewed in Erdmann et al. 1997; McNew and Goodman 1996; Subramani 1996, 1997); these are summarized in Table 1. Some of the peroxins share sequence homology with other, previously studied, protein families. Interactions between peroxins have been uncovered through two-hybrid screens and coimmunoprecipitation experiments. Despite these clues, little about the function of these proteins is understood.

Early screens for peroxisome biogenesis mutants in yeasts revealed that two classes of mutants were defective in the import of either PTS1 proteins or PTS2 proteins; most of the mutants were unable to import either type of protein (reviewed in Erdmann et al. 1997; McNew and Goodman 1996; Subramani 1996; Subramani 1997). This suggests models for peroxisome biogenesis in which there are two separate pathways for import, the PTS1 and the PTS2 pathways, that converge at some point and share some components in common.

pex5 mutants are defective in the import of PTS1 proteins only (McCollum et al. 1993; Van der Leij et al. 1993). PEX5 encodes a protein that lacks predicted transmembrane domains and whose carboxyl terminus contains seven tetratricopeptide repeat (TPR) domains (Fransen et al. 1995; McCollum et al. 1993; Nuttley et al. 1995; Szilard et al. 1995; van der Klei et al. 1995; Van der Leij et al. 1993). Binding studies have shown that the TPR domain interacts with peptides containing PTS1-like peroxisomal targeting signals, but peptides lacking the PTS1 tripeptide do not bind (Brocard et al. 1994; McCollum et al. 1993; Terlecky et al. 1995). Thus, based on the specificity of Pex5p for the PTS1 targeting signal, Pex5p was designated the PTS1 receptor. Although Pex5p's ability to bind PTS1 sequences has been confirmed by several laboratories, there is no clear consensus on Pex5p's localization (see references in Table 1). Pex5p has been localized to the cytosol in *P. pastoris* and in *S. cerevisiae,* to the cytosol and peroxisomal matrix in *H. polymorpha*, and exclusively to the peroxisomal matrix in *Yarrowia lipolytica*. Pex5p appears to be cytosolic and peroxisome-associated in humans.

Similarly, the isolation of a yeast strain defective specifically in the import of PTS2 proteins led to the identification of the PTS2 receptor, Pex7p (Braverman et al. 1997; Marzioch et al. 1994; Rehling et al. 1996; Zhang and Lazarow 1995; Zhang and Lazarow 1996). This protein is composed almost entirely of WD-40 (β-transducin related) repeats. Pex7p binds specifically to PTS2 signals in vitro and interacts with thiolase in both two-hybrid and coimmunoprecipitation experiments. Interestingly, in *S. cerevisiae* Pex7p also interacts with Pex5p, suggesting an indirect role for Pex5p in PTS2 protein import. As with Pex5p, the Pex7p sequence contains no recognizable transmembrane domains, and the protein has been localized to several subcellular locations (see refs. in Table I). Pex7p was found to be both cytosolic and intraperoxisomal in *S. cerevisiae*, but strictly cytosolic in *Mus musculus*.

Several models have been suggested to account for the various subcellular localizations of the PTS1 and PTS2 receptors (shown in Figs. 1–3; see Olsen 1998; Rachubinski and Subramani 1995; Waterham and Cregg 1997). In one model (Figs. 1, 3), cytosolic peroxisomal import receptors bind proteins destined for the peroxisomal matrix and accompany their cargo to the peroxisomal membrane. Included in this model are two hypotheses to explain the fate the receptor-peroxisomal protein complex at the membrane: (a) the receptor could deliver the cargo to the translocation apparatus, release the protein, and then recycle to the cytosol (shown for PTS1 proteins in Fig. 1A and for PTS2 proteins in Fig. 3); or (b) the receptor could be transported through the translocation machinery while still bound to the peroxisomal matrix protein (shown in Fig. 1B for a PTS1 protein). If receptors do enter the matrix of the organelle, they could be degraded or exported out of the organelle to the cytoplasm in a manner similar to nuclear import receptors. It should be noted that export of proteins out of peroxisomes has not been reported. (Further discussion of these models may

Table 1. Peroxins[a]

Name[b]	Proposed function/characteristics	Subcellular location	Ref.[c]
Pex1p	homologous to NSF proteins; contains two AAA-ATPase domains; interacts with Pex6p; 117–127 kDa	cytoplasm; peripheral association with non-peroxisomal vesicles	1
Pex2p	contains C_3HC_4 zinc-finger motif; 35–52 kDa	peroxisomal membrane (integral)	2
Pex3p	peroxisomal membrane synthesis; peroxisome proliferation; interacts with Pex19p; mPTS in first 40 amino acids targets to both peroxisomes and endoplasmic reticulum; 51–52 kDa	peroxisomal membrane (integral)	3
Pex4p	ubiquitin-conjugating protein; 21–24 kDa	peroxisomal membrane (peripheral, cytosolic face)	4
Pex5p	PTS1 receptor; contains TPR motifs; interacts directly with Pex13p, Pex14p, and Pex7p (probably), indirectly with Pex17p; 64–69 kDa	cytoplasm; peroxisomal matrix; peroxisomal membrane	5
Pex6p	homologous to NSF proteins; contains two AAA-ATPase domains; some homology to myosin; interacts with Pex1p; 112–127 kDa	cytoplasm; peripheral association with non-peroxisomal vesicles	6
Pex7p	PTS2 receptor; interacts with Pex14p and Pex5p (probably); contains WD-40 motifs; 37–42 kDa	cytoplasm; peroxisomal matrix; peroxisomal membrane	7
Pex8p	contains both PTS1 and PTS2; 71–81 kDa	peroxisomal matrix; peroxisomal membrane (peripheral, matrix face)	8
Pex9p	contains cysteine-rich region; 42 kDa	peroxisomal membrane (integral)	9
Pex10p	peroxisome proliferation; lumen formation; contains C_3HC_4 zinc-finger motif; 34–48 kDa	peroxisomal membrane (integral)	10
Pex11p	peroxisome division; homodimerizes; 27–32 kDa	peroxisomal membrane (peripheral, matrix face)	11
Pex12p	contains C_3HC_4 zinc-finger motif; 40–48 kDa	peroxisomal membrane (integral)	12
Pex13p	putative docking protein for Pex5p; also interacts with Pex14p; contains SH3 domain; 40–43 kDa	peroxisomal membrane (integral)	13
Pex14p	interacts with both Pex5p and Pex7p; also interacts with itself, Pex13p and Pex17p; putative point of convergence of PTS1 and PTS2 pathways; 38–39 kDa	peroxisomal membrane (integral, peripheral, cytosolic face)	14
Pex15p	peroxisome proliferation; phosphorylated; O-glycosylated; mPTS in carboxyl-terminal (lumenal) tail is necessary, but not sufficient for targeting to peroxisome; 43.6 kDa	peroxisomal membrane (integral)	15
Pex16p	peroxisome proliferation; 44.5 kDa	peroxisomal membrane (peripheral, matrix face)	16
Pex17p	interacts directly with Pex14p, indirectly with Pex5p; 23 kDa	peroxisomal membrane (peripheral, cytosolic face)	17
Pex19p	farnesylated; interaction with Pex3p is dependent upon farnesylation; 39.7 kDa	cytoplasm; peroxisomal membrane (peripheral, cytosolic face)	18
Pas22p	DnaJ homolog; 48 kDa	cytoplasm	19

[a] Adapted from similar tables in (Distel, Erdmann, 1996; Erdmann, Veenhuis, 1997; McNew and Goodman, 1996; Olsen, 1998; Subramani, 1997; Waterham and Cregg, 1997) and updated as necessary.

[b] Based on the unified nomenclature (Distel, Erdmann, 1996); proteins which are involved in peroxisome biogenesis are termed peroxins (acronym, PEX)

[c] References: 1. Erdmann et al. 1991; Faber et al. 1998; Heyman et al. 1994; Reuber et al. 1997; 2. Berteaux-Lecellier et al. 1995; Eitzen et al. 1996; Flaspohler et al. 1997; Shimozawa et al. 1992; Tsukamoto et al. 1991; Waterham et al. 1996; Tsukamoto et al. 1994b; 3. Baerends et al. 1996; Wiemer et al. 1996; 4. Crane et al. 1994; Wiebel and Kunau 1992; 5. Dodt et al. 1995; Fransen et al. 1995; Kalish et al. 1996; McCollum et al. 1993; Nuttley et al. 1995; Szilard et al. 1995; Terlecky et al. 1995; van der Klei et al. 1995; van der Leij et al. 1993; Wiemer et al. 1995; 6. Faber et al. 1998; Nuttley et al. 1994; Spong and Subramani 1993; Tsukamoto et al. 1995; Voorn-Brouwer et al. 1993; Yahraus et al. 1996; 7. Braverman et al. 1997; Marzioch et al. 1994; Motley et al. 1997; Rehling et al. 1996; Zhang and Lazarow 1995; Zhang and Lazarow 1996); 8. Liu et al. 1995; Smith et al. 1997; Waterham et al. 1994; 9. Eitzen et al. 1995; 10. Kalish et al. 1995; Tan et al. 1995; 11. Erdmann and Blobel 1995; Marshall et al. 1995; Sakai et al. 1995; 12. Chang et al. 1997; Kalish et al. 1996; 13. Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996; 14. Albertini et al. 1997; Brocard et al. 1997; Komori et al. 1997; 15. Elgersma et al. 1997; 16. Eitzen et al. 1997; 17. Huhse et al. 1998; 18. Götte et al. 1998; 19. Subramani 1997

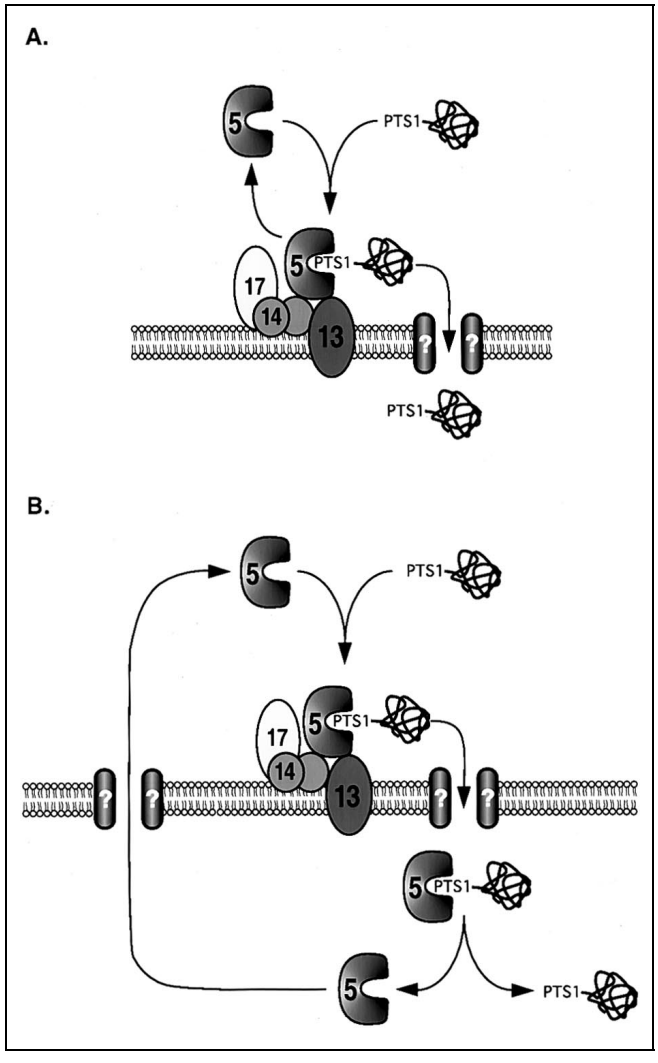

Fig. 1A,B. Models of PTS1 protein import with Pex5p shuttling between the cytosol and peroxisome. A) Cytosol to membrane shuttle. The PTS1 receptor, Pex5p, binds to the PTS1 protein in the cytoplasm. The Pex5p-PTS1 protein complex then docks on a membrane-bound peroxin complex that may include Pex13p, Pex14p and Pex17p. Pex5p dissociates from the PTS1 protein and is recycled back to the matrix as the PTS1 protein is translocated across the membrane. B) Cytosol to matrix shuttle. The Pex5p-PTS1 protein recognition and docking events occur as in A). In this model, however, the Pex5p-PTS1 protein complex translocates across the membrane. Dissociation of the complex occurs in the matrix; Pex5p may be degraded or may recycle back to the cytoplasm. *5*, Pex5p; *13*, Pex13p; *14*, Pex14p; *17*, Pex17p

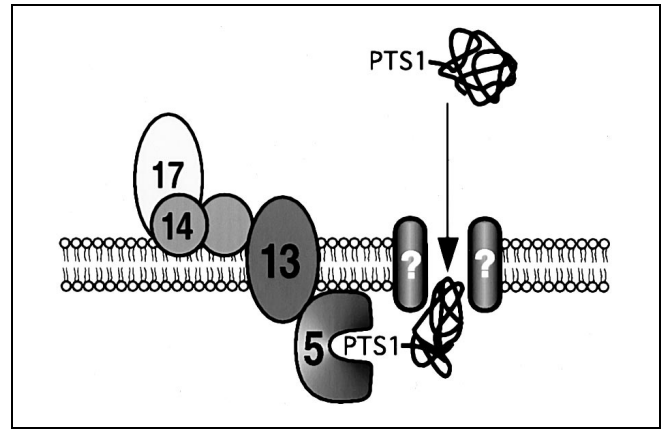

Fig. 2. Model of PTS1 protein import with Pex5p localized only inside the peroxisome. In this model the PTS1 receptor, Pex5p, is peripherally bound to the matrix face of the peroxisomal membrane. The PTS1 protein translocates across the membrane and is bound by Pex5p as soon as it is exposed to the matrix. This binding prevents the PTS1 protein from slipping back through the translocation machinery, driving the transport event forward. *5*, Pex5p; *13*, Pex13p; *14*, Pex14p; *17*, Pex17p

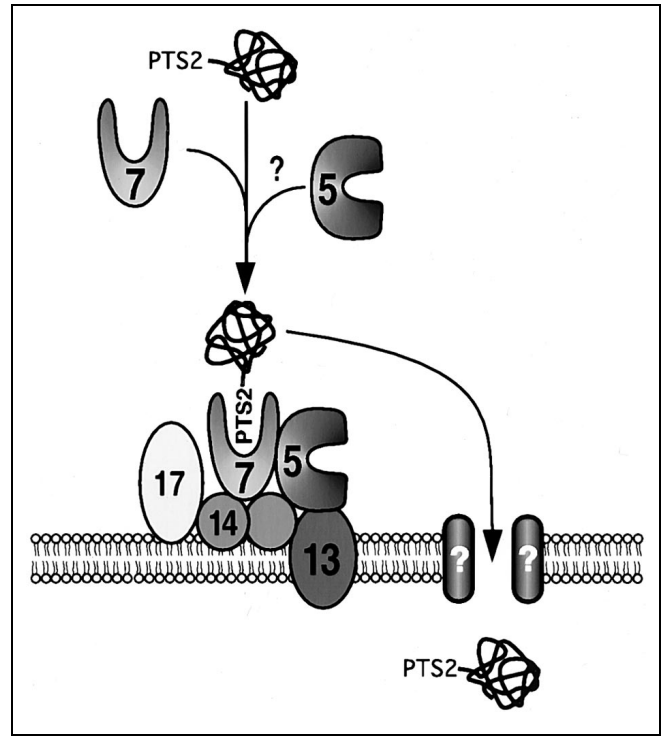

Fig. 3. One possible model for PTS2 protein import. The PTS2 receptor Pex7p binds to the PTS2 protein in the cytoplasm. In some species Pex5p may then join the complex by binding to Pex7p. This complex then docks on the membrane bound peroxin complex, and the PTS2 protein is directed through the translocation machinery. This is comparable to the model shown in Fig. 1A for PTS1 proteins; other models, similar to those shown in Figs. 1B and 2 are also possible. A direct interaction between Pex5p and Pex7p may not always occur. *5*, Pex5p; *7*, Pex7p; *13*, Pex13p; *14*, Pex14p; *17*, Pex17p

be found in Dodt and Gould 1996; Elgersma et al. 1996; Erdmann and Blobel 1996; Erdmann et al. 1997; Gould et al. 1996; Marzioch et al. 1994; Wiemer et al. 1995.)

In another model, the receptor is restricted to the organelle matrix or to the inner surface of the peroxisomal membrane (shown in Fig. 2 for a PTS1 protein) (see also Szilard et al. 1995; van der Klei et

al. 1995; Zhang and Lazarow 1996). The receptor is predicted to bind to the peroxisomal protein as soon as part of the targeted protein protrudes into the matrix. Binding would prevent the targeted protein from slipping back out of the apparatus and therefore would drive the translocation reaction forward. This "pulling" or "ratchet" mechanism is similar to the proposed function of mitochondrial hsp70s in facilitating mitochondrial protein import (Glick 1995; Martinus et al. 1995; Stuart et al. 1994).

Thus, based on findings with *pex5* and *pex7* mutants, it appears that there are two separate pathways for the initial steps of matrix peroxisomal protein import, one for each type of peroxisomal targeting signal. This raises the question of whether there are also PTS1- and PTS2-specific import channels or whether PTS1 and PTS2 proteins share components of a common import apparatus. Yeast peroxisome biogenesis mutants have again helped address this issue. Pex14p, which may function as a dimer, is a membrane protein that interacts with the PTS1 and PTS2 receptors (Albertini et al. 1997; Brocard et al. 1997; Komori et al. 1997). Based on these interactions and the inability of *pex14* mutants to import both PTS1 and PTS2 proteins, Pex14p is thought to be a peroxisomal membrane docking protein for both receptors (see Figs. 1–3). Pex14p may therefore represent a point of convergence between the PTS1 and PTS2 import pathways.

Pex14p has also been shown to interact weakly with Pex13p, a peroxisomal integral membrane protein that contains an SH3 (Src homology 3) domain (Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996). As with *pex14* mutants, *pex13* mutants fail to import both PTS1 and PTS2 proteins into peroxisomes. Pex13p interacts with the PTS1 receptor but may not recognize the PTS2 receptor (compare Figs. 1A, 3; see Erdmann and Blobel 1996). Although the *pex13* phenotype suggests that this strain is a general peroxisomal import mutant, it is not yet clear what role Pex13p plays in the PTS2 pathway.

Screens for yeast peroxisome biogenesis mutants have identified genes encoding several additional peroxisomal membrane proteins thought to be components of the translocation machinery (Figs. 1–3). Pex17p is a cytosol-facing, peripheral membrane protein that interacts directly with Pex14p and indirectly with Pex5p; the Pex17p-Pex5p interaction is mediated by the presence of Pex14p (Huhse et al. 1998). Pex2p and Pex12p are integral membrane proteins with zinc-finger motifs; *pex2* and *pex12* mutant yeast strains accumulate the PTS1 receptor at the peroxisomal membrane (Chang et al. 1997; Eitzen et al. 1995; Kalish et al. 1996; Waterham et al. 1996). There are more than a dozen additional peroxins whose functions are poorly understood (see Table 1). Some of these peroxins may not be directly involved in import. Instead they may indirectly influence matrix protein import by perturbing peroxisome division or proliferation.

Chaperones

Protein import into organelles often requires cytosolic and/or organelle-specific chaperones. Chaperones, typicallyheat-shock proteins (Hsps), are involved in controlling protein folding in many cellular reactions (Hartl 1996; Miernyk 1997; Rassow et al. 1997). Chaperones such as Hsp70 (70-kDa class of heat-shock proteins) bind to proteins soon after synthesis in the cytoplasm. Chaperone binding prevents the nascent polypeptide from folding and thus maintains the protein in a loosely folded conformation. This extended conformation is often necessary to facilitate translocation across organellar membranes (Rassow and Pfanner 1995).

Hsp70 chaperones are probably involved in peroxisomal protein import. Antibodies against cytosolic Hsp70 have been shown to inhibit import of peroxisomal proteins. Microinjection of bovine Hsp73 antibodies into mammalian fibroblast cells inhibits peroxisomal protein import, but import is restored by the addition of exogenous Hsp70 (Walton et al. 1994). Results from our laboratory also indicate the importance of Hsp70s in peroxisomal protein import (Crookes and Olsen 1998). We have found that addition of antibodies directed against cytosolic wheat germ Hsp70 to in vitro import assays (Brickner et al. 1997; Brickner and Olsen 1998; Olsen 1998) lowers peroxisomal protein import levels. These antibodies also successfully immunoprecipitate two peroxisomal proteins, suggesting that Hsp70s and peroxisomal proteins interact directly.

Proteins related to Hsp70s have been localized to peroxisomes. PMP73, a 73-kDa peroxisomal integral membrane protein, is immunorelated to the Hsp70 family (Corpas and Trelease 1997). Another study has shown that proteins that crossreact with Hsp70 antibodies are recruited to the peroxisomal membrane during import; this Hsp70 has been localized to the outer leaflet of the peroxisomal membrane (Walton et al. 1994). Finally, a recently cloned watermelon Hsp70 is a putative peroxisomal matrix chaperone (Wimmer et al. 1997). The gene apparently possesses two in-frame translation initiation sites; synthesis from the second site would produce a protein containing a PTS2.

Thus, Hsp70s may participate in peroxisomal protein import at several subcellular locations, and they may play multiple roles. Cytosolic Hsp70 might maintain the targeted matrix protein in an import-competent state. In addition, cytosolic Hsp70 might stabilize the region of the protein containing the PTS until it is bound by the appropriate soluble PTS receptor (Subramani 1996). Intraperoxisomal Hsp70 might function as a "ratchetlike" protein, analogous to its putative role in mitochondrial protein import (Glick 1995; Martinus et al. 1995; Stuart et al. 1994), to assist in translocation of the protein across the membrane.

There is also evidence indicating the involvement of other chaperones, in addition to cytosolic Hsp70, in peroxisomal protein import. Since Hsp40s are chaperones that enhance Hsp70s' endogenous ATPase activity, it is not surprising that a prenylated, membrane-bound DnaJ (Hsp40) homolog has also been localized to the peroxisomal membrane (Preisig-Muller et al. 1994). No function has been attributed to this particular protein, however. Furthermore, Pas22p has been identified as a cytosolic DnaJ homolog (Subramani 1997).

In addition, it has been reported that a member of the Hsp60 family of chaperonins is located within the peroxisomal matrix (Velez-Granell et al. 1995). These proteins fold the transported polypeptide into its active conformation after translocation across mitochondrial and chloroplast membranes (Hartl 1996; Martinus et al. 1995; Stuart et al. 1994) and may play a similar role in peroxisomal protein assembly after transport.

Experiments from our laboratory have also shown that Hsp90 antibodies inhibit peroxisomal protein import in vitro (Crookes and Olsen 1998). Hsp90s function in a "super-chaperone" complex with Hsp70s to prime mammalian steroid receptors for ligand binding (Jakob and Buchner 1994; Pratt 1993; Xu and Lindquist 1993). Hsp90s also influence the assembly of protein complexes. Therefore the role of Hsp90 in peroxisomal protein import could be to prime the cytosolic PTS receptors for binding to recently synthesized peroxisomal proteins. It may also control the assembly state of the targeted protein or of the import complex prior to import.

Protein folding and assembly

The participation of molecular chaperones in peroxisomal protein import suggests that the folding state of the targeted peroxisomal protein could influence that protein's import competence. Several groups, however, have shown that oligomeric and fully folded substrates can be imported into peroxisomes (Hausler et al. 1996; Walton et al. 1995; Walton et al. 1994; Wendland and Subramani 1993). Examples of folded substrates that gain access to the peroxisomal matrix include albumin crosslinked to PTS1 peptides (Wendland and Subramani 1993) and dihydrofolate reductase–PTS1 fusion proteins stabilized in their folded state by aminopterin (Hausler et al. 1996). One of the most surprising results is that 9-nm gold particles decorated with PTS1 signals enter the peroxisomal matrix (Walton et al. 1995).

Several laboratories have also used "piggy-backing" experiments to show that protein subunits are assembled in the cytosol prior to import (Glover et al. 1994; Lee et al. 1997; McNew and Goodman 1994). In these experiments an epitope-tagged peroxisomal protein construct lacking its PTS is coexpressed with the wild-type form of the peroxisomal protein (possessing a PTS). The epitope-tagged construct cannot reach the peroxisomal matrix without coexpression of the wild-type form, indicating that the two constructs probably associate prior to translocation. Although these studies cannot rule out that the assembled peroxisomal protein subunits dissociate at the membrane just before translocation, they demonstrate that an association between subunits must occur in the cytosol.

In contrast, it appears that some peroxisomal proteins are not assembled until after translocation across the peroxisomal membrane. For instance, assembly of octameric alcohol oxidase in *H. polymorpha* requires a peroxisomal factor (Evers et al. 1996). In addition, piggy-backing experiments similar to those described above have shown that *P. pastoris* alcohol oxidase cannot initiate oligomerization in the cytosol (Waterham et al. 1997). Therefore, import of alchohol oxidase into peroxisomes must precede oligomerization. Moreover, studies from our laboratory suggest that the question may not be whether proteins that are assembled in the cytosol can reach the matrix, but rather how efficiently the assembled proteins are translocated (Crookes and Olsen 1998). Whereas tetrameric isocitrate lyase can be imported into pumpkin peroxisomes in vitro, import of monomeric isocitrate lyase is much more efficient. Therefore, although folded and assembled substrates may be competent for import, loosely folded or monomeric substrates may be the preferred substrates for translocation.

Several models describing possible mechanisms for import of folded substrates have been proposed (see Erdmann et al. 1997; McNew and Goodman 1996; Subramani 1996; Waterham and Cregg 1997). In one model, peroxisomal proteins are imported predominantly as monomers, in a loosely folded conforma-

tion. This mechanism is similar to that described for mitochondrial protein import (Hartl and Neupert 1990; Neupert et al. 1990). A second possibility is that oligomeric peroxisomal proteins are assembled in the cytosol and then disassembled or partially unfolded at the surface of the peroxisomal membrane. A chloroplast outer envelope membrane Hsp70 may provide chloroplast membranes with similar unfolding activity (Boston et al. 1996). This could also be the role for the putative membrane-bound Hsp70 and Hsp40 in peroxisomes. Another proposal is that assembled, oligomeric proteins are imported intact, i.e., without dissociation at the peroxisomal membrane. Finally, Goodman and colleagues have suggested that the peroxisomal membrane may invaginate into the matrix, enveloping assembled peroxisomal proteins, and then "bud off" into the peroxisomal matrix (McNew and Goodman 1994; McNew and Goodman 1996). The membrane vesicles would then degenerate and release their contents into the organellar matrix. Some support for this model is provided by a report in which internal membranes were observed in rat liver peroxisomes (Fahimi et al. 1993).

Conclusions

The peroxisome biogenesis models which have emerged over the past 10 years are far more complex than originally imagined: multiple targeting signals direct proteins to peroxisomes, at least two receptors interact with the targeted proteins, membrane components may bind to one receptor but not the other, additional membrane components may bind to both types of receptors, chaperones facilitate import, and folded substrates can be translocated. Over the past several years new peroxins have been rapidly identified. A few of these have been localized; several have been used in screens for interacting proteins. Studies on the localization and interactions of peroxins have given researchers a glimpse of how these proteins may function. There are, however, still many unanswered questions about the peroxisomal protein import pathway. Contributions from research programs utilizing yeast, mammalian, and plant systems will provide us with new information as we begin to unravel the surprisingly complex mechanisms of peroxisome biogenesis.

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