Studies on peroxisome biogenesis

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CHAPTER 3

THE ER-PEROXISOME CONNECTION: THE PEROXISOMAL MEMBRANE PROTEINS PEX13P AND PMP70 ARE FOUND IN SPECIALIZED ER DOMAINS, LAMELLAE, AND PEROXISOMES IN MOUSE DENDRITIC CELLS

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Chapter 3

ABSTRACT

The authoritative review by Lazarow and Fujiki (1985) on peroxisome biogenesis set the stage for the notion that peroxisomes are autonomous organelles that multiply by growth and division. More recently, various literature reports suggest a contribution of the endoplasmic reticulum to peroxisome formation. Our studies on the location of peroxisomal membrane and matrix proteins in mouse dendritic cells by immuno electron microscopy provide direct evidence for such an ER-peroxisome connection.

The peroxisomal membrane protein Pex13p is found in specialized regions of the ER, in peroxisome reticula and occasionally in globular peroxisomes. The peroxisome ABC transporter protein PMP70 is found in peroxisome reticula and in peroxisomes, while the matrix enzymes thiolase and catalase are only present in the latter structures. These results indicate that in dendritic cells peroxisomal membranes are formed from the ER, that additional membrane proteins are acquired subsequently and that matrix proteins are taken up as a last step in this maturation pathway.

INTRODUCTION

Eukaryotic cells differ from prokaryotic cells by the presence of membrane bounded organelles creating micro environments suitable for carrying out different functions. This compartmentalisation enables the segregation of complex cellular processes and allows potentially harmful activities to be confined within membranous compartments so that no damage can be done to other parts of the cell. This is certainly true for lysosomes and perhaps also for peroxisomes. Peroxisomes are organelles which are involved in a variety of processes (Van den Bosch et al., 1992). Their importance is clearly demonstrated by the existence of peroxisomal disorders, which have the most severe presentation when peroxisomal biogenesis is disturbed (Wanders et al., 1999). To try and elucidate the mechanisms underlying peroxisome assembly, comparison of peroxisomes with other organelles might be informative. Basically membrane bounded organelles can be divided into two main groups, based on their appearance and the way they communicate with the rest of the cell.

The first group consists of single-membrane bounded organelles which can communicate with each other via vesicular membrane structures. This group consists of organelles involved in secretion or endocytosis like the
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endoplasmic reticulum (ER), the Golgi apparatus, lysosomes and endosomes. The origin of these organelles lies in the ER. The ER is the site of phospholipid and membrane synthesis and the other organelles receive their membranes via vesicular transport from this donor organelle.

The second group of organelles is characterised by the presence of their own DNA and they are bounded by a double membrane. Members of this group are mitochondria and plastids, like chloroplasts, they multiply as autonomous organelles by growth and division. The origin of these organelles lies in an early endosymbiotic event between a guest (organelle) and a host cell (rest of the cell).

Where do peroxisomes fit in? Peroxisomes have features of both groups which complicates the understanding of their origin and thus also of their biogenesis. Based on their appearance and properties (single membrane and no DNA) peroxisomes resemble organelles of the first group, whereas the fact that they can import their own proteins is similar to that of organelles of the second group. Basically, the most intriguing question in studies on peroxisome biogenesis has always been the possible ER involvement in this process. Although early electron microscopic studies revealed that peroxisomes were often in close proximity to the ER (Novikoff and Shin, 1964), an ER involvement could not be proven. An autonomous mechanism of peroxisome formation was proposed (Lazarow and Fujiki, 1985), based on the idea that each cell inherits at least one peroxisome which can grow and by fusion can give rise to new peroxisomes. This hypothesis was supported by the findings that both peroxisomal matrix proteins and membrane proteins are synthesised on free poly ribosomes in the cytosol and are post-translationally imported into peroxisomes (Fujiki et al., 1984; Imanaka et al., 1996). However the origin of peroxisomal membranes remains hard to explain with this hypothesis.

In the mean time more and more clues for a direct ER involvement in peroxisome biogenesis have been reported (reviewed in Stroobants, 2001). Several articles appeared in which a peroxisome-forming sheet is described (Fahimi et al., 1993; Gorgas, 1987; Ohno and Fujii, 1990; Zaar et al., 1987), suggesting an ER association with peroxisomes. For example, close lateral membrane associations of peroxisomes with ER are a common feature in bovine kidney cortex epithelial cells (Zaar et al., 1987). The ER enzyme glucose-6-phosphatase was found in these peroxisome-associated membrane structures and was indicative for their derivation.
from the ER. The latest indications for involvement of the ER in peroxisome formation in yeast are described by Titorenko and Rachubinski (1998). They showed N-glycosylation (an ER specific event) of 2 peroxins in *Yarrowia lipolytica*. Mullen et al. (1999) provided a clue for ER involvement in plants by localisation and membrane-insertion studies of peroxisomal ascorbate peroxidase (APX) in tobacco cells. By immunofluorescence they were able to show that the integral membrane protein (APX) was present both in peroxisomes (containing catalase) and in reticular/circular membrane structures. These reticular structures were interpreted as a specialised region of the ER since they could be marked with the stain DIOC<sub>6</sub>. Their results suggest that plant peroxisomal APX is inserted into a specialised region of the ER membrane, which is a possible pre-peroxisomal compartment, before it can be sorted to the peroxisome.

Here we describe high resolution immuno EM observations which provide additional evidence for the involvement of the ER in peroxisome biogenesis. In mouse dendritic cells peroxisomes were surrounded by a complex of membranes, including characteristic lamellar structures. These lamellae showed membrane continuities with the ER, and contained the peroxisomal membrane proteins Pex13p and PMP70. The lamellae were also continuous with a anastomosing reticulum from which peroxisomes seemed to be formed at distended portions. These observations suggest a biogenetic link between the ER and peroxisomes.

**MATERIALS AND METHODS**

D1 cells were derived from immature splenic DCs from C57BL/6 mice, and were cultured as described (Winzler et al., 1997). In one experiment, mouse bone marrow-derived DCs were used for comparison. For immuno-cytochemistry, we used polyclonal antibodies against mouse I-chain luminal part (S22, kind gift of Dr. N. Koch, University of Bonn, Germany), mouse langerine (Valladeau et al, 1999), human PDI (kindly provided by Dr Braakman, University of Utrecht, the Netherlands), bovine catalase (Tager et al., 1985), rat thiolas (Heikoop et al., 1990), human Pex13p, and rat PMP70 (described below). Mouse monoclonals were against protein disulfide isomerase PDI tail (Vaux et al.,1990), and MHC class II (la-b) kindly provided by Dr Rudensky, (Dept. Of Immunology and Howard Hughes Medical Inst., Univ. Washington, Seattle, USA), and a rat monoclonal against the mouse I-chain cytoplasmic tail (ln-1; Dr. N. Koch, see above).
Anti-Pex13p was raised against the SH3 domain of human Pex13p (aa 275-454) fused at the N-terminus to a DHFR tag. The DNA encoding the SH3 domain was amplified from human cDNA introducing a 5' Bgl II site and a 3' Hind III site and was cloned into PQE13 (Quiagen). The fusion protein was expressed and purified according to the QIA express system before it was injected in a rabbit for antiserum production. For competition experiments with EM the DHFR-Pex13pSH3 fusion protein was further purified twice on a Centricron YM-10 column from Amicon according to the manufacturers instructions, in order to loose SDS which might disturb the immuno EM. Anti-PMP70 was made against its ATP-binding domain. The 3' part of rat PMP70 cDNA (from the Bcl I site to the stop-codon) was cloned behind a 6 x His tag. The resulting protein was expressed in SG13009 cells, was purified on a Ni-NTA column (Quiaex), and was used to immunise rabbits.

The specificity of anti-Pex13p for mouse Pex13p was tested as follows. Lysates were made of cultivated mouse D1 cells (25 cm²), of wild-type human fibroblasts and of fibroblasts of a Pex13p deficient patient (kindly provided by R. Wanders, University of Amsterdam, the Netherlands, Shimozawa et al., 1999) by washing the cells with PBS and lysing them by scraping in 0.5 ml (PBS 0°C) containing 1% triton X-100 and 1 mM PMSF. After centrifugation at 13,000 x g in an eppendorf centrifuge, the pellets were resuspended in Leamli sample buffer and equal amounts of protein (as determined by a Bradford assay, Bradford, 1976), were analysed by SDS-PAGE and Western blotting (Sambrook et al., 1989) with the anti-Pex13p as primary antibody and goat-anti-rabbit alkaline phosphatase as the secondary antibody. On the Western blot the mouse lysate showed a clear band at the same height (46 kD) as the band of human Pex13p, which was absent in the lysate of patient cells. This demonstrates that the Pex13p anti-bodies raised against human Pex13p also specifically recognise mouse Pex13p. The specificity of the other antibodies for mouse proteins (PMP70, catalase, and thiolase) was also determined by SDS-PAGE and western blotting of mouse dendritic cell lysates.

For immunocytochemistry, cells were washed by centrifugation in FCS-free medium and fixed in 2% paraformaldehyde and 0.2% glutar-aldehyde. The cells were then processed for immuno-electronmicroscopy (IEM) as described (Liou et al,1997). Briefly, the fixed cells were washed in PBS with 50 mM glycine at room temperature to quench free aldehydes, embedded in 10% gelatin and cryosectioned. For fluorescence microscopy,

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approximately 100 nm thin sections were indirectly immunolabeled with goat anti-rabbit Cy3 (Jackson Immuno Research Laboratories, Inc, Westgrove PA, USA), and viewed in a Reichert Polyvar fluorescence microscope. For IEM, ultrathin sections were indirectly single- or double-immunolabeled with 5 nm gold, or 10 and 15 nm gold particles, respectively (Geuze et al., 1981, Liou et al., 1996).

Quantitation of immunogold for Pex13p and PMP70 on the peroxisomal complex, i.e. peroxisomes, peroxisomal reticula and lamellae, was done in 20 random electronmicrographs with a final magnification of 25,000. In total 304 gold particles were counted for Pex13p and 232 for PMP70. Relative membrane surface areas of the peroxisomal complex subdomains were determined by putting a transparent overlay with a squared lattice of 1 cm spaced lines on top of the same micrographs as used for gold counting. Labeling densities of Pex13p and PMP70 in the peroxisomal complex subdomains were calculated by determining the ratio's of the respective percentages of gold particles over the percentages of intersections between the lines and the various membranes involved.

RESULTS

Immature dendritic cells (DCs) are specialized in capturing antigens (pathogens) by endocytosis and phagocytosis from their environment in peripheral tissues like the skin. To proteolytically process the antigens and present resulting peptides to T-lymphocytes, the cells contain a well developed vacuolar system. Moreover they have numerous peroxisomes. Immature cells of the murine DC cell line D1 show the peculiar feature of peroxisomal reticula (Yamamoto and Fahimi, 1989; Schrader et al, 1999) and other peroxisome-associated membranes, providing an excellent opportunity to study the distribution of peroxisomal matrix and membrane proteins in these cells with high resolution IEM on cryosections. We studied the peroxisomal matrix proteins catalase and thiolase, which contain different targeting signals to enter the peroxisome (PTS1 and PTS2 respectively, Hettema et al., 1999), and two integral peroxisomal membrane proteins, Pex13p, which is a component of the protein import complex (Erdmann and Blobel, 1996; Elgersma et al., 1996; Gould et al., 1996), and the ABC transporter PMP70 (Gartner et al., 1998). By indirect immunofluorescence (Figure 1), catalase (A) and thiolase (B) labeling in semi-thick cryosections appeared as numerous tiny spots as
Figure 1. Peroxisomal matrix and membrane proteins show different distribution patterns. Immunofluorescence microscopy of 100 nm cryo-sections. Catalase (A) and thiolase (B) staining patterns are punctate, as expected for peroxisomes. No fluorescence can be seen outside the peroxisomes. On the other hand, Pex13p (C) and PMP70 (D) show in addition to puncta, fluorescent strands and dots, and some labeling in the cytoplasm. Bar, 24 micron.
expected for matrix proteins of peroxisomes at this level of resolution. However, when we stained for the peroxisome membrane proteins Pex13p (C) and PMP70 (D), a pattern of much larger spots and strands was seen in addition to the puncta. These observations prompted us to investigate these distributions in greater detail by using IEM. We have used our recently improved immunogold labeling technique of thin cryosections, which allows precise delineation of membranes (Liou et al., 1997).

**Peroxisomes are closely associated with membranous lamellae enriched in Pex13p and PMP70.**

The peroxisomes occurred in clusters throughout the cells. Besides typical globular peroxisomes, the cells showed many tubular and reticular peroxisomal structures, which have previously been described as peroxisomal reticulum (Yamamoto and Fahimi, 1987; Schrader et al., 2000). Both the peroxisomes and reticula were positive for catalase and thiolase (Figures 2A and B, Figure 3B). The clusters of peroxisomes and reticula were closely associated with characteristic flattened tubules, of which the limiting membranes were tightly apposed forming rigid lamellae with a characteristic internal striping (Figures 3 and 4). The lamellae were often seen in continuity with the peroxisomal reticula, which in turn seemed to give rise to peroxisomes by budding (Figures 3B and C). The lamellae resembled so-called Birbeck granules in Langerhans' cells. However Birbeck granules were thicker, had a different striping pattern, and were associated with the plasma membrane and endosomes. In addition, Birbecks strongly labeled with an antibody against the Birbeck marker Langerine, while the peroxisome-associated lamellae were negative (not shown). The lamellae, on the other hand, contained abundant label for Pex13p Figures 3A, and C) and PMP70 (Figure 3B), which were absent from Birbeck granules. Both membrane proteins were also present on the limiting membrane of peroxisomes, and peroxisomal reticulum (Figures 3B and C), albeit with different relative intensities. A quantitative evaluation of the gold labeling patterns showed that of all Pex13p labeling in the peroxisomal system, i.e. peroxisomes, reticulum and lamellae, 64 % was present in the lamellae, 27 % in the reticulum, and only 6 % in the peroxisomes. For PMP70 these figures were 12 %, 62 %, and 26 %, respectively. Thus, by far the majority of Pex13p was located outside the typical globular peroxisomes, in particular in the lamellae. For PMP70, this distribution was reverse, i.e
Figure 2. The peroxisomal lamellae are ER subdomains. A) A peroxisome immunolabeled for catalase is surrounded by a lamella that shows membrane continuity with the ER. At the transition, the ER lumen is narrowed (arrowhead). B) Like in A, but immunolabeled for thiolase. Bar for A and B, 120 nm. C) Like in A and B, but immunolabeled for the ER protein PDI.
Figure 3. Pex13p and PMP70 have differential distributions in the peroxisomal system. A. Pex13p labeling is predominantly associated with the lamellae, and is almost absent from the peroxisomes (P). Note that the striped lamellae at the right is continuous with the ER at the arrowhead. Bar, 100 nm. B. PMP70 labeling is present on both peroxisomes and lamellae. The upper and lower peroxisomes seem to be connected with the lamellae via a reticulum (arrowheads). Bar, 125 nm. C. Peroxisomal system showing thiolase in its subcompartments, except in the lamella. Pex13p is present in the lamella and in the peroxisomal reticulum (R). Bar, 110 nm. D. A Pex13p-positive and striped lamella, shows a transition with the ER at a nexus (arrowhead). Only the peroxisomes are labeled for thiolase. Bar, 80 nm.
Figure 4. The abundant ER protein li is present in peroxisomal lamellae. This cryosection is double-immunogold labeled with antibodies against the cytoplasmic N-terminal domain of li (IN) and against the C-terminus of li (IC) with gold sizes in nm as indicated on the figure. As expected, both epitopes of li are present in the ER, but only IN can be seen in endosomes (E). Like in the ER, both epitopes can be found in the lamellae associated with peroxisomes (P) (arrowheads). Note the fine longitudinal striping in the lamellae. Bar, 125 nm.

Table I Differential distributions of Pex13p and PMP70 in the peroxisomal complex

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shifted towards the reticulum and peroxisomes (Table I). When we related the gold labeling to the membrane surface areas involved to reveal labeling densities (Table I), the highest density of Pex13p occurred in the lamellae, and that of PMP70 in peroxisomes. For both proteins, the peroxisomal reticulum took an intermediate position. The scarce presence of gold particles for Pex13p and PMP70 outside the peroxisomal clusters did not allow to allocate them to the ER or cytosol. The narrow lamellae were devoid of labeling for catalase and thiolase, which were only
detectable in the reticula and peroxisomes. These differential labeling patterns for content and membrane proteins at the EM level explain the differences seen by immunofluorescence microscopy as shown in Figure 1. Immunogold particles for Pex13p and PMP70 present over the lamellae, reticulum and the peroxisomes were counted and expressed as percentages of the total gold in the peroxisomal complex. Gold densities were derived from the ratios of gold percentages over the relative membrane surface areas of the compartments (see materials and methods).

**Peroxisome-associated lamellae are a subdomain of the ER.**

Figures 2 and 3 show connections between the rough ER and the lamellae. The sudden transition between an ER cisterna and a lamella was morphologically characterized by the absence of ribosomes from the lamellae, and the narrowing of the lumen in the lamellae in which 2 longitudinal electron-dense stripes were present (Figures 2 and 3). The ER could be identified both by the presence of adhering ribosomes and by the presence of the ER protein PDI (Figure 2C). The relation between the ER and the lamellae was further indicated by the low, but consistent presence of invariant chain (Ii) in the lamellae (Figure 4). Ii is a transport chaperone of MHC class II, and is abundantly present in the ER of DCs. The absence of ribosomes from the lamellae and their continuity with the ER is reminiscent of the transitional elements at the ER, which represent the COP II-mediated exit sites for newly synthesized secretory, lysosomal and membrane proteins for transport from the ER to the Golgi complex. However, the lamellae were negative for COP II, as well as for COP I (not illustrated). Both COPs were present in the Golgi areas in the same sections. Accordingly, the lamellae lacked any morphologically detectable coat on their cytoplasmic surface (see Figures 3 and 4).

**DISCUSSION**

An unresolved question lingering in the field of cell biology relates to the origin of peroxisomes in eukaryotic cells. This question can be posed with regard to the present: how does a daughter cell derive its peroxisomes from the mother cell, or with regard to the past: how did the progenitor of modern eukaryotic cells obtain its peroxisomes. We hope that our surprising findings in a differentiated cell type, the dendritic cell of the
immune system, that was never used in studies on peroxisome biogenesis before, can shed some light on both questions.

The specific mouse dendritic cell line, D1, cultivated in vitro, shows remarkable subcellular, tubular structures that were often in close apposition with mature, ovoid peroxisomes. This observation prompted us to investigate the nature of these tubules in more detail using EM immuno-cytochemistry in combination with antibodies raised against authentic peroxisomal proteins. A highly unexpected labeling pattern was seen using the antibody directed against human Pex13p. Pex13p in yeast and man is an SH3 containing integral peroxisomal membrane protein with a role in import of matrix proteins into peroxisomes. With this antibody gold particles were seen on specialized extensions of the ER, on tubules with similar morphology as the specialized ER extensions that often surrounded the ovoid, mature peroxisomes in a decreasing gradient of gold particle density. With an antibody raised against the peroxisomal membrane protein PMP70, a member of the ABC transporter protein family, gold particles were found on the tubules surrounding peroxisomes and on peroxisomes themselves. Finally, catalase and thiolase were found in the lumens of the mature peroxisomes.

Although our morphological observations on the peroxisomal system present a static picture, it is tempting to interpret them in a more dynamic perspective (Figure 5). We propose that certain membrane proteins, among which Pex13p, insert into the ER and induce the formation of specialized ER domains characterized by typical morphological features such as internal striping, rigid, rod-like appearance and lack of ribosomes. At a certain stage this domain is separated from the ER. This does not involve the components required for budding of vesicles in the secretory pathway. Peroxisome formation is not inhibited by Brefeldin A or a dominant-negative version of Sar1p in mammalian cells (Voorn-Brouwer et al., unpublished results; South et al., 2000) and we did not observe association of COP I or II proteins to the specialized ER. In the subsequent reticulaa other membrane proteins are taken up such as PMP70. Finally, the matrix proteins catalase and thiolase appear in the distensions of the reticula and in mature peroxisomes. In this model the peroxisomal membrane is largely ER-derived and at a certain maturation state a functional import machinery for matrix proteins is assembled classifying peroxisomes as semi-autonomous organelles in contrast to completely autonomous organelles such as mitochondria and chloroplasts.
The implication of this scenario is that certain peroxisomal proteins with a direct role in the biogenesis of the organel start their functional life in the ER and that the ER is a major donor of the lipid membrane constituents of peroxisomes. In this respect peroxisomes are thus part of the differentiated and dynamic vacuolar system also comprising the ER, Golgi, and vacuoles/lysosomes/endosomes. Within this group of organelles, peroxisomes show the greatest degree of autonomy. This is exemplified by the post-translational import of finished, often (partially) folded peroxisomal proteins and a protein import machinery of its own for matrix proteins. The possibility of fission of mature peroxisomes into new ones cannot be excluded (Hoepfner et al.). Nevertheless, it is likely that the ER is the ultimate origin of new peroxisomes. This is nicely illustrated by the fact that S. cerevisiae pex mutants, such as pex3 or pex19, in which residual peroxisomal structures can no longer be observed, form peroxisomes even after generations of growth as mutant after transformation with the corresponding wild type PEX gene.

The observed connection between ER and peroxisomal membranes can also be used as new information to consider the evolutionary origin of peroxisomes. Are they remnants of endosymbionts like mitochondria and chloroplasts that have lost all their tell-tale features such as DNA of their former life? Or are they superspecializations from already pre-existing membrane structures?
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This last possibility becomes more plausible in view of the observed ER connection. Based on phylogenetic studies using as markers the highly conserved heat shock family of molecular chaperone proteins (HSPs) it was argued that nucleus and ER are of ancient origin and that ER and cytosolic HSP70s are related to each other and originated from a gene duplication event early in the history of the eukaryotic cell. The chaperones present in other organelles such as mitochondria and chloroplasts are not close homologs of their nuclear/ER-cytosolic counterparts supporting a different endosymbiotic origin of these organelles. Interestingly, peroxisomes seem to be fully dependent on the cytosolic chaperone system: (1) no evidence for the presence of chaperone proteins in the matrix of peroxisomes based on in silico or in vitro analysis has yet been reported; (2) folding of peroxisomal matrix proteins takes place in the cytosol and (partially) folded proteins can be translocated across the peroxisomal membrane. Assuming that a merger between an archaebacterium and a eubacterium was the basis for modeling the first nucleus-containing eukaryote, these considerations suggest that peroxisomes co-evolved with the ER and are a superspecialization of this organel. It might thus rank as one of the oldest among the organel family that gave rise to the typical eukaryotic cell.

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REFERENCES

Chapter 3


ER-peroxisome connection


