Mechanisms of selective peroxisome degradation in Hansenula polymorpha

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Animals, plants and fungi are multi-cellular organisms build up from eukaryotic cells. Eukaryotic cells characteristically contain organelles, which are small, membrane-bound structures that each performs a specialized task. For example, the nucleus is a double membrane bound structure that encloses the hereditary material (DNA) of the cell. The mitochondrion is responsible for energy generation in the cell and protein secretion is executed via multiple intracellular membrane structures mediated by the endoplasmic reticulum (ER) and Golgi apparatus. In the vacuole cellular components are degraded and recycled. Microbodies (glycosomes, hydrogenosomes, glyoxysomes, and peroxisomes) represent an exceptional class of organelles because they carry out highly diverse functions, which are often reflected in their name. For instance, glycosomes of Trypanosomes (sleeping disease causing parasites) contain enzymes of the glycolytic pathway; glyoxysomes of plants and fungi contain the key enzymes of the glyoxylate cycle and hydrogenosomes, observed in anaerobic fungi, carry out hydrogen metabolism. Peroxisomes are specialized microbodies that are involved in hydrogen peroxide metabolism and contain at least one hydrogen peroxide producing enzyme as well as catalase to metabolize this highly toxic compound.

In yeast, peroxisomes are involved in the metabolism of a range of unusual carbon and organic nitrogen sources, because they contain key enzymes involved in the oxidation of these compounds. Examples of such compounds are oleic acid, alkanes, methanol, urate and primary amines. In man, peroxisomes are most abundant in liver cells and contain enzymes involved in the oxidation of very long chain fatty acids. Human peroxisomes are also essential for the synthesis of special lipids (the ether-linked lipids) and bile acids. Defects in peroxisome formation or function are the cause of several inherited human diseases, most of which leading to severe neurological problems. The most severe forms of peroxisomal diseases result in an early death (e.g. in Zellweger syndrome), illustrating the importance of these organelles.

Peroxisomes are ubiquitously present in eukaryotes. Although microbodies perform a diverse array of functions, the mechanisms underlying their formation and degradation are highly conserved among eukaryotes. Therefore, studying these mechanisms in relatively simple model organisms such as fungi or yeast contributes to understanding of these processes in higher eukaryotes.

Yeast peroxisomes are highly inducible. Upon transfer of cells into growth media which require the function of peroxisomal enzymes, peroxisome proliferation is induced. This process results in an enhanced abundance of peroxisomes because the organelles grow and multiply by fission in the new environment. The opposite process, directed organelle degradation, can occur when the cells are exposed to fresh media in which the organelles are no longer needed for growth. Removal of the organelles occurs by degradation of complete organelles in the vacuole. The degradation products (predominantly amino acids, the building blocks of proteins/enzymes) are reused by the cells for the synthesis of new components.

In methylotrophic yeast species (*Hansenula polymorpha, Pichia pastoris*) the processes of peroxisome proliferation and degradation can be readily induced by ma-

nipulation of the growth conditions. This property renders these yeast species ideal model organisms to study the molecular mechanisms of these processes. The work described in this thesis involves studies on peroxisome degradation in *H. polymorpha*. Two different processes that result in degradation of peroxisomes have been described in this yeast, namely microautophagy and macropexophagy. When *H. polymorpha* cells experience nitrogen limitation, organelles (including peroxisomes) and components of the cell fluid (called cytosol) are degraded by microautophagy. Microautophagy is a non-specific degradation process, in which a portion of the cell content is swallowed by the vacuole. During microautophagy the vacuolar membrane forms large protrusions ("vacuolar arms"), which surround the material to be degraded. Next, the membranes that form the vacuolar arms fuse with each other in such a way that cellular components are trapped and finally end up inside the vacuole. An alternative mechanism includes the uptake of cytoplasmic components by an invagination process. These incorporated cell components subsequently decay, because of the direct contact with digestive vacuolar enzymes.

The process of macropexophagy is observed in *H. polymorpha* cells when cells, pre- grown on methanol-containing media, are transferred to growth media containing glucose or ethanol. During growth on methanol numerous large peroxisomes are formed in the cells. These organelles are crucial to allow growth on methanol, because they contain key enzymes of methanol metabolism. Upon transferring these cells to glucose media, these organelles become redundant for growth, because the enzymes of methanol metabolism are no longer required. Instead the cells need enzymes that metabolize glucose (the glycolytic pathway), which are not localized in organelles but are localized in the cytosol.

In macropexophagy peroxisomes are degraded in a highly selective way. Individual peroxisomes are marked (tagged) by a yet unknown mechanism and degraded sequentially, one by one. Each tagged peroxisome is enwrapped by multiple layers of sequestering membranes. Upon completion, this membrane structure, designated autophagosome, fuses with the vacuolar membrane resulting in selective incorporation of peroxisomes into the vacuole. Again, vacuolar hydrolytic enzymes break down the delivered content.

The work described in this thesis aims to understand peroxisome degradation in the yeast *H. polymorpha*. An overview of our current knowledge of the protein factors involved in peroxisome degradation is given in **CHAPTER 1**.

In *H. polymorpha* the mode of peroxisome degradation (microautophagy or macropexophagy) is determined by the nutritional signal (nitrogen depletion versus addition of glucose). In studies using baker's yeast (*Saccharomyces cerevisiae*) peroxisome degradation is generally induced by applying excess glucose and nitrogen starvation to the cells at the same time. This prompted us to investigate what happens when two distinct modes of peroxisome degradation are induced in *H. polymorpha* at the same time. In **CHAPTER 2** we describe that exposure of methanol-grown cells of wild type *H. polymorpha* to nitrogen depletion and excess glucose cause the onset of both processes simultaneously. Therefore, neither of the processes dominates the other.

In **CHAPTER 3** we describe the isolation of the *H. polymorpha ATG25* gene that encodes a novel coiled-coil protein, essential for selective peroxisome degradation by macropexophagy. Our studies indicate that the protein encoded by *ATG25*, Atg25, is not necessary for non-selective microautophagy. Unexpectedly, deletion of the *ATG25* gene results in constitutive degradation of peroxisomes by microautophagy. In contrast, mild overexpression of *ATG25* negatively regulates microautophagy. These data indicate that Atg25 plays a regulatory role in microautophagy and macropexophagy. Although under special circumstances macropexophagy and microautophagy can be induced simultaneously (see chapter 1), during normal vegetative growth of the cell both processes are tightly controlled in *H. polymorpha*. We propose that Atg25 plays an important function in this regulatory process.

In **CHAPTER 4** the cloning and characterization of the *H. polymorpha ATG8* gene is described. Like previously shown for *P. pastoris* and *Saccharomyces cerevisiae* Atg8, we found that *H. polymorpha* Atg8 is also involved in both microautophagy and micropexophagy. However, electron microscopy studies indicated that in a constructed *ATG8* deletion strain, peroxisomes were still sequestered by membranes upon induction of macropexophagy. Careful electron microscopical analysis of serial sections revealed that these membranes did not fully close and never fuse to vacuoles.

The location of *H. polymorpha* Atg8 was analyzed using a strain producing a fusion protein consisting of green fluorescent protein (GFP) and Atg8. Fluorescence microscopy revealed that during normal growth on methanol, Atg8 is concentrated at a spot in the vicinity of the vacuole. Most likely, this spot represents the PAS structure, a special structure in the cells that is required for macroautophagy processes. However, after induction of macropexophagy, GFP fluorescence was observed at extended structures that protruded around peroxisomes. These findings suggest that Atg8 is present in sequestering membranes during macropexophagy, but not essential for the formation of these membranes. Most likely Atg8 plays an important in closing the sequestering membranes prior to fusion with the vacuole.

To get more insight into one of the final steps of macropexophagy, namely fusion of sequestering membranes with the vacuolar membrane, we studied the possible function of two proteins, Vam7p and Vam3p, which are known to be involved in membrane fusion processes (**CHAPTER 5**). In *S. cerevisiae* it has been shown that these so-called SNARE proteins are components of a protein complex that is essential for fusion of autophagosomal membranes with the vacuolar membrane. Analysis of constructed *H. polymorpha VAM3* and *VAM7* deletion strains revealed that macropexophagy normally proceeded in cells lacking Vam3p, but not in cells that did not produce Vam7p. Remarkably, deletion of the *VAM7* gene also resulted in the formation of unusual peroxisomes that contained multiple membranes and membrane extensions. The nature of these membranes is still unknown.