BioMol Concepts, Vol. 2 (2011), pp. 353–364 • Copyright © by Walter de Gruyter • Berlin • Boston. DOI 10.1515/BMC.2011.031

Review

Membrane elongation factors in organelle maintenance: the case of peroxisome proliferation

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Abstract

Separation of metabolic pathways in organelles is critical for eukaryotic life. Accordingly, the number, morphology and function of organelles have to be maintained through processes linked with membrane remodeling events. Despite their acknowledged significance and intense study many questions remain about the molecular mechanisms by which organellar membranes proliferate. Here, using the example of peroxisome proliferation, we give an overview of how proteins elongate membranes. Subsequent membrane fission is achieved by dynamin-related proteins shared with mitochondria. We discuss basic criteria that membranes have to fulfill for these fission factors to complete the scission. Because peroxisome elongation is always associated with unequal distribution of matrix and membrane proteins, we propose peroxisomal division to be non-stochastic and asymmetric. We further show that these organelles need not be functional to carry on membrane elongation and present the most recent findings concerning members of the Pex11 protein family as membrane elongation factors. These factors, beside known proteins such as BAR-domain proteins, represent another family of proteins containing an amphipathic α-helix with membrane bending activity.

Keywords: amphipathic α-helix; DRP1/DLP1; FIS1; membrane remodeling; peroxisome proliferation; Pex11.

Introduction

Eukaryotic life relies on the arrangement of specialized intracellular microenvironments, the organelles, with several advantages including an increase in efficiency of metabolic activities. To ensure such functionality, processes exist that control the number, size and shape of organelles as well as their positioning during cell cycle progression. The molecular mechanisms triggering these events depend on specialized proteins, such as anchoring factors for the cytoskeleton, motor proteins or membrane shaping factors.

The above-mentioned processes share a common aspect: they require membrane remodeling and thus proteins that have the ability to shape the organelle. Proteins exist that affect membrane curvature, their specialized domain bends the phospholipid bilayer, thereby stabilizing the charged concave surface of the membrane. In the absence of such morphogenic factors, the endoplasmic reticulum (ER) would be misshaped, mitochondria or peroxisomes would be unable to divide and vesicular trafficking, endocytosis or neuronal function would not be possible.

Evidently, the field of membrane remodeling is very broad and we are unable to cover it entirely in only few pages. Therefore, we point at excellent overviews on endocytosis and vesicular trafficking involving factors such as BAR proteins (1–7). Here, we focus on processes that ensure proper maintenance of peroxisomes for cellular homeostasis. We elaborate particularly on proteins involved in the elongation of the peroxisomal membrane.

The peroxisome, a dynamically shaped organelle

Peroxisomes integrate into the organellar system in all eukaryotic organisms to perform a variety of tasks mostly associated with lipid metabolism, e.g., β-oxidation in *S. cerevisiae*, methanol oxidation in *Y. lipolytica* as well as α- and β-oxidation of very long chain fatty acids or plasmalogen synthesis in mammals, and detoxification of reactive oxygen species (ROS) (8–11). A role for peroxisomes in ageing and inflammation response has also been suggested (12–14). Consequently, the absence of functional peroxisomes causes severe diseases eventually leading to early death, e.g., Zellweger spectrum diseases such as the Zellweger syndrome, neonatal adrenoleukodystrophy or infantile Refsum disease (15–17). Similarly, yeast mutant cells lacking peroxisomes are unable to grow on media containing fatty acids as the sole carbon source, but they can easily ferment if the culture medium is supplemented with sugars such as glucose (18, 19).

To perform their wide-ranging tasks, peroxisomes are adaptable organelles. Indeed, they exchange material with the endoplasmic reticulum (ER) and the mitochondria (20–24). They also adjust their size, shape, number and even their protein content according to the organism, the tissue or the environmental conditions (8, 25). To ensure such high versatility the maintenance of the peroxisomal compartment must be precisely regulated. Regulatory steps include the selective degradation of superfluous or elderly peroxisomes

via micro and macropexophagy, a mechanism conserved throughout kingdoms (26, 27). In addition, tight regulation of peroxisome inheritance during cell division was shown to occur in yeast through the function of specialized proteins controlling peroxisome positioning in the mother cell or in the bud (28). Furthermore, when their function is required peroxisomes can proliferate. Their propagation is either constitutive during cell cycle progression or inducible upon environmental pressure, e.g., growth of yeasts on fatty acids; fibrate supply for rodents or UV-light, high-levels of ROS, and xenobiotics in mammals (8, 11, 29, 30).

Biogenesis of peroxisomes, a need for membrane proliferation

What is the origin of the peroxisomal compartment? The peroxisome field has been highly studied and debated over the last decades but the mechanistics of peroxisome biogenesis and proliferation still requires investigation. However, owing to the characterization of mutant cells, the use of GFP- or photoactivatable GFP-fused proteins *in vivo*, it is now clear that two main routes lead to peroxisome formation: (i) *de novo* biogenesis from the ER and (ii) growth and division from existing peroxisomes (19, 31–39).

Studies either report on *de novo* biogenesis or on growth and division, yet focusing on only one side of peroxisome proliferation. However, the two pathways leading to formation of peroxisomes might not be controlled by completely independent mechanisms. How could the growth and division model possibly work without membrane recruitment? Although a role for the ER in the import of peroxisomal membrane proteins has been suggested (40, 41), little is known on how peroxisomes exchange material with the ER or acquire their membrane lipids.

Generally, most proteins involved in peroxisome biogenesis and proliferation belong to the group of *PEX* genes-encoded peroxins, most of which act as part of the peroxisomal matrix protein import machinery (42). Only a subset of peroxins, to which the Pex11-protein family belongs, controls the size, shape and number of peroxisomes. Conceptually, peroxisome proliferation can be divided into five steps: (i) organellar polarization, (ii) membrane protrusion, (iii) membrane elongation, (iv) protein import and (v) membrane scission (43). While the Pex11 proteins have been suggested to control the first steps (44), the actual peroxisomal membrane scission is performed by factors also known to operate in mitochondrial fission (45–47).

The Pex11 protein was first identified in the yeast *S. cerevisiae*. Deletion of the *PEX11* gene led to the occurrence of fewer and enlarged peroxisomes and upon overexpression of Pex11p, the cells contained more and smaller peroxisomes than wild type cells (48). Homologues of *Sc*Pex11p are known in most eukaryotic organisms and these usually contain more than one Pex11 protein (44, 49–60). Depending on the species, up to three proteins of the Pex11 family were identified in yeasts, e.g., Pex11p, Pex25p and Pex27p in *S. cerevisiae*; Pex11p, Pex11Cp and Pex25p in *H. polymorpha*; Pex11p and Pex11Cp in *Y. lipolytica*; and Pex11p in *P. pastoris* (61). Plants typically contain five Pex11 proteins, PEX11a to -e, whereas mammals harbor three, namely PEX11α, PEX11β and PEX11γ. Noteworthy, PEX11α, PEX11β and PEX11γ are related to *Sc*Pex11p only, and no homolog has been identified for *Sc*Pex25p or *Sc*Pex27p in mammals, so far.

Dynamin-related proteins are involved in mitochondrial and peroxisomal fission

The role of Pex11 proteins in peroxisome proliferation was strengthened by the results of several studies notably showing that human PEX11β was able to interact with hFis1, a component of the peroxisomal fission machinery (43, 62). In human, the peroxisomal fission apparatus consists of hFis1, a tail-anchored recruitment factor, and the dynamin-related protein DRP1/DLP1, the actual scission factor (62–67). Recently, a new protein, Mff (mitochondrial fission factor), has been identified that acts in both, mitochondrial and peroxisomal fission processes (68). Furthermore, in knockdown studies *Mff* RNAi seemed to have a stronger effect than *hFis1* RNAi. Similar to DRP1 knockdown, they induced tubulation of peroxisomes suggesting that Mff is an important player in the process of peroxisome proliferation (68, 69).

Similarly, in plant proteins of the dynamin family, DRP3A/B and DRP5, were identified as proliferation factors and shown to be accountable for peroxisome fission (70–73). Again, these are recruited to the peroxisomal membrane by FIS1 proteins, homologues of the mammalian hFis1 (70, 72, 74). In biomolecular fluorescence complementation assays with split YFP, FIS1B interacted with all five plant Pex11 proteins (70).

In yeasts, the dynamin-like protein Dnm1p was identified as the peroxisomal membrane scission factor. Dnm1p is recruited by Fis1p through adaptor proteins, either Mdv1p or its paralogue Caf4p (45, 75–79). In addition, a second and apparently independent pathway was identified relying on the function of another dynamin-related protein, Vps1p, as fission factor (80–82). In contrast to plant or human, no interaction between the scission factors and Pex11 proteins could be established in yeast, so far. Yet, a very recent study in *S. cerevisiae* reported the characterization of peroxin 34, a peroxisomal membrane protein (83). Its interaction with the Pex11 proteins as well as with Fis1p was illustrated in yeast-two-hybrid assays establishing the first link between Pex11 proteins and the fission machinery in yeast. Noteworthy, Pex34p seems to only exist in yeasts and no homolog could be identified in higher eukaryotes (83).

Together with the fission machinery, the cytoskeleton also plays a crucial role in organellar maintenance. Indeed, peroxisomes attach to the cytoskeleton and move along cytoskeletal tracks i.e., microtubules in human (84, 85) or actin in plant (86) and yeast (87). Additionally, it has been shown that organellar fission depends on the cytoskeleton as exemplified by Dnm1p-dependent scission of mitochondria in

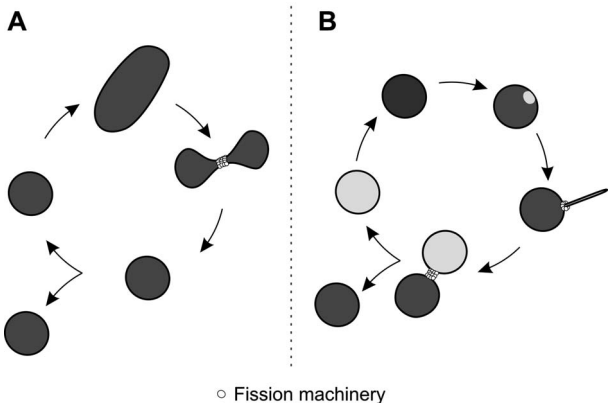

Figure 1 Stochastic versus asymmetric peroxisome proliferation. (A) In a simplified model for peroxisome proliferation, a peroxisome grows and elongates and, upon a critical size, the membrane is constricted and divides through fission. Herein, the inheritance of membranes and proteins is stochastic. (B) In asymmetric proliferation, the peroxisome becomes polarized and its membrane elongates at a specific site reorganizing membrane proteins. The fission machinery assembles at the site of membrane protrusion and import of new matrix proteins assembles a daughter peroxisome which separates from the mother organelle through membrane scission.

S. pombe (88). In human cells, functional microtubules and dynein motors were shown to be essential for peroxisome biogenesis (89).

Interactions of Pex11 proteins with fission factors give some insight into a molecular mechanism for peroxisomal proliferation; however, many questions remain unanswered. How does the peroxisomal membrane arrange for scission? What are the factors involved in the membrane remodeling process? Do proteins of the Pex11 family organize this whole process and why do most organisms contain more than one Pex11 protein? In the following sections we integrate the most recent findings that tackle these questions.

Mechanistic aspects of peroxisome division, Pex11 steering membrane elongation

Although several modes of proliferation are possible for organelles, the peroxisome relies on an apparently simple growth and division process. A simplified model depicts a single round-shaped peroxisome starting to elongate (Figure 1A). Once a critical size is reached, the membrane tightens and constricts until scission occurs through the action of the fission machinery. This leads to stochastic distribution of lipids and proteins between the two newly formed organelles. Evidently, this model is questionable: how does a typically round-shaped organelle start to elongate and what are the factors that squeeze the membrane and generate the constriction? In a more realistic model, extension of the peroxisome would be controlled in a concerted manner such that both, membrane elongation and assembly of the fission machinery take place at the site of membrane protrusion. Then, scission would occur across the axe of elongation generating a new daughter organelle (Figure 1B). Here, two alternatives can be foreseen, namely (i) non-polarized elongation equally dividing the peroxisomal matrix content or (ii) polarized elongation of the membrane followed by protein import at the site of membrane outgrowth. In such a model the peroxisome does not require a constriction factor *per se* since the thin membrane protrusion already fulfills the criteria for scission i.e., suitable membrane diameter to adapt the fission factors. Nevertheless, in both models proposed the membrane must elongate and factors are required to initiate its outgrowth. The findings that the Pex11 proteins interact with the fission machinery in plant and mammal suggest that they act as recruitment factor for the fission apparatus. But, this does not explain how the peroxisomal membrane arranges for fission.

Assessment of the information known about the fission machinery, especially proteins of the dynamin family, might allow for mechanistic assumptions. Dynamin proteins, including DRP1, are self-assembling and self-activating large GTPases. They typically carry three distinct domains, an N-terminal GTPase domain, a middle domain and a GTPase effector domain (GED) at their C-terminus (90). These three domains arrange into an evolutionary conserved structure: the middle domain and the GED region form a neck and a trunk, respectively, whereas the GTPase domain lies on the top. All dynamin-related proteins dimerize along their GTPase domain, further stabilized by their GED region (91, 92). This dimerization step seems to correlate with nucleotide binding and was proposed to arrange the catalytic machinery for GTP hydrolysis (93, 94). Recent structural data however, suggest that the dynamin dimers build spirals around the membrane in its GDP-bound form, which implies that GTP hydrolysis is not the trigger for membrane fission (95). The exact structure of the dynamin spiral is still a matter of discussion. Nonetheless, it creates such high curvature and instability in the membrane that the sudden breakdown of the spiral through GDP dissociation is ultimately resolved by membrane fission (96, 97). Electron microscopy analyses showed that Dnm1p-spirals are exactly fitting mitochondrial constriction sites exhibiting a diameter of about 110 nm. *In vitro*, high non-physiological levels of Dnm1p were able to elongate liposomes (1 μm in diameter) to 110 nm wide tubules (98). Elegant experiments making use of giant unilamellar vesicles (GUVs) demonstrated that dynamin polymerization requires high membrane curvature. The authors demonstrated that adsorption of dynamin monomers to the bare tubes did not significantly affect curvature of the membrane, however, clusters of dynamins occurred by pulling tubes from these GUVs thereby decreasing the tube radius (99). In agreement, at physiological concentrations, dynamin proteins were shown to only assemble and function on already curved membranes (100, 101). In fact, BAR-domain proteins were reported to prepare the membrane and target the function of dynamin such as amphiphysin in the scission of clathrin coated vesicles (101). No BAR-domain protein has been identified that acts on the peroxisomal membrane.

The conformation of dynamin proteins appears to be regulated through GTP hydrolysis performed by the intrinsic GED region thought to function as internal GTPase-activat-

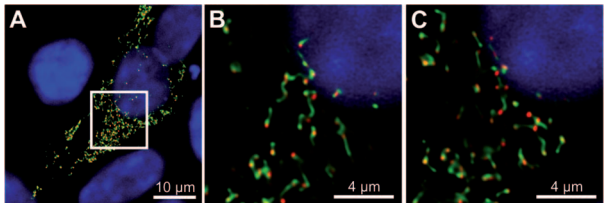

Figure 2 Pex11 induces peroxisome elongation.
(A) Maximum intensity projection of a confocal microscopic image showing the effect of ectopic expression of EGFP–HsPEX11β (green channel) on peroxisomes in human embryonic kidney cells (HEK293T). The elongated peroxisomal membrane shows segregation of the matrix marker, mCherry–Px (red channel). (B, C) Single z-layers from the insert region indicated in panel (A).

ing protein (GAP). However, Lee et al. reported a role for phospholipase D as external GAP for dynamin increasing its GTPase activity in a more effective manner than the inherent GED. The molecular mechanism appears similar to that of other GAPs based on the positioning of an arginine finger (102). Interestingly, Erdmann and colleagues showed in *S. cerevisiae* that Lpx1p, a phospholipase, is targeted to peroxisomes (103). Although this enzyme was suggested to have a metabolic function, the authors report drastic changes in peroxisome morphology including membrane invaginations and formation of intra-peroxisomal vesicles in mutant cells lacking *LPX1*. It is thus tempting to speculate that besides its metabolic activities Lpx1p influences the remodeling of the peroxisomal membrane during proliferation.

Several studies connected the function of Pex11 proteins not only to the recruitment of the fission machinery, but also to peroxisomal membrane remodeling, elongation, prior to fission (52, 104, 105). In previous studies we showed that overexpression of Pex11 proteins from yeast, plant and human resulted in elongation and thereafter clustering of peroxisomes in human cells (43). Peroxisome clustering had already been reported for *Hs*PEX11γ only (60). Close analysis of the peroxisomal clusters in 3D-reconstitutions and electron microscopy revealed that these are composed of individual, elongated peroxisomes that intertwined in a superstructure that we called juxtaposed elongated peroxisomes (JEP). Fluorescence recovery after photobleaching (FRAP) experiments demonstrated that the membranes of the individual peroxisomes in JEPs did not share components (43). Furthermore, we observed an evident separation of matrix and membrane proteins, with the matrix proteins accumulating at one or both extremities of the tubular peroxisomes (Figure 2). In parallel, Schrader and coworkers described the formation of tubular peroxisome accumulations after overexpression of PEX11β tagged with YFP at its extreme C-terminus (106). The authors also state the separation between matrix proteins in the tubular peroxisomes and report the differential localization of some peroxisomal membrane proteins. Interestingly, the early peroxisome biogenesis factors, PEX3, PEX16 and PEX19, were rather found on the stretched and elongated part of the peroxisome, whereas other membrane proteins, e.g., PMP70, PMP22 localized to the globular part. A very recent study in *H. polymorpha* on differential localization of various peroxisomal membrane proteins during membrane elongation showed that the spatiotemporal dynamic of membrane proteins ultimately depends on Pex11p function (107).

Asymmetric division of peroxisomes – segregation of the matrix protein content

The finding that upon Pex11 overexpression matrix proteins were unequally distributed alongside JEP cast some doubts about the current view that peroxisome division is stochastic. The observation could be merely due to a dilution effect with low amounts of matrix proteins in the elongated structures being below the detection limit in fluorescence microscopy. Alternatively, during the process of membrane protrusion matrix proteins could be sequestered leading to their exclusion from the thin tubular elongation. To differentiate between the two possibilities we measured repetitive fluorescence decay after photoactivation (rFDAP) of photoactivatable-GFP targeted either to mitochondria (paGFP-Mito) or to peroxisomes (paGFP-Px). A small region in mitochondria was photoactivated and the GFP signal was monitored in living mammalian cells. Mitochondria constantly fuse and divide giving them a network-like appearance. Hence, the paGFP-Mito signal could quickly diffuse through the mitochondrial network (Figure 3A, B). In contrast, in cells co-expressing mRFP-*Hs*PEX11β and paGFP-Px the activated GFP signal did not decline with time suggesting that paGFP-Px remained static and sequestered at one side of the elongated peroxisomal membrane (Figure 3C, D). In agreement with this observation, using the HALO-tag, Delille et al. demonstrated that the matrix content in the globular part of the elongated peroxisomes was present before membrane elongation occurred (106). In summary, under the effect of PEX11 peroxisomes elongate in a polarized fashion leaving their matrix content trapped at its original position although we cannot exclude that limited diffusion of small amounts of matrix content occurs during the elongation process. Hence, elongation of the peroxisomal membrane seems to create a matrix protein gradient, thereby segregating the 'old' matrix from the 'new' membrane. Segregation of matrix proteins during peroxisome elongation could ensure that old and possibly damaged proteins do not populate the new organelle. New matrix proteins would then target to the tip of the new membrane thereby inflating the new peroxisome and modeling the membrane constriction required for fission.

The observations by Delille et al. upon expression of a PEX11β-YFP suggest that the chimera inhibits peroxisomal fission while allowing their elongation. We showed that PEX11-driven peroxisomal elongation and even JEPs could be dissolved by providing high amounts of hFis1 to the cells (43). Interestingly, overexpression of the dynamin protein, DRP1, led to the appearance of elongated peroxisomes or to an increase in JEP size in cells expressing PEX11 proteins rather than to fission. These findings place hFis1 as limiting factor in the process of peroxisomal fission and highlight the importance of PEX11 as recruitment factor.

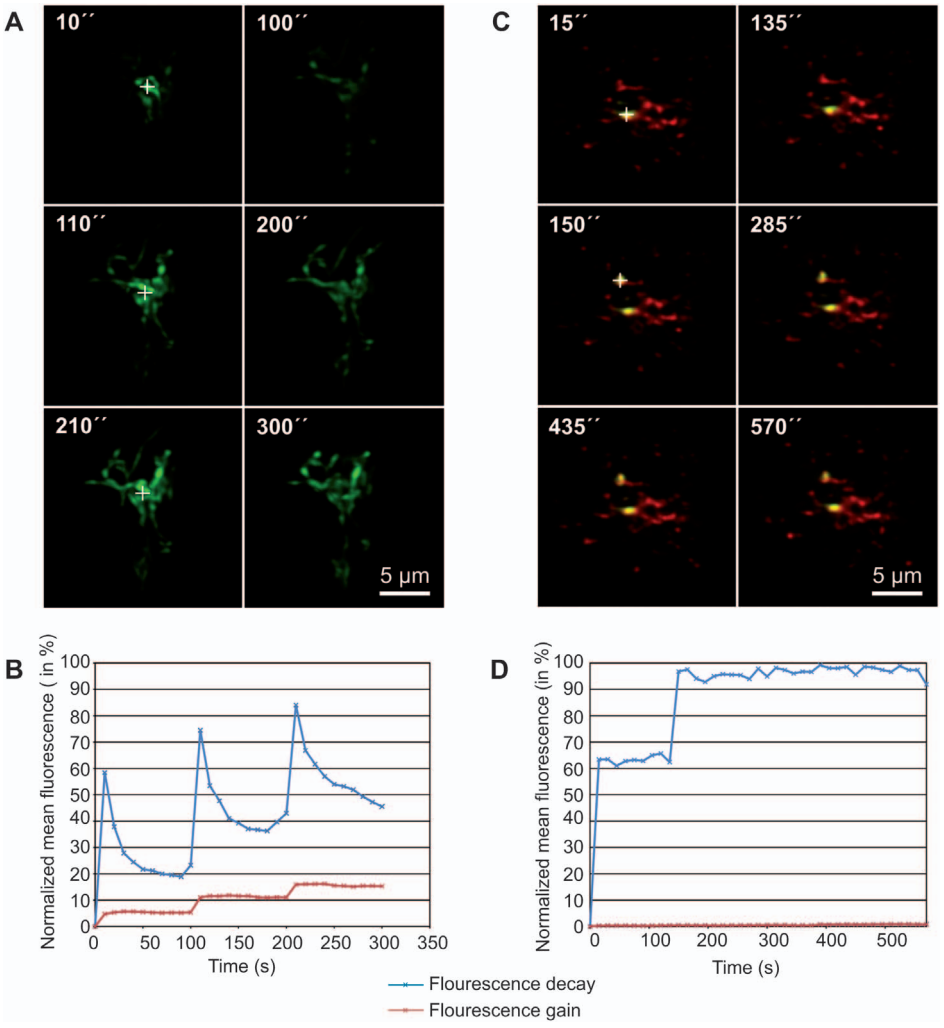

Figure 3 Peroxisomal matrix proteins are kept back during peroxisome elongation.
HEK293T cells expressing either the mitochondrial matrix marker, paGFP-mito (A, B), or the peroxisomal matrix marker, paGFP-Px and mRFP-*Hs*PEX11γ (C, D) were analyzed 48 h after transfection. Diffusion of matrix proteins was analyzed in repetitive fluorescence decay after photoactivation (rFDAP) experiments. paGFP was activated in a small area and fluorescence was monitored for decay along with measurement of fluorescence gain in the rest of the cell. (A) For mitochondria, repetitive activation of a single area (white crosses) led to rapid diffusion of the paGFP signal throughout the mitochondrial network. (B) Quantification of (A) showing fluorescence decay in the activated region (blue line) and gain of fluorescence in the non-activated region (red line). (C) paGFP was activated in JEPs caused by overexpression of mRFP-*Hs*PEX11γ. Since no decay was measured in the activated region (white cross 15″), a second area was activated (white cross 150″). (D) Quantification clearly shows that no signal was lost during acquisition and no diffusion took place. Image acquisition parameters: LSM DuoScan (ApoChromat 63x1.4; settings: paGFP (489 nm, MBS490, BP 500–525), mRFP (532 nm, MBS 535, BP 560–675); activation: 405 nm).

Recent experiments on mitochondrial fission described that Mff, another tail-anchored protein, is the ultimate recruiter of hFis1 for membrane fission (69). Although this latter study focused on mitochondrial fission, it had been shown earlier that Mff also played a role in peroxisome proliferation (68). Indeed, mammalian cells transfected with *Mff* RNAi presented peroxisomes that were more elongated than peroxisomes in cells depleted for hFis1. In consequence, assuming that the interplay between Mff, hFis1 and DRP1 is comparable in mitochondrial and peroxisomal fission, hFis1 might rather modulate DRP1 function than act as recruitment factor. In the light of these new observations it would be intriguing to test whether Pex11 proteins interact with Mff. Interaction with hFis1 only would suggest that Pex11 proteins act as membrane elongation factors, which stimulate the fission machinery. But, interaction with both, Mff and hFis1, would strengthen the role of Pex11 proteins in powering fission of the peroxisomal membrane.

Pex11 proteins elongate membranes *in vitro*

All these findings strongly point at the involvement of the Pex11 proteins in the membrane elongation event. A first

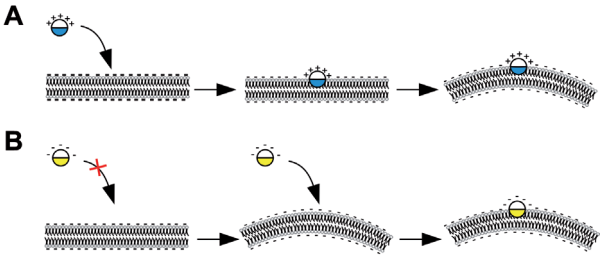

Figure 4 Amphipathic helices as membrane curvature sensors or inducers.
(A) A positively charged amphipathic helix leads to membrane bending upon insertion into one leaflet of the lipid bilayer. The energy cost for helix insertion can be compensated through electrostatic interactions. (B) If the amphipathic helix displays a negatively charged surface, it cannot deform the membrane, and acts as membrane curvature sensor.

hint about the molecular function of Pex11p was presented by Opalinski et al. (2010). The authors report the presence of an amphipathic α-helix at the N-terminus of several Pex11 proteins from yeast to mammal (108). Incubation of peptides containing the Pex11 amphipathic region with small unilamellar vesicles (SUVs) clearly showed an ability to restructure membranes. The initially round SUVs elongated and formed tubules in the presence of the Pex11 peptides. Similar results were obtained using the purified first 95 amino acids of *P. chrysogenum* Pex11p. The size and shape of the elongated SUVs could be altered by introducing bulky tryptophan residues in the amphipathic peptide. Changes in the peptide composition, such as introduction of negative charges or proline residues, annihilated the effect on membrane elongation. *In vivo*, expression of a mutated Pex11p protein lacking this alpha-helix was unable to protrude the peroxisomal membrane suggesting a mechanistic role for this helical structure in membrane elongation.

Amphipathic helices have been reported in a variety of proteins, well-known examples being the BAR proteins (1, 109–112). It has been suggested that two types of amphipathic helices exist namely, curvature sensors or inducers (113). Upon insertion of the helix into one leaflet of the lipid bilayer, the space requirement of this leaflet increases with respect to the other, which leads to membrane bending (Figure 4). This insertion requires the lipids to be pushed aside. If the energy cost is compensated by the presence of positively charged amino acids, it favors interaction between the charged head groups of the lipids and the polar face of the amphipathic helix, the helix can actively curve the membrane (Figure 4A). Alternatively, the helix contains mainly negatively charged residues, which hinder its insertion into a flat membrane. Hence, such helices are unable to induce membrane curvature and require a membrane already curved to insert. These amphipathic helices are membrane curvature sensors (Figure 4B). Evidently, this mechanism depends on the nature of the membrane including its lipid composition and local enrichment in specific lipids. Indeed, the often neglected physical properties of membrane lipids might determine the limits in which proteins can act (114). A well-studied example of curvature sensors is the ArfGAP1 lipid packing sensor (ALPS) motif, which contains numerous serine and threonine residues that favor its adsorption onto membranes with strong positive curvature (113). Curvature inducers are for instance the BAR domain proteins. The N-BAR domain e.g., in endophilin adopts a banana-wedge shape that bends the membrane to give it a curved form. Interestingly, mathematic modeling suggests that induction of membrane curvature relies on the sole property of the amphipathic helix and not on the entire N–BAR domain (115).

Consequently, amphipathic helices play pivotal roles in a plethora of intracellular processes and their presence in Pex11 proteins seems to be crucial for proliferation of the peroxisomal membrane. The generation of high curvature in the peroxisomal membrane could explain the redistribution of peroxisomal membrane proteins along the peroxisome tubules. Recent quantitative fluorescence microscopy analyses showed that membrane curvature as such can account for redistribution of integral or membrane anchored proteins (116). In the context of peroxisome proliferation such reorganization could lead to (i) attraction of the fission machinery and (ii) redistribution of membrane proteins including the import machinery to ensure efficient transport of matrix proteins into the newly formed peroxisome. Because the polar face of the Pex11 amphipathic helix contains lysine and arginine residues, it seems to rather induce membrane curvature. However, membrane curvature still needs to be tightly regulated. No polarized outgrowth would occur if all Pex11 amphipathic helices equally distributed and inserted into the peroxisomal membrane. Therefore, spatiotemporally confined protrusion has to be established to ensure elongation of the peroxisomal membrane. Thus, a strict control is required for Pex11 protein positioning on the membrane or for molecular interactions. This could arise through post-translational modifications. A study in the yeast *S. cerevisiae* showed that Pex11p is modified through phosphorylation. Cells expressing a phospho-mimicry mutant of Pex11p displayed more and smaller (S → D, 'phosphorylated') or less and bigger (S → A, 'non-phosphorylated') peroxisomes than wild type cells (117).

Several Pex11 proteins interact to orchestrate peroxisome proliferation

The interplay of the various Pex11 proteins in organisms that contain more than one Pex11 protein remains to be elucidated. Earlier studies showed the homodimerization properties of several Pex11 proteins including the human PEX11β and *Sc*Pex11p (62, 118). In addition to *Sc*Pex11p homodimerization, yeast-2-hybrid analyses showed homo-dimerization of *Sc*Pex25p and *Sc*Pex27p, respectively, but no hetero-oligomerization (56). In human cells, all three Pex11 proteins homo-oligomerized and both, PEX11α and PEX11β were shown to interact with PEX11γ. Co-immunoprecipitation experiments also revealed that the three proteins interacted with the fission machinery (43). *In vitro* binding

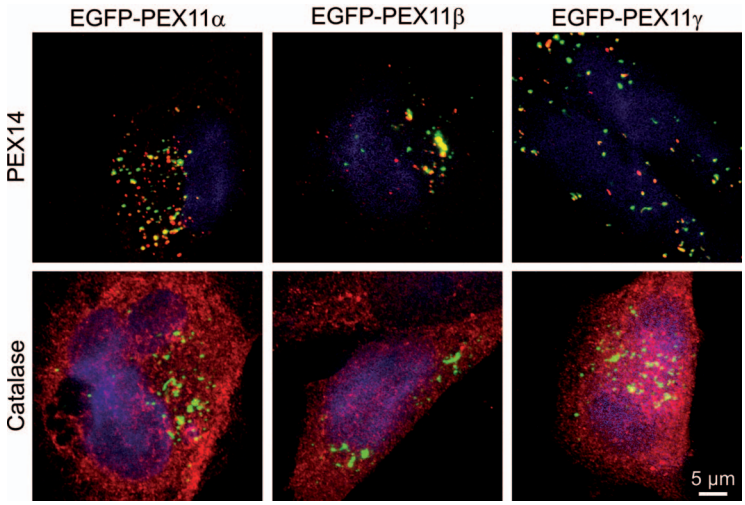

Figure 5 Pex11 membrane elongation factors do not require peroxisomal matrix content to function.
Analysis of mutant fibroblast cells with mutated PEX5 containing empty peroxisomal membranes for the effect of ectopic expression of GFP-tagged human Pex11 proteins. Pex11 proteins elongated the peroxisomal membrane in the absence of matrix content as demonstrated by immunofluorescent stainings for the peroxisomal membrane protein PEX14 (red channel, upper panel) and the matrix protein catalase (red channel, lower panel). Images represent maximum intensity projection of confocal images acquired on a LSM510META [objective 100×1.45; settings: EGFP (488 nm, MBS 488, BP 500–525), AlexaFluor594 (561 nm, MBS 561, LP 585)].

assays demonstrated a direct interaction between *Hs*PEX11β and hFis1 (62). Importantly, all these experiments were performed using digitonin, a mild detergent that preserves lipid environment, and the addition of Triton X-100 abolished interactions. This implies the requirement of membrane lipids for interactions. The orientation of several Pex11 proteins has been studied based on differential cell permeabilization with digitonin or protease accessibility of their extreme termini (50, 52, 60, 105, 119) however, their exact topology in the membrane remains to be elucidated. Hence, such information would be important to comprehend the mutual influence of Pex11 proteins and the fission machinery.

It is still unclear whether all Pex11 proteins are equally important for peroxisome proliferation. In yeast, the absence of Pex11p resulted in reduced growth of the cells on oleic acid (48) and in the abscence of Pex11p, Pex25p and Pex27p cells were unable to grow on oleate-containing medium. Interestingly, Pex25p alone was able to rescue the oleate non-utilizing phenotype of the *pex11Δpex25Δpex27Δ* mutant cells (56). In mammal, while PEX11α expression is inducible, PEX11β is constitutively present in the cell (49, 50, 120, 121). Knockout mouse models showed that in the absence of PEX11β mice developed pathologies similar to those of Zellweger patients and the number of peroxisomes per cell was significantly decreased (121, 122). Deletion of *PEX11α* did not have a phenotype neither did it worsen the condition in PEX11$\alpha^{-/-}$/$\beta^{-/-}$ mice (120). These data suggest that in mammal, two routes exist for peroxisome proliferation, one inducible and one constitutive, driven by either PEX11α or PEX11β, respectively. Both ways might require the function of PEX11γ. Homodimerization, interaction with PEX11γ or both could allow for recruitment of the fission machinery. Analysis of PEX11β suggested that its C-terminus was required to interact with hFis1 (62). Proteineaceous interactions were proposed to depend on one of the tetratricopeptide repeat (TPR) regions of hFis1 (123). Peptide-scan analyses demonstrated that proline-rich peptides efficiently bind hFis1, specifically in the TPR region (124). Interestingly, plant and human Pex11 proteins contain proline-rich regions, among which some resemble a Fis1 binding site. In contrast, none of the *S. cerevisiae* Pex11 family member contains such motif suggesting that in this species Pex11 proteins might not directly recruit Fis1 to peroxisomes.

Oligomerization of Pex11 proteins could regulate their activity. In *S. cerevisiae*, dimerization of Pex11p was suggested to act as molecular switch. Considering that *Sc*Pex11p was localized to the inner surface of the peroxisomal membrane it could easily be influenced by the peroxisomal redox state. Hence, redox-sensitive dimerization of Pex11p could represent a signal for proliferation (118). A redox-sensitive dimerization of Pex11 proteins has not been reported in mammalian cells. However, a recent study investigated the mammalian peroxisomes redox-balance using a redox-sensitive variant of EGFP and an artificial light-triggered ROS-induction protein. The authors demonstrate that although peroxisomes resist to an oxidative stress produced elsewhere in the cell, the intraperoxisomal redox status is strongly affected by the environmental growth conditions. Interestingly, the redox state of peroxisomes did not correlate with their age (125).

To address whether the peroxisomal matrix content exerts an influence on the function of Pex11 proteins we assessed whether PEX11 could act on empty peroxisomal membranes (remnants) in cells expressing a mutated PEX5, a receptor for peroxisomal matrix proteins (126). Most peroxisome remnants elongated and formed JEPs upon overexpression of either of the human Pex11 proteins (Figure 5). This observation points to an independent mode of regulation for per-

oxisome function and proliferation. Nevertheless, the expression of some Pex11 proteins is tightly regulated, which allows for coordination of the proliferation machinery and the metabolic state of peroxisomes. Alternatively, matrix proteins could affect the properties of the peroxisomal membrane thereby modulating proliferation of the organelle as already suggested for the peroxisomal enzyme acyl-CoA oxidase in *Yarrowia lipolytica* (127).

Perspectives

Recent reports placed the Pex11 proteins as key actors in the process of peroxisomal membrane remodeling. These proteins elongate the peroxisomal membrane. It will be important to test how their positioning selects the site of membrane protrusion, and how they interact with the fission machinery to coordinate membrane scission. Future experiments will be required to determine whether Pex11 proteins represent a new family of amphipathic alpha-helix-containing proteins with membrane bending activities.

Furthermore, evidence exists that peroxisome elongation is polarized. Asymmetric division of the matrix protein content during membrane elongation might allow for import of new material at the site of membrane growth. We propose this mechanism to ensure selective retention instead of dilution of old matrix content. Whether the selective degradation of peroxisomes via pexophagy is specifically targeted to old organelles is an attractive question.

Although the distribution of matrix proteins seems to be highly regulated, the action of the Pex11 proteins does not depend on the functionality or maturity of the peroxisomes. As shown in our experiments, overexpressed Pex11 acts on the membrane obviously without requiring feedback from the matrix. It remains to be elucidated whether the function of the Pex11 proteins is directly or indirectly influenced by the metabolic state of the cell.

The Pex11 interactome was shown to require the integrity of the peroxisomal membrane. Thus, understanding the membrane topology of Pex11 proteins is important in order to gain insight in its role as membrane elongation factor. Eventually, structural studies will deliver the missing elements to understand how these proteins act at the molecular level.

In conclusion, the two pathways leading to peroxisome formation, *de novo* biogenesis and growth and division, are presumably connected at the stage of membrane uptake. Consequently, with Pex11 proteins as membrane shaping factors, it would not be surprising that some of these proteins also contribute to *de novo* peroxisome biogenesis from the ER. Interestingly, a very recent study on the identification of peroxisome biogenesis factors in the yeast *H. polymorpha* revealed the importance of Pex25p for the reintroduction of peroxisomes in mutant cells lacking these organelles (128). Noteworthy, an interaction between the rat PEX11 and Arf1/coatomer has been reported and coatomer inhibition in temperature sensitive CHO-mutant cells correlated with the occurrence of tubular peroxisomes (119).

Alterations in peroxisomal metabolism and peroxisome proliferation cause neurodegenerative diseases and might also represent a trigger for cellular ageing. Understanding how peroxisomes proliferate and, more specifically, generate membrane protrusion to facilitate scission, will have a major impact on understanding the dynamics of biological membranes. The concept of organelle polarization and asymmetric membrane growth and division might engage the re-investigation of the proliferation of other organellar membranes.

Highlights

- Most organisms contain more than one Pex11 protein and all Pex11 proteins act on the peroxisomal membrane
- Pex11 proteins are regulated at transcriptional, and post-translational levels through modifications as well as homo- and heterodimerization
- Pex11 proteins influence the shape of the peroxisomal membrane
- Pex11 proteins coordinate the fission machinery shared between peroxisomes and mitochondria
- Pex11 proteins contain an amphipathic alpha-helix suggested to bend the peroxisomal membrane
- Pex11 proteins act as membrane elongation factors regardless of whether peroxisomes are functional
- Asymmetric inheritance of peroxisomal matrix proteins during peroxisome proliferation might lead to rejuvenation of the peroxisome pool in the cell

Acknowledgments

The authors declare having no financial or other conflicts of interest regarding the work presented in this publication. We are grateful to Ronald Wanders (University of Amsterdam, The Netherlands) and Ralf Erdmann (Ruhr–University Bochum, Germany) for the PEX5 deficient fibroblast cell line. The authors wish to thank Josef Gotzmann (MFPL, Austria) for technical assistance. This work was funded by a grant from the Austrian Science Fund (FWF) P-20803 to C.B. C.B. is supported by the Elise-Richter-Program (V39-B09) of the Austrian Science Fund (FWF, http://www.fwf.ac.at/) and the Austrian Federal Ministry for Science and Research (BMWF, http://www.bmwf.gv.at/).

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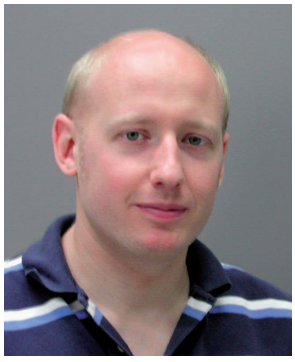
Received May 13, 2011; accepted June 21, 2011



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