Peroxisome assembly: matrix and membrane protein biogenesis

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The biogenesis of peroxisomal matrix and membrane proteins is substantially different from the biogenesis of proteins of other subcellular compartments, such as mitochondria and chloroplasts, that are of endosymbiotic origin. Proteins are targeted to the peroxisome matrix through interactions between specific targeting sequences and receptor proteins, followed by protein translocation across the peroxisomal membrane. Recent advances have shed light on the nature of the peroxisomal translocon in matrix protein import and the molecular mechanisms of receptor recycling. Furthermore, the endoplasmic reticulum has been shown to play an important role in peroxisomal membrane protein biogenesis. Defining the molecular events in peroxisome assembly may enhance our understanding of the etiology of human peroxisome biogenesis disorders.

Introduction

Peroxisomes are single membrane-enclosed organelles of eukaryotic cells harboring two fundamental processes: diverse reactions involved in lipid metabolism, and defense systems for in situ scavenging of peroxides and reactive oxygen species (Alberts et al., 2002). Mammalian peroxisomes are involved in the catabolism of very long chain fatty acids, branched chain fatty acids, D-amino acids, polyamines, and the biosynthesis of plasmalogens, which are ether phospholipids essential for the normal function of mammalian brains and lungs (Wanders and Waterham, 2006). Other known peroxisomal functions include the glyoxylate cycle in glyoxysomes of germinating seeds, photorespiration in plant leaves, glycolysis in glycosomes of certain parasites (e.g., Trypanosomes), and methanol and/or amine oxidation and assimilation in some yeasts (van der Klei and Veenhuis, 1997; Brown and Baker, 2008). Peroxisomes in mammalian cells also serve as signaling platforms that are activated by the peroxisomally localized mitochondrial antiviral signaling protein during antiviral defense. This activation results in a rapid interferon-independent response followed by sustained antiviral interferon-dependent signaling at mitochondrial membranes (Dixit et al., 2010).

Proteins that control peroxisome assembly, division, and inheritance are named peroxins (encoded by *PEX* genes). Over a dozen peroxins are conserved from yeasts to mammals and are essential for normal human development. Dysfunction of peroxins causes fatal human peroxisome biogenesis disorders (PBDs), which include the Zellweger syndrome spectrum (ZSS) disorders and rhizomelic chondrodysplasia punctata (RCDP) type I (Table I; Steinberg et al., 2006; Wanders and Waterham, 2006; Ebberink et al., 2010). Mutations in the *PEX7* gene are responsible for the latter disorder, whereas mutations in any one of many other *PEX* genes cause the ZSS disorders. A recent analysis of cells from >600 patients with PBDs showed that dysfunction of *PEX1* and *PEX6* accounts for the majority of the ZSS disorders and suggested that most, if not all, *PEX* genes that cause these disorders are now known (Ebberink et al., 2010). However, additional human genes are likely to be involved in peroxisome biogenesis, particularly peroxisomal membrane protein (PMP) biogenesis via the ER, peroxisome division, movement, and inheritance. Mutations in these genes may not yet have been identified as the etiological cause of traditional PBDs or may have eluded detection because of embryonic lethality in humans.

Many excellent reviews have appeared on peroxisome biogenesis (Léon et al., 2006a; Platta and Erdmann, 2007; Brown and Baker, 2008; Tabak et al., 2008). We focus here on recent advances in our understanding of protein translocation into the matrix of this organelle and how PMPs are assembled.

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Abbreviations used in this paper: DRP, dynamin-related protein; mPTS, membrane PTS; PBD, peroxisome biogenesis disorder; PMP, peroxisomal membrane protein; PTS, peroxisomal targeting signal; RADAR, receptor accumulation and degradation in the absence of recycling; RING, really interesting new gene; TPR, tetratricopeptide; ZSS, Zellweger syndrome spectrum.

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Table I. ***PEX* mutations associated with the ZSS disorders**

Mutation	Frequency of *PEX* gene defects in ZSS disorders	Function
	%	
PEX1	58	Peroxisome biogenesis and PTS receptor recycling to the cytosol
PEX2	4	E3 ligase; PTS receptor ubiquitination
PEX3	<1	PMP biogenesis and Pex19 receptor
PEX5	2	PTS1 receptor for peroxisomal matrix protein import
PEX6	16	Peroxisome biogenesis and PTS receptor recycling to the cytosol
PEX10	3	E3 ligase; PTS receptor ubiquitination
PEX12	9	E3 ligase; PTS receptor ubiquitination
PEX13	1	Peroxisomal matrix protein import
PEX14	<1	Component of translocon for peroxisomal matrix protein import
PEX16	1	PMP biogenesis
PEX19	<1	PMP biogenesis; budding of pre-peroxisomal vesicles from the ER
PEX26	3	Peroxisomal membrane receptor for Pex6

ZSS, a main subgroup of PBDs, is comprised of the following diseases: Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. Mutations in the *PEX7* gene are responsible for the second PBD subgroup, called rhizomelic chondrodysplasia punctata type I. This analysis is reproduced here from Ebberink et al. (2010), with some modifications, with permission from John Wiley & Sons, Inc.

Unique features of peroxisome biogenesis

The uniqueness of peroxisomes in comparison with other organelles, as well as their links to human PBDs, has piqued the attention of cell biologists (Alberts et al., 2002). Because peroxisomes lack their own DNA, all peroxisomal matrix and membrane proteins are nuclear-encoded. Newly synthesized peroxisomal matrix proteins are directly targeted to the organelle lumen (Purdue and Lazarow, 2001a). However, most, if not all, PMPs are targeted to peroxisomes via the ER (ER-to-peroxisome pathway; Hoepfner et al., 2005; Kim et al., 2006; van der Zand et al., 2010). This is in contrast to endosymbiont-derived organelles, such as mitochondria and chloroplasts, which are autonomous organelles that also encode some proteins from their organelle genomes.

Distinct machineries sort matrix and membrane proteins of the peroxisomes. Because the assembly of the peroxisomal matrix protein translocation machinery (translocon) in the membrane is necessary for matrix protein import, a block in PMP assembly impairs both membrane and matrix protein assembly, but a block in matrix protein import alone allows the assembly of PMPs into empty or partially empty membranous structures known as ghosts or remnants.

Unlike the translocation of unfolded polypeptides across the membranes of the ER, mitochondria, and chloroplast (Schnell and Hebert, 2003), peroxisomes can transport cargoes in a folded, cofactor-bound, and/or oligomeric state (Léon et al., 2006a). In this respect, peroxisomal protein import has parallels to protein export systems, such as the twin-arginine translocator pathway of bacteria and the thylakoid membrane of chloroplasts (Gutensohn et al., 2006), as well as nuclear protein import (Görlich, 1997).

In contrast to the division of mitochondria and chloroplasts by fission of preexisting organelles, new peroxisomes can be generated by growth and fission of preexisting peroxisomes (Purdue and Lazarow, 2001a) or by de novo biogenesis from the ER (Hoepfner et al., 2005). Interestingly, peroxisomes and mitochondria or chloroplasts share common division machinery components (Kuravi et al., 2006; Motley et al., 2008; Zhang and Hu, 2010) despite their distinct evolutionary origins.

Peroxisomal matrix protein biogenesis

Peroxisomal targeting signals (PTSs) and their receptors. The targeting of matrix cargo depends on two distinct peroxisomal targeting signals: PTS1 and PTS2. PTS1 is located at the C terminus of the protein, is composed of a non-cleavable tripeptide SKL or its conserved variants, and is used by most peroxisomal matrix proteins (Gould et al., 1989).

PTS1-containing cargoes in the cytoplasm are recognized posttranslationally by the receptor protein Pex5, which contains a conserved C-terminal domain composed of 6–7 tetratricopeptide (TPR) motifs and a divergent N-terminal domain (Fig. 1; Stanley and Wilmanns, 2006). The C-terminal TPR domains interact with the PTS1 of peroxisomal cargo, although, in a few cases, it is the N-terminal half of Pex5 that mediates the binding to peroxisomal cargoes lacking canonical PTS1 sequences (Klein et al., 2002; Gunkel et al., 2004). The crystal structure of the Pex5 TPR domains shows that this protein undergoes dramatic conformational changes upon cargo binding, switching from an open, snail-like conformation into a closed, circular conformation (Stanley et al., 2006).

The N-terminal region of Pex5 is less conserved and is disordered (Stanley and Wilmanns, 2006), but this region of Pex5 interacts with other peroxins including Pex8, Pex13, and Pex14. Moreover, the extreme N-terminal region (comprising key Cys and Lys residues) is essential for Pex5 recycling from peroxisomes and for its degradation through monoubiquitination and polyubiquitination of distinct amino acids, respectively (Platta et al., 2007; Williams et al., 2007; Grou et al., 2008).

The PTS2 is a nonapeptide (R/K)(L/V/I/Q)XX(L/V/I/H/Q)(L/S/G/A/K)X(H/Q)(L/A/F) near the N terminus of a smaller subset of peroxisomal matrix proteins (Swinkels et al., 1991; Petriv et al., 2004). The targeting signal of several, but not all, PTS2 proteins is cleaved by proteolytic enzymes—Deg15 in plants and TYSND1 in mammals—in the peroxisome lumen; these enzymes are targeted to the peroxisome by PTS1 sequences (Helm et al., 2007; Kurochkin et al., 2007).

Delivery of PTS2 proteins to peroxisomes requires the cooperation of the PTS2 receptor, Pex7, with its co-receptor:

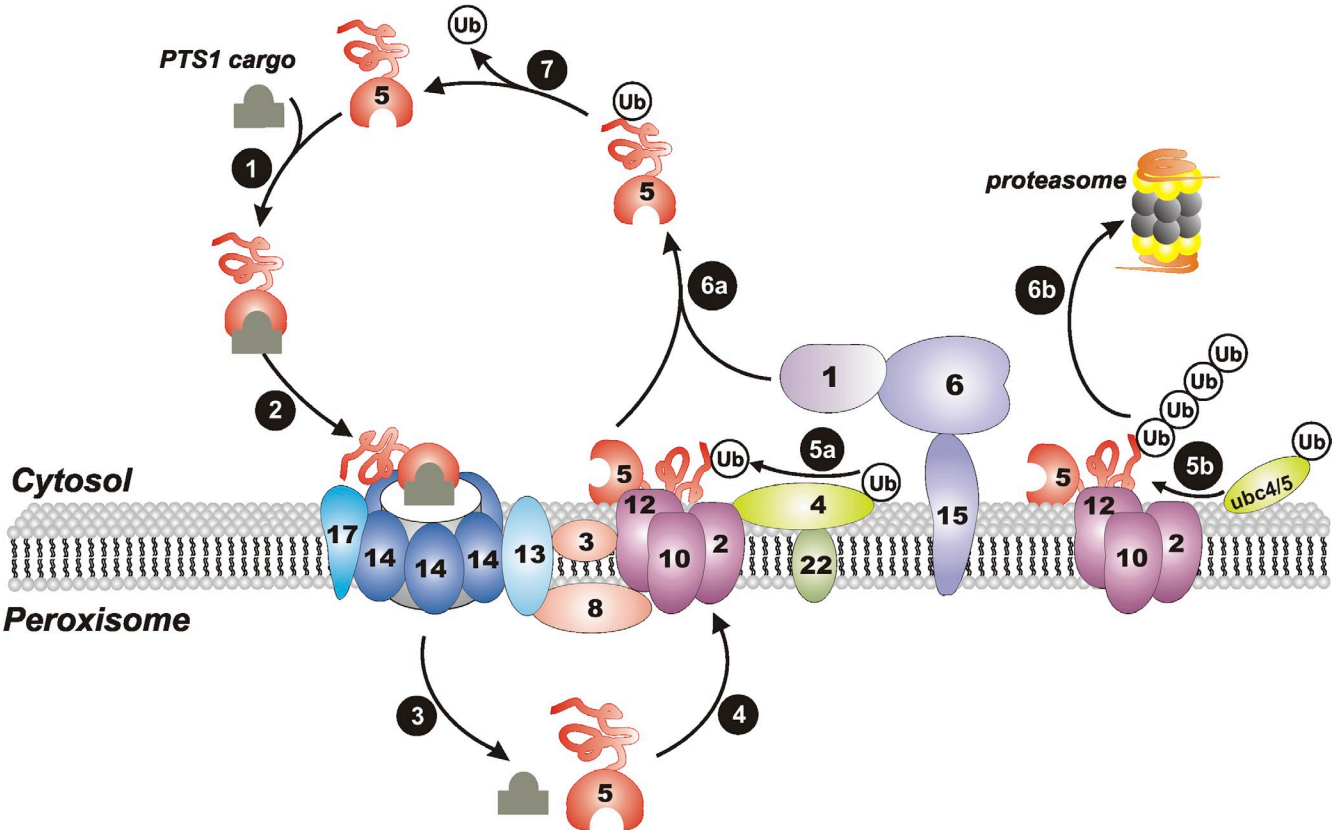

Figure 1. **The import of peroxisomal matrix proteins.** The process may be divided into distinct steps (white numbers in closed black circles). Bold numbers indicate corresponding Pex proteins. The steps are: (1) Receptor–cargo interaction in the cytosol (PTS2 pathway is not depicted). (2) Receptor–cargo docking at the peroxisomal membrane with the docking subcomplex, inducing the assembly of the translocon. (3) Translocation of the receptor–cargo complex across the membrane followed by the dissociation of the receptor–cargo complex; i.e., cargo release. (4) Export of cargo-free receptors from the peroxisome matrix to the membrane. (5a) Monoubiquitination of the receptor on a cysteine by Pex4 and Pex2 (for receptor recycling) or (5b) polyubiquitination of the receptor on a lysine by Ubc4/5 and Pex10/12 (for degradation by the RADAR pathway). (6a) Receptor recycling from the peroxisome membrane back to the cytosol by the action of the AAA ATPases (Pex1 and Pex6) and ATP hydrolysis, or (6b) degradation of a receptor that is blocked from recycling via the RADAR pathway involving the proteasome. (7) Deubiquitination of the receptor before the next round of import. The squiggly line on Pex5 denotes its disordered N-terminal segment.

Pex18 and/or Pex21 in *Saccharomyces cerevisiae* (Purdue et al., 1998) or Pex20 in other fungi (Titorenko et al., 1998; Otzen et al., 2005; Léon et al., 2006b), or Pex5L (an alternative mRNA splice isoform of Pex5) in plants and mammals (Braverman et al., 1998; Lee et al., 2006). Like Pex5, the N-terminal region of Pex20 is required for its recycling from peroxisomes and for its proteasomal degradation (Léon et al., 2006b; Léon and Subramani, 2007).

Peroxisomal importomer and its associated receptor recycling machinery. The importomer is a set of protein subcomplexes located in the peroxisomal membrane, and is responsible for protein translocation into the peroxisome lumen and partly responsible for PTS receptor recycling. Components of the yeast importomer include two subcomplexes bridged by other peroxins. One of these is the docking subcomplex (Pex14, Pex13, and Pex17), which recruits the receptor–cargo complex to the peroxisome membrane and includes the translocon component Pex14. A second subcomplex is comprised of three E3-like proteins (Pex2, Pex10, and Pex12) containing proteins with really interesting new gene (RING) domains (RING subcomplex) and is involved partly in facilitating PTS receptor recycling and degradation. Finally, the importomer also includes peroxins (Pex8 and Pex3) that bridge the docking and RING subcomplexes (Hazra et al., 2002; Agne et al., 2003).

Associated with the importomer is the receptor recycling machinery (Pex4, a homologue of E2 ubiquitin-conjugating enzyme, with its anchor protein Pex22, and two AAA ATPases—Pex1 and Pex6—anchored to Pex15/Pex26). This machinery recycles PTS receptors/co-receptors from the peroxisome membrane to the cytosol after each round of matrix protein import.

In yeast and mammals, the lack of any component of the importomer is characterized by the cytosolic mislocalization of peroxisomal matrix proteins. By a series of ordered interactions between the receptors and components of importomer, the receptors deliver cargoes into the peroxisome matrix and return to the cytosol for subsequent rounds of import using the receptor recycling machinery (Purdue and Lazarow, 2001a; Platta and Erdmann, 2007; Ma and Subramani, 2009). Among the components of the importomer, Pex17 and Pex8 are not conserved in all organisms. In mammals, the role of Pex4 is substituted by a cytosolic E2 enzyme (Grou et al., 2008). Whether Pex3, or some other peroxin, bridges the docking and RING subcomplexes in higher eukaryotes is still unknown.

The import of peroxisomal matrix proteins may be divided into seven distinct steps (Fig. 1). We address recent advances in several of these areas.

Receptor–cargo complex translocation across the peroxisome membrane. How the receptor–cargo complex translocates across the peroxisome membrane is a major unresolved question in the field, particularly because large, folded, oligomeric cargoes cross this membrane without compromising the permeability barrier. Current data favor a protein-conducting channel model over pinocytosis or vesicle fusion. Components of the minimal translocon and evidence for the protein-conducting channel emerged only recently.

The minimal peroxisomal translocon. Because every component of the importomer was implicated genetically in the targeting of all peroxisomal cargoes, it was assumed that the peroxisomal translocon might be more intricate than those identified in other membrane compartments. However, the minimal translocon does not require the entire importomer (Zhang et al., 2006; Ma et al., 2009).

In recent studies in the yeast *Pichia pastoris*, we analyzed the peroxisomal matrix import of Pex8, a special cargo, containing both PTS1 and PTS2, to elucidate whether the entire importomer is a prerequisite for protein translocation (Zhang et al., 2006; Ma et al., 2009). By using fluorescence microscopy, subcellular fractionation, and protease protection assays, we showed that despite the fact that Pex8 has PTS sequences like other matrix cargoes, its entry into peroxisomes is quite unlike that of other cargoes in that it requires only PTS receptors and Pex14, but not the other two components (Pex13 and Pex17) of the docking subcomplex, and neither the preexisting intraperoxisomal Pex8 nor the RING subcomplex. The receptor recycling machinery is indirectly involved in cargo import by maintaining the stability of PTS receptors, but its role in cargo import can be bypassed by using stabilized receptors. Thus, we proposed that Pex5 and Pex14 represent the minimal translocon for matrix protein import.

In agreement with the in vivo data obtained from *P. pastoris*, Erdmann's group demonstrated in *S. cerevisiae* that the membrane-bound Pex5 and Pex14 are the primary components of the peroxisomal translocon by reconstituting the affinity-purified Pex5/Pex14-containing subcomplex in vitro into proteoliposomes and analyzing for protein channel activity using planar lipid bilayers (Meinecke et al., 2010). They showed that the translocon is transiently assembled upon the induction by the receptor–cargo complex and that its size expands to a diameter of 9 nm when presented with a 750-kD complex containing Pex5 and its cargo, Fox1. This is in accordance with previous experiments demonstrating that in mammalian cells, 9-nm gold particles coupled to the PTS1 are imported into peroxisomes (Walton et al., 1995). It was concluded that Pex5 and its partner Pex14 are the primary components of the peroxisomal translocon, despite the fact that other known peroxins, such as Pex13 and Pex17, were present in small amounts in their reconstitution reactions (Meinecke et al., 2010).

However, it is unclear whether Pex5 or Pex14 oligomers constitute the central channel because both have been proposed to form transient pores (Erdmann and Schliebs, 2005; Cyr et al., 2008; Meinecke et al., 2010). How the peroxisome membrane maintains its impermeability while importing large receptor/cargo complexes through a protein-conducting channel remains an enigma.

Cargo release. Several models seek to describe how peroxisomal matrix cargo is released, but this problem remains unresolved. In the first model, a pH gradient, which induces a conformational transition in the Pex5–cargo complex, is required for cargo release in the peroxisome lumen. This model is based on earlier findings in the yeast *Hansenula polymorpha* that the oligomeric states of Pex5 switch from a cargo-bound tetramer at neutral pH (7.2) to a cargo-free monomer at acidic pH (6.0), and the fact that the intraperoxisomal pH of *H. polymorpha* is ∼5.8–6.0 (Nicolay et al., 1987; Wang et al., 2003). However, this model cannot be reconciled with the finding of unimpaired import of PTS1 proteins into peroxisomes of fibroblasts from RCDP patients in which the pH gradient between the cytosol and peroxisome has been dissipated (Dansen et al., 2001).

In the second model, the dissociation of PTS1 cargo from Pex5 inside the peroxisome is coupled to the interaction between the N-terminal region of Pex5 and Pex8, which form a heterodimeric complex. This interaction may induce a conformational change in the cargo-binding domain of Pex5 and trigger cargo release. In an in vitro experiment using fluorescence correlation spectroscopy, it was found that in the presence of Pex8, the amount of PTS1 peptide bound to Pex5 decreased around 35%, which suggests that Pex8 plays a role in cargo release (Wang et al., 2003). However, the limited extent of this release raises the question of whether this effect is sufficient for cargo release in vivo, and whether other factors enhance this process. Another problem is that this model would be inapplicable for mammals and plants, wherein no Pex8 homologues exist.

In higher eukaryotic cells, the affinity between Pex5 and its cargo is significantly decreased when the receptor/cargo complex arrives at the peroxisome membrane and interacts with the docking subunit, Pex14 (Otera et al., 2002; Madrid et al., 2004). Another component of the docking subcomplex, Pex13, interacts more strongly with cargo-free, relative to cargo-bound, Pex5. Therefore, a third model has been proposed: the interactions between the N-terminal region of Pex5 with Pex14, Pex13, Pex17, and/or Pex8 could have an effect on the conformation of the TPR domain, which may switch from a closed conformation back to an open, snail-like conformation, resulting in cargo release (Stanley et al., 2006). Alternatively, an unidentified peroxisomal protein could also trigger cargo release before Pex5 ubiquitination (Alencastre et al., 2009). However, much of this remains speculative and needs further investigation.

PTS receptor recycling and the RADAR pathway. After cargo release, the transient intraperoxisomally (or membrane) localized, cargo-free receptors enter the peroxisome membrane either for shuttling back to the cytosol for another round of import (receptor recycling) or for degradation by the proteasome (receptor accumulation and degradation in the absence of recycling [RADAR] pathway) when there is some dysfunction in receptor recycling (Léon et al., 2006a; Platta and Erdmann, 2007). The export of the PTS2 receptor, Pex20, to the peroxisome membrane requires the RING subcomplex

(Léon et al., 2006b), whereas PTS receptor/co-receptor recycling from peroxisomes requires a ubiquitination step followed by an ATP-driven dislocation step catalyzed by Pex1 and Pex6 (Miyata and Fujiki, 2005; Platta et al., 2005). Both Pex5 and Pex18/Pex20 can be modified by monoubiquitination (linkage of a single ubiquitin molecule) or polyubiquitination (conjugation of at least four ubiquitin molecules), which serve as mandatory signals for receptor recycling or proteasomal degradation, respectively (Purdue and Lazarow, 2001b; Kragt et al., 2005; Léon et al., 2006b; Platta et al., 2007).

The ubiquitination pathway requires an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) to conjugate ubiquitin to its target protein (Kerscher et al., 2006). Associated with peroxisomes are one E2 ubiquitin-conjugation enzyme (Pex4 in plants and lower eukaryotic cells, but absent in mammals) and three E3 ligases (Pex2, Pex10, and Pex12). The monoubiquitination of Pex5, which occurs on a conserved cysteine residue near the N terminus of Pex5 homologues, depends on Pex4 in yeast and in plants, and on UbcH5a/b/c, a cytosolic counterpart of Pex4, in mammals, respectively (Williams et al., 2007; Grou et al., 2008). Recently, in *S. cerevisiae*, the E3 ligase involved in monoubiquitination was shown to be Pex12 (Platta et al., 2009). Interestingly, the PTS2 co-receptor, Pex20, contains a conserved cysteine residue near its N terminus, and this residue is essential in *P. pastoris* for its recycling from the peroxisome to the cytosol (Léon and Subramani, 2007). However, whether it is monoubiquitinated on this cysteine is unknown.

The polyubiquitination of Pex5 and Pex20, which occurs on one or more lysines near their N termini, occurs in cells lacking any component of the receptor recycling machinery (Platta et al., 2004; Kiel et al., 2005; Kragt et al., 2005; Léon et al., 2006b) and in proteasomal degradation mutants (Platta et al., 2004). In wild-type cells, there is very little receptor turnover by the RADAR pathway under peroxisome biogenesis conditions. However, we speculate that Pex5 and Pex20 may be polyubiquitinated under certain physiological conditions; for example, either when they are dysfunctional after multiple rounds of recycling or when the AAA ATPases Pex1 and Pex6 are transiently nonfunctional because of low levels of ATP. It seems that the mono- and polyubiquitination reactions do not share common E2 and E3 enzymes. In *S. cerevisiae*, the polyubiquitination of Pex5 depends on the cytosolic E2 enzyme Ubc4 and the E3 ligase Pex10 (Williams et al., 2007). However, Platta et al. (2009) showed that Pex2, with the assistance of Pex10, is the E3 ligase that mediates Ubc4-dependent polyubiquitination of Pex5.

Among the unresolved issues are how PTS receptors/co-receptors are recognized and regulated for monoubiquitination or polyubiquitination, and which E3 ligases are necessary for these reactions on the PTS2 co-receptor.

Peroxisome membrane protein biogenesis

Contribution of the ER in peroxisome biogenesis. Biochemical and morphological studies on peroxisome formation over four decades have yielded conflicting conclusions regarding the membrane origin for peroxisomes. Electron microscopic investigations suggested that peroxisomes originate from the ER (Novikoff and Novikoff, 1972; Geuze et al., 2003) and some enzymes were found to appear first in the ER and later in glyoxysomes during seed germination (Gonzalez and Beevers, 1976). Subsequently, it was found that the peroxisomal enzymes and even a few PMPs were synthesized on free polyribosomes and imported posttranslationally into the organelle (Rachubinski et al., 1984). These data suggested that peroxisomes are autonomous organelles that multiply by growth and division (Purdue and Lazarow, 2001a). Identification of the PTS1, PTS2, and membrane PTSs (mPTSs) and the discovery that peroxisomes possess their own protein-import machinery strongly supported the autonomous organelle hypothesis. As expected from this model, proteins lacking PTS1 and PTS2 sequences are cytosolic.

However, this model was difficult to reconcile with later findings made when genetic mutants blocked in peroxisome assembly were described in yeast and in mammalian cells. In certain mutants (e.g., *pex3* or *pex19*) lacking peroxisomes or even remnants, the organelles reappear upon complementation with the wild-type gene (Höhfeld et al., 1991; Subramani, 1998). Several independent studies published in the last decade, including several in the past year, provide compelling genetic, biochemical and cell biological support for the involvement of the ER in de novo peroxisome biogenesis (Tam et al., 2005; Hoepfner et al., 2005; Motley and Hettema, 2007; Motley et al., 2008; Perry et al., 2009; van der Zand et al., 2010).

Although previous studies ruled out a direct role for COPI- and COPII-mediated vesicular transport in peroxisome biogenesis (South et al., 2001; Voorn-Brouwer et al., 2001), other studies showed that several ER-associated secretory proteins were necessary for peroxisome assembly. The *SEC238* and *SRP54* genes of *Yarrowia lipolytica* are necessary for the exit of Pex2 and Pex16 from the ER and for peroxisome assembly (Titorenko and Rachubinski, 1998). Repression of the ER-associated proteins Sec20, Sec39, and Dsl1, which form a complex at the ER, resulted in cytosolic mislocalization of the peroxisomal matrix protein, Pot1. Additionally the PMP chimera Pex3-GFP was mislocalized to tubular-vesicular structures in these cells (Perry et al., 2009). Therefore, these ER proteins may play a pivotal role in the exit of Pex3-containing structures from the ER to their final destination, the peroxisomes.

In plant systems, a replication protein, p33, of tomato bushy stunt virus is targeted from the cytosol to peroxisomes and subsequently traffics to the peroxisomal/ER subdomain (McCartney et al., 2005), which suggests that a retrograde route most likely returns certain proteins to the ER (Yan et al., 2008), but this needs to be confirmed.

Trafficking of PMPs through the ER. Biochemical evidence supporting the trafficking of peroxisomal proteins via the ER is provided by the N- or O-linked glycosylation of different peroxins. In *Y. lipolytica*, the trafficking of two PMPs—Pex2 and Pex16—to the peroxisome occurs via the ER and results in the N-linked glycosylation of both PMPs in the ER lumen. These PMPs do not transit through the Golgi as an intermediate step. Further, mutations in the *SEC238*, *SRP54*, *PEX1*, and *PEX6* genes delayed or prevented the exit of these PMPs from the ER, while also impairing the assembly

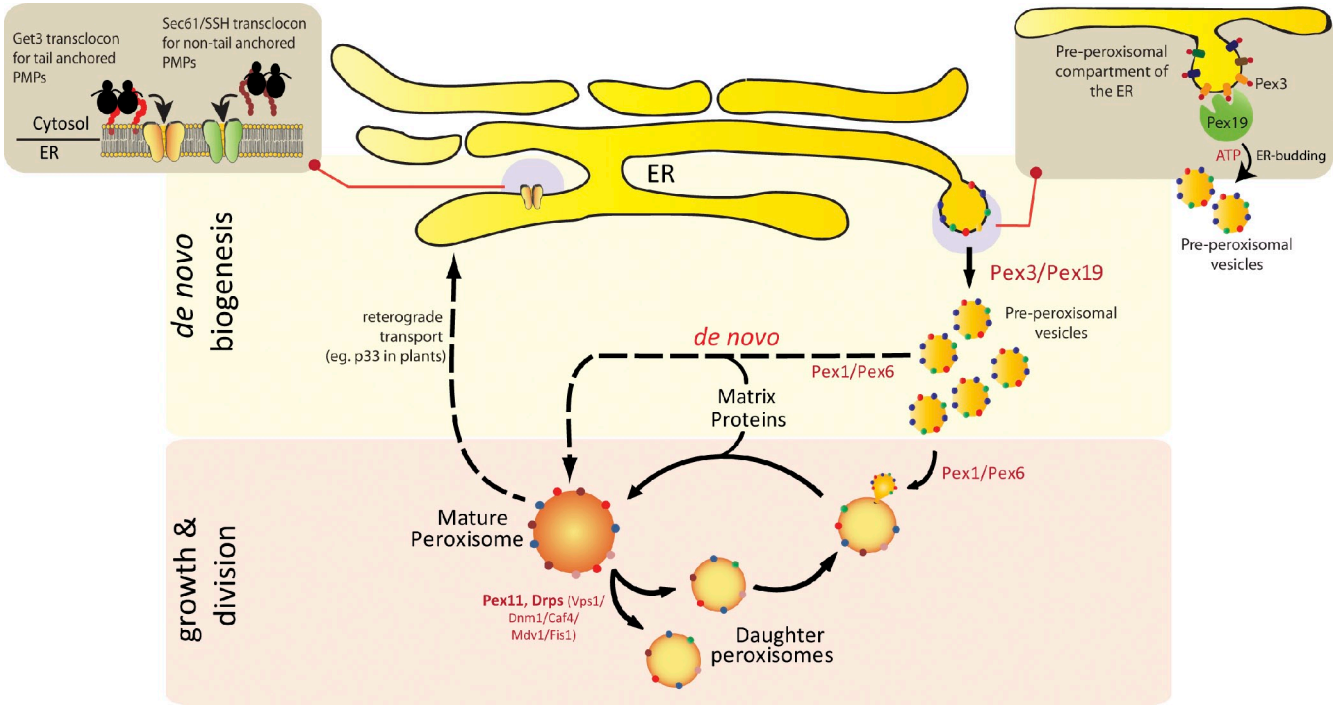

Figure 2. **Contribution of the ER to peroxisome biogenesis.** Most, if not all, PMPs are first imported into the ER through the Sec61/SSH1 translocon or the GET3 complex (left inset), are sorted into a pre-peroxisomal compartment, and bud out in a Pex3/Pex19-dependent manner to form pre-peroxisomal vesicles (right inset). These vesicles can form mature peroxisomes after fusion, dependent on Pex1/Pex6 (Titorenko and Rachubinski, 1998) and matrix protein import (de novo pathway). The de novo pathway repopulates cells with peroxisomes in the biogenesis mutants (e.g., *pex3Δ/pex19Δ*) lacking the organelle when corresponding genes are reintroduced (Elgersma et al., 1997; Fang et al., 2004; Tam et al., 2005; Hoepfner et al., 2005; Motley and Hettema, 2007; Motley et al., 2008; Perry et al., 2009; van der Zand et al., 2010). Alternatively, the pre-peroxisomal vesicles fuse with divided peroxisomes generated from preexisting mature peroxisomes. Peroxisome division requires Pex11 and a specific set of DRPs. In plants, retrograde trafficking from peroxisomes to the ER has been described (McCartney et al., 2005).

of functional peroxisomes (Titorenko and Rachubinski, 1998). The ER-lumenal tail of another PMP, Pex15, a tail-anchored peroxin, is O-mannosylated (an ER-specific modification) in *S. cerevisiae* (Elgersma et al., 1997). Because Pex15 does not undergo Golgi-specific mannose chain elongation, it is clear that it does not pass through the Golgi on its way from the ER to the peroxisomal membrane. Pex15 enters the ER in a Get3-dependent manner, a mechanism common to tail-anchored proteins (Fig. 2; Schuldiner et al., 2008).

Further evidence that PMPs may get to peroxisomes via the ER (Gonzalez and Beevers, 1976; Bodnar and Rachubinski, 1991) came from pulse-chase fluorescence microscopy monitoring the reappearance of peroxisomes in peroxisome-free *S. cerevisiae* (Hoepfner et al., 2005). Membrane-anchored, fluorescently labeled Pex3 was demonstrated to first appear in the ER, concentrated in one or two dots; later, these dotted structures detached from the ER in a Pex19-dependent manner to form import-competent peroxisomes. Likewise, when a 46–amino acid N-terminal fragment of Pex3 was expressed in *pex3* cells as a GFP fusion protein, it localized to a subdomain of the ER and initiated the formation of a pre-peroxisomal compartment, leading to de novo peroxisome biogenesis (Tam et al., 2005). Similarly, in *P. pastoris*, the Pex19-dependent peroxisomal trafficking of Pex30 and Pex31 (peroxins that regulate the number and size of peroxisomes) occurs via the ER (Yan et al., 2008). In mammalian cells, additional evidence suggesting the role of ER in peroxisome biogenesis came from mouse dendritic cells where Pex13 and PMP70 were found in reticular structures apparently connected to the smooth ER (Geuze et al., 2003). Recently, a photoactivated form of GFP fused to Pex16 was localized solely in peroxisomes and the ER, but no cytosolic pool was found. Additionally, in Pex19-deficient cells lacking peroxisomes and in N-terminal Pex16 truncation mutants that lack the mPTS, Pex16 remained exclusively in the ER. Furthermore, a novel pulse-chase strategy showed that the ER plays a central role in both the origin and maintenance of mammalian peroxisomes (Kim et al., 2006).

The growth and division of peroxisomes was linked with the ER-derived biogenesis model by showing the fusion of ER-derived membrane structures with preexisting peroxisomes in yeast cells (Fig. 2; Motley and Hettema, 2007). However, ER-derived de novo peroxisome biogenesis occurred only when preexisting peroxisomes were absent because of peroxisome segregation defects, and the process was slower than peroxisome multiplication in wild-type cells. Furthermore, peroxisome biogenesis in the cells carrying preexisting peroxisomes was dependent on dynamin-related proteins (DRPs), namely Vps1 and Dnm1, whose absence markedly reduced the number of peroxisomes. Subsequently, they also implicated Dnm1-dependent Caf4, Mdv1, and Fis1 proteins in peroxisome fission (Motley et al., 2008). In contrast, the de novo process was DRP independent, which suggests that the fission of preexisting peroxisomes, but not the exit of pre-peroxisomal structures, requires DRPs (Fig. 2; Motley and Hettema, 2007).

Peroxisomes as an intrinsic member of the cellular endomembrane system. Cells lacking peroxisomes use the ER as a donor for essential membrane constituents required for the de novo synthesis of peroxisomes, making this organelle an intrinsic member of the endomembrane family (Hoepfner et al., 2005). The magnitude of the contribution of the ER toward peroxisome biogenesis was recently evaluated in *S. cerevisiae* (van der Zand et al., 2010). A comprehensive set of 16 PMPs was demonstrated to enter the ER, en route to peroxisomes, irrespective of their functions and topologies. Fluorescence pulse-chase experiments showed an initial localization of these peroxins at the ER as distinct punctate structures within 2 h followed by multiple dots, which became independent of the ER at ∼6 h. At this time, the independent punctate structures became competent for import of PTS1 proteins. The entry of these PMPs into the ER was dependent on the Sec61 translocon, except for the tail-anchored protein Pex15, which requires Get3 for its ER entry (see "Trafficking of PMPs through the ER"). PMP exit from the ER was dependent on Pex3 and Pex19 (Fig. 2).

This trafficking pathway operates in dividing wild-type cells where the peroxisome population needs to be maintained, as well as in mutant cells lacking peroxisomes, in which new peroxisomes form after complementation with the wild-type gene. Thus, the basic framework for creation of an import-competent peroxisomal membrane with the PMPs is via the ER. A consensus sequence that imports these PMPs to the ER is still unknown, but a region containing the transmembrane segment was identified as the ER-targeting domain of the tail-anchored protein Pex15 (Elgersma et al., 1997).

The role of the mPTS in PMP biogenesis. The exact roles of the mPTS and Pex19 are quite distinct in two models proposed for PMP import. In the ER-derived peroxisome model, PMPs would be inserted into the ER membrane even without Pex19, but Pex19 action, presumably via its interaction with mPTSs of PMPs, would be necessary for the budding of ER-derived vesicles. Indeed, we ascribed this type of role to Pex19, suggesting that it acts at the membrane after protein insertion, as a chaperone to assemble PMPs or their complexes (Snyder et al., 1999). In experiments done with mammalian Pex16 lacking its mPTS, the protein remained in the ER and was not transported to peroxisomes, which suggests that there is requirement of the mPTS for Pex16 exit from the ER (Kim et al., 2006). Recent studies show an indispensable role for Pex19 in the budding of pre-peroxisomal vesicles, supporting the view that Pex19 could bind to the ER-inserted PMPs and then facilitate their budding from the ER (Fig. 3; Lam et al., 2010; unpublished data).

In contrast, in the alternative model involving direct post-translational insertion of PMPs from the cytosol into the peroxisome membrane, Pex19 has been invoked as the PMP receptor that interacts with mPTSs on PMPs and shuttles them to the peroxisome membrane, where Pex19 interacts with its anchor Pex3, and inserts these proteins into the membrane (Fig. 3; Fang et al., 2004; Jones et al., 2004; Fujiki et al., 2006). In the light of the more recent data on the transit of most PMPs to the peroxisomes via the ER and the role of Pex19 in the budding of ER-derived pre-peroxisomal vesicles, the concept of an mPTS as a signal for targeting PMPs to the peroxisomal membrane has to be questioned.

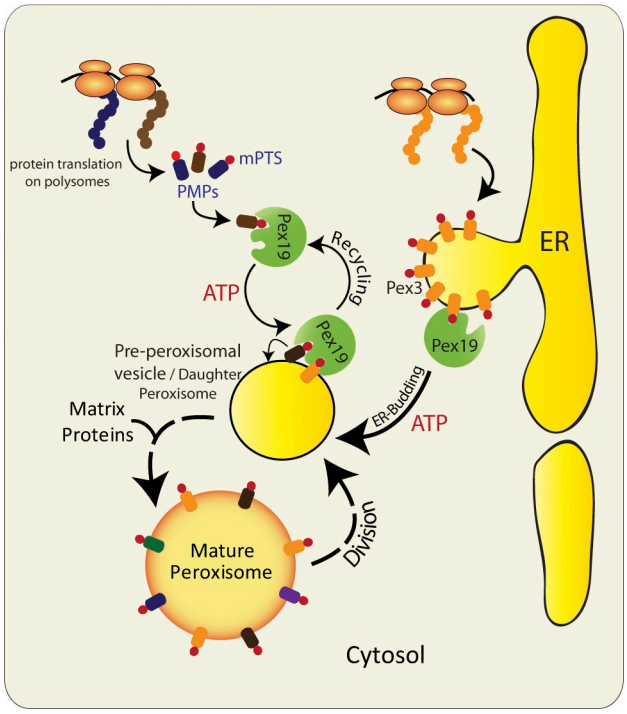

Figure 3. **Alternative roles of Pex19 in the insertion of PMPs into the peroxisomal membrane.** The role of Pex19 in peroxisome biogenesis and import of various PMPs has been clearly established in yeast and mammals, but its mechanism of action is still a matter of debate (Snyder et al., 1999; Sacksteder et al., 2000). Previous studies implicated Pex3 and Pex19 in the posttranslational insertion of PMPs. Pex19 serving as a chaperone binds and stabilizes newly synthesized mPTS-containing PMPs in the cytoplasm, and transports them to peroxisomes by docking to Pex3 present in the peroxisomal membrane (Muntau et al., 2003; Fang et al., 2004; Jones et al., 2004; Matsuzono and Fujiki, 2006; Matsuzono et al., 2006). However, subsequent studies in yeast show the requirement of Pex19 for the exit of most, if not all PMPs, including Pex3, from the ER (Fig. 2 B; Hoepfner et al., 2005; Lam et al., 2010; van der Zand et al., 2010; unpublished data). In the light of the Pex19-independent insertion of most PMPs into the ER and the role of Pex19 in mediating the budding of pre-peroxisomal vesicles, the role of Pex19 in the posttranslational import of PMPs is questionable for all PMPs that go to peroxisomes via the ER.

Emerging areas of interest and future directions

Several new studies highlight novel areas that contribute to and impact our overall understanding of peroxisome biogenesis in the cellular context. The description of vesicles emanating from mitochondria and providing a vesicular trafficking pathway from mitochondria to peroxisomes (Neuspiel et al., 2008; Braschi et al., 2010) is intriguing, but of unknown physiological significance. The emerging understanding of global regulators of peroxisome biogenesis in simple unicellular organisms will shed light on signaling and regulatory events coordinating peroxisome biogenesis (Smith et al., 2007; Saleem et al., 2008, 2010). The use of proteomic approaches to fully catalog and characterize this compartment and its specialized forms will bridge gaps in our understanding of peroxisomal metabolic pathways, while also uncovering novel functions (Saleem et al., 2006; Managadze et al., 2010). Also of interest is the mechanism of peroxisome division, especially the sharing of this machinery with mitochondria and chloroplasts (Hettema and Motley, 2009). Studies on the dynamics and inheritance of

this organelle are yielding interesting insights (Fagarasanu et al., 2010). Finally, the new advances in peroxisome turnover by autophagy address the question of how peroxisome homeostasis is regulated (Manjithaya et al., 2010). As the study of new proteins involved in these processes broadens our mechanistic understanding of peroxisome biogenesis, it seems likely that mammalian counterparts of these proteins will be discovered and, additionally, mutations in some of these proteins in humans will be found to cause PBDs.

With time, our understanding of peroxisomes is evolving with remarkably rapidity. From their initial description as simple eukaryotic organelles containing enzymes that generate and degrade hydrogen peroxide, we now consider a broader definition for peroxisomes as a cellular compartment involved in several metabolic, as well as signaling and developmental processes. Recent studies, providing compelling evidence for the ER's involvement in peroxisome biogenesis, challenge the earlier dogma that peroxisomes only replicate autonomously. Moreover, the ER appears to be essential both for de novo biogenesis of peroxisomes (Hoepfner et al., 2005; van der Zand et al., 2010) and for normal peroxisome growth and division (Motley and Hettema, 2007; Yan et al., 2008).

From an evolutionary perspective, we are embracing the concept of the peroxisome as an ER-derived member of the endomembrane system (Gabaldón, 2010). This new vision also raises several fundamental questions. For example, how are peroxisomal proteins sorted to the ER membrane? How is the ER-derived pre-peroxisomal compartment generated? How are PMPs and other machinery sorted from and to the ER using anterograde and retrograde pathways? The in vitro systems that recapitulate cytosol-dependent peroxisome assembly from the membrane constituents supplied by the ER promise answers to these questions (Lam et al., 2010; unpublished data).

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