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Sorting and function of peroxisomal membrane proteins

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Abstract

Peroxisomes are subcellular organelles and are present in virtually all eukaryotic cells. Characteristic features of these organelles are their inducibility and their functional versatility. Their importance in the intermediary metabolism of cells is exemplified by the discovery of several inborn, fatal peroxisomal errors in man, the so-called peroxisomal disorders. Recent findings in research on peroxisome biogenesis and function have demonstrated that peroxisomal matrix proteins and peroxisomal membrane proteins (PMPs) follow separate pathways to reach their target organelle. This paper addresses the principles of PMP sorting and summarizes the current knowledge of the role of these proteins in organelle biogenesis and function. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Contents

1. Introduction

Microbodies (peroxisomes, glyoxysomes) represent the last class of organelles that have been discovered [1,2]. They are present in virtually all eukaryotic cells and are characterized by an unprecedented functional versatility, which varies with the organism in which they occur and with the environmental conditions to which cells are exposed. The observation that in man a group of genetically distinct, mostly fatal, inborn peroxisomal disorders exists [3] has strongly stimulated the interest in the biogenesis and function of these organelles. In particular, the use of yeast and Chinese hamster ovary cells as model systems has been instrumental in attempts to characterize the mo-

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lecular details of peroxisome assembly. Many mutant strains deficient in peroxisome biogenesis (designated *pex* mutants) have been isolated, the corresponding genes (*PEX* genes) cloned and their protein products (termed peroxins) analyzed (unified nomenclature after [4]). These and other studies have revealed that the principles of microbody biogenesis are highly conserved between yeasts and higher eukaryotes, including man. At present, 22 peroxins have been described; most of them are components of the peroxisomal membrane (see Table 1 and Fig. 1).

Recently, in silico screening of the *Saccharomyces cerevisiae* genome database for protein patterns (i.e. containing the peroxisomal targeting signals; PTS1 or PTS2) or gene expression (i.e. induced in response to oleate) is applicable for the identification of new peroxins [5]. In addition, a comparison of transcript profiles (serial analysis of gene expression) from yeast grown under peroxisome-requiring and non-requiring conditions was also performed [6]. However, these studies indicated that the transcripts encoding known peroxins have a low abundance and are hardly or not induced under peroxisome-requiring conditions, and thus have not yet been the expected source to identify new proteins involved in peroxisome biogenesis [6].

Conceptually, the development of peroxisomes involves a series of events that have to be tuned carefully. These include the recruitment of (phospho)lipids, the synthesis, sorting and assembly/activation of matrix and membrane proteins, as well as organelle inheritance and fission. The peroxisomal membrane mainly consists of phosphatidylcholine and phosphatidylethanolamine and largely resembles that of the endoplasmic reticulum (ER) (reviewed in
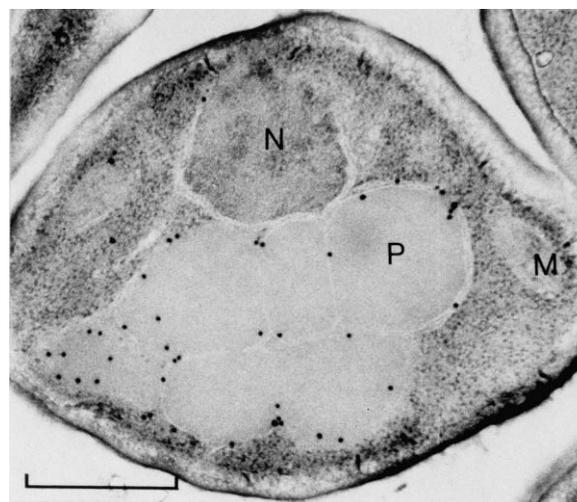

Fig. 1. Immunocytochemical localization of the PMP Pex2p in methanol-grown *P. pastoris* wild-type cells, using antibodies against Pex2p (in collaboration with Dr. J.M. Cregg). [aldehyde-unicryl-uranyl acetate] Abbreviations: N, nucleus; P, peroxisome; M, mitochondrion. The bar represents 0.5 μm.

[7]). How the lipid components of these membranes are recruited is still largely unknown. Only recently, the first experimental data became available which suggest that the ER plays a role in this process and requires vesicle trafficking and fusion events [8–12].

The sorting of microbody matrix proteins is now rather well understood. These proteins are encoded by nuclear genes and, after their synthesis in the cytosol, are recognized by specific soluble receptor molecules. The recognition is mediated by specific targeting signals that reside

Table 1
List of membrane-bound peroxins

Peroxin	Molecular mass (kDa)	Characteristics[a]	References
Pex1p	117–143	AAA-type ATPase; contains two AAA domains; interacts with Pex6p	[1–8]
Pex2p	31–52	IPMP; C-terminal Zn-finger motif; *N*-glycosylated; sorted via ER[b]	[9–17]
Pex3p	42–52	IPMP; *pex3Δ* cells do not contain ghosts; interacts with Pex19p	[18–21]
Pex4p	21–24	PMP; ubiquitin-conjugating enzyme	[22–24]
Pex6p	112–127	AAA-type ATPase; contains two AAA domains; interacts with Pex1p	[6–8,25–30]
Pex8p	71–81	PMP facing matrix; contains PTS1 and PTS2	[31–33]
Pex9p	45	IPMP	[34]
Pex10p	34–48	IPMP; C-terminal Zn-finger motif	[35–38]
Pex11p	24–31	IPMP; in mammals two isoforms (α/β); α-form contains dilysine motif, binds coatomer/ARF; dimerizes in baker's yeast and trypanosomes	[39–48]
Pex12p	40–46	IPMP; C-terminal degenerate Zn-finger motif	[49–51]
Pex13p	40–43	IPMP; cytosolic SH3 domain; interacts with Pex5p, Pex7p and Pex14p	[52–54]
Pex14p	38–41	PMP; facing cytosol; phosphorylated; interacts with Pex5p, Pex7p, Pex13p, Pex17p	[55–59]
Pex15p	44	IPMP; phosphorylated	[60]
Pex16p	39–44	PMP; facing matrix; *N*-glycosylated; sorted via ER[b]	[61–63]
Pex17p	23	PMP; facing cytosol; interacts with Pex14p	[64]
Pex19p	33–40	PMP; facing cytosol; farnesylated; in baker's yeast mainly cytosolic; interacts with Pex3p and Pex10p	[65–69]
Pex22p	23	IPMP; C-terminus cytosolic; interacts with Pex4p	[70]

[a](I)PMP: (integral) peroxisomal membrane protein.
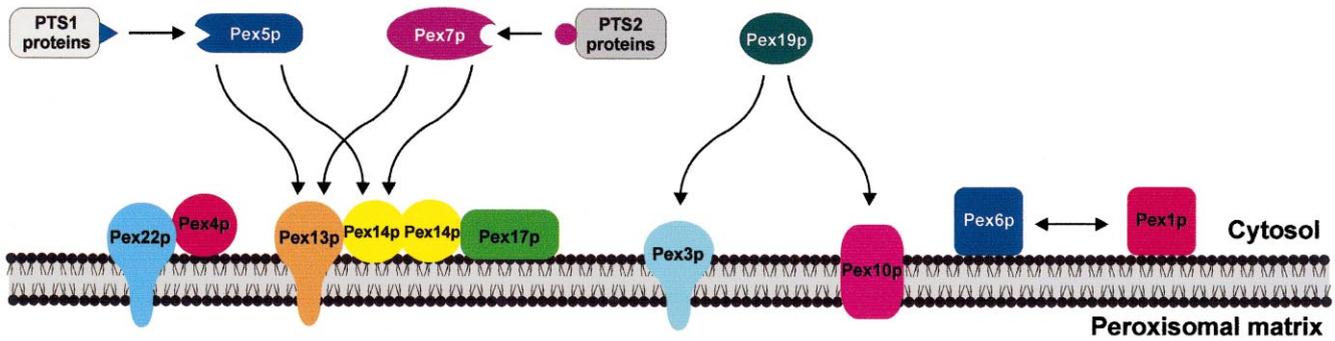[b]In the yeast *Y. lipolytica*.

Fig. 2. Schematic representation of interactions between soluble and membrane-bound peroxins, as demonstrated by two hybrid analysis or co-immuno-precipitation experiments.

either at the extreme C-terminus (PTS1, recognized by the cytosolic receptor Pex5p) or in the N-terminus of the matrix protein (PTS2, recognized by the cytosolic receptor Pex7p). Most likely, binding of the cargo molecule induces a conformational change in the receptor molecule, which allows the cargo/receptor complex to be recognized by a specific docking protein residing at or in the peroxisomal membrane. At present, two putative docking proteins have been identified, namely Pex13p and Pex14p (see Fig. 2) (reviewed in [13,14]). After release of the cargo protein, the receptor molecule is thought to be recycled to the cytosol. In the methylotrophic yeast *Hansenula polymorpha*, recycling of the PTS1 receptor is dependent on the function of a peroxisomal ubiquitin conjugation enzyme, Pex4p [15]. Whether or not the receptor functionally enters the peroxisomal matrix before recycling is still a matter of debate.

Peroxisomal membrane proteins (PMPs) do not use the sorting machinery of matrix proteins to reach their target organelle, but follow alternative pathways. This paper summarizes the current knowledge on this topic and discusses the role of membrane proteins in peroxisome biogenesis and function.

2. Peroxisome biogenesis

2.1. PMPs involved in organelle biosynthesis and proliferation

Upon induction of peroxisome biogenesis, the synthesis of PMPs was shown to precede that of matrix components [16–18]. In contrast to various inducible matrix proteins, genes that encode essential PMPs are expressed during repressing growth conditions (e.g. on glucose) and are only moderately induced, even when peroxisomes are present at high levels (e.g. in methanol-induced *H. polymorpha*) [19]. This characteristic phenomenon suggests that basic levels of these proteins are necessary for organelle maintenance and inheritance. The finding that the levels of various essential membrane proteins do not increase very much relative to the strong increase in membrane

surface could imply that these proteins are present in functional complexes that are donated to newly formed organelles upon peroxisome fission [20]. The major exception to this is Pex11p; in *S. cerevisiae*, the synthesis of Pex11p is strongly induced (over 30-fold) by oleate and, like many peroxisomal matrix enzymes, is controlled by the oleate-inducible transcription factors Pip2p and Oaf1p [6,21,22].

Both in yeast and in other organisms, Pex11p is essential for normal peroxisome proliferation. This function is proposed from the finding that in oleate-induced cells of a *S. cerevisiae pex11Δ* strain, only a few enlarged peroxisomes are observed, while in cells overexpressing *PEX11*, enhanced numbers of relatively small organelles are found [23]. Similar phenotypes were reported when *PEX11* was overexpressed in trypanosomes and mammals, implying similar roles for Pex11p in these organisms [23–26]. *S. cerevisiae* and *Trypanosoma brucei* Pex11p were shown to form homodimers [24,27]. Marshall et al. proposed that Pex11p in its 'monomeric' form is active in proliferation, while increasing oxidative metabolism within maturing peroxisomes causes dimer formation, which inhibits further organelle division [27]. In mammals, two Pex11p isoforms have been identified, encoded by *PEX11α* and *PEX11β* [26,28]. Of these, only *PEX11α* was shown to be induced by peroxisome proliferators, such as clofibrate, whereas *PEX11β* was constitutively expressed [26,28,29]. Interestingly, mammalian Pex11αp's contain a dilysine motif at their C-termini, which is absent in Pex11βp's [26,28]. Cosson and Letourneur have shown that such dilysine motifs may be involved in binding specific coat proteins (coatomers, COP I vesicles) when exposed to the cytosolic face of the membrane and play a role in the generation of vesicles for the retrieval of ER-resident membrane proteins [30]. Indeed, using purified rat peroxisomes, the group of Just showed that the dilysine motif is able to bind the ADP-ribosylation factor (ARF) and coatomer in a strictly guanosine GTP-γS (5'-O-(3-thiotriphosphate))-dependent manner [25]. This result led them to suggest that by ARF- and coatomer-dependent vesiculation of the peroxisomal membrane, peroxisomes divide or generate nucleation centers for new organelles [25]. However, other mechanisms are likely to exist, since the C-

terminal dilysine motif is not conserved in all Pex11p's identified so far. Also, discrepancies exist in the reported locations of different Pex11p's. In *S. cerevisiae*, Pex11p was suggested to be a membrane-associated protein facing the peroxisomal matrix, whereas in rat and trypanosomes, the N- and C-termini of Pex11p are believed to be exposed to the cytosol [24,25,27]. Detailed biochemical studies will be required to solve these apparent contradictions.

Apart from Pex11p, other peroxins may be involved in peroxisome proliferation, namely Pex10p and Pex16p [31,32]. Peroxisome proliferation can also be regulated by varying the *PEX3* expression level in *H. polymorpha* [33,34]. In constructed conditional mutants of *H. polymorpha* in which the Pex3p levels can be modulated, the size/number of peroxisomes ranged from one or a few large organelles to highly enhanced numbers of small organelles relative to lowered or enhanced Pex3p levels [33,34]. This induction of peroxisome proliferation can occur independent from peroxisome-inducing conditions (extracellular stimuli), since Pex3p overproduced in glucose-grown *H. polymorpha* cells also resulted in proliferation of very small peroxisomes [33]. Essentially, similar results were obtained with *H. polymorpha* Pex14p [35].

Pex deletion strains characteristically contain peroxisomal membrane remnants ('ghosts') in which various peroxisomal membrane components accumulate. The major exception is *pex3Δ*, since in the absence of Pex3p, no peroxisomal remnants were detected [36,37]. Recently, a predominantly cytosolic peroxin, Pex19p, was characterized which was shown to interact with Pex3p (Fig. 2) [38–40]. Also in *pex19* deletion strains, membrane remnants were either completely absent (in *S. cerevisiae*) or morphologically distinct from the late remnants found in other *pex* mutants (in *Pichia pastoris*) [38,39], suggesting that these two proteins are involved in an early stage of peroxisome biogenesis.

2.2. Sorting of PMPs

Heterologous expression experiments have demonstrated that the sorting machinery for PMPs is conserved [24,41–45]. Like peroxisomal matrix proteins, PMPs are encoded by nuclear genes and synthesized on free polysomes [46–48]. Consequently, they may insert directly from the cytosol into the peroxisomal membrane. Evidence for this came from both in vitro (i.e. mammalian PMP22 and Pex2p) [49,50] and in vivo insertion experiments (i.e. PMP70) [51]. However, other sorting mechanisms may also exist. This can be concluded from the recent findings that several peroxins undergo N- or O-linked glycosylation, thus suggesting that these proteins are sorted to peroxisomes via the ER. The most convincing result in this respect is that in the yeast *Yarrowia lipolytica*, peroxisomal Pex2p and Pex16p were shown to be *N*-glycosylated. In this yeast, the secretory pathway and peroxisome biogenesis seem to be in part converging

[12,52]. At present, in no other organism, neither *N*-glycosylation (e.g. human Pex16p) [53,54] nor convergence of the secretory pathway and peroxisome biogenesis have been observed making *Y. lipolytica* an ideal organism to study the process of sorting of PMPs.

Other studies also indicate that the ER may play an essential role in peroxisome biogenesis, although none of them is as yet conclusive. Firstly, Elgersma and coworkers demonstrated that radioactive-labeled mannose was bound to overproduced Pex15p, indicating that the protein is *O*-mannosylated in *S. cerevisiae* [55]. Additionally, deletion of 30 amino acids from the C-terminus of Pex15p resulted in mislocalization of the truncated protein to the ER [55]. Secondly, we demonstrated that the first 16 N-terminal amino acids of *H. polymorpha* Pex3p are able to direct a reporter protein to the ER [36], suggesting that Pex3p, like Pex2p, Pex15p and Pex16p, is sorted to peroxisomes via the ER. Also peroxisome biogenesis in *H. polymorpha* cells was partially inhibited by brefeldin A, a fungal toxin that interferes with coated vesicle formation. Newly synthesized PMPs (i.e. Pex3p) as well as certain peroxisomal matrix proteins reversibly accumulated in or at the ER [9].

Targeting signals of PMPs (termed mPTSs) have first been identified in *Candida boidinii* PMP47. PMP47 is an integral component of the peroxisomal membrane and a member of the family of mitochondrial solute carriers, containing six transmembrane domains. The mPTS is present at an internal position in a loop between membrane-spanning domains 4 and 5 and contains a stretch of positively charged amino acids [56]. In addition, the mPTS of rat PMP70 was delineated to an internal region around amino acids 190–235 [51]. Subsequently, comparable mPTSs have been identified in Pex3p and Pex15p [37,55,57].

Some general characteristics of the described mPTSs can be defined. In all cases, the mPTS is in close proximity of (a) hydrophobic region(s). The mPTS sequences are clearly distinct from the two targeting sequences so far characterized for peroxisomal matrix proteins, the C-terminal PTS1 and the N-terminal PTS2 (reviewed in [13]). Based on the proposed topologies of PMP47, Pex3p and Pex15p in the peroxisomal membrane, their mPTSs are exposed to the matrix side of the peroxisomal membrane. Furthermore, its precise location is fairly arbitrary, since they have been found in the N-terminal (Pex3p), C-terminal (Pex15p) and an internal (PMP47) position within the primary sequence of the protein.

It may be concluded that distinct classes of mPTSs exist for proteins of different functions. One of these (i.e. in PMP22, PMP47 and PMP70) directs functional proteins (e.g. solute carriers) from the cytosol directly to the peroxisomal membrane. Alternatively, proteins involved in organelle biosynthesis (i.e. Pex2p, Pex3p, Pex15p and Pex16p) may contain sequences directing them to the peroxisome via the ER (Fig. 3).
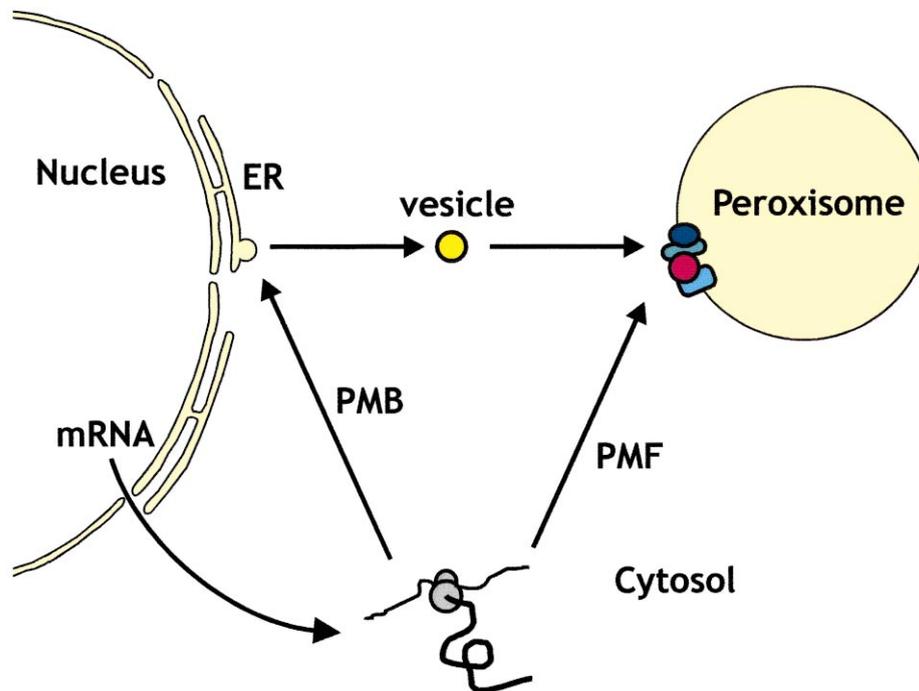
Fig. 3. Hypothetical pathways of the PMP sorting. Membrane proteins are synthesized on free polysomes in the cytosol. The model discriminates between membrane proteins essential for the biogenesis of organelles (PMBs) and those involved in the organellar function (PMFs, e.g. transporters). PMBs (e.g. Pex2p, Pex3p, Pex15p and Pex16p) may initially be transported to the ER and, eventually vesicle-mediated, be sorted to the growing peroxisome; PMFs may be transported directly to the organelle. Adapted from [9].

At present, no components have been identified which are involved in the recognition of the mPTSs and the subsequent insertion of PMPs into the membrane.

2.3. Post-translational modifications

Several PMPs undergo post-translational modifications. Of these, *H. polymorpha* Pex14p and *S. cerevisiae* Pex15p have been shown to be phosphorylated. However, the functional relevance of these modifications is not yet understood [55,58]. As discussed before, also glycosylation has been observed (see Section 2.2). Human and *S. cerevisiae* Pex19p are farnesylated, a modification that may promote interaction of the protein with the peroxisomal membrane and, furthermore, has been shown to be essential for the proper functioning of the protein in peroxisome biogenesis [38,59]. Apart from membrane anchoring, farnesylation can facilitate protein–protein interactions [60]. This might in fact represent the most probable function in Pex19p since deletion of the farnesylation site strongly affects the interaction with the PMP Pex3p [38]. However, since farnesylation of *P. pastoris* Pex19p is questionable [39], the importance of this modification for peroxisome biogenesis in general needs further investigation. On the other hand, in the C-termini of yeast Pex19p's, the tetrapeptide cysteine-lysine-glutamine-glutamine (CKQQ) is conserved; this sequence was shown to be the best substrate for S-farnesyl transferase, the first enzyme involved in farnesylation [61].

2.4. Insertion of PMPs

In a series of in vitro experiments using purified mammalian peroxisomes, it has been shown that the insertion mechanism of the PMPs Pex2p, PMP22 and PMP70 proceeds in two distinct steps; namely (1) binding to the peroxisomal membrane and (2) integration of the protein into the membrane [49–51]. The insertion of PMP22 and PMP70 did not require ATP or GTP, whereas insertion of Pex2p did require ATP-binding, but not hydrolysis [49–51]. Also, insertion of PMP22 and PMP70 is dependent on (a) proteinaceous component(s) at the peroxisomal membrane which is insensitive to *N*-ethylmaleimide. Cytosolic factors were shown to be involved in the binding and/or insertion of PMP22 and PMP70 [51,62]. These factors most probably represent molecular chaperones or polypeptide chain-binding proteins stabilizing the hydrophobic regions of the proteins thus allowing them to properly insert into the peroxisomal membrane [62]. On the other hand, these factors could represent cytosolic receptors involved in the targeting of the membrane proteins to their target organelle.

3. Peroxisome function

As already predicted from the, often inducible, functional versatility, the peroxisomal membrane must be able to readily adapt in order to allow new metabolic processes to

proceed. A number of the functions that are attributed to the peroxisomal membrane are listed below.

1. The peroxisomal membrane contains several enzymes involved in lipid metabolism (reviewed in [7]).
2. Systems must exist for the recruitment of (phospho)lipids for membrane assembly [63].
3. Association of peroxisomes with the cytoskeleton was shown to be involved in peroxisome morphology, movement and possibly segregation [64–66].
4. The membranes harbor mechanisms that specify recognition and insertion of membrane proteins or import of peroxisomal matrix proteins (e.g. Pex13p, Pex14p, Pex18p and Pex21p) [13,67].
5. Selective degradation of peroxisomes is specified by membrane component(s) [68].
6. Various transport mechanisms (e.g. carriers, transporters) must exist to allow passage of substrates, metabolic intermediates and products across the peroxisomal membrane (reviewed in [69]) as well as cofactors for the peroxisomal enzymes (e.g. FAD, NADH).

3.1. Metabolite and solute transport

The peroxisomal membrane is impermeable in vivo. This was evident from the observation that peroxisomes of *H. polymorpha* are acidic, which suggests that a proton gradient must exist across the peroxisomal membrane [70,71]. As a consequence of this impermeability, various small molecules like NADH, NADPH and acetyl-CoA have to be transported actively. To this purpose, several metabolite shuttles across the membrane have been proposed (e.g. the glycerol-3-phosphate/dihydroxyacetone phosphate and the malate/aspartate shuttles). Instead of transporting the compound itself, they mediate the transfer of metabolic intermediates, which are converted back to the original compound after transport across the membrane [69,72,73]. However, the essential carriers/transporters in these shuttles which transport the intermediates

across the peroxisomal membrane have not yet been identified [20,69].

Among the PMPs identified so far, several members are thought to carry out solute transport (see Table 2). The *C. boidinii PMP47* gene encodes a 47-kDa integral PMP which is homologous to mitochondrial solute carriers present in the mitochondrial inner membrane (e.g. ADP/ATP exchanger) [41,74]. The N- and C-termini of PMP47 were shown to be exposed to the peroxisomal matrix [75]. Thus, the proposed topology is inverted in comparison to mitochondrial carriers, because their termini extend into the mitochondrial intermembrane space [76]. Deletion of PMP47 in *C. boidinii* wild-type cells causes a defect in the transport and folding of the peroxisomal enzyme dihydroxyacetone synthase (DHAS), whereas other peroxisomal matrix proteins were normally sorted and active. Since deletion of PMP47 in peroxisome-deficient strains did not result in reduced activity of DHAS, it is likely that PMP47 does not catalyze folding directly but is involved in the transport of the DHAS cofactor, thiamine pyrophosphate [77]. Recently, putative human and mouse homologues of *C. boidinii* PMP47, designated PMP34, have been identified. Similar to PMP47, six transmembrane domains were predicted in these proteins [78]. However, the precise function of PMP34 is not yet known.

In addition to the PMP34/PMP47 proteins, another peroxisomal member of the mitochondrial solute carrier family has recently been identified in rabbit, namely a 53-kDa peroxisomal Ca^{2+}-dependent solute carrier [79]. The N-terminus of this protein is exposed to the cytosol and is able to bind Ca^{2+}. Based on the prediction of the presence of six transmembrane domains in the protein and similarity to mitochondrial solute carriers, the authors proposed that the N- and C-termini face the cytosol. Again, the function of the protein is unknown [79].

3.2. ABC transporters

Four ATP-binding cassette (ABC) transporters have

Table 2
PMPs (other than peroxins)

PMP	Molecular mass (kDa)	Characteristics	Reference[a]
PMP20	20	C-terminal PTS1; induced on methanol in yeast	[100–102]
PMP22	22	Pore-forming structure	[103]
PMP34	34	Homologous to mitochondrial carrier family; PMP47 homolog	[78]
PMP47	47	Homologous to mitochondrial carrier family; involved in transport/folding of DHAS	[41,77]
CDSC	53	Ca^{2+}-dependent solute carrier	[79]
PMP70	75	ABC transporter; C-terminal ATP-binding domain; heterodimer	[82,83]
PMP70R	73	ABC transporter; C-terminal ATP-binding domain; heterodimer	[85–87]
ALDP	75	ABC transporter; C-terminal ATP-binding domain; heterodimer	[80,81]
ALDRP	80	ABC transporter; C-terminal ATP-binding domain; heterodimer	[84]
Pxa1p	100	ABC transporter; C-terminal ATP-binding domain; heterodimer (Pxa2p)	[91–94]
Pxa2p	97	ABC transporter; C-terminal ATP-binding domain; heterodimer (Pxa1p)	[91–94]

[a]See references.

been identified in the peroxisomal membrane in mammals: the adrenoleukodystrophy (ALD) protein (ALDP) [80,81], a 70-kDa PMP (PMP70) [82,83], an ALD-related protein (ALDRP) [84] and the PMP70-related protein (PMP70R) [85–87]. ABC transporters are members of the superfamily of membrane proteins involved in transport of various compounds across membranes, ranging from ions to peptides [88]. The transporters are found either as complete transporters (generally 12 transmembrane domains) or as half transporters (generally six transmembrane domains) which can homo- or heterodimerize to form an active transporter [88]. The peroxisomal ABC transporters are all half transporters, which are composed of two functional domains: a transmembrane domain with multiple (six) transmembrane segments and a nucleotide-binding domain with Walker A and B consensus motifs [89] in the C-terminus.

Mutations in the ALD gene cause X-linked ALD, a disorder in peroxisomal β-oxidation resulting in the accumulation of very long chain fatty acids [80]. Although mutations in PMP70 genes of Zellweger syndrome patients have been identified, the specific role of mutant PMP70 in this disease is still speculative [83,90]. ALDRP is most similar to ALDP, whereas PMP70R is most similar to PMP70 [84,85]. The function of both proteins is unknown.

In the genome of the yeast *S. cerevisiae*, only two peroxisomal ABC half transporters have been identified, namely Pxa1p and Pxa2p. These proteins are highly similar to PMP70 and ALDP and were shown to form a heterodimer [91–94]. *S. cerevisiae* Pxa1p and Pxa2p are involved in the transport of long chain fatty acids into peroxisomes [91–93].

Based on the observations in baker's yeast, the mammalian peroxisomal ABC transporters were suggested to mediate transport of fatty acids or enzymes required for β-oxidation into the peroxisomal matrix. The formation of homodimers or different heterodimers by various combinations of the four half transporters could provide different transport facilities towards fatty acids of different saturation states and/or lengths into peroxisomes [91–93]. However, the specific transport function(s) of and physical interaction(s) between the various mammalian or yeast peroxisomal ABC transporters remain to be elucidated. Also, for neither of the half transporters has the topology in the peroxisomal membrane been established [95].

4. Concluding remarks

In the past few years, our knowledge on the biogenesis of peroxisomes has significantly advanced. In particular much has been learned about the mechanisms of matrix protein import which involve novel proteins and new principles. However, major gaps in our knowledge still exist, for instance (1) do PTS receptors functionally enter the peroxisomal matrix and how are they recycled, (2) what is the actual translocation machinery, (3) are protein import and membrane development coupled processes and (4) is the ER involved in sorting of a specific subset of PMPs and/or phospholipids?

The progress of this work would strongly benefit from the development of an in vitro import system. Although some progress has been made in this field using an in vitro membrane protein insertion assay, a reliable matrix protein import system, in particular for yeast, is not yet available. These studies could also shed light on the basic problem why some proteins can be imported in folded and oligomeric forms (e.g. thiolase) [96] where others are not (e.g. alcohol oxidase) [97].

Another fundamental problem that requires a thorough analysis is the suggestion that peroxisomes may form de novo [5,54,98]. This hypothesis is mainly based on the kinetics of the re-appearance of peroxisomes in specific mutant cells which lack peroxisomal ghosts but in which the originally defective protein is reactivated/reintroduced. As, strictly spoken, de novo synthesis conflicts with the dogma that 'membranes arise from membranes', the alternative explanation is that re-assembly of peroxisomes in transformed *pex* mutants that originally lacked ghosts initiates at the ER. It can be envisaged that upon reintroduction of the corresponding protein, e.g. Pex3p, a peroxisomal import site can be formed at the docking site of Pex3p at the ER, that can act as a pre-peroxisome with the potential to develop into a normal organelle. In line with this reasoning, the ER, or specific regions of the ER, should contain specific docking sites for such peroxins.

Finally, one major line of research that urgently requires exploration concerns peroxisome function. The genetic studies of the group of Tabak have provided evidence that the peroxisomal membrane is not permeable for specific metabolites in vivo [69]. Also, the function of peroxisomes in the metabolism of various substrates (e.g. fatty acids, methanol, primary amines and D-amino acids) predicts that specific transport systems must exist. One of the challenges of the near future is to isolate these carriers and test their function in reconstituted membrane vesicles. Attempts using yeast peroxisomes for in vitro studies failed so far, since the purified organelles are invariably leaky after isolation. Since the protein causing this leakage phenomenon has been identified [99], this aspect can also be addressed.

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