Peroxisome degradation in hansenula polymorpha

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Summary

In eukaryotic cells various functions are organised in special compartments, termed organelles. Prokaryotes basically lack such compartments but nevertheless can grow fast under a wide range of conditions. This raises the question of the possible advantages for eukaryotic cells in maintaining such compartments since complex regulatory mechanisms are involved in generating these organelles and control their abundance to precisely meet the cell's requirements. The data known so far lend support to the notion that the advantage of compartmentalisation lies in the ability to control specific cellular processes. Especially the efficiency of multi-step metabolic pathways may be increased by bringing together sets of enzymes that allow a rapid and optimal passage of metabolic intermediates or by creating unique microenvironments with specific (bio) chemical properties. Thus, by precisely controlling the abundance and function of different organelles (termed homeostasis), the cell can directs its resources optimally towards specialised functions and survival. An analysis of the principles of these processes is one of the main challenges of modern cell biology.

Among eukaryotic organelles, peroxisomes provide attractive model systems for such an analysis. The organelles are of significant metabolic importance for cell growth and viability and are characterised by a remarkably simple construction. Microbodies were first discovered in 1954. The organelles consist of a single membrane that encloses a proteinaceous matrix that may contain crystalline inclusions and measure up to 1 – 1.5 μm. Their functional variability is unprecedented and ranges from crucial roles in cholesterol metabolism in man, photorespiration in plant to essential roles in the primary metabolism of various unusual carbon and nitrogen sources in yeast. Their importance is probably best illustrated by the description of several inherited peroxisomal disorders in man, which generally cause an early death. Although these are rare, their discovery has strongly stimulated peroxisome research.

Yeasts are ideal models for studies on peroxisomes because they can grow in the absence of functional microbodies. This property has led to the isolation of various mutants (*pex* mutants) affected in peroxisomes assembly and to the cloning and characterization of the corresponding genes. Methylotrophic yeasts provide an additional advantage in that the processes of peroxisome development and turnover can be readily controlled by manipulation of the growth conditions. For instance, in *Hansenula polymorpha* a most pronounced peroxisome development is observed during growth on methanol.

Also the opposite occurs: when methanol-grown cells are transferred into fresh cultivation media in which peroxisomes are redundant for growth (e.g. glucose or ethanol), the organelles originally present in the methanol-grown cells are rapidly and selectively degraded (pexophagy). This process is morphologically characterized by three subsequent steps namely i) tagging, followed by sequestration of the organelle that is to be degraded by multiple membranous layers, ii) fusion of the sequestering membranes with the vacuolar membrane and iii) proteolytic turnover of the organelle contents in the vacuole. Various mutants (termed *pdd* mutants) of *H. polymorpha* affected in pexophagy have been isolated.

The work described in this thesis focuses on one aspect of peroxisome homeostasis, namely an analysis of the factors that control peroxisome degradation. Emphasis was placed on the intriguing question whether the processes of peroxisome biogenesis and degradation would overlap at any stage.

Summary

In chapter 1 an overview is presented of the current knowledge of the mechanisms of selective peroxisome degradation in various yeast species.

In yeast cells, the vacuole may be considered as wastebasket able to recycle redundant cell components. Various components that are no longer useful (soluble proteins, organelles) are taken up by these organelles and are proteolytically processed into their building blocks. During pexophagy, also peroxisomes that are destined for degradation are delivered to the vacuole. In *S. cerevisiae* various vacuolar proteolytic activities (e.g. proteinase A (PrA), proteinase B (PrB), carboxypepdidaseY (CPY) and aminopeptidase I (API)) have been characterized. These proteases are synthesized as inactive precursors in the cytosol and are activated after entrance into the vacuole. PrA and PrB are responsible for the processing/activation of precursors of several vacuolar hydrolases. In the methylotrophic yeast *Pichia pastoris,* strains lacking both PrA and PrB are impaired in peroxisomes degradation. We decided to study the role of the vacuolar protease carboxypeptidase Y (CPY) in pexophagy in *H. polymorpha* (chapter 2). The CPY gene was cloned and a disruption mutant was constructed (Δ*cpy*). Enzyme activity measurements and western blot analysis of peroxisomal marker proteins indicated that CPY is not a major protease component in the turnover of peroxisomal constituents during pexophagy in *H. polymorpha.*

The *S. cerevisiae* Vps34p is a phosphatidylinositol (PtdIns) 3-kinase that is required for sorting of specific vacuolar proteins (VPS pathway) and endocytosis. It is bound to membranes via the integral membrane protein ScVps15p, a protein kinase that is essential for activation of Vps34p. Mutations mapping in the *H. polymorpha PDD1* gene affect the initial step of peroxisome degradation. The gene product of H. polymorpha *PDD1* is the functional homologue of ScVps34p. We investigated in *Pichia pastoris* whether the *VPS15* gene was also involved in pexophagy (chapter 3). The Pp*VPS15* gene was cloned and a *VPS 15* deletion mutant was constructed (Δ*vps15).* Biochemical and ultrastructural studies on methanol-grown Δ*vps15* cells revealed that peroxisomal degradation was impaired in these cells. As in *H. polymorpha pdd1* mutants, pexophagy was blocked at an early stage. Studies in *S. cerevisiae* suggest that the activity of the Vps15/Vps34 complex results in the formation of PtdIns 3-kinase patches on the membrane that recruit proteins essential for membrane trafficking and fusion processes of the endosome. In pexophagy we hypothesize that these patches may be formed on the peroxisomal membranes and function as tags that may recruit proteins that initiate the organelle sequestration process.

Apart from selective degradation also a non-selective degradation machinery exists, that is induced when the cells are exposed to nitrogen deprivation. In *H. polymorpha* this process is characterized by the seemingly random uptake of cytosol and organelles by invagination of the vacuolar membrane without prior sequestration. We aimed to elucidate whether random autophagy and pexophagy were independent processes or may share common steps (chapter 4). This question was tackled using available *pdd* mutants that were affected in different steps of pexophagy. After exposure of methanol-grown pdd1 cells to N-limitation, non-selective-autophagy was not observed suggesting that the two processes overlap. However, in pdd2 mutant cells that are affected in the second step of pexophagy, namely the vacuolar fusion process, non-selective-autophagy normally took place. This indicates that pexophagy requires also unique genes not involved in random autophagy.

H. polymorpha Pex14p is a peroxisomal membrane protein essential for peroxisome biogenesis. Previous observations indicated that pexophagy was inhibited in Δ*pex14* cells. Apparently, two oppositely directed processes, namely organelle development and turnover,

converge at Pex14p. To further understand the function of Pex14p as a possible molecular switch, various truncated forms of Pex14p were constructed and analyzed for their function (chapter 5). The data indicate that the function of Pex14p in pexophagy resided in the extreme N-terminus of the protein. How the discrimination is made between peroxisome biogenesis and degradation is still speculative. Possible options may include i) conformational changes of HpPex14p (oligomerisation from monomers to dimers), ii) alteration of the location of the N-terminus from the inside to the outside of the organelle thus enabling specific interactions with other proteins.

Previous studies of the peroxisomal membrane protein Pex3p supported the idea that this protein is essential in the assembly of the peroxisomal membrane. Notwithstanding this generally accepted function, we obtained evidence in the course of our degradation studies that Pex3p behaved differently from other membrane proteins upon glucose-induced pexophagy. This was particularly evident in *pdd1* cells that were able to degrade Pex3p although peroxisome turnover was prevented (chapter 6). Experiments performed on WT *H. polymorpha* cells confirmed these data and clearly indicated that the degradation of Pex3p occurred much faster than could be explained from the turnover of whole peroxisomes. Subsequent experiments, carried out in the presence of proteasome inhibitors, indicated that the removal of Pex3p was dependent on the function of the proteasome. The proteasome can be considered as an intracellular factory with the sole function to degrade redundant and/or malfunctional proteins. Also, pexophagy was fully prevented when the cells were exposed to excess glucose in the presence of proteasome inhibitors. The removal of Pex3p from the membrane of peroxisomes to be degraded was confirmed by immunocytochemical experiments, using antibodies against Pex3p. These methods allow to visualize- and thus localize- protein molecules in ultra thin sections prepared from the cells. Our data led us to conclude that Pex3p removal from the membrane was a prerequisite to allow pexophagy. Remarkably, pexophagy was inhibited in *H. polymorpha Δpex3* cells that were functionally complemented using the *S. cerevisiae PEX3* gene. Most likely, this is related to conformational properties of ScPex3p that, although peroxisome biogenesis is restored, prevent interaction with other components essential for the initial steps of pexophagy.

One of the explanations for the degradation of peroxisomes under these conditions may be inactivation of peroxisome function. However, lowering the temperature to 18°C hardly affected the activities of the methanol dissimilatory pathway so that the observed cold-induced pexophagy in *H. polymorpha* remains to be