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Review

Protein import machineries of peroxisomes ☆

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ABSTRACT

Peroxisomes are a class of structurally and functionally related organelles present in almost all eukaryotic cells. The importance of peroxisomes for human life is highlighted by severe inherited diseases which are caused by defects of peroxins, encoded by PEX genes. To date 32 peroxins are known to be involved in different aspects of peroxisome biogenesis. This review addresses two of these aspects, the translocation of soluble proteins into the peroxisomal matrix and the biogenesis of the peroxisomal membrane. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

Peroxisomes or microbodies are structurally and functionally related organelles of eukaryotic cells. They are mostly spherical, 0.1 to 1 μm in diameter and surrounded by a single lipid bilayer membrane [1]. The proteinaceous organellar matrix is electron-dense and contains no DNA. The peroxisome-family consists of peroxisomes, glyoxysomes of plants and fungi, glycosomes of trypanosomes, and Woronin-bodies of filamentous fungi [2]. With the exception of Woronin-bodies, whose sole function is to plug septal pores in case of hyphal injury [3], peroxisomes fulfil a variety of biochemical functions [4]. Foremost of these is fatty acid ß-oxidation which exclusively takes place in peroxisomes of fungi and plants. In mammals very long chain fatty acids are oxidized in peroxisomes. In addition, peroxisomes are involved in the synthesis of plasmalogens, cholesterol and bile acids [5–8] as well as the oxidation of alcohols, catabolism of purines and polyamines, metabolism of prostaglandins, photorespiration in plants and penicillin synthesis in fungi [1,9–11]. The importance of peroxisomes for human life is highlighted by severe inborn diseases (peroxisomal biogenesis disorders) like the Zellweger-Syndrome, Neonatal Adrenoleucodystrophy or Infantile Refsum's disease which are caused by defects of PEX genes [12]. At present, 32 different PEX genes have been discovered which are required for the biogenesis and maintenance of functional peroxisomes [13,14].

2. Import of matrix proteins

Proteins designated for import into the peroxisomal matrix or insertion into the peroxisomal membrane, follow distinct pathways.

Abbreviations: AAA, ATPase associated with various cellular activities; PTS, peroxisomal targeting signal; RING, really interesting new gene; Ub, ubiquitin

☆ This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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As peroxisomes neither contain DNA nor transcription/translation machineries, all peroxisomal proteins are encoded by the nuclear genome. Without exception, all peroxisomal matrix proteins are synthesized on free polyribosomes in the cytosol and imported post-translationally [15]. Thereby the involved import-receptor molecules pass through a cycle starting from the recognition by import receptors in the cytosol [16,17]. The receptor–cargo complex is then targeted to a docking complex at the peroxisomal membrane. Later on, the cargo is delivered to the peroxisomal matrix via a translocation pore and the receptor is released from the membrane [18]. Finally, the receptor is recycled for another round of import or removed by proteasomal degradation.

2.1. Targeting signals and recognition-factors

The sorting of proteins to peroxisomes depends on signal sequences, known as peroxisomal targeting signal (PTS) type I and type II. The PTS1, used by the majority of peroxisomal matrix proteins, is located at the extreme C-terminus and was initially discovered in firefly luciferase as the tripeptide SKL [19]. Based on mutagenesis experiments, amino acid permutations and sequence comparisons between different species, the PTS1 generally fits the consensus sequence (S/A/C)-(K/R/H)-(L/M) [20]. For most of the matrix proteins the presence of a PTS1 is sufficient for their proper targeting to the peroxisomal matrix. However, in some cases, additional interactions of the cargo protein with the receptor are required, which are provided by amino acid residues adjacent to the PTS1. Accordingly, the PTS1 has been redefined as C-terminal dodecamer [21].

In the cytosol, the PTS1 is recognized by the predominantly soluble protein Pex5p [22,23]. Pex5p is composed of two domains, a C-terminal domain that contains six tetratricopeptide repeats (TPRs) and provides high affinity PTS1-binding sites, and an N-terminal domain that functions in receptor docking and recycling [24].

The usage of the PTS2 for peroxisomal protein import varies from species to species. While in mammals only a few proteins are targeted to peroxisomes via the PTS2-pathway, in plants, approximately one third of peroxisomal proteins harbour a PTS2 [25]. In the yeast *Saccharomyces cerevisiae*, only 3-ketoacyl thiolase and the NAD^+-dependent glycerol 3-phosphate dehydrogenase (Gpd1p) have been identified as PTS2-proteins [26,27]. Remarkably, for *Caenorhabditis elegans* the PTS2-pathway does not play any role at all [28].

The PTS2 was first identified as a conserved sequence which is located near the N-terminus of rat liver thiolase and which in some species is comprised within a pre-sequence that is cleaved off after import into the peroxisomal matrix [29,30]. Sequence comparisons of the signal sequences of thiolases derived from different species, watermelon malate dehydrogenase, amine oxidase of *Hansenula polymorpha* and *Trypanosoma brucei* aldolase defined the PTS2 as the conserved nonapeptide R-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/A) [31]. PTS2-harbouring proteins are recognized by the soluble protein Pex7p [32]. It consists of six tryptophan-aspartic acid (WD) repeats, preceded by a distinct N-terminal region. Unlike Pex5p, the Pex7p-mediated import pathway requires species-specific auxiliary proteins also known as co-receptors: Pex18p and Pex21p in *S. cerevisiae* [33], Pex20p in *Yarrowia lipolytica, Pichia pastoris, H. polymorpha*, and *Neurospora crassa* [34–37] or a longer splice variant of the PTS1-receptor Pex5p in plant and mammals [38–41]. These co-receptors form a ternary complex with the cargo-loaded import receptor in the cytosol and direct the complex to the peroxisomal membrane [26,42].

So-called non-PTS proteins do neither contain a PTS1 nor a PTS2. Examples thereof are acyl-CoA oxidase from *S. cerevisiae* and *Y. lipolytica*, the alcohol oxidase from *H. polymorpha* as well as castor bean isocitrate lyase [43]. Different mechanisms are known for non-PTS proteins to reach the peroxisomal matrix [44]. For piggy-back transport, proteins without a PTS hijack onto the peroxisomal targeting pathways by binding to PTS-containing proteins. As peroxisomes can accommodate folded and even oligomeric proteins, these non-PTS proteins can reach the peroxisomal lumen in complex with PTS-proteins [45]. Other non-PTS proteins contain internal, not well-defined targeting signals. Interestingly, these proteins still directly bind to the PTS1-receptor albeit to regions distinct from the PTS- recognition sites. Thus, peroxisomal targeting of this kind of non-PTS-proteins depends on Pex5p but cargo recognition occurs in a PTS1-independent fashion [44].

2.2. The docking-complex and formation of the Importomer

After the receptor–cargo complex has assembled in the cytosol, the next stage in the cascade of events is the association of this complex with the peroxisomal membrane. This step is facilitated by the docking-complex, which consists of Pex13p and Pex14p and in bakers yeast also Pex17p [46]. Pex13p is an integral peroxisomal membrane protein (PMP) that exposes both its N- and C-terminus to the cytosol [47] and binds Pex5p via its cytosolic C-terminal Src-homology-3 (SH3) domain [48–50] and Pex7p by its N-terminal domain [51]. Pex14p forms a complex with Pex13p and also binds both import receptors. Pex14p also provides the binding platform for Pex17p [52–55]. Although Pex17p is part of this complex, it is does not significantly contribute to the structural integrity of the docking complex [18,56] and seems to be absent from higher eukaryotes. Thus, its functional significance still awaits clarification. Interestingly, an *in silico* approach predicted the existence in filamentous fungi of a chimeric protein consisting of an N-terminal Pex14p-like domain and a C-terminal Pex17p-like domain [14].

The docking complex associates with other components, including the RING-(really interesting new gene)-finger complex (composed of Pex2p, Pex10p and Pex12p) to form the assembled import-competent state of the peroxisomal protein import machinery, the importomer.

2.3. Cargo translocation and release

Peroxisomes import their matrix enzymes in a folded and even oligomerized manner [45,57,58]. Remarkably, even gold particles with an average diameter of 9 nm can traverse the peroxisomal membrane, when decorated with a peroxisomal targeting signal [59]. This fact distinguishes the peroxisomal translocon from that of mitochondria, chloroplasts and the endoplasmatic reticulum, which only import unfolded polypeptides [60]. However, our knowledge of how peroxisomes import large protein complexes without disruption of the metabolic compartmentalization is still scarce. Some models proposed the presence of an aqueous pore in the peroxisomal membrane [60–62]. Indeed, large conductance channels have been identified in membranes of mammalian peroxisomes [63,64], but either the identity of pore-forming proteins or its relationship to the protein-translocation machinery remained unclear. Based on increasing evidence for a significant contribution of the cycling import receptors, it was proposed that the translocation pore would be transient in nature and that the import receptors themselves might play an important role in its formation [62]. In fact, the PTS1 receptor Pex5p proved to have many properties expected for a transient-pore-forming protein. A considerable portion of the membrane-bound fraction of Pex5p behaves as an intrinsic membrane protein and forms a stable complex with components of the docking complex [65]. Evidence for the nature of the translocon was also provided by the observation that the peroxisomal matrix import of the intraperoxisomal Pex8p only requires the PTS receptors and Pex14p [61].

Recently, the importomer from yeast peroxisomal membranes was isolated by affinity purification of a tagged version of Pex5p [18]. Pex5p-complexes turned out to be present in three subcomplexes, a high-molecular mass complexes greater than 800 kDa (complex III), a complex spanning between 600 and 800 kDa (complex II) and a Pex5p–cargo complex (complex I) with a size of 300 k. When

reconstituted into liposomes, ion channel activity was detected with complexes II and III, but channel incorporation occurred only at low frequency, and channels showed a large variety of conductance states and ion selectivity.

Assuming that the putative Pex5p-dependent pore forms only transiently and is subject to continuous disassembly or degradation of core constituents, *PEX8* gene was deleted, which prevents association of the docking complex with the export machinery [56] and causes stabilization of the translocon. Moreover, PEX18 and PEX21 were deleted which disabled the PTS2-pathway and avoided disturbances. Now, the Pex5p-complex exhibited the main conductance of a pore with 3.8 nm in diameter. This pore can transiently expand to more than 9 nm when Pex5p is associated with large oligomeric cargo proteins [18], as suggested by the previously observed import of PTS1-decorated gold particles [59]. Taken together, Pex5p shuttles between a soluble form in the cytosol, where it functions as PTS1-receptor in cargo recognition and an integral membrane-bound form at the peroxisomal membrane, where it contributes to pore formation and presumably translocation.

At some point of the import cascade, the cargo has to be released from the import receptor. However, this step is still not well characterized. It has been suggested that Pex8p is involved in this process. This assumption is based on the presence of a PTS1 and PTS2 signal within this peroxin [66,67] and the observation that it causes dissociation of a Pex5p–PTS1–peptide complex by means of *in vitro* assays [68]. However, Pex8p has been identified only in yeast and whether a functional orthologue exists in higher eukaryotes is unclear. Moreover, mutations of the PTS-sequences do not affect Pex8p function. Thus, the mechanism of cargo release remains one of the open questions regarding peroxisomal protein import.

2.4. Receptor release and its degradation or recycling

Once the cargo is released into the peroxisomal lumen, the receptor has to be liberated from peroxisomal membrane to the cytosol. The discovery of an ubiquitination machinery and specific dislocases as central components of an elaborate peroxisomal export machinery brought forward our understanding of the release step in the receptor cycle (Fig. 1). In contrast to the import event, which was demonstrated to be ATP-independent, the dislocation of the receptor requires ATP at two different stages, the export complex and the ubiquitination machinery. The export complex contains Pex1p and Pex6p, two members of the AAA-protein family (ATPases Associated with diverse cellular Activities family) [69–71]. The AAA-peroxins are partially cytosolic; a portion is also attached to the peroxisomal membrane. This peroxisomal localization is facilitated by the integral peroxisomal membrane protein Pex15p (or Pex26p in mammals) that provides binding sites for Pex6p which in turn recruits Pex1p to the peroxisomal membrane [72,73]. The Pex1p/Pex6p interaction depends on the presence of ATP. Moreover, it was demonstrated that the AAA-complex provides the ATP-dependent driving force for the export of Pex5p back to the cytosol [69,70]. The mechanism of this event is still unsolved but it is known that ubiquitination of the receptor molecule plays a crucial role [71,74]. In general, ubiquitination is the attachment of the 76 amino acid ubiquitin (Ub) moiety to a target protein facilitated by a three-step enzyme-cascade [75]. The Ub is activated in an ATP-consuming manner by an ubiquitin activating enzyme (E1) and subsequently transferred to the ubiquitin conjugating enzyme (E2). In the final step, a protein-ubiquitin ligase (E3) binds both E2 as well as substrate and thereby facilitates the conjugation of Ub moiety with substrate protein. Pex5p was demonstrated to be mono- as well as polyubiquitinated.

Polyubiquitination of Pex5p appears in strains affected in late stages of the import cascade, especially receptor recycling reflected by defects in the export machinery (Pex1p, Pex6p, Pex15p) or components required for mono-ubiquitination (Pex4p, Pex22p). Polyubiquitination of the PTS1-receptor modification is not a prerequisite for its function in peroxisomal protein import but might be a crucial step of a quality control system for the disposal of dysfunctional Pex5p [76–78]. It was demonstrated that the polyubiquitination of Pex5p primarily depends on the E2 protein Ubc4p, which upon deletion can be partly replaced by

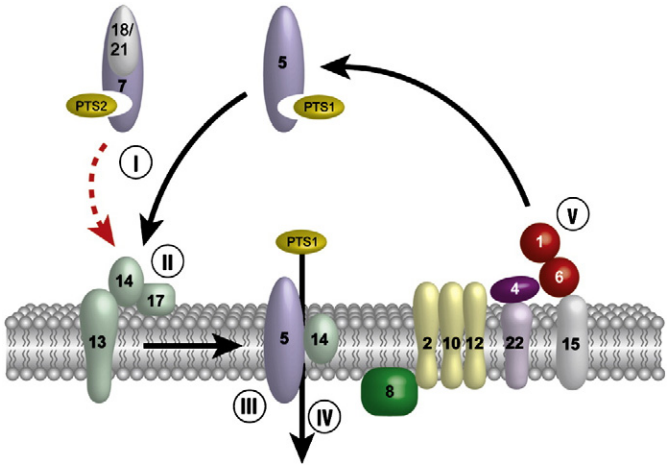

Fig. 1. The receptor cycle. According to the model of the cycling receptor, the peroxisomal protein import conceptually can be divided in five steps: (I) cargo recognition in the cytosol and (II) docking of the receptor–cargo complexes to the peroxisomal membrane. (III) Cargo-translocation into the peroxisomal matrix. (IV) Disassembly of the receptor–cargo complex and (V) export of the receptor back to the cytosol. PTS1-containing proteins are recognized by the soluble import receptor Pex5p in the cytosol. Proteins harbouring the PTS2 are recognized by Pex7p and the cofactors Pex18p and Pex21p in *S. cerevisiae*, the orthologous Pex20p in other fungi or Pex5L in plants and mammals. After this step, the receptor–cargo complex targets to and associates with the peroxisomal membrane via the docking complex consisting of Pex14p, Pex13p and Pex17p. The transport of PTS1-proteins across the membrane is facilitated by formation of a pore mainly consisting of Pex14p and Pex5p. Pex8p connects the RING-complex to the docking complex. The three ubiquitin ligases Pex2p, Pex10p and Pex12p form the RING-complex and together with ubiquitin-conjugating enzymes like Pex4p are responsible for receptor ubiquitination. In the last step of the cycle, the receptor Pex5p is exported back to the cytosol by the two AAA-peroxins Pex1p and Pex6p and is enabled for the next round of import.

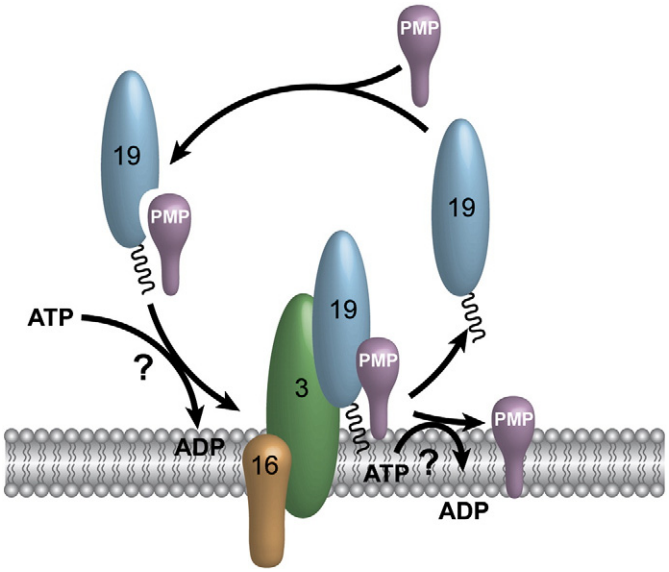

Fig. 2. Pex19p-dependent import of PMPs. Class I peroxisomal membrane proteins (PMPs) harbour a peroxisomal membrane protein targeting signal (mPTS) which is recognized in the cytosol by the import receptor and/or PMP-specific chaperone Pex19p, a farnesylated, mostly cytosolic protein with a small portion of the protein found associated with the peroxisomal membrane. In the next step, the cargo-loaded Pex19p docks to the peroxisomal membrane via association with its docking factor Pex3p. Then the PMP is inserted into the membrane in an unknown manner but presumably with assistance of Pex19p, Pex3p and, in some organisms, Pex16p. The requirement of ATP for this process is not clear. Finally, Pex19p shuttles back to the cytosol where it might start a new round of import.

Ubc5p or Ubc1p [76,77,79]. Two components of the RING-finger complex, Pex2p and Pex10p, have been implicated to act as E3-ligase in Pex5p-polyubiquitination [80,81]. As mutation or truncation of Pex10p only reduces Pex5p-polyubiquitination [80,81], whereas this receptor modification is completely absent when Pex2p is affected [80], it is more likely that Pex2p is the crucial ubiquitin ligase for Pex5p-polyubiquitination. Thus, the specific role of Pex10p still remains unclear.

In contrast to polyubiquitination, Pex5p-monoubiquitination primes the receptor for its export back to the cytosol [71,74,82]. Remarkably, monoubiquitination of the receptor occurs on a cysteine instead of a lysine-residue [83], which results in the formation of a thioester instead of a thioether bond and is facilitated by the E2 protein Pex4p (Ubc10p) in yeast or the Pex4p-like UbcH5a/b/c in humans [74,81,82]. The third RING-finger complex constituent, Pex12p, acts as ubiquitin ligase responsible for Pex5p monoubiquitination and thus represents a central part of the receptor cycle [80].

Interestingly, ubiquitination was also observed for components of the PTS2-pathway. The PTS2-co-receptors Pex18p of *S. cerevisiae* and Pex20p of *P. pastoris* are ubiquitinated at the peroxisomal membrane [46]. At least for Pex20p this modification turned out to be essential for its recycling from the membrane to the cytosol [37]. Future experiments have to clarify whether the same ubiquitination-cascade acting on Pex5p is also responsible for the PTS2-co-receptor modification.

Once the functional receptor has been exported to the cytosol, the ubiquitin needs to be removed prior to the initiation of a new receptor cycle. The cleavage of ubiquitin from a substrate protein is generally carried out by a specific enzyme class, the ubiquitin hydrolases also known as deubiquitinating enzymes (DUBs) [84]. Recent *in vitro* data obtained from rat indicated that the mono-Ub moiety of Pex5p might be cleaved off in two different ways. The thioester bond between Pex5p and mono-Ub could be released in a non-enzymatic manner by a nucleophilic attack of glutathione or enzyme-catalyzed by an ubiquitin hydrolase which still needs to be identified [85].

3. Topogenesis of peroxisomal membrane proteins

The import of peroxisomal membrane proteins (PMPs) is distinct from the import machinery of peroxisomal matrix proteins [48,50]. This is supported by the fact that most pex-mutants are characterized by an impaired import of matrix proteins but the import of PMPs is still functional. In these mutants the PMPs are imported in peroxisomal remnants, so called ghosts [13,86,87]. Only few mutants were characterized by the complete absence of detectable peroxisomal membrane ghosts. Functional complementation of these mutants led to the identification of Pex3p, Pex19p and in some organisms Pex16p which are involved in the biogenesis of the peroxisomal membrane [88–95] (Fig. 3).

3.1. Membrane biogenesis factors

Pex16p is an integral membrane protein which is mainly found in higher eukaryotes and in the yeast *Y. lipolytica*. It was first identified in 1998 by functional complementation of Zellweger patient cell lines [93]. The function of this protein is still not clear and seems to differ between mammals and yeast. Although the proteins from the different kingdoms show a sequence identity of 24% their topology is completely different. While the mammalian Pex16p is an integral membrane protein with the C- as well as the N-terminus facing the cytosol [96], the yeast Pex16p is a membrane associated protein facing the peroxisomal lumen [89]. More strikingly, the proteins seem to perform different functions in peroxisome biogenesis. The mammalian Pex16p is required for the topogenesis of membrane proteins and functions in the very early stages of peroxisome biogenesis while the yeast Pex16p is more likely a negative regulator of peroxisomal fission [89,97].

Pex19p is a farnesylated, mostly cytosolic protein with a small portion of the protein found associated with the peroxisomal membrane [91,98]. Pex19p has the ability to interact with most PMPs [99–104]. Structurally, Pex19p consists of an unstructured N-terminal- and a structured C-terminal domain [105]. The N-terminal domain is responsible for the membrane targeting of Pex19p as it mediates the interaction with the PMP Pex3p. The C-terminal domain harbors the binding sites for most PMPs [105–108]. The crystal structure of the folded C-terminal part of the receptor reveals a globular domain that binds PMP-targeting signal (mPTS) sequences. The structural arrangement of the N-terminal and C-terminal domains in Pex19p resembles a similar division in the Pex5p receptor which might allow separation of cargo recognition and peroxisomal targeting [109]. The farnesylation of Pex19p plays a critical role for the function of Pex19p. Recently, it was shown that the farnesylation contributes to the structural integrity of Pex19p and is important for the ability of Pex19p to interact with its binding partners [110]. Several functions have been proposed for Pex19p. First, due to its capability to interact with most of the PMPs and based on its dual localization at the peroxisomal membrane and in the cytosol, Pex19p is thought to represent a soluble import receptor for newly synthesized PMPs [111,112]. Accordingly, Pex19p binds PMPs in the cytosol and directs them to the peroxisomal membrane by docking to its membrane anchored binding partner Pex3p (Fig. 2). Second, Pex19p is also supposed to function as a PMP-specific chaperone. Accordingly, Pex19p possesses the ability to bind and stabilize PMP by the formation of a soluble complex and thus preventing aggregation of the PMP [105,113]. Third, Pex19p might act as an insertion factor during PMP import [99,104] or function as an assembly/disassembly factor for peroxisomal membrane complexes at the peroxisomal membrane [114]. Finally, it was shown that Pex19p is required for the transport of Pex3p from the endoplasmic reticulum to the peroxisomal membrane [115].

Pex3p is an integral membrane protein at the peroxisomal membrane with a topology differing throughout species [90,116–118].

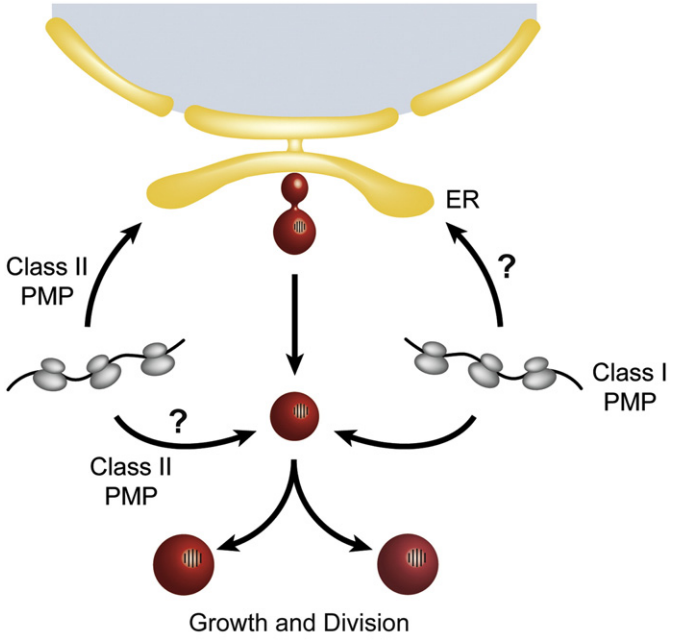

Fig. 3. Topogenesis of peroxisomal membrane proteins. Two routes are proposed for the targeting of peroxisomal membrane proteins (PMPs). Class I proteins are directly imported into existing peroxisomes. Class II proteins are first targeted to ER where they concentrate in pre-peroxisomal vesicles which then are targeted to existing peroxisomes or function as an origin for de novo formation of peroxisomes. Currently, it is controversially discussed whether class I PMPs are also targeted to the ER and whether class II PMPs are also targeted to existing peroxisomes.

In *S. cerevisiae*, Pex3p possesses an N-terminal transmembrane region and a large C-terminal domain facing the cytosolic side of the peroxisome [92]. Pex3p plays a central role in the import of PMPs where it serves as a docking factor at the peroxisomal membrane and functions as binding partner for Pex19p-PMP-complexes during import of the PMPs [106,107,119]. Recently, an unprecedented role for Pex3p in peroxisome motility and inheritance was unravelled in *S. cerevisiae*. In this context, Pex3p turned out to function as peroxisomal receptors for class V myosin as well as for the peroxisome retention factor Inp1p [120,121]. Pex3p also plays an important role for the de novo formation of peroxisomes as it is thought to represent the starting point for this peroxisome forming process (see below).

3.2. Import of peroxisomal membrane proteins

The import pathway for peroxisomal membrane proteins is thought to be completely independent from the import pathways of peroxisomal matrix proteins. Peroxisomal membrane proteins can be divided into two classes: Class I PMPs are imported via a Pex19p-dependent pathway, Class II PMPs target independent of Pex19p to peroxisomes [111].

Most of the proteins imported into the peroxisomal membrane are class I PMPs. The recognition of these PMPs by Pex19p in the cytosol is the first step of the import pathway. In this context, Pex19p functions as a soluble import receptor and/or chaperone which binds newly synthesized PMPs during or directly after their synthesis in the cytosol. Pex19p-targeted PMPs contain a Pex19p-binding site which is an integral part of their peroxisomal membrane targeting signal (mPTS) [122]. The Pex19p-binding site is characterized by the presence of basic and hydrophobic amino acids. The introduction of a proline leads to a complete block of Pex19p-binding, thus the binding site is supposed to acquire an alpha-helical conformation. Although the binding sites of PMPs from different species throughout the kingdom show some similarities, a reliable consensus sequence could not yet be deduced. However, based on the limited information available an algorithm for the prediction of Pex19p binding sites was developed, which currently is used successfully [102,122].

In addition to the Pex19p-binding site, the mPTS of type 1 PMPs contains a transmembrane sequence for their integration into the peroxisomal membrane [111,122–126]. Interestingly, also some peripheral membrane proteins, for instance *S. cerevisiae* Pex17p, are targeted to peroxisomes via the Pex19p-dependent pathway [127]. These proteins also harbor a Pex19p-binding site. However, since these proteins lack a transmembrane domain, anchoring to the peroxisomal membrane requires the association with other peroxisomal membrane protein [127]. Thus, their mPTS comprise the Pex19p binding site and a protein interaction domain. Accordingly, Class I PMPs are targeted to peroxisomes via the Pex19p-dependent pathway and their mPTS comprises a Pex19p-binding motif and a membrane anchor sequence which might be a transmembrane domain or protein interaction site [127].

After recognition of the PMPs, the complex of Pex19p and the PMP is targeted to the peroxisomal membrane where Pex3p functions as a docking factor for this complex [119,122,128]. This docking step is promoted by a higher affinity of the Pex19p-PMP-complex to Pex3p than Pex19p alone [129]. After docking of the complex, the PMP is integrated into the bilayer by an unknown mechanism. Existing data show that in analogy to the PTS1- and PTS2-receptors also Pex19p cycles between the cytosol and the peroxisomal membrane. Pex19p partially integrates into the peroxisomal membrane and after cargo release, it is exported back to the cytosol. The energy requirement of PMP-targeting and insertion is still a matter of debate. Evidence has been provided that the PMP-integration step is ATP-driven whereas the export of Pex19p to the cytosol is not [130]. However, the peroxisomal insertion of some PMPs into the peroxisomal membrane seems not to require ATP, at least in vitro [129,131]. The proteins which are responsible for ATP consumption or the factors required for the Pex19p-export are still unknown.

While most of the peroxisomal membrane proteins are class I proteins, a minor portion belongs to the group of class II PMPs. These are targeted to peroxisomes independent of Pex19p. The few known class II PMPs are Pex3p, Pex16p (for review [132]) as well as Pex22p [133], the peroxisomal membrane anchor of the E2 Pex4p, which is required for the import of peroxisomal matrix proteins (see above) [134]. Class II PMPs are supposed to be targeted to the ER prior to their transport to the peroxisome. The mPTS of these proteins is located in their N-terminal regions and consists of a transmembrane region but lacks a binding site for Pex19p [116,133]. The targeting signal of Pex3p and Pex22p share high similarities and are functionally interchangeable [133]. For Pex16p it has been demonstrated that the protein is imported co-translationally into ER-membranes and then traffics to existing peroxisomes [97]. The mechanism of how class II PMPs are imported into the ER is still not clear. Early studies indicated that Sec61p, the major translocon for ER-membrane proteins is not required for ER-targeting of class II PMPs [135]. Recent data, however, suggest 1) that the Sec61p translocon plays an essential role for the ER-targeting of PMPs and 2) that the Get3p-complex is required for the ER-targeting of peroxisomal tail-anchored proteins [136].

Currently, it is not known how class II PMPs are transported from the ER to peroxisomes. An elaborate vesicle-mediated transport from the ER to peroxisomes has been described [137]. However, the nature of these vesicles still needs to be disclosed, especially as their transport is not affected by inhibitors of COPI and COPII that block vesicle transport in the early secretory pathway [138,139]. Recently, first evidence for an ER-associated secretory machinery involved in peroxisome biogenesis has been provided. Essential components of the secretory pathway (Sec20p, Sec39p, and Dsl1p) have been identified as also being required for Pex3p-exit from the ER and thus being involved in the early stages of the de novo synthesis of peroxisomes [140].

3.3. The involvement of the ER

For a long time the origin of the peroxisomal membrane was controversially discussed. Early models proposed that the peroxisomal membrane originate from the endoplasmic reticulum which was deduced from the morphological appearance of peroxisomes and the ER in electron microscopic pictures showing both organelles in close proximity [141]. Later it was found that peroxisomal matrix proteins as well as PMPs are synthesized on free ribosomes in the cytosol and are posttranslationally imported into preexisting peroxisomes [142]. This gave rise to the proposal of the growth and division model with the central assumption that peroxisomes are autonomous organelles which import proteins and multiply in a similar way as chloroplasts and mitochondria [15]. This model, however, was difficult to reconcile with later findings. For example, the reintroduction of Pex3p in Pex3p-deficient cells, which lack peroxisomal membrane ghosts, leads to the formation of new peroxisomes, raising the question of the membrane origin of newly formed organelles [115,143]. Several lines of evidence indicate that the ER is involved in this de novo formation of peroxisomes. First implications were made from data which showed that in the yeast *Y. lipolytica* the peroxins Pex2p and Pex16p are N-glycosylated [137]. This glycosylation step is exclusively located at the ER indicating that in *Y. lipolytica* these two PMPs route to peroxisomes via the ER. Biochemical and ultrastructural findings suggested that the nuclear membrane is the donor membrane for the de novo-formation of preperoxisomal vesicles [144]. Studies in mouse dendritic cells showed a localization of Pex13p as well as the ABC-transporter PMP70 in specialized subdomains from the ER in connection with a so called peroxisomal reticulum [145]. N-glycosylation of a tagged Pex3p and cleavage of an introduced ER-targeting signal suggested

that ER-targeted Pex3p routes via the ER to peroxisomes [146]. Finally, using time-lapsed fluorescence microscopy it was shown that after reintroduction Pex3p first localizes to the ER, concentrates in specialized subdomains of this organelle and then buds off in a Pex19p-dependent manner [115]. Based on these findings the "de novo biogenesis model" was proposed which not only claims that many PMPs are targeted to peroxisomes via the ER but also that peroxisomes represent a new branch of the endomembrane system [147]. It is now well accepted in the field that de novo synthesis involves the ER and the discovery of the de novo formation of peroxisomes upon ER-targeting of Pex3p in cells lacking peroxisomal membrane ghost was a major breakthrough in our understanding of peroxisome biogenesis. However, a model proposing a general involvement of the ER in the biogenesis of peroxisomes is not without doubt as the major question whether Pex3p is also targeted to ER in the presence of peroxisomes has not yet been conclusively solved. In fact, evidence has been provided for a direct targeting of Pex3p and other PMPs to existing peroxisomes. At least in higher eukaryotes, Pex3p is imported directly into the peroxisomal membrane via a Pex19p-Pex16p dependent pathway [148]. It was also demonstrated that the "growth and division" as well as "de novo biogenesis" pathways both can exist in one organism. In yeast, peroxisomes mainly multiply by growth and division and in cells lacking peroxisomal membranes the ER functions as a donor for essential membrane constituents for the de novo synthesis of peroxisomes [149,150].

4. Concluding remarks

The recent identification of a peroxisomal pore complex with properties suitable for the import of oligomeric proteins has brought forward our understanding of the peroxisomal protein import mechanism. However, a number of aspects still need to be addressed. The identification of the protein import pore of the PTS2-pathway is a major challenge, with Pex18p being a good candidate. Pex5p/Pex14p are core components of the peroxisomal import pore in the PTS1-pathway, raising the question about contribution of Pex8p, Pex13p and Pex17p, which without doubt play an essential role in peroxisomal protein import. There is still room for many important mechanistic aspects which will keep the field busy. For example: In light of the many binding factors for the import receptors at the membrane, what is the order of interaction in the import cascade, how is the pore assembled, are gating-factors required, and most importantly what provides the driving force for the cargo translocation? With respect to the receptor cycle, the mechanism of cargo-liberation, the identification components of the ubiquitination machinery of the PTS2-pathway, the nature of putative de-ubiquitinating enzymes that prepare the receptors for a new round of import as well as the mechanism of receptor dislocation from the peroxisomal membrane, especially the mechanistic function of the AAA-peroxins Pex1p and Pex6p in this process still await elucidation.

Our knowledge on the topogenesis of peroxisomal membrane proteins is still scarce. Pex19p is known to interact with a number of membrane proteins and was thus designated as import receptor and/or chaperone for this type of proteins. Pex3p acts as membrane anchor protein for Pex19p. The function of Pex16p in PMP-targeting is not fully understood and so far it is also not solved how membrane proteins are inserted into the peroxisomal lipid-bilayer. A milestone was the recognition of the contribution of the ER and especially ER-localized Pex3p to the de novo formation of peroxisomes. However, the question is still open whether the ER represents a common route for at least some PMP`s or whether it displays a rescue system for cells that have lost peroxisomes. Finally, the mechanisms underlying sorting of Pex3p to the ER and its observed concentration in distinct foci upon de novo formation of peroxisomes remain to be elucidated.

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