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Peroxisomes: minted by the ER

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Peroxisomes are one of numerous organelles in a eukaryotic cell; they are small, single-membrane-bound vesicles involved in cellular metabolism, particularly fatty acid degradation. Transport of metabolites and co-factors in and across the membrane is taken care of by specific transporters. Peroxisome formation and maintenance has been debated for a long time: opinions swinging from autonomous to ER-derived organelles. Only recently it has been established firmly that the site of origin of peroxisomes is the ER. It implies that a new branch of the endomembrane system is open to further characterization.

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Introduction

Organelles in a cell are distinguished according to their evolutionary origin. Whereas most organelles form a single endomembrane system that communicates through vesicular transport and derives from the endoplasmic reticulum (ER), the mitochondria and chloroplasts originate from an endosymbiont prokaryote precursor [1]. For peroxisomes this origin has remained a mystery, until recently [2•,3•]. Since the organelle was discovered [4], peroxisome biogenesis concepts have toggled between descent from the ER and an autonomous nature. This last concept of multiplication by independent growth and division would be in line with an endosymbiont origin of the organelle. The past 3 years have seen enormous progress in the resolution of this conflict, which is the focus of the second part of this review.

Knowledge about peroxisomes has lagged behind that of other organelles. Even after half a century of research (Box 1), and despite the identity of the components being known for decades, the structure and mechanism of action of the protein import machinery is still unsolved [5]. Major reason for this is the absence of an *in vitro* assay in which import of proteins can be studied in isolated organelles, as has been done for all other protein importing organelles. This issue of latency and its implications will be our first focus. The aim of this review is to outline two major conceptual transformations in peroxisome research: the impermeability of the peroxisomal membrane to small molecules and proteins and the origin of peroxisomal membrane proteins (PMPs) from the ER.

Latency

Subcellular compartmentalization enables the eukaryotic cell to maintain specific niches for its chemical transactions. These niches or organelles derive their distinctive properties from unique localization of proteins/enzymes and the impermeability of the surrounding membrane. Protein translocation machineries are required to allow entry of proteins into the inner space of organelles and transporters allow water-soluble metabolites and cofactors to traffic in and out of the organelle. It therefore came as a surprise that isolated peroxisomes showed no latency when they were isolated [4]. It means that substrates and cofactors can reach the enzymes present in the peroxisomal matrix and that the surrounding membrane poses no barrier for their interaction. Similar enzymatic assays carried out with other isolated organelles require detergent or other means to disrupt this membrane permeability barrier.

What is the explanation for this behavior? Did peroxisomes sustain damage during opening of the cell and their subsequent enrichment by biochemical fractionation, or is this an intrinsic property of the peroxisomal membrane? For instance because the peroxisomal membrane contains one or more pore forming proteins? These then would be comparable to the nuclear pore complexes of the nuclear envelope or the pores present in the outer membrane of mitochondria, both allowing free passage to small molecules. Attempts to identify such pore forming proteins have failed thus far. Studies on peroxisomal metabolism in intact cells, particularly in *Saccharomyces cerevisiae*, rather indicate that the peroxisomal membrane is impermeable to small molecules (for an extensive discussion of this topic see [6]). See Box 2 for peroxisomal proteins mentioned in this review.

Evidence from substrates

Almost all peroxisomes have the capacity to degrade fatty acids. Long-chain fatty acids are esterified to CoA esters in the cytosol by chain-length specific acyl-CoA synthetases [7]. This modification makes the molecule more

Box 1 Historical perspective of peroxisome research

In 1954 the Swedish graduate student Rhodin observed small (0.5–1.0 μm) single membrane bounded vesicles in renal tissue in his microscope, which later turned out to be peroxisomes. Their breakthrough in cell biology, however, came with the biochemical fractionation studies aimed at enriching organelles from cells, carried out by the group of De Duve. Specific fractions of an equilibrium density gradient contained a group of H_2O_2-producing oxidases and catalase, suggesting that these enzymes were contained in a new type of organelle. Based on these enzymatic properties the name peroxisome was chosen.

Similar organelles (microbodies) were found in other organisms including plants, Protozoa (*Tetrahymena pyriformis*), and Trypanosomes, but initially they went undercover by different names: glyoxysomes and glycosomes, owing to their differences in enzymatic content. Later research focussing on genes (*PEX* genes) coding for proteins involved in organelle formation and maintenance indicated that these microbodies form a coherent group with a single root in evolutionary history. For instance, they all import their newly synthesized enzymes from the cytosol with the support of conserved Pex proteins.

Failure of peroxisomes to function properly leads to clinical symptoms varying in severity. Lack of a single enzyme function may have a relatively mild effect (for instance premature kidney stone formation in Primary Hyperoxaluria type I). When *PEX* genes are affected, sometimes leading to the total absence of the peroxisomal compartment, the most serious conditions result and life expectancy is severely compromised.

From the cell biological point of view it is highly remarkable that cellular life without peroxisomes is possible. Even more remarkable is the experimental observation that introducing the correct *PEX* gene in such a *pex* mutant, restores the peroxisome population in the transfected cell. Where do these new peroxisomes come from? Recent research has solved this riddle and this review provides the present state of the art to understand how peroxisomes function, are formed and maintained in multiplying cells.

polarized preventing it from passing through membranes. In *S. cerevisiae* the β-oxidation of fatty acids occurs exclusively in peroxisomes. A deficiency in β-oxidation therefore prevents growth of *S. cerevisiae* on a fatty acid like oleate as sole carbon source. Growth of *S. cerevisiae* on oleate is not only abrogated in mutant strains with defective β-oxidation enzymes but also in strains harboring mutations in the genes coding for two peroxisomal ABC half transporter proteins (Pat1p/Pat2p or Pxa1p/Pxa2p) [8,9]. ABC transporters are membrane proteins that enable a variety of small, polar molecules to traverse membranes. Experimental evidence supports the proposal that Pat1p/Pat2p transfers long-chain acyl-CoA esters from the cytosol into the peroxisome (Figure 1). Surprisingly, a member of the acyl-CoA synthetase family, Faa2p, which acts specifically on acyl chains of medium length, is located on the inner leaflet of the peroxisomal membrane [10]. Considering the capacity of medium chain length free fatty acids to enter membranes and flop in or out again to either side, peroxisomal matrix located Faa2p is likely to irreversibly capture the medium-chain fatty acids through their esterification to CoA. This is an ATP requiring reaction, however, and leads to the important implication that ATP must be available within the peroxisome.

Evidence from transporters

Ant1p was initially discovered in a proteomic screen for peroxisomal proteins. Its amino acid sequence shows similarity with a family of mitochondrial transporters, in particular with the mitochondrial ADP/ATP translocator [11••]. The *ant1* mutant in *S. cerevisiae* is deficient in growth when medium-chain fatty acids are the exclusive diet in the medium but it can still grow on long-chain fatty acids (Figure 1). The mutant strain also shows diminished peroxisomally located luciferase activity *in vivo*, an ATP requiring reaction [12]. Finally, purified Ant1p was reconstituted in liposomes and extensively studied *in vitro*, providing proof for it being a bona fide adenine nucleotide transporter [11••]. It is the only peroxisomal transporter for which such in-depth analysis has been carried out and at the moment the best evidence for the notion that peroxisomes are impermeable to small molecules.

Considering the metabolic reactions taking place in peroxisomes and the interaction with the cytoplasm required to sustain these reactions, more transporters must exist. For instance, CoA must be available inside the peroxisome to allow Faa2p to work. Or is sufficient CoA released from long-chain CoA esters that enter the organelle for their degradation? Such interdependence between medium- and long-chain fatty acid degradation generates additional balancing problems. A CoA carrier would be the more simple solution, but there is no hint for its existence thus far. Breakdown of fatty acids generates a surplus of reducing equivalents that need to be transported to the cytosol and mitochondria for energy conservation and utilization. Specific metabolite shuttles have been proposed to take care of this, such as a malate-oxaloacetate shuttle comparable to the one that transfers reducing equivalents across the mitochondrial membrane. Evidence for the existence of such peroxisomal transporters is still lacking however.

Cofactors such as FAD, NAD and NADP also are required intraperoxisomally. FAD as cofactor for acyl-CoA oxidase could enter the peroxisome together with the enzyme [13]. This is a possible consequence of the remarkable property that matrix proteins are synthesized and folded in the cytosol and are imported into the organelle without complete unfolding [14–16]. Whether this could be a more general mechanism to also get NAD and NADP into peroxisomes is doubtful considering the low affinity of these cofactors for their corresponding enzymes.

Although the impermeability of the peroxisomal membrane to small molecules seems to be a reasonable point of departure, there is still a lot to be learned about transporters. Mammalian peroxisomes for instance contain

Box 2 Peroxisomal proteins mentioned in the review

Peroxisomal protein	Function	Human disease
Enzyme (gene)		
*Sc*Faa2p	Medium chain fatty acyl-CoA synthetase	
Thiolase (ACAA1, *ScPOT1/FOX3*)	Breakdown of fatty acids	
Acyl-CoA oxidase (ACOX1)	Breakdown of fatty acids	Acyl-CoA oxidase deficiency
Glyoxylate cycle enzymes	Converts fat into carbohydrates	
Malate synthase (*ScMLS1*)		
Citrate synthase (*ScCIT2*)		
Catalase (CAT, *ScCTA1*)	Breakdown of H_2O_2 into water and oxygen	Acatalasemia
Alanine-glyoxylate aminotransferase (AGT)	Converts oxalate to glycine	Primary Hyperoxaluria type I
Transporters		
*Sc*Pat1p/Pat2p or *Sc*Pxa1p/Pxa2p	Import of long chain acyl-CoA esters	
ALD (ABCD1)	Proposed: import of very long chain fatty acids and/or fatty acyl-CoAs	X-linked adrenoleukodystrophy (XALD)
*Sc*Ant1p	Transport of adenine nucleotides	
Pex proteins	Peroxisome biogenesis and maintenance	Zellweger syndrome Neonatal adrenoleukodystrophy (Infantile) Refsum disease Rhizomelic chondrodysplasia punctata type 1 (RCDP1)

For a complete list of peroxisomal proteins and their relation to disease, see http://www.peroxisomedb.org/; *Sc*: *Saccharomyces cerevisae* gene.

several ABC transporters, one of which is linked to disease: adrenoleukodystrophy (ALDP). Unfortunately, despite intensive research in this field solid evidence identifying the molecules they transport is still lacking [6].

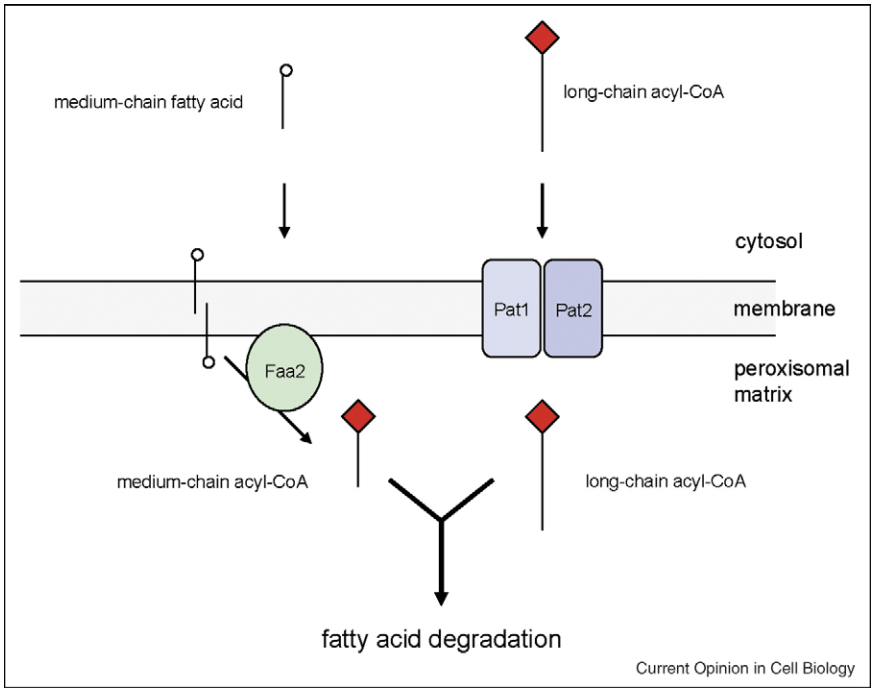
Formation of peroxisomes: historical context

Vicissitudes in views

For the past two decades the prevailing concept has been that peroxisomes multiply by growth and division and should therefore be considered autonomous organelles [17]. The evidence supporting this proposal was mainly based on the observation that peroxisomal enzymes are synthesized on free polyribosomes and after completion of synthesis are imported directly into peroxisomes. These experiments were carried out with mammalian cells and were given priority over discrepant observations in plants. When castor beans are soaked in water to start germination they develop glyoxysomes, which mobilize fat resources and turn them into carbohydrates with enzymes of the glyoxylate cycle. Biochemical fractionation showed that two of these enzymes, malate synthase and citrate synthase, were recovered initially in the ER fraction [18]. A few days later, when glyoxysomes had been formed, the enzymes were found in the glyoxysome fractions [18]. Moreover, glyoxysomes contain glycosylated proteins, a modification they could have received only while passing through the ER [19]. These results fully warranted the conclusion that glyoxysomes are derived from the ER and underpinned earlier morphological observations by electron microscopy that peroxisomes were often seen in close association with the ER and occasionally showed membrane continuities between the two organelles [20]. These discrepancies were 'resolved' when Lazarow and Fujuki reviewed the field and pushed the concept that microbodies are autonomous organelles multiplying by growth and division [17]. The model received strong support with the discovery of peroxisomal targeting signals (PTS1 and PTS2) by which newly synthesized matrix proteins are directed into the organellar matrix [21].

The tools for studies up until the nineties consisted mostly of peroxisomal enzymes. This changed when emphasis was given to various yeasts as model organisms, which allowed application of genetic screens to discover new peroxisomal proteins. At the same time, the urge increased to understand the molecular basis of diseases linked to peroxisome deficiencies. Of particular academic interest was the observation that in the most severe diseases the complete peroxisome compartment was missing. These efforts brought the group of Pex proteins to light: proteins with a function in formation and maintenance of peroxisomes. Some of these Pex proteins, mostly membrane located, constitute a protein import complex (importomer) through which PTS1 and PTS2 containing proteins enter the organelle [22], an additional tribute to the apparent autonomy of peroxisomes [23]. Although the properties of some proteins supported the current model, others showed behavior that was difficult to explain within its context. For instance, in yeast strains with deficient alleles of *PEX3* or *PEX19* all peroxisomes

Figure 1



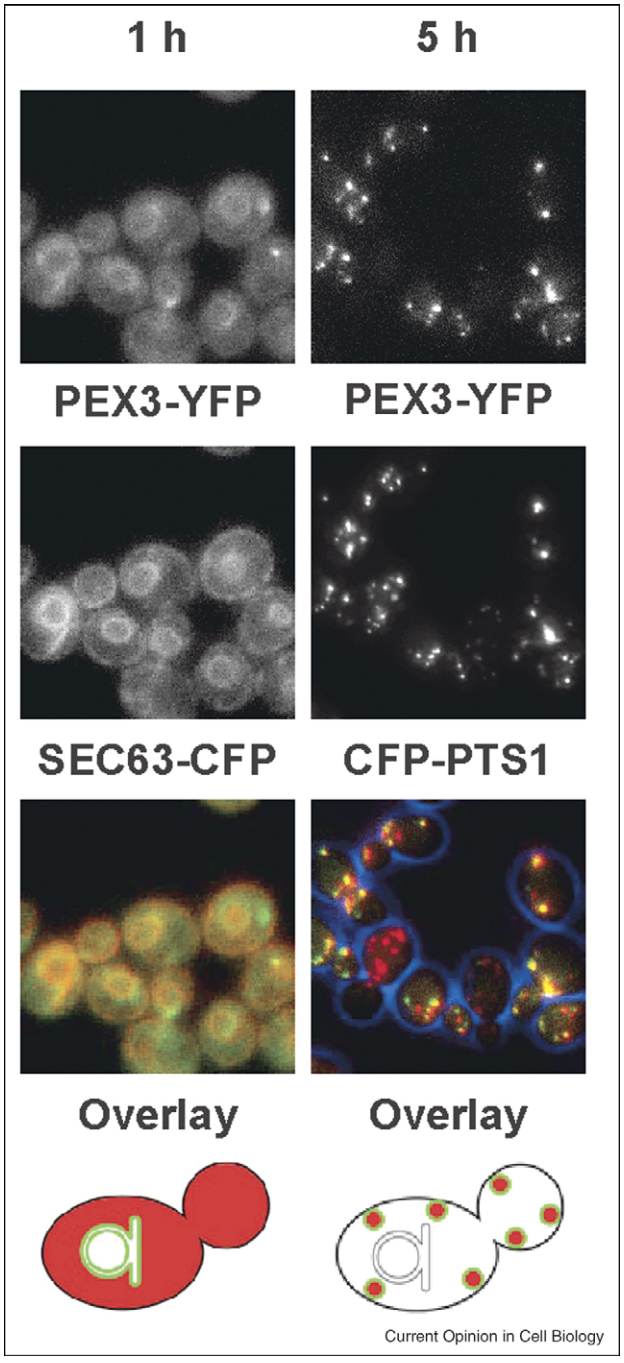
Fatty acid degradation in peroxisomes. Long-chain fatty acids enter peroxisomes as CoA esters via the ABC transporter complex Pat1p/Pat2p (also called Pxa1p/Pxa2p). Medium-chain fatty acids are CoA esterified inside the organelle by the fatty acid CoA synthetase Faa2p. Once inside they are degraded by the fatty acid degrading enzymes.

were gone, a phenotype resembling some of the severe diseases [24,25]. Surprisingly, when the wild-type genes were reintroduced in these mutants the peroxisomes reappeared [24]. So, where did these new peroxisomes come from and how to solve this paradox? According to the dictum: 'omnis membrana e membrana' [26] a membrane system must be present that is capable of regenerating the peroxisome population. No wonder that the ER reappeared at the horizon. Attempts to implicate the ER in peroxisome formation resulted in both negative and positive answers. Experiments aiming to demonstrate involvement of proteins serving in the secretory pathway such as Sar1p, COPII coat proteins, or the Sec61 protein import complex remained negative [27,28]. Positive indications however were open to criticism owing to possible contamination of biochemical fractions or mislocalization of overexpressed peroxisomal proteins.

The ER gets a foothold

The balance started to tip in the direction of the ER with the demonstration that Pex2p and Pex16p in pulse-labeled wild-type *Yarrowia lipolytica* cells first encountered the ER before arriving in peroxisomes, and that all Pex2 and Pex16 proteins underwent posttranslational glycosylation [29]. Later, immuno-EM studies in mouse dendritic cells showed the presence of Pex13p in specialized regions of the ER and in lamellar structures (peroxisomal precompartments) embracing mature peroxisomes [30]. Only the mature ovoid-shaped peroxisomes contained enzymes such as thiolase and catalase [30]. Three-dimensional reconstructions using electron tomography indicated that membrane continuities exist between the three compartments (specialized ER, lamellae and peroxisomes) [31]. Combined with the protein distribution over these compartments – Pex proteins in precompartments, enzymes in mature organelles – this suggested a developmental pathway leading from ER to peroxisomes [31]. To strengthen this interpretation we set out to transfigure the morphological EM stills into dynamic pictures taken from living cells. An experimental set up was designed in *S. cerevisiae* to visualize the reappearance of peroxisomes when a *pex3* or *pex19* mutant is rescued by the introduction of the wild-type version of the corresponding gene [32••]. A strain was constructed in which the *PEX3* gene was put under the control of the conditional *GAL1* promoter and the 5′ end was extended with the DNA coding for YFP. One strain contained in addition Sec63p-CFP to indicate the presence of the ER, another contained CFP-PTS1 as a marker for peroxisomes and reporter for the capacity of peroxisomes to import PTS1 containing proteins. When grown on glucose or raffinose these strains have the *pex3* mutant phenotype lacking peroxisomes. When the strains are transferred to galactose and the formation of Pex3p is

Figure 2



Peroxisome formation from the ER. Development of peroxisomes is followed by fluorescence microscopy in living *Saccharomyces cerevisiae* cells. Peroxisome formation is induced in a *Δpex3* mutant by expressing Pex3p-YFP from the conditional *GAL1* promoter. Pex3p (green) first appears in the ER (panel A) and five hours later is present in peroxisomes (panel B). Sec63p-CFP (red) is used to mark the ER, whereas CFP-PTS1 (red) marks the presence of protein import-competent peroxisomes. For details see ref. [32].

induced the cells develop peroxisomes capable of importing CFP-PTS1 within five hours (Figure 2). Importantly, Pex3p-YFP first colocalizes with the Sec63p-CFP marked ER before appearing in peroxisomes [32••]. This route is not only taken in mutant cells but in wild-type cells as well [32••]. Despite the presence of functional peroxisomes the ER is the organelle of choice taken by Pex3p. Similar results have been reported by others in various types of cells [33,34,35,36••,37,38•] and the concept that the ER contributes to peroxisome formation has found its way into the textbooks [39].

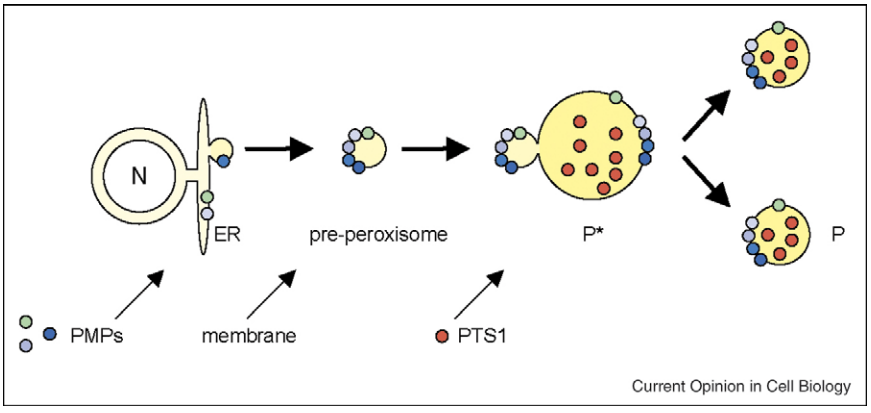
Reinterpretation of existing views

Does this new concept impinge on prevailing views of peroxisome biogenesis? Indeed it does. We recently showed that PMPs first target to the ER independent of Pex3 and Pex19 before arriving in peroxisomes. These results challenge the current model that PMPs are imported directly into the peroxisomal membrane with the help of the membrane located Pex3p and the cytosolic 'chaperone' Pex19p. This current model is mainly based on the phenotypes displayed by the *pex3* and *pex19* deletion mutants: absence of peroxisomes and distinctly lower levels of PMPs in these cells. The crucial test would be to show *in vitro* that isolated peroxisomes can indeed take up PMPs in the membrane in a Pex3p and Pex19p dependent manner. Unfortunately, as we describe above, peroxisomes are easily damaged during isolation and such reconstitution experiments are very difficult to perform and have not been reported thus far. The household protein import complexes of the ER taking care of insertion of PMPs into the membrane has opened a new role of Pex3p and Pex19p.

Implications

The new model is a stimulus to explore its implications in further detail (Figure 3). After entering the ER the PMPs must be grouped together and segregated from the ER resident proteins before leaving the ER. How is this departure organized? Are small vesicles budding from the ER or are larger parts of specialized ER severed from the donor compartment. Being part of the endomembrane system one could envisage that proteins taking care of intermembrane communication such as SNARES, COPs, NSF, and rabs could be involved but older results argue against this. Is there still room for new principles to emerge or are we dealing with already known players with additional new functions? Candidates here are Pex3p and Pex19p. While it is unlikely that they have a role in import of PMPs into the peroxisomal or ER membrane, they certainly are required for exit of PMPs from the ER. It has been shown that Pex19p can bind specific motifs (called mPTS) in PMPs [40]. Pex19p, through its interactions with PMPs and with Pex3p, may help assemble PMPs into packageable groups for transport out of the ER, or Pex3p and Pex19p may assist in a budding/fission pro-

Fig. 3



Dynamic view on peroxisome formation and maintenance. Peroxisomal membrane proteins (PMPs) enter the ER, are sequestered in specialized regions and bud from the ER to form peroxisomal precompartments. These become capable of importing PTS1/PTS2 containing proteins and develop into mature peroxisomes or fuse with existing peroxisomes. Enlarged peroxisomes can divide by fission.

cess required to sever the peroxisomal pre-compartment from the ER.

With the contribution of the ER comprising not only the lipid bilayer but also the PMPs it is puzzling that the final peroxisomal membrane is such a fragile entity after cell breakage and isolation. Vesicles derived from the ER reseal, are relatively impermeable to small molecules, and are suitable for *in vitro* reconstitution experiments such as protein import and folding studies. Apparently, segregation of lipids and proteins of the peroxisomal pre-compartment from the bulk of the ER changes the peroxisomal membrane such that reconstitution experiments are no longer feasible. By studying membrane properties of peroxisomal precompartments at various stages of development, accumulated in suitable pex mutants, it may be possible to find out the basis for this lack of latency *in vitro*. When we find the cause of this fragility, improvements of it will add a new dimension to peroxisome investigations.

Given the view that peroxisomes are part of the endomembrane system, why do peroxisomes possess their own importomer complex to fill up the organellar matrix with enzymes synthesized in the cytosol? It may be of interest to make a comparison here between peroxisomes and lysosomes, because a separate protein import route for soluble matrix proteins is not unique within the endomembrane system. In *S. cerevisiae* two enzymes, alpha-mannosidase and aminopeptidase 1, reach the vacuole via a different route than the majority of lysosomal proteins [41]. They are picked up in the cytoplasm, enwrapped in vesicles and delivered to the vacuole by the so-called 'cytoplasm to vacuole targeting' (Cvt) pathway, while the other vacuolar proteins travel via the ER and Golgi [42].

Conclusions

The recent advances have brought an exponential increase in our understanding of peroxisomes and of many published and unpublished data that did not fit the reigning models. The concrete role for the ER in peroxisome biogenesis as well as maintenance, and the realization that peroxisomes, like all other organelles of the endomembrane system, are impermeable to small molecules, has merged many apparently conflicting observations. Now that their place in the cell has been more firmly established, general principles derived from studies on peroxisomes will turn out to apply to other organelles as well, placing this 'orphan organelle' for the first time on the general playing field of cell biology.

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