Minireview

Peroxisomal Biogenesis: Multiple Pathways of Protein Import*

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The purpose of this review is to bring together and reflect upon recent experimental results concerning the molecular mechanisms underlying the biogenesis of peroxisomes and other microbodies. These organelles, which are nearly ubiquitous in eucaryotic cells, carry out a variety of essential reactions in lipid metabolism as well as other important biochemical functions (1). The burgeoning interest in peroxisomal biogenesis, galvanized by the successful application of yeast genetics, is reflected by the recent appearance of a number of other review articles (2-5). The identification and delineation of peroxisomal targeting sequences (PTSs) are being complemented by the identification of components of the cellular peroxisomal protein import machinery, predominantly through analysis of peroxisomal biogenesis mutants. Such mutants have now been established in mammalian CHO cells (6-8) and in four types of yeast, Saccharomyces cerevisiae (9-12), Pichia pastoris (13, 14), Hansenula polymorpha (15), and Yarrowia lipolytica (16). Genes that complement many of these mutants have been cloned and characterized, including several peroxisome assembly (PAS)/peroxisome biogenesis (PEB) genes from S. cerevisiae (4, 17-20), Candida tropicalis (21, 22), one from Y lipolytica (23), and a rat (24) and homologous human (25) gene which complement a CHO peroxisome-deficient cell line. One of the tasks ahead is to relate knowledge concerning PTSs to that of components of the cellular import machinery. Peroxisomes are similar to mitochondria in that biogenesis proceeds by division of pre-existing organelles and the post-translational import of proteins. Although peroxisomes lack DNA and are enclosed by only a single membrane, the observations that they are present in almost all eucaryotic cells and do not arise de novo have encouraged speculation that they are a subset of their microbody proteins. However, as detailed in Table 1, the range of permissible sequences has shown an unexpected degree of species specificity.

Mammals—Several mammalian peroxisomal proteins end with tripeptides within the originally described consensus (S/A/C)-(W/R/K)-(L) for peroxisomal targeting of luciferase in vivo in CV1 cells (28). In the case of one such protein, rat acyl-CoA oxidase, an in vitro import system has provided independent evidence for the importance of the terminal -SKL in targeting to peroxisomes (32).

Yeast—In the yeast S. cerevisiae, peroxisomal citrate synthase is targeted by a C-terminal -SKL sequence, which is absent from the mitochondrial isozyme (33). In addition, the peroxisomal multifunctional fatty acid β-oxidation protein terminates in -SKL (34), as do malate synthase (35) and isozyme 3 of malate dehydrogenase (36); the latter two activities have been detected by cell fractionation in S. cerevisiae peroxisomes (37).

Targeting Sequences of Matrix Proteins—Variations on Three Themes

C-terminal PTSs—Tripeptides Outside the Mammalian Consensus Are Functional in Yeasts and Trypanosomes

Numerous peroxisomal proteins from a wide range of species are targeted by C-terminal tripeptides. The first such PTS to be characterized was the C-terminal -Se-Lys-Leu-COOH(-SKL) tripeptide responsible for targeting of transgenically expressed firefly luciferase to monkey kidney CV1 cell peroxisomes (27). This tripeptide and conservative variants thereof (see Table I) are sufficient to target reporter proteins to peroxisomes in these cells (28).

The observation that an insect PTS functioned efficiently in cultured mammalian cells intimated the evolutionary conservation of this PTS across the eukaryotic kingdom for its recognition. This was confirmed by peroxisomal localization of luciferase expressed in plant and S. cerevisiae cells (29). Further evidence for conservation of this sorting pathway derived from the observation that antibodies against a peptide terminating with -SKL immunolabeled microbody proteins of P. pastoris, Neurospora crassa, and Trypanosoma brucei, as well as plants and mammals (30, 31). All species analyzed to date appear to employ C-terminal tripeptide PTSs to target a subset of their microbody proteins. However, as detailed in Table 1, the range of permissible sequences has shown an unexpected degree of species specificity.

It may be that individual species utilize a more limited range of tripeptide sequences than the full spectrum of functional PTSs defined by mutagenesis and heterologous expression studies. For instance, antibodies raised against a peptide terminating -AKI recognize multiple peroxisomal proteins from C. albicans, C. tropicalis, and Y lipolytica but show no reaction with rat liver peroxisomal proteins, consistent with -AKI not falling within the mammalian consensus.

N-terminal PTSs—Cleaved and Uncleaved

In all organisms in which peroxisomal biogenesis has been studied, there exist proteins whose C termini clearly fall outside the appropriate consensus to be considered PTSs of the class described above. For one such rat protein, 3-ketothiolase, it has been demonstrated that peroxisomal targeting is achieved through the

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first 11 amino acids of an N-terminal peptide consisting of 26 amino acids, which is removed proteolytically after import (45, 46). Similar sequences are present in thioldases of humans and several yeasts and in a peroxisomal isozyme of watermelon malate dehydrogenase. The first 16 residues (MSQFtLQSIKDHLVLSA) of plant enzyme into lytically cleaved in watermelon but not in the protein termini. Two redundant internal PTSs have been located in peroxisomes, which is removed proteolytically after import (45, 46). Similarly, the conserved arginine and feature, are nonfunctional. The asterisks refer to the fact that -N& and -SQL (outside the mammalian consensus, but not directly tested) have been found at the C termini of mammalian peroxisomal proteins. Uppercase, underlined, not yet found on a peroxisomal protein in that species.

| Mammals | S. cerevisiae | H. polymorpha | C. albicans | Trypanosomes |
|---------|---------------|---------------|-------------|-------------|
| SKI     | SKI           | SKI           | SKL         | SKI         |
| SKL     | SKI           | SKI           | SKL         | SKI         |
| SRL     | SKL           | SKI           | SKL         | SKI         |
| SRF     | SKL           | SKI           | SKL         | SKI         |

**Import Machinery**—The Complexity of Peroxisomal Biogenesis

The accumulated data on PTSs have raised interesting questions concerning the machinery and mechanisms responsible for the import of matrix proteins into peroxisomes. Distinct classes of PTS substrates may account for the existence of the two import receptors as shown in Fig. 1. Phenotypic and genetic analysis of peroxisome biogenesis mutants is proving exceptionally powerful in unraveling the complexity of this import machinery, revealing a picture that shows remarkable correspondence to the PTS data. For example, within the collections of both human and yeast complementation groups, mutants that mislocalize proteins targeted by C-terminal tripeptides (but not by the deamidated N-terminal -AKL sequence and not C-terminal tripeptide targeted proteins) have been identified (10, 11, 62). This is consistent with the hypothesis that the C-terminal and thiolase PTSs will have distinct receptors (see Fig. 1). The postulation that yeast acyl-CoA oxidase (internal PTS) may need yet another receptor is supported by the observation that this protein is correctly sorted in yeast mutants that mistake either thiolase or catalase (60). Many mutants (yeast and human) appear to missort all matrix proteins (as discussed below), indicating the existence of shared components, possibly in the form of a central translocation machinery, as shown in Fig. 1. Other shared components might be found in the cytosol or within the organelle (chaperons, perhaps?). Genetic complementation of the carefully characterized peroxisome biogenesis mutants is now beginning to define this molecular structure of the import machinery (4). A second receptor in H. polymorpha interacts with SKL, NKL, and ARF (see Table I)? If so, how has this extra tolerance (relative to mammalian cells) of tripeptide sequence evolved without compromising the specificity of peroxisomal protein import? Could a family of tripeptide receptors with different specificities be operating in some species? These questions remain unanswered, but two recent papers have begun the process of unraveling this situation. First, McCollum et al. (21) have cloned the gene (PAS8) that complements P. pastoris pas8 mutant cells. These mutant cells have lost the ability to import transgenically expressed firefly luciferase into peroxisomes while retaining the ability to import thiolase, suggesting that the affected gene product may be an -SKL receptor. This is supported by biochemical experiments showing that the product of the PAS8 gene (Pas8p) binds a dodgedependent targeting signal in -SKL but not a control nonapeptide lacking the -SKL sequence (21). Other proteins mislocalized to the cytosol in pas8 cells include catalase (the sequence of which is unknown in this species), dihydroxyacetone synthase, and methanol oxidase.
N-terminal import of SKL-conjugated albumin is saturable and could be mediated by cytosolic receptors, both of which end with -SKL. They showed that the import block could be overcome by the addition of fresh cytosol. Possibly the receptors could be peripheral membrane proteins, or even shuttle between cytosol and peroxisome. The conjecture for a shared translocation machine is supported by the many mutants of S. cerevisiae that appear to be defective in the import of all matrix proteins. However, the existence of receptor-specific translocation subunits, or totally independent translocation machineries remain valid alternatives; not all the shared machinery implied by the mutants need be membrane-associated.

Figure 1: A model for the import of matrix proteins into peroxisomes. A. A section of the peroxisomal membrane is shown, in which are embedded receptors for each of the three known types of peroxisomal targeting features. Above, in the cytosol, are newly synthesized peroxisomal proteins, each with a different PTS. The receptors are shown feeding into a common translocation machinery. The ATP-binding site reflects the requirement for ATP hydrolysis in translocation (69, 74). B, an alternative version of the model with soluble, cytosolic receptors, which might dock at the suggested sites on the translocation machinery. Possibly the receptors could be peripheral membrane proteins, or even shuttle between cytosol and peroxisome. The conjecture of a shared translocation machine is supported by the many mutants that appear to be defective in the import of all matrix proteins. However, the existence of receptor-specific translocation subunits, or totally independent translocation machineries remain valid alternatives; not all the shared machinery implied by the mutants need be membrane-associated.

(which terminate -DKL and -ARF, respectively). Although there is no information on the targeting sequences of these latter P. pastoris peroxisomal proteins, the fact that the same H. polymorpha enzymes are targeted via their C termini (-NKL and -ARF, respectively) (41) suggests that this is true in P. pastoris. All this points toward the P. pastoris PAS8 gene product (Pas8p) being a tripeptide PTS receptor with broad specificity, recognizing -SKL, -DKL, and -ARF. Pas8p is associated with peroxisomes (21), and it is stated in a recent review that it resides on the cytoplasmic face of the peroxisomal membrane (5). Corroborating evidence derives from analysis of a S. cerevisiae PAS mutant (pas10), which is complemented by PAS10, the S. cerevisiae homologue of P. pastoris PAS8 (68). S. cerevisiae pas10 cells fail to import several peroxisomal proteins, including multifunctional enzyme and transgenically expressed luciferase, both of which end with -SKL.

By analyzing import in semi-intact cells, Wendland and Subramani (69) gathered evidence for a mammalian cytosolic SKL-bindng protein required for peroxisomal import. They showed that import of SKL-conjugated albumin is saturable and could be blocked by preincubation with peptides terminating in -SKL; the block could be overcome by the addition of fresh cytosol.

Machinery Required Specifically for the Import of Proteins with N-terminal PTS6—Yeast mutants pas7 and pep1 specifically fail to package thiolase but do import most other peroxisomal proteins (10, 11). Cells from patients with the human genetic disease rhizomelic chondrodysplasia punctata are similar, in that morphologically normal peroxisomes are present, and targeting by C-terminal tripeptides is normal, but thiolase biogenesis is defective (70). Cloning of the genes that complement these mutants should prove very useful in unraveling the import pathway of thiolase. One or more of these genes might encode a thiolase import receptor.

Machinery Required for the Import of All Matrix Proteins—The original biochemical characterization of Zellweger syndrome showed that numerous proteins, including thiolase and proteins with C-terminal tripeptide PTSs, fail to be packaged into peroxisomes (1). Moreover, AGT (which appears to have internal targeting) is not packaged into peroxisomes in Zellweger syndrome (71). Thus the defect in Zellweger cells, which do assemble peroxisomal membranes, appears to be a generalized failure to import matrix proteins (60). This conclusion has been extended by Metley et al. (62), who expressed luciferase and choramphenicol acetyltransferase fused to the rat thiolase targeting presequence in control and Zellweger fibroblasts. Three of the four Dutch Zellweger complementation groups (II, III, V) could not package either protein into peroxisomes. Thus at least three of the four complementation groups fail for the import of both proteins with -SKL PTSs and proteins with thiolese-type PTSs. For one group (V), the defective gene product is peroxisome assembly factor 1 (PAF-1), a 35-kDa integral membrane protein of unknown function (72). Additional American complementation groups do not package SKL-targeted proteins and have not been tested yet for their ability to package thiolase or AGT (60).

Many S. cerevisiae mutants fail to package all enzymes tested, including thiolase (4, 9–12). Genes that complement pas1, pas2, pas3, and pas8 have been cloned, but definitive functional assignments for the respective gene products have not been possible. The PAS3 gene product (Pas3p) is associated with peroxisome membranes (19), and thus, like PAF-1, is positioned such that it could contribute to translocation or possibly to the docking of receptors. Pas2p, a member of the family of ubiquitin-conjugating enzymes, may be a peripheral membrane protein (18). Pas1p (17), which is hydrophilic, and Pas8p (20), which has two possible membrane-spanning domains, are predicted to belong to a large family of ATPases with diverse cellular functions (4). P. pastoris Pas3p (22) and Y. lipolytica Pay4p (23) also belong to this family. The functions of these proteins may be determined by future biochemical analyses of the defects in peroxisomal biogenesis which they cause, perhaps exploiting established in vitro peroxisome assembly assays (32, 73, 74) and permeabilized cell systems (69).

Studies on permeabilized mammalian cells show a requirement for cytosol in peroxisomal protein import (69), but many essential factors are organellar-associated. Fibroblasts from six U. S. complementation groups of peroxisome assembly patients had fully functional cytosol but nonfunctional organelles (63).

There is evidence that import may be a two-step process, with translocation being preceded by an ATP-independent binding interaction (74). Bearing this in mind, some mutations in components of the import machinery, such as in the postulated translocation subunits, might not interfere with the binding of newly made peroxisomal proteins to receptors on the outside surface of peroxisomes (and/or the docking of soluble receptors to the peroxisome surface) (Fig. 1). Thus small amounts of newly made proteins might be associated with the outside surface of peroxisome ghosts in these mutants (for example, see Ref. 62).

Conservation and Divergence in the Evolution of Peroxisomal Biogenesis Mechanisms

The conservation of sorting by C-terminal -SKL is diverse species encourages the idea that at least part of the fundamental molecular basis of peroxisome biogenesis may be conserved. The conservation of N-terminal targeting of thiolase in yeast and rat, and of malate dehydrogenase in watermelon as well, further supports this view. Moreover, there is a remarkable similarity in the spectrum of phenotypes between the naturally occurring human peroxisome biogenesis mutants and the generated yeast mutants; some mutations prevent the import of most if not all matrix proteins, whereas other mutations are selective and appear to dis-
PASIPEB genes have yet been cloned. The putative receptor for SKL is reported to be on peroxisomal membranes, whereas a yeast isozyme terminates in -SKL. In H. polymorpha, sorting of catalase is fully dependent on a C-terminal tripeptide PTS, whereas the S. cerevisiae enzyme contains a second, internal PTS. Such evolutionary divergence cues caution in the interpretation of heterologous targeting studies and raises questions concerning the evolutionary origin of the several types of PTSs.

Comparison of the mammalian and yeast genes/proteins implicated in peroxisomal biogenesis thus far gives no evidence of conserved components of the cellular biogenesis machinery. Homologues of the two mammalian peroxisomal membrane proteins that have been shown (PAF-1 (25) and postulated (PMP70) (75) to be defective in Zellweger syndrome patients have not yet appeared in the collections of yeast pas/pes mutants, although PAF-1 has been assigned to the same family of zinc finger proteins as S. cerevisiae Pas1p (41). Likewise, no mammalian homologues of yeast PAS/PEB genes have yet been cloned. The putative P. pastoris receptor for SKL is reported to be on peroxisomal membranes, whereas a mammalian SKL binding activity required for import via this PTS appears to be cytosolic. It is possible that a receptor might shuttle from cytosol to peroxisome, and the steady-state distribution might vary between species, thus explaining the apparent differences.

There is clearly a good way to go toward understanding peroxi-some biogenesis and the similarities and differences of this process between species. Current progress promises that many of the questions posed here will be resolved in the near future, a prospect that is not only intellectually satisfying but also of enormous importance in the drive toward an understanding of the primary defects in Zellweger syndrome.

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