Protein Translocation into Peroxisomes*

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Peroxisomes, as well as the signals and the machinery that target proteins to this organelle, are conserved in most eukaryotic organisms. Different peroxisomal targeting signals (PTSs),1 acting in concert with specific PTS receptors, account for the specific accumulation of proteins in the peroxisomal matrix and membrane. This review focuses only on the signals, proteins, and mechanisms involved in peroxisomal protein import and peroxisome biogenesis, emphasizing features unique to this organelle. The relevance of these import mechanisms to human peroxisomal disorders is reviewed elsewhere (1, 2). The gene and protein names used in this article conform to the new nomenclature system adopted recently (3).

The absence of DNA in peroxisomes requires that all peroxisomal proteins be encoded by the nuclear genome. There is very strong evidence that many, if not all, peroxisomal matrix proteins are synthesized on free polysomes and then imported post-translationally from the cytosol directly to the peroxisomes (1, 2). Matrix proteins devoid of a PTS, or containing a non-functional PTS, are generally cytosolic, with the exception that certain matrix protein subunits lacking a PTS can enter peroxisomes by association with other subunits possessing a functional PTS (4, 5).

Most, but not all, peroxisomal membrane proteins (PMPs) are believed to be synthesized on free polysomes and then transported from the cytosol directly to the peroxisomes. In vivo evidence for direct import from the cytosol to peroxisomes exists, to my knowledge, only for one protein, PMP70 (6). A few PMPs have been shown to be translated on free polysomes (7–9), but direct transfer from the cytosol to peroxisomes is either inferred or supported by in vitro experiments for only two proteins, PMP22 and PMP70 (6, 10). The implications of these studies are discussed later.

Pathways for Import of Matrix and Membrane Proteins into Peroxisomes

Multiple PTSs (generally one/protein) direct proteins to the peroxisomal membrane. The most widely used of these is the conserved, C-terminal tripeptide (SKL and its functional variants) named PTS1 (1, 2). This sequence requires the –COOH group of the last amino acid and consequently functions only at the C termini of proteins and not at internal locations. Its location explains the post-translational nature of peroxisomal protein import for PTS1-dependent polypeptides. The PTS1 sequence is recognized specifically and with high affinity (Kd = 460–500 nM) by a receptor encoded by the PpPEX5 (former name, PpPAS8) gene of the yeast Pichia pastoris (11) and the HsPEX5 (former names, PTS1R and PXR1) gene in humans (12–14). Homologues of these genes have also been described in Saccharomyces cerevisiae (15), Hansenula polymorpha (16), and Yarrowia lipolytica (17).

A second PTS, used by a smaller subset of peroxisomal matrix proteins, is the conserved N-terminal nonapeptide ((R/K)(L/V/I)(X)5(H/Q)(L/A)) (1, 2). In contrast to the PTS1 sequence, this sequence also functions as a PTS at internal locations in proteins.2 The PTS2 motif in proteins is recognized by its cognate receptor encoded by the ScPEX7 (former names, ScPAS7 and ScPEB1) gene of S. cerevisiae (18–21).

Other sequences from peroxisomal matrix proteins, fulfilling the criterion of being sufficient for peroxisomal targeting of a reporter protein, have been described (cited in Ref. 2). With one exception (22), it is not yet clear, however, that these are true PTSs in the sense that there are distinct receptors for them. They are poorly defined, and their use by other peroxisomal proteins is unconfirmed.

Sequences responsible for the sorting of proteins to the peroxisomal membrane have been identified only recently. These membrane PTSs (mPTSs) have been defined in only two proteins. Goodman and colleagues (23, 24) have narrowed an mPTS to a 20-amino acid loop facing the peroxisomal matrix in the peroxisomal membrane protein, PMP47, from Candida boidinii. This loop is located between putative transmembrane domains (TMDs) in a protein postulated to have six TMDs (23, 24). Another mPTS has been defined in the first 40 amino acids of P. pastoris PpPex3p (former name, PpPas2p) and also does not include any TMDs (25). Receptors for these mPTSs are not known.

Mutants affecting protein import via the PTS1 (11, 12, 14–17) or PTS2 (18, 19) pathways alone as well as mutants deficient in protein import via both of these pathways (reviewed in Ref. 23) are known. In many of these mutants peroxisomal remnants or membrane “ghosts” are seen, suggesting that membrane proteins are sorted via a different route. Mutants deficient in the import of PMPs are expected to have no peroxisomal ghosts and should be compromised in both peroxisome biogenesis and matrix protein import (25).

Involvement of the PTS Receptors in Peroxisomal Protein Import

There is genetic and biochemical evidence that the PTS receptors are essential for import of proteins to the matrix, but not the membrane, of peroxisomes. Mutations in the PTS1 receptor (PEX5) gene (or its homologues) in yeasts and humans exhibit a specific impairment in the import of PTS1- but not of PTS2-containing proteins (11, 12, 14–17). Conversely, mutations in the PTS2 receptor (PEX7) gene compromise only the import of PTS2-containing proteins (18, 19).

In biochemical experiments, the yeast and human PTS1 receptors bind the PTS1 peptide specifically (14, 26, 27). Antibodies to HsPex5p (human PTS1R) inhibit the peroxisomal import of a PTS1-containing reporter protein in permeabilized cells (14). Interactions have been detected in a yeast two-hybrid

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1 The abbreviations used are: PTS, peroxisomal targeting signal; ER, endoplasmic reticulum; mPTS, membrane peroxisomal targeting signal; PMP, peroxisomal membrane protein; TMD, transmembrane domain.
2 K. N. Faber and S. Subramani, unpublished data.
system between yeast and human PTS1 receptors, on the one hand, and PTS1-containing proteins on the other (13, 22). Similar interactions have been detected between the PTS2 receptor and PTS2-containing proteins (20, 21). Evidence for this ranges from genetic suppression of a temperature-sensitive mutation in a yeast PTS2 sequence by overexpression of the receptor ScPex7p (previously ScPas7p) to two-hybrid interactions between the receptor and PTS2-containing proteins and coimmunoprecipitation of the receptor-ligand complex (20, 21). Proof of direct binding between the PTS2 receptor and its ligand is not yet available.

Consistent with the ability of the PTS2 sequence to function as a targeting signal in vivo when placed at internal locations in proteins,2 the PTS2 receptor (ScPex7p) of S. cerevisiae binds to a Gal4p-thiolute fusion in which the PTS2 is located internally (21).

An earlier study (14) had noted that a human patient with a nonsense mutation in the PTS1R gene was deficient in the import of both PTS1- and PTS2-containing proteins, suggesting a connection between the PTS1 and PTS2 import pathways. Because proteins containing tetratricopeptide repeats are known to interact with polypeptides possessing WD40 repeats, this connection had been explained by a possible association between the HsPex5p (a tetratricopeptide repeat protein) and the human PTS2 receptor (HsPex7p, a putative WD40 protein based on homology with ScPex7p) (1, 27). Proof of such an association between the PTS1 (ScPex5p) and PTS2 receptors (ScPex7p) of S. cerevisiae was obtained recently in a yeast two-hybrid system (21).

Controversial Subcellular Locations of the PTS1 and PTS2 Receptors

In cell fractionation studies, the PTS1 receptor of P. pastoris, PpPex5p, behaves like an integral PMP though no TMDs are obvious in its sequence. It faces the cytosol and binds the PTS1 sequence directly with high affinity, both as a purified recombinant protein and when it is associated with purified peroxisomes (26). However, multiple subcellular locations for this homologue have been found for its homologues from other species. The S. cerevisiae homologue, ScPex5p, is mainly cytosolic (28), the HpPex5p (former names, HpPah2p and HpPer3p) from H. polymorpha is cytosolic and intraperoxisomal (16, 29), and YlPex5p (former name, YlPay32p) from S. cerevisiae is principally intraperoxisomal (17). However, multiple subcellular locations for this homologue have been found for its homologues from other species. The S. cerevisiae homologue, ScPex5p, is mainly cytosolic (28), the HpPex5p (former names, HpPah2p and HpPer3p) from H. polymorpha is cytosolic and intraperoxisomal (16, 29), and YlPex5p (former name, YlPay32p) from Y. lipolytica is principally intraperoxisomal (17). HpPex5p (former names, PTS1R and PXR1) has been described as being mainly cytosolic and only partially peroxisomal (12, 14), as well as completely peroxisome-associated (13).

The location of the PTS2 receptor in S. cerevisiae is equally equivocal. Kunau and colleagues (18, 21) report that an epitope-tagged and overexpressed version of ScPex7p is mainly cytosolic and partially peroxisomal, whereas Zhang and Laz-arov (19, 20) find that a different epitope-tagged version of the same protein is intraperoxisomal and that the protein has a PTS near its N terminus.

Shuttling of Receptors between the Cytosol and the Peroxisomes

The varying subcellular locations of the PTS1 and PTS2 receptors have resulted in a model that proposes shuttling of the receptors between the cytosol and the peroxisomes (1). It is clear that both the PTS1 and PTS2 receptors are capable of binding their respective PTSs in the absence of peroxisomes (11, 12–14, 21, 26, 27). Thus, cytosolic PTS receptors would be capable of binding PTS-containing proteins. Following this initial interaction, the receptor(s) would transport the PTS-containing protein for delivery to the translocation machinery in the plane of the peroxisomal membrane (18). The sequence of events after this step remains a mystery. Several scenarios can be entertained. 1) The receptor might be recycled to the cytosol for another round of import. 2) The receptor might enter the peroxisome with its cargo and then be degraded within the matrix following release of the cargo. 3) The receptor might deliver its cargo in the peroxisomal matrix and then be recycled out of the peroxisome. Experimental tests need to be designed to address which of these possibilities is correct.

In Vitro Systems for the Analysis of Import

The import of radiolabeled matrix proteins translated in vitro into rat liver and yeast peroxisomes has been monitored using binding and protease protection assays (30–33). The import was time-, temperature-, ATP-, and signal-dependent.

Import of PTS1-containing proteins into permeabilized mammalian cells has shown, in addition to the features described above, that the process is cytosol-dependent and sensitive to N-ethylmaleimide (34, 35).

One report of in vitro import of a PTS2-containing protein into a subcellular fraction enriched in peroxisomes has appeared (33), but the specific requirements have not been elucidated.

Quantitative cytosol-dependent in vitro import assays are not yet available. The fluorescence-based assays in permeabilized cells are not quantitative, and the import assays using proteins radiolabeled by in vitro translation are cytosol-independent.

PMPs can also be imported post-translationally into purified rat liver peroxisomes in vitro (6, 10). Insertion in the membrane was evaluated using extractability with alkaline sodium carbonate. The insertion of PMP22 and PMP70 into rat liver peroxisomes is time- and temperature-dependent but independent of ATP and N-ethylmaleimide-sensitive factors (6, 10). It is not yet clear, however, whether the topology of the proteins inserted in the membrane mimics that observed in vivo. The mPTTs are unknown for the proteins studied, so the signal dependence of import could not be assessed.

Cytosolic and Membrane Proteins Involved in Import

Cytosolic hsc70 (the homologue of Escherichia coli DnaK) and hsc40 (homologue of E. coli DnaJ) are required for import of PTS1-containing proteins in permeabilized cells (36). Because protein unfolding (see later) is not essential for import of peroxisomal matrix proteins, the requirement for these factors has been explained in terms of alternative models (2), which do not require the stabilization of fully unfolded proteins by these chaperones.

It is likely that in mammalian cells, and in several yeasts, the PTS1 and PTS2 receptors shuttle between the cytosol and the peroxisomal membrane, transporting their cargo to the translocation machinery in the peroxisomal membrane. A docking protein, Pex13p, for the yeast PTS1 (Pex5p) receptors has been found in S. cerevisiae and P. pastoris. It is an integral PMP and contains an SH3 domain (28, 37, 38). Proteins involved in preventing entry of the receptors into the peroxisomal matrix or others playing a role in receptor recycling to the cytosol have been postulated, but no candidates have been identified yet (1).

There appears to be a role for a 35-kDa zinc-binding PMP in the import of peroxisomal matrix proteins. The function of this protein and its relationship to several zinc-finger proteins known to reside in the peroxisomal membrane (listed in Ref. 23) are under investigation.4

The genes for several integral PMPs involved in peroxisome

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1 W. Nuttley and S. Subramani, unpublished data.
2 S. Terlecky and S. Subramani, unpublished data.
protein biogenesis and/or import have been characterized, but it is not really clear whether they are affected mainly in peroxisome biogenesis or import, or both. The identities of PMPs constituting the translocation channel are likely to be derived from a combination of genetic and biochemical studies. Functional data are not yet available to show that the PMPs already characterized are directly involved in the peroxisomal protein translocation machinery.

**Folded State of Peroxisomal Matrix Proteins during Their Import**

PTS1- and PTS2-containing proteins can be imported into peroxisomes in an oligomeric state that allows subunits lacking a PTS to be imported into peroxisomes in association with other subunits that do have a PTS (4, 5). Furthermore, gold particles conjugated to a peroxisomally targeted protein are imported into the organelle membrane (39). These results show that protein unfolding is not essential for the targeting of proteins to the peroxisomal matrix. Similar results are observed for glycosomes, which are related to peroxisomes (40). The path taken by folded, oligomeric proteins across the peroxisomal membrane may either be through a gated pore or channel, or may involve an endocytosis-like mechanism at the peroxisomal membrane (2). Protein unfolding requirements for PMPs have not been addressed.

**Biogenesis of Peroxisomal Membrane Proteins**

It is believed that PMPs are synthesized on free polysomes and then imported post-translationally to the peroxisome, without any involvement of the ER in the biogenesis. This fact has been documented for two mammalian proteins, PMP22 and PMP69/70 (7, 9). Import of PMP70 from the cytosol to the peroxisomes has been confirmed recently by pulse-chase experiments (6). Furthermore, PMP70 and PMP22 have been shown to be targeted to purified peroxisomes in *vitro*, following translocation of these proteins in a rabbit reticulocyte lysate (6, 10). PMP26 is synthesized on free polysomes, but there is no evidence in *vivo* or *in vitro* that it is imported to peroxisomes directly from the cytosol (8).

In contrast, mammalian PMP50 is localized both to the ER and to peroxisomes, and is synthesized on membrane-bound polysomes (9).

These data are insufficient to rigorously rule out an involvement of the ER in peroxisome biogenesis. In fact, several recent experiments suggest the possibility that a subset of PMPs may reach the peroxisomes via the ER.

Overexpression of the *S. cerevisiae* Pas21p (name not assigned in Pex nomenclature) has been found to result in a pronounced proliferation of intracellular membranes that appear to be continuous with the nuclear ER and also contain ScPas21p. A truncated version of ScPas21p is targeted to the plasma membrane of *S. cerevisiae* (41), and a fusion of the first 16 amino acids of HpPex3p (old HpPer9p) with a reporter protein causes its accumulation in the ER (42). Furthermore, treatment of *H. polymorpha* cells with brefeldin A results in the localization of several peroxisomal membrane and matrix proteins to the ER (cited in Ref. 42). On the one hand, these new data may represent artifacts of a natural process gone awry as the result of overproduction of a protein, perturbation of the cells with drugs, or synthesis of polypeptides in which cryptic targeting signals for the secretory pathway are exposed. However, on the other hand, these results may also reflect the presence of a novel mechanism for the generation and/or growth of peroxisomes. Such a mechanism might involve cotranslational, or even post-translational, insertion of a subset of PMPs into some part of the (smooth) ER membrane. Budding and release of vesicles containing these key PMPs would create a peroxisome precursor that could either fuse by heterotypic fusion to a preexisting peroxisome to allow organelle growth or undergo homotypic fusion to generate larger vesicles, which would continue to import the matrix proteins and the other PMPs directly from the cytosol to become functional peroxisomes.

An obvious question is whether there exist reasonable precedents for various aspects of this model. As stated above, PMP50 is localized to the ER and the peroxisomes and is synthesized on membrane-bound polysomes lending credence to the suggestion that cotranslational insertion of a protein into the ER might lead eventually to residence of the protein in the peroxisome (9). Alternatively, post-translational insertion of membrane proteins such as cytochrome *b*$_5$ (44, 45), cytochrome *b*$_6$ reductase (46), and the SNARE protein, synaptobrevin (47), into the ER membrane suggests that the post-translational insertion of certain PMPs into the ER membrane is plausible. Recent results also support a role for vesicles and vesicular fusion events in peroxisome biogenesis.

Several features of the proposed involvement of the ER in peroxisome biogenesis are worth noting: 1) The model explains, at least in part, the heterogeneity of peroxisomes in terms of their import competence and their constituents. The vesicles and their fusion intermediates may exhibit different physical properties (such as densities on gradients), import competencies, and protein compositions relative to mature peroxisomes. 2) The model provides a mechanism for generating peroxisomes from the ER, even when all pre-existing peroxisomes are lost. The prevalent model, in which new peroxisomes arise by budding and fission of pre-existing ones, has to invoke *de novo* synthesis of the organelle to explain this phenomenon (48). This would violate the proposal by Palade (49) that cellular membranes cannot arise *de novo* but are derived instead from other pre-existing membranes. 3) We predict that there may be two classes of PMPs distinguishable by the targeting signals they possess: those that are targeted initially to the ER and are involved in the earliest stages of peroxisome biogenesis and others that are targeted directly to import-competent peroxisomes. It will be interesting to see whether these two classes of PMPs exist and whether they use different mPTTs. 4) The involvement of the ER in peroxisome biogenesis would explain why overproduction of certain Peroxins causes proliferation of ER-like membranes or of vesicles (41, 50). 5) The model would provide the means to supply lipids to the peroxisomal membrane and account for the ability to proliferate peroxisomes rapidly in response to nutritional cues.

It should be possible to devise experimental tests for various steps of the model to see if they are correct. The proposal that both peroxisomal matrix and membrane proteins might arise from parts of the smooth ER was actually made over three decades ago by Novikoff and Shin (43) but was discarded primarily because of incontrovertible data showing that many peroxisomal matrix, and a few membrane, proteins are imported directly from the cytosol to the peroxisome. It will not be surprising if peroxisomes still have some novel lessons to teach us.

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