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Peroxisomes: flexible and dynamic organelles

Ida J van der Klei* and Marten Veenhuis

Peroxisome development is a dynamic process that may involve organelle fusion and fission events. Cells contain different types of peroxisomes that vary in protein composition and capacity to incorporate membrane and matrix proteins. The protein import machinery is highly flexible and includes a cycling receptor that passes the peroxisomal membrane.

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Abbreviations

COP coat protein
mPTS targeting signal of PMPs
PEX gene encoding peroxin
Pexp translation product of PEX gene
PMP peroxisomal membrane protein
TPR tetratricopeptide repeat

Introduction

Peroxisomes are ubiquitous cell organelles that strongly vary in abundance and function between cells. Characteristic is their extremely high matrix-protein concentration, sometimes leading to crystalline inclusions. The matrix is packed with enzyme molecules that catalyse diverse oxidative and biosynthetic reactions [1,2]. Fungal Woronin bodies represent an exceptional class of peroxisomes in that they display a structural function in sealing septal pores of damaged hyphae [3] (Figure 1).

Because the peroxisomal membrane is semi-permeable *in vivo*, the metabolic function of the organelle requires the activities of several transporters. So far, however, only a few are known. Their identification is severely hampered by the exceptionally low abundance of peroxisomal membrane proteins (PMPs) [4]. The fact that peroxisomal membranes are leaky *in vitro* also obstructs their functional characterisation. Only recently, the first peroxisomal transporter was analysed for its transport activities and substrate specificities, which was achieved upon functional reconstitution in liposomes [5].

The identification of proteins involved in peroxisome biogenesis has been more successful. During the past decade, various genes involved in matrix-protein import, membrane biogenesis, organelle fission and movement have been identified, and the first details on their molecular functions are emerging. Recent breakthroughs in this fascinating field are discussed in this paper.

Peroxisome development

The prevailing model of peroxisome biogenesis, proposed by Lazarow and Fujiki in 1985 [6], predicts that the organelle grows by uptake of new components from the cytosol and multiplies by division (Figure 2a). Little is known on the incorporation of lipids into the peroxisomal membrane. It has been postulated that they are transported from the ER to peroxisomes in vesicles, together with specific PMPs that reach peroxisomes via the ER (Figure 2b). Most PMPs, however, are thought to be sorted directly to the peroxisomal membrane [7].

Different mPTSs — targeting signals of PMPs — have been identified [7]. Interestingly, individual PMPs can contain more than one independent mPTS [8]. The mPTSs known so far lack conserved amino acid motifs but often contain a group of positively charged amino acids. Mutational analysis of the mPTS region of *Hansenula polymorpha* Pex3p revealed that only part of these charges are essential for targeting, however [9].

Three peroxins (Pex3p, Pex16p and Pex19p) have been implicated in PMP targeting/insertion [10–12]. Pex19p was initially proposed to be the mPTS receptor. Later studies challenged this view and pointed to a role for Pex19p in formation/maintenance of PMP complexes [13,14]. Moreover, the finding that in *Yarrowia lipolytica* peroxisomes are formed in the absence of Pex19p argues against a role for Pex19p as the mPTS receptor [15].

Matrix protein import

For matrix proteins, two peroxisomal targeting signals (PTS1 and PTS2) are known that are recognised by the cytosolic receptors Pex5p and Pex7p, respectively. Both receptors bind their cargo proteins in the cytosol and guide them to a docking site at the peroxisomal membrane. Most peroxisomal proteins contain a PTS1, and their import has been extensively studied, and it is most likely that the PTS2 pathway follows similar principles (for an excellent review, see [7]).

Dammai and Subramani [16] recently presented compelling evidence that human Pex5p is a cycling receptor, which, upon binding to a PTS1–cargo protein, associates with the peroxisomal membrane, translocates across it, and finally, upon release of its cargo, recycles to the cytosol (Figure 3). This so-called ‘extended shuttle model’ was first proposed for the yeast *H. polymorpha* [17,18]. Other evidence came from studies by Dodt and Gould [19], who showed that Pex5p molecules do cycle between the cytosol and peroxisomes *in vivo*.

The importance of the recent work of the Subramani laboratory [16] is that it unequivocally showed that

Figure 1

Ultrathin sections of fungal cells showing (a) fission of a Woronin body from a normal peroxisome in *Penicillium chrysogenum* and (b) a hexagonal Woronin body in *Neurospora crassa*. KMnO_4 fixation. Bar = 0.5 μm . M, mitochondrion; N, nucleus; P, peroxisome.

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Pex5p molecules functionally enter the organelle. This has major implications in that a protein-export machinery must exist for Pex5p, and that in case Pex5p dissociates from the inner surface of the membrane, a Pex5p sorting machinery exists in the organellar matrix. Both aspects are, as yet, completely unresolved. It is also not known whether import and export require separate machineries or use one and the same. In this context, it is tempting to speculate that some peroxins proposed to function in PTS1 import in fact may function in Pex5p export. Given the binding properties to Pex5p (see below), Pex13p is a plausible candidate. Another candidate is Pex8p, which might play a role in intraperoxisomal sorting of the PTS1 receptor to the export site [20].

Molecular mechanisms of Pex5p-dependent import

A remarkably high number of proteins specifically interact with Pex5p (Figure 3). PTS1 binds to tetratricopeptide repeats (TPRs) in the carboxy-terminal half of the protein. The three-dimensional structure of this domain of human Pex5p containing a PTS1 peptide revealed that two clusters of three TPRs (TPR1–3 and TPR 5–7) almost completely enclose the PTS1, while TPR4 forms a hinge region [21], which does not play a direct role in PTS1 binding [22].

Pex8p, Pex12p, Pex13p and Pex14p interact with the amino-terminal half of Pex5p. Some of them also interact with each other or other peroxins, leading to an extensive network of interactions. There is still debate on the nature of these interactions. Some are only detected in one or a few species, and conflicting data have been presented on the domains involved. It seems unlikely that these discrepancies solely are related to species differences, as the mechanisms of peroxisome biogenesis are strongly conserved. Instead, they may reflect differences in experimental approaches. Indicative for this are the major differences observed in the strength of peroxin interactions. For instance, stable complexes containing Pex5p and several of its interacting partners could be isolated from rat and yeast peroxisomal membranes [23–25]. Other studies, however, pointed to dynamic, reversible interactions of Pex5p with other peroxins [26,27].

According to the extended shuttle model, Pex5p undergoes a series of protein association and dissociation events during one cycle of PTS1 import. This implies that the affinity of Pex5p for the different peroxins must vary, and possibly depends on protein context, conformation or modifications (e.g. phosphorylation, as observed for Pex14p [28,29]). These changes in affinities may not (completely) occur under the experimental conditions used to analyse peroxin interactions (e.g. in two-hybrid studies, *in vitro* binding studies, or when small portions of the protein are used). Indeed, it has been reported that the interaction of Pex5p with only the Src homology 3 (SH3) domain of Pex13p is much stronger when only the SH3 domain is used relative to full-length Pex13p [26]. Also, the very strong interaction [30] observed between small peptides of Pex5p and the amino-terminal domain of Pex14p is unlikely to occur *in vivo*, where Pex5p easily dissociates from the membrane upon altering the energy status of the cell [19].

Recent *in vitro* binding studies [26,27] indicated that Pex5p interacts with various partner peroxins in a spatio-temporally differentiated manner: cargo-bound Pex5p had highest affinity for Pex14p, whereas unloaded Pex5p preferentially bound Pex13p. Other data suggest that binding of cargo-bound Pex5p causes dissociation of the Pex13p–Pex14p interaction [27], which also points to a dynamic import machinery.

The order of the different Pex5p interactions is still highly speculative. Epistasis analysis revealed that Pex13p, Pex14p, Pex10p, Pex12p, Pex2p and Pex8p function at the initial stage of PTS1 import, followed by Pex1p/Pex6p and subsequently Pex22p and Pex4p [31]. The latter is in line with previous data that revealed a role for Pex4p in Pex5p export and recycling [32]. Most current models group Pex13p, Pex14p and Pex17p as docking proteins and Pex2p, Pex10p and Pex12p as the actual protein translocation machinery (translocon). However, the experimental basis for these roles is still limited and mainly comes from the observation that human cells defective in Pex2p, Pex10p or Pex12p are able to recruit Pex5p to the peroxisomal membrane [33]. However, in *H. polymorpha* Pex14p is not essential for PTS1 import or Pex5p docking, but for the efficiency of

Figure 2

Hypothetical model containing different proposed pathways of peroxisome formation. **(a)** The classical growth and division model proposed by Lazarow and Fujiki [6], with the exception that the temporary matrix-protein import competence, as observed in yeast, is included [18]. This model predicts that newly synthesized peroxisomal matrix proteins (MPs) and membrane proteins (PMPs) are incorporated in pre-existing, nascent peroxisomes (NP). When a certain size is obtained, a new peroxisome buds off, resulting in the formation of a small nascent peroxisome together with a large, mature one (M) that has lost the capacity to take up additional matrix proteins. **(b)** The peroxisome maturation model proposed by Titorenko and Rachubinski [2*,44,45]. According to this model, preperoxisomal vesicles (P1 and P2) are derived from the ER, which fuse into P3 vesicles that develop via P4 and P5 into mature peroxisomes (M). Each type of organelle has specific properties with respect to protein import competence and protein composition [2*]. **(c)** The model of South and Gould [12] for re-introduction of peroxisomes into cells of Pex16p-deficient human cell lines. First, Pex16p is targeted to preperoxisomal structures (pp) that do not derive from the ER. Next, other PMPs are incorporated into these structures, which transforms the organelle into nascent matrix-protein import-competent peroxisomes. **(d)** A schematic representation of *H. polymorpha pex3* in which the first 50 amino-terminal residues of Pex3p (N₅₀.Pex3p) fused to green fluorescent protein (GFP) is produced, which leads to the formation of several small 'preperoxisomal' structures from the nuclear membrane [48*]. Upon subsequent production of full-length Pex3p, part of these structures is transformed into normal NPs.

PTS1 import [34]. Unexpectedly, *H. polymorpha* Pex14p has a dual role and functions both in peroxisome formation and in the opposite process: namely, selective peroxisome degradation [35*].

Figure 3

Schematic representation of the extended shuttle model of PTS1 protein import. Upon binding to a newly synthesised PTS1-containing cargo protein, Pex5p (5) associates with a putative docking site at the peroxisomal membrane, which may contain Pex13p (13), Pex14p (14) and Pex17p (17). Subsequently, this Pex5p-cargo complex is translocated across the membrane by a so far unknown mechanism that may involve the ring finger proteins Pex2p (2), Pex10p (10) and Pex12p (12). In the organellar matrix, the Pex5p-cargo complex dissociates, followed by export of Pex5p, processes that might be mediated by Pex8p (8), Pex1p/Pex6p (1/6) and Pex4p/Pex22p (4/22).

A plausible model for a dynamic PTS1 matrix-protein import machinery is that upon arrival of the Pex5p-cargo complex at the peroxisomal membrane, a docking complex assembles, which subsequently dissociates, paralleled by association of another subset of peroxins, into a dynamic translocation pore. At later stages, other complexes may be formed that are involved in cargo dissociation and Pex5p export/recycling. The complexes that are assembled and disassembled may contain common elements [36], which explains the extensive network of protein-protein interactions that have been discovered.

Differential PTS1 protein import?

Hypothetical models on PTS1 protein import generally propose a single import pathway. However, this route might be much more versatile. For instance, certain PTS1 proteins are only imported as oligomers, whereas translocation of others is restricted to the monomeric conformation [37*,38*]. This observation already divides the PTS1 pathway into two separate, but overlapping, tracks. It is most likely that there are additional overlapping PTS1 pathways. Illustrative for this are the differences observed in the import of the three major PTS1 proteins in mutant strains of methylotrophic yeasts (Table 1), where each protein apparently has its own requirements in addition to the general import components. Importantly, discrepancies on data of PTS1 protein import may therefore be related to differences in the marker

Table 1

Differential import of the three major PTS1 proteins in methylotrophic yeasts.

Protein	Mature conformation	Imported conformation	Wild type	$\Delta pmp47$	<i>ass3</i>	$\Delta pex14$	$\Delta pex14::P_{AOX}PEX5$
AO	Octamer	Monomer	+	+	–	–	+
DHAS	Dimer	Dimer	+	–	+	–	+
CAT	Tetramer	?	+	+	+	–	–

In wild-type cells of methylotrophic yeasts (*Candida boidinii*, *H. polymorpha*, *Pichia pastoris*) the three major PTS1 proteins alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT) are exclusively located in peroxisomes. AO is imported as a monomer, whereas DHAS is only imported as a dimer [38]. *C. boidinii* cells lacking the transporter protein PMP47 ($\Delta pmp47$) are specifically blocked in import of DHAS, whereas AO and CAT are normally imported [49]. In the *H. polymorpha* *pex14*-null mutant ($\Delta pex14$), import of all three PTS1 proteins is blocked. However, upon overproduction of Pex5p ($\Delta pex14::P_{AOX}PEX5$) import of AO and DHAS is restored, whereas CAT remains mislocated in the cytosol [34]. The recently isolated *H. polymorpha* AO assembly mutant (*ass3*) is specifically defective in AO import [50].

proteins used. In this respect, localisation experiments using artificial model proteins, such as green fluorescent protein (GFP)–SKL, should be interpreted with care.

Peroxisome division

Two proteins, Pex11p and VPS1, have been proposed to function in peroxisome fission. Pex11p overproduction leads to the formation of numerous small organelles, whereas in the absence of Pex11p only one large peroxisome is formed per cell [39,40]. It has been suggested that Pex11p functions in medium-chain fatty acid (MCFA) transport, rendering its role in proliferation as indirect [41]. This view is questioned, however, because the Pex11p-induced proliferation effect is also observed in cells that do not perform MCFA β -oxidation [42].

In *Saccharomyces cerevisiae*, the dynamin-related protein Vps1p was recently shown to be required for peroxisome division [43•]. It seems possible that Pex11p also participates in this process. Alternatively, Pex11p molecules might be involved in a coat-mediated budding process.

Alternative modes of peroxisome formation

In a great series of ground-breaking experiments, Titorenko and colleagues were the first to provide direct evidence that peroxisome development in *Y. lipolytica* involves membrane fusion [2•,44,45] (Figure 2b). These studies revealed that peroxisomes develop by a multistep process that starts with the formation of pre-peroxisomal vesicles, thought to arise from a subdomain of the ER. These structures harbour distinct subsets of membrane proteins, as well as components of coat protein II (COPII) vesicles, and transform into early peroxisomal precursors, designated P1 and P2, as a result of the uptake of additional membrane proteins and release of the COPII elements. P1 and P2 peroxisomes are competent to incorporate distinct sets of matrix proteins and fuse in a Pex1p/Pex6p-dependent way to generate P3 peroxisomes that develop into mature peroxisomes by a multistep assembly pathway via P4 and P5 peroxisomes (Figure 2b).

In peroxisome-deficient human fibroblasts also, evidence was obtained for a multistep peroxisome-assembly pathway

to form new peroxisomes upon reintroduction of *PEX16* into cells of a Pex16p-defective cell line [12] (Figure 2c). In this system, first Pex16p is incorporated in a preperoxisome, followed by the insertion of other PMPs that enables subsequent matrix-protein import. These preperoxisomes are autonomous structures that do not arise from the ER and assemble into nascent peroxisomes independent of COP proteins [46] or the ER translocon [47].

Recently, however, it was shown that in *H. polymorpha* *pex3* cells the endomembrane system might serve as a template for the formation of new peroxisomes [48•]. Upon synthesis of the initial 50 amino acids of Pex3p (N₅₀.Pex3p) in *H. polymorpha* *pex3*, various vesicles where formed that arose from the nuclear envelope (Figure 2d). These vesicles showed peroxisomal characteristics and contained, apart from N₅₀.Pex3p, other peroxisomal membrane proteins. Upon subsequent synthesis of full-length Pex3p, a portion of these vesicles developed into normal peroxisomes [48•].

As concepts, the models proposed for *Y. lipolytica* (Figure 2b) and *H. polymorpha* *pex3* (Figure 2d) display comparable properties, as they suggest that (re-) introduction of peroxisomes initiates at the endomembrane system. However, the *Y. lipolytica* model (Figure 2b) proposes that this pathway occurs in wild-type cells upon induction of peroxisome formation. The re-introduction models (Figure 2c,d) explain how peroxisomes assemble in cells that were fully devoid of peroxisomes due to genetic defects. It is unclear whether such a mechanism is also operative in cells that grow normally at peroxisome-inducing conditions. In *H. polymorpha*, the ‘normal’ pathway of growth and division became operative upon prolonged cultivation in cells in which peroxisome formation initially was started by N₅₀.Pex3p-induced vesicles (Figure 2a,d). Thus, in this organism the above mechanism of peroxisome recovery may represent a rescue mechanism that becomes functional in case peroxisomes are lost — for example, owing to failure in inheritance.

Conclusions

Despite the progress made in the isolation and characterisation of various proteins essential for peroxisome biogenesis, our

knowledge on their precise function is still remarkably poor. New approaches, including biophysical techniques, are essential to resolve the molecular details of matrix-protein import and the origin and synthesis of the peroxisomal membrane. However, a major problem concerns the striking differences in the proposed functions for specific peroxins. As outlined by Purdue and Lazarow [7•] for the highly diverse functions suggested for Pex1p and Pex6p (vesicle fusion versus matrix-protein import), it is difficult to envisage that these discrepancies are simply due to species differences or are methodology related. A possible strategy to resolve these questions is to make room for a more prominent position of physiology in the field. At present, the bulk of the progress is based on molecular genetic approaches. However, especially for yeasts the physiology (growth conditions, substrates, growth phase) of the examined cells may differ largely. Obviously, these variations are reflected in cell performance and thus also in the kinetics of processes involved in peroxisome biogenesis. It seems therefore advisable for the field to normalise cultivation conditions, preferably by using chemostat cultures, as these predict cells of equal performance at various separate cultivations. The use of normalised cells also seems crucial for comparison of data of future genomic/proteomic approaches.

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