Studies on peroxisome biogenesis
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CHAPTER 6

GENERAL DISCUSSION
One of the most intriguing questions in studies on peroxisome biogenesis has always been: is the ER involved in this process? Although many indications for ER involvement have been reported, this concept is not generally accepted yet. Our studies on the biogenesis of peroxisomes and the possible link with the ER described in this thesis were based on two observations. The first was the observation made in earlier studies by Elgersma et al. (1997) that in S. cerevisiae overexpression of Pex15p led to extension of what was thought to be the ER membrane and to targeting of Pex15p to these membranes. The second observation was made in collaboration with the group of Prof. Dr. Geuze (University of Utrecht): in mouse dendritic cells a peroxisomal marker could be traced from the ER to mature peroxisomes.

The first observation implied that Pex15p might be sorted to peroxisomes via the ER and was studied in more detail. By co-expressing Pex15p and an ER marker we were able to show with immuno EM cytochemistry that the Pex15p-containing membranes were extensions of the ER membrane. However, in an attempt to verify whether this meant that Pex15p travels to peroxisomes via the ER, we encountered some problems. We tried to use the observation made by Cox et al. (1997) that karmellae formation (extension of ER membranes) and the unfolded protein response (UPR) were controlled via the same protein Ire1p and that deletion of IRE1 resulted in cell death when cells were challenged via the UPR. Our working hypothesis was that overexpression of Pex15p in an ire1Δ mutant would lead to cell death. Although this turned out to be true under certain conditions, additional controls made interpretation of this result rather difficult. Firstly, because we expected that only overproduction of ER-resident or ER-passing membrane proteins and not overproduction of proteins with a different origin would result in ire1 cell death. However, additional controls with overexpression of membrane proteins which are not sorted via the ER gave the same result. Karmellae could even be formed in the absence of IRE1. Secondly, we noticed that when yeast was grown on oleate instead of on glucose, overexpression of membrane proteins in the absence of IRE1 was not lethal. These results indicated that the picture presented by Cox et al. (1997) was far from complete. The fact that Pex15p overexpression led to karmellae formation could very well be an artefact. This protein is quite hydrophobic and as such has a tendency to stay in a membrane-rich environment. Another finding also indicated that extension of ER membranes is independent of
peroxisome formation: even in the absence of Pex3p (a peroxin essential for an early step in peroxisome biogenesis) karmellae could be formed. Besides, since Pex15p (at its physiological level) is usually degraded in the absence of Pex3p, the observation that overexpressed Pex15p was not degraded in the absence of Pex3p was not expected and indicates that overexpression of this protein might cause an artefact. Taking these results together, the idea that Pex15p travels via the ER is far from proven. Thus, unfortunately, these results have an open end where peroxisome formation is concerned.

Thanks to the second observation made in mouse dendritic cells, however, we feel confident to suggest that the ER has a role in peroxisome biogenesis. Tubular structures which were continuous with the ER could be labelled with anti-bodies against the peroxisomal membrane protein Pex13p. Similarly labelled tubular and lamellar structures were continuous with peroxisomes (which were shown to contain thiolase and catalase). These tubular and lamellar structures could also be labelled with anti-bodies against the integral peroxisomal membrane protein Pmp70p, confirming that these structures are peroxisomal. Furthermore, by tomography continuity could be shown between the lamellar structures and peroxisomes (J. L. Murk, personal communication). This suggests that there is membrane transport from the ER to peroxisomes. Matrix proteins could only be detected in the ovoid-shaped peroxisomes and therefore import of proteins seems to be a late event in peroxisome formation, which is in line with other studies (see chapter 1).

Of course, some critical notes are in order. For example, we have to keep in mind that the observations represent static situations and that the direction of movement from the ER to peroxisomes could not be actually visualised, although there are several reasons to support this idea. Besides, the reason why the continuity between ER and peroxisomes could be visualised specifically in these cells remains unexplained. The observation made in dendritic cells resembles the results of Mullen et al. (1999) that in plant cells APX is inserted in a specialised region of the ER before it is sorted to peroxisomes. These two findings reinforce each other in the idea that peroxisomal membranes are derived from the ER and that this is a general mechanism in higher eukaryotes. The strongest indication for ER involvement in peroxisome formation in yeast thus far was provided by Titrenko et al. (1998), who showed that two *Y. lipolytica* peroxins could be N-glycosylated.
Although Pex15p did not provide new insight into a connection between the ER and peroxisome biogenesis in S. cerevisiae, further studies on this protein yielded new perspectives. The first link of Pex15p with other peroxins was provided by the discovery of its ability to interact with the AAA peroxin Pex6p, which in its turn interacts with another AAA peroxin, Pex1p. The N-terminal part of Pex6p is essential for interacting with Pex15p: its first AAA domain is the least conserved of the two and is suggested to stimulate association of Pex6p to Pex15p, and its second ATPase domain stimulates dissociation of these two peroxins (chapter 4). The dynamics of the interaction between Pex15p and Pex6p gave rise to speculation about the functions of these two peroxins. Pex6p, being a member of the AAA family, might be like some of its closest relatives (NSF and Vps4p) involved in ATP-dependent dissociation of a protein complex. When Pex1p and Pex6p where compared with other AAA family members, they proved to be not only functionally but also in primary amino acid sequence most similar to the NSF-like ATPases, as seen by phylogenetic analysis of the AAA-proteins (http://yeamob.pci.chemie.uni-tuebingen.de/AAA/Tree. html). Especially Pex6p seems to contain similar building blocks, although in a different order than NSF. The NSF N-terminus is involved in binding to SNAREs; its first AAA-domain is the most conserved of the two and is the regulator of dissociation of the SNARE complex, while its less conserved AAA-domain is important for oligomerisation. Continuing the comparison, the least conserved ATPase domain of Pex6p is necessary for the interaction with Pex1p (B. Metzig unpublished results), while binding of Pex6p to Pex1p is comparable to oligomerisation since these proteins are quite similar. Furthermore, the ATP-dependent binding to an integral membrane protein and its ATP-hydrolysis-driven dissociation from this protein are possible for both NSF and Pex6p. In the case of NSF the integral membrane protein is a SNARE, while in the case of Pex6p, the integral membrane protein is Pex15p. Although the structural homology between SNAREs and Pex15p is very low, the general idea of dissociation of a complex as the driving force supporting a particular process deserves attention. The question is what process Pex6p (possibly together with Pex1p) is able to drive. In other words: what is the consequence of the dissociation of Pex6p from Pex15p?

Recent literature about peroxisome biogenesis provides two options. The first is the theory presented by Titorenko and Rachubinski which states
that ATP hydrolysis by Pex1p and Pex6p is the signal for fusion of preperoxisomal vesicles (Titorenko et al., 2000; Titorenko and Rachubinski, 2000). This holds true for Y. lipolytica, but whether this is a common event in peroxisome formation remains to be established. In this hypothesis, the function of Pex6p is in line with that of several of its AAA family members. Dissociation of Pex6p from the peroxisomal membrane anchor Pex15p might lead to membrane fusion of peroxisomal vesicles. Pex15p might be a target SNARE which indicates to which membrane a vesicle should fuse. Furthermore, Pex15p seems to be activated by release of Pex6p. In this context, it is interesting that activation of a SNARE can be achieved by dissociation of a protein complex by an ATPase in the vacuolar fusion system in yeast (Seals et al., 2000).

In a second theory supported by Gould and colleagues (Hauser et al., 1998; Sacksteder and Gould, 2000), Pex6p is believed to be involved in the import of matrix proteins, possibly in the recycling of the PTS1 receptor. This hypothesis is based on the presence of large peroxisomal ghosts in S. cerevisiae and in human pex6 mutants, and on the instability of the PTS1 receptor in human and P. pastoris pex6 mutants. In this view, dissociation of Pex6p from Pex15p might result in import of matrix proteins, possibly by recycling of the PTS1 receptor. This is supported by the findings that there is a PTS1-specific import defect and that Pex5p seems to be more peroxisome-bound when Pex6p is unable to hydrolyse ATP (addendum to chapter 4).

Neither of these theories can be rejected or confirmed with the present knowledge. And although it is possible that the Pex6p-driven mechanism of peroxisome biogenesis in Y. lipolytica is different from that in higher eukaryotes, a situation in which protein import and membrane fusion take place simultaneously should be considered too. Examples of such processes have been proposed, such as import of proteins into peroxisomes via endocytosis at the peroxisomal membrane (McNew and Goodman, 1996) or via an autophagy-like process (Teter and Klionsky, 1999). However, none of these models seems very likely: neither the membrane recruitment of peroxisomes nor the topology of the import complex can be explained. Besides, the presence of internal vesicles is something that has not been observed frequently. Another possible mechanism by which membrane fusion and protein import are combined is presented in figure 1. Fusion of a maturing peroxisome with a membrane vesicle triggers protein import. Presumably the pre-
peroxisome and the vesicle contain different (incomplete) sets of proteins or unactivated proteins of the import complex. By fusion of the membrane structures, the import complex can be fully assembled or activated, which leads to import of matrix proteins. In this model both the hypothesis presented by Titorenko and Rachubinski and that proposed by Gould fit. Moreover, this model is in line with the observations made in mouse dendritic cells. Undoubtedly, future studies will shed more light on the situation.

In all mentioned cases Pex15p is the peroxisomal membrane anchor that binds Pex6p which fulfils its task there. However, one thing is hard to explain by these hypotheses: the rescue of the growth defect of cells in which Pex6p is mutated in its second ATPase domain by overexpression of Pex15p. The observations described in chapter 4 allow us to suggest that Pex6p mutated in this second domain is unable to dissociate from Pex15p with the consequence that peroxisome formation is blocked. This means that the mutated Pex6p will block only part of the Pex15p molecules when Pex15p is overexpressed. The rescue of the growth defect in this situation implies that free Pex15p (not bound to mutated Pex6p) is necessary for the maintenance of peroxisomes. This indicates that Pex15p might have a signalling function. The knowledge that Pex15p can be phosphorylated (Elgersma et al., 1997) strengthens this idea. The hard part to understand in this case is that Pex15p is the effector protein while Pex6p has an inhibitory function.

If Pex6p would only be an inhibitor, then why is the disturbance of the
interaction between Pex15p and Pex6p lethal when yeast is grown on oleate? The answer might lie in a dual function of Pex6p. We know that Pex6p has another binding partner, Pex1p. Disturbance of the interaction between Pex6p and Pex1p in humans leads to the Zellweger syndrome and thus the interaction between these two peroxins must be very important for peroxisome maintenance in man. Also in yeast the importance of this interaction was demonstrated. We showed that the interaction between Pex15p and Pex6p is independent of Pex1p, but we did not show yet whether or not the interaction between Pex1p and Pex6p is independent of Pex15p. Possibly Pex6p has to bind Pex15p before it is able to interact with Pex1p. If this is the case then disturbance of the Pex6p-Pex15p interaction would not only lead to an inability of Pex6p to prevent Pex15p from 'signalling' but would also lead to another serious defect in the maintenance of peroxisomes. This leads to another explanation of the rescue of pex6 mutant growth by Pex15p overexpression. Possibly Pex1p can partially take over the function of Pex6p when sufficient Pex15p is present. The rescue of pex6 mutants by Pex1p and of pex1 mutants by Pex6p has been demonstrated (Geisbrecht et al., 1998; Faber et al., 1998). Future efforts to study this topic should, I feel, be aimed at the interplay among these three peroxins, the effect of ATP binding to Pex1p and hydrolysis by Pex1p, and the effect of phosphorylation of Pex15p.

Coming back to the models for peroxisome biogenesis presented in figure 2 of the general introduction, my personal favourite is a combination of models C and D for several reasons: (1) The evidence for involvement of ER-derived particles is accumulating. (2) Peroxisomes can be formed in the absence of peroxisomal membrane structures (model C). (3) However, under normal circumstances it is likely that model D is preferred, since it is energetically more favourable for a cell to inherit peroxisomes than to build them anew.

Looking back at the formation of the eukaryotic cell and its organelles, and integrating herein the knowledge we have at present, we come to the conclusion that peroxisomes probably originate from the ER.
REFERENCES


