Pex14p is Not Required for N-Starvation Induced Microautophagy and in Catalytic Amounts for Macropexophagy in Hansenula polymorpha

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Pex14p is Not Required for N-Starvation Induced Microautophagy and in Catalytic Amounts for Macropexophagy in *Hansenula polymorpha*

**ABSTRACT**

We showed before that the two oppositely directed processes of peroxisome biogenesis and selective peroxisome degradation (macropexophagy) converge at the peroxisomal membrane protein Pex14p. Here we show that this protein is not required for peroxisome degradation during nitrogen starvation-induced general autophagy, thereby limiting its function to the selective degradation process.

Pex14p is present in two forms, namely an unmodified (Pex14p) and a phosphorylated form (Pex14p\(\text{P_i}\)) that are differently induced during peroxisome proliferation. The data suggest that Pex14p is required for peroxisome biogenesis during organelle proliferation and Pex14p\(\text{P_i}\) in macropexophagy. Finally, we show that macropexophagy is not coupled to normal peroxisome assembly, because Pex14p is required in only catalytic amounts to allow initiation of the selective peroxisome degradation process.

**INTRODUCTION**

Peroxisomes are essential organelles of eukaryotic cells. Characteristically, these organelles are inducible in nature and may be involved in various metabolic processes.\(^1,2\) Defects in peroxisome biogenesis cause severe diseases in man that in some cases are lethal (e.g., Zellweger syndrome).\(^3\) Yeast cells are attractive model organisms to study the principles of peroxisome homeostasis. In these organisms peroxisome proliferation and turnover are readily inducible by manipulation of the growth conditions.\(^4,5,6\)

In *Hansenula polymorpha* two peroxisome autophagy processes have been described that degrade peroxisomes either selectively (macropexophagy) or non-selectively (microautophagy). Macropexophagy is induced when the organelles have become dys-functional or redundant for growth after glucose- or ethanol-induced catabolite inactivation of methanol metabolism.\(^7\) So far, microautophagy has only been observed in *H. polymorpha* when cells are exposed to nitrogen starvation conditions; at these conditions peroxisomes are degraded together with other cytoplasmic constituents.\(^8\) Interestingly, the processes of peroxisome biogenesis and selective organelle degradation converge at the peroxin Pex14p.\(^9\) Pex14p has been initially described to serve a function in docking of the cytosolic matrix protein receptors of PTS1 and PTS2 proteins, Pex5p and Pex7p.\(^10,11\) However, also macropexophagy is crucially dependent on the function of Pex14p.\(^9\) It was proposed that the Pex14p N-terminus is involved in recognition of peroxisomes by the autophagy machinery and allows initiation of the organelle sequestration process.\(^9\) This hypothesis may imply that probably only minor amounts of Pex14p may be sufficient to initiate degradation, relative to the amounts required for normal peroxisome biogenesis. To analyze this, we have constructed a strain that produces Pex14p at strongly reduced levels and analyzed the effects on peroxisome biogenesis and degradation. The results of these experiments are detailed in this paper.

**MATERIALS AND METHODS**

Micro-organisms and growth conditions. The *Hansenula polymorpha* strains used in this study are listed in Table 1. The cells were grown at 37°C in YPD media (1% yeast extract, 1% peptone, 1% glucose), selective media containing 0.67% Yeast Nitrogen Base without amino acids (DIFCO) supplemented with 1% glucose (YNB) or mineral media (MM)\(^12\) supplemented with 0.5% glucose or a mixture of 0.1% glycerol + 0.5% methanol in the presence of 0.25% ammonium sulphate as sole nitrogen source. For macropexophagy and N-starvation induced autophagy, cells were extensively precultivated and grown to the mid-exponential growth phase in MM supplemented with glucose.

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**KEY WORDS**

peroxisome, biogenesis, autophagy, macropexophagy, yeast, GFP, phosphorylation

**ACKNOWLEDGEMENTS**

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and ammonium sulphate and subsequently shifted to MM supplemented with methanol and glycerol. For N-starvation-induced autophagy, the cells were collected by centrifugation and resuspended in MM containing methanol, but lacking ammonium sulphate, leucine and yeast extract. As controls, cells were resuspended in media plus ammonium sulphate. For macroautophagy, the cells were resuspended in fresh glucose-containing media. Samples were taken at regular time intervals after the shift of cells into the new environment. When required, media were supplemented with 30µg/ml leucine or 100 µg/ml zeocin. For growth on plates 2% agar was added to the media. For cloning purposes, Escherichia coli DH5α (GibcoBrl, Gaithesburg, MD) was grown at 37˚C in LB media (1% trypton, 0.5% yeast extract, 0.5% NaCl), supplemented with 100 µg/ml ampicillin, 50 µg/ml kanamycin or 25µg/ml zeocin when required.

**Table 1: Hanselula polymorpha strains used in this study**

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<th>Strain</th>
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<tr>
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<td>This study</td>
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**Table 2: Plasmids used in this study**

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<td>This study</td>
</tr>
<tr>
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</table>

**RESULTS**

**Pex14p is not required for N-starvation induced autophagy.** The finding that Pex14p plays a crucial role in peroxisome degradation by macroautophagy<sup>9</sup> raises the question whether this protein is also involved in N-starvation induced general autophagy. To analyze this, glycerol/methanol grown pex14::P<sub>AOX</sub>-PEX5<sup>mc</sup> cells were transferred to fresh media lacking any nitrogen source. Western blots prepared of crude extracts of samples taken after regular time intervals and decorated with α-Pex10p antibodies revealed that the levels of this peroxisomal membrane protein declined with time in a similar fashion as WT control cells, grown at identical conditions (Fig. 1). In contrast, in H. polymorpha atg1 cells, defective in N-starvation induced autophagy, Pex10p levels did not decrease during nitrogen starvation (Fig. 1). These data demonstrate that Pex14p is not essential for degradation of peroxisomes in N-starving cells.

**Phosphorylated Pex14p is rapidly degraded after the onset of macroautophagy.** Pex14p of methyloptrophic yeast species may be phosphorylated in vivo.<sup>18,19</sup> However, a physiological function of the phosphorylated protein (Pex14p<sup>5</sup>) is not yet known. We first examined the kinetics of Pex14p induction during adaptation of cells to peroxisome-inducing growth conditions. As shown in Figure 2, Pex14p is rapidly induced after transfer of repressed, glucose-grown cells into methanol-containing media, conditions that maximally induce peroxisome proliferation. Total Pex14p levels are maximal at the mid-to late exponential growth phase and decrease during prolonged cultivation. Remarkably, Pex14p and Pex14p<sup>5</sup> show different induction patterns. Nonphosphorylated Pex14p is highest in the initial stages of growth (maximal at 7 hours of incubation) whereas Pex14p<sup>5</sup> reaches its maximal level in the mid-to late exponential growth phase (8–16 hours of induction). During further cultivation the levels of Pex14p<sup>5</sup> decrease gradually until 20% of the highest level. However, nonphosphorylated Pex14p levels are below the limit of detection in cells of cultures that entered the stationary growth phase (Fig. 2A and B).
Figure 1. Pex14p is not required for N-starvation induced autophagy in H. polymorpha. WT, atg1 and pex14::PAOX.PEX5inc cells were grown on glycerol/methanol until OD660 = 1.9 and subsequently exposed to nitrogen (N-) starvation conditions. As control, cells were grown in the presence of nitrogen (N+). Blots were decorated with α-Pex10p antibodies. In both WT and pex14::PAOX.PEX5inc cells Pex10p levels decrease at N-starvation conditions (N-), but not in N+ controls. In atg1 controls Pex10p levels do not decrease during N-starvation. Samples were taken at the indicated time points (in hours). Equal amounts of protein were loaded per lane.

Subsequently, macrophagophagy was studied in cells in which total Pex14p levels were maximal (Fig. 2C and D). Cells induced for 10 hours on methanol were exposed to glucose excess conditions. Western blotting experiments showed that macrophagophagy normally occurred, based on the degradation of AO protein (not shown). Blots decorated with α-Pex14p antibodies revealed that specifically Pex14pP\(^i\) levels rapidly declined in the first 20 minutes of induction in the presence of glucose, whereas the non-phosphorylated Pex14p displayed a relatively slow reduction typical for peroxisome degradation. The subsequent induction of Pex14p is due to the formation of new organelles on glucose.

We also analyzed the kinetics of Pex14p induction by fluorescence microscopy. To this end, a strain was constructed in which the original PEX4 gene was replaced by PEX4.GFP, and that also contained P\(^{AX}\) driven DsRed.SKL. Cells of this strain, when grown on glucose, contained a single green spot (Fig. 3) indicating that these cells contain generally a single peroxisome located in the vicinity of the cell membrane.\(^{20}\) First DsRed fluorescence was observed after four hours of incubation and colocalized with the GFP fluorescence suggesting that the original organelle in the glucose-grown cell accumulated DsRed. Remarkably, at later growth stages Pex14p.GFP was predominantly observed as a spot at the organelle membrane. At 6-8 hours of incubations the first organellar fission events were observed. Also, at the site of fission (6 h sample, arrow) invariably GFP fluorescence accumulated. After subsequent organellar fission peroxisomes were observed that did not or hardly contain any GFP fluorescence. If detectable, the GFP-dependent fluorescence was frequently located at the sites of organellar association. The number of organelles in which Pex14p.GFP fluorescence was below the limit of detection gradually increased. Invariably only few (generally two to three) organelles showed distinct Pex14p.GFP fluorescence at any stage of prolonged methanol cultivation (Fig. 3). These data are consistent with the biochemical data and suggest that heterogeneity exists between individual organelles of one cell regarding their Pex14p levels.

Characterization of the P\(^{PEX}\)Pex14 strain. As shown before\(^9\) macrophagophagy and peroxisome biogenesis in H. polymorpha converge at Pex14p. To investigate whether macrophagophagy and organelle biogenesis are coupled processes, we constructed a strain in which the levels of Pex14p are strongly reduced. To this end the PEX4 gene was placed under control of the PEX4 promoter (P\(^{PEX}\)). P\(^{PEX}\) represents the weakest H. polymorpha promoter known so far.\(^{21}\) The constructed pex14::P\(^{PEX}\)Pex14 strain grew normally on compounds that do not require peroxisome function (glucose, glycerol) but failed to grow on methanol as sole source of carbon and energy (data not shown).

Western blot analysis of crude extracts, prepared from glycerol/methanol-grown pex14::P\(^{PEX}\)Pex14 cells and decorated with α-Pex14p antibodies, revealed that the Pex14p levels were indeed strongly reduced, relative to the Pex14p levels observed in WT controls (Fig. 4). As expected, in extracts of pex14 control cells, Pex14p was not observed. The levels of two other peroxisomal membrane proteins, Pex3p and Pex10p, were not (Pex3p) or only slightly reduced (Pex10p) in the pex14::P\(^{PEX}\)Pex14 cells, relative to WT. Also, the levels of alcohol oxidase (AO), catalase (CAT) and dihydroxyacetone synthase (DHAS) were comparable in the three strains analyzed (Fig. 4).
The defect in growth on methanol suggests that the PTS1 protein import machinery is affected in the constructed strain, because the major peroxisomal enzymes of methanol metabolism AO, CAT and DHAS are all PTS1 proteins.

Electron microscopy showed that the glycerol/methanol-grown \( p_{\text{PEX4}}^{\text{pex14}} \) cells contained relatively small peroxisomes, frequently observed in conjunction with a cytosolic AO crystalloid (Fig. 5A). This morphology is characteristic for cells that display a defect in AO import.\(^{22} \) This was confirmed by immunocytochemical analysis. Incubation of ultrathin sections of Unicryl-embedded \( p_{\text{PEX4}}^{\text{pex14}} \) cells with \( \alpha \)-AO antibodies and GAR gold revealed that the specific labeling was present on peroxisome profiles as well as on the cytosol (Fig. 5B). Incubations, using \( \alpha \)-CAT and \( \alpha \)-DHAS antisera, gave similar results (data not shown). As expected, in WT controls the labeling of these enzymes was confined to peroxisomes (not shown). Also GFP.SKL produced in \( p_{\text{PEX4}}^{\text{pex14}} \) cells resulted fluorescence located in peroxisomes and in the cytosol, whereas in WT controls fluorescence was confined to peroxisomes (Fig. 6). Taken together, these data indicate that \( p_{\text{PEX4}}^{\text{pex14}} \) cells display a severe PTS1 protein import defect.

Selective degradation of peroxisomes is not disturbed in \( p_{\text{PEX4}}^{\text{pex14}} \) cells. Subsequently we analyzed whether the defect in peroxisome development in \( p_{\text{PEX4}}^{\text{pex14}} \) cells was accompanied by a defect in selective organelle degradation (macropexophagy). To investigate this, glycerol/methanol grown \( p_{\text{PEX4}}^{\text{pex14}} \) cells were exposed to glucose excess conditions. To address macropexophagy in these cells, AO could not be used as marker protein since the portion of this protein that is localized in the cytosol is not subject to degradation via macropexophagy.\(^{9,23} \) For this reason Pex10p was used as the peroxisomal marker protein. Western blot analysis of crude extracts of samples of \( p_{\text{PEX4}}^{\text{pex14}} \) cells taken at various time points after the shift of cells to glucose, demonstrated that the levels of Pex10p decreased at rates similar to those observed in WT controls (Fig. 7). Pex10p reduction was not observed in \( p_{\text{PEX4}}^{\text{pex14}} \) cells, which are known to be disturbed in macropexophagy.\(^{23} \) This suggests that macropexophagy is not blocked in \( p_{\text{PEX4}}^{\text{pex14}} \) cells.

Figure 3. Pex14p.GFP is not evenly distributed over individual peroxisomes in WT cells. WT cells, producing \( P_{\text{PEX4}}^{\text{PEX14}} \)-driven Pex14p.GFP and \( P_{\text{AOX}}^{\text{PEX14}} \)-driven DsRed.SKL were grown on glucose to the mid-exponential growth stage and subsequently shifted to fresh methanol media. Glucose-grown cells, in which \( P_{\text{AOX}} \) is fully repressed, contain a single peroxisome spot visualized by Pex14p.GFP (0 h). Four hours after the shift of cells the first DsRed fluorescence is observed that coincides with Pex14p.GFP. Note that Pex14p is present in a focal peroxisomal spot (4 h). After 6 h, the first organelle fission events are observed that are associated with the presence of two Pex14p.GFP spots, the strongest one of which coincides with the site of fission (arrows). At 12 h the cells contain generally 3-4 peroxisomes two of which contain a GFP spot (12 h). Pex14p.GFP is undetectable in the other large organelle. A similar distribution pattern is observed after 24 h of incubation; only two organelles display Pex14p. GFP fluorescence. The bar represents 1 µm [valid for all pictures].

Figure 4. Peroxisomal protein levels in \( p_{\text{PEX4}}^{\text{pex14}} \) cells. Western blot analysis of crude extracts prepared from glycerol/methanol-grown \( p_{\text{PEX4}}^{\text{pex14}} \) cells, using WT and pex14 cells as control. Blots were decorated with antisera against Pex14p, Pex10p, Pex3p, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). The data show that reduction of Pex14p in \( p_{\text{PEX4}}^{\text{pex14}} \) cells does not affect other peroxisomal protein levels. Equal amounts of extracts were loaded per lane.
DISCUSSION

This paper describes the function of Pex14p in peroxisome degradation during N-starvation induced autophagy (microautophagy) and the effect of reduced levels of this peroxisomal membrane protein on selective peroxisome degradation (macropexophagy). Our data demonstrate that Pex14p is not required for peroxisome degradation by microautophagy. Hence, Pex14p is only involved in the selective autophagy process (macropexophagy). In case of macropexophagy, we show that at Pex14p levels that are too low to support normal peroxisome development, macropexophagy is unaffected.

Pex14p was first identified as a main component of the docking machinery for the cytosolic matrix protein receptors, Pex5p and Pex7p.10,11 Two other components of this putative docking complex are Pex13p and Pex17p.24-26 Apparently, as indicated also by Agne et al.25 for bakers yeast peroxisomes, the stoichiometry of these components is rather fixed for normal function, as overexpression but also reduced expression (this paper) of PEX14 strongly interferes with normal peroxisome development. Recently, we showed that apart from its function in organelle biogenesis Pex14p also plays a crucial role in macropexophagy.9 From the analysis of specific H. polymorpha peroxisome-deficient (pex) mutants Veenhuis et al23 demonstrated that the mechanisms involved in tagging peroxisomes for degradation via macropexophagy are directed against components of the peroxisomal membrane. Two of such components have now been identified. It is shown that both Pex3p and Pex14p are essential for macropexophagy.27 First, Pex3p has to be removed from the membrane in order to allow degradation to initiate. It was suggested that this Pex3p-dependent organelle de-stabilization is associated with the exposure of the Pex14p N-terminus for attack by the macropexophagy machinery.7

In vivo Pex14p is present in both a phosphorylated (Pex14p\(^{P_i}\)) and nonphosphorylated form.18,19 H. polymorpha Pex14p phospho-
rulation is localized to the extreme C-terminus of the protein, although the amino acids that are involved in phosphorylation are unknown. Our data on the kinetics of Pex14p induction during growth of cells on methanol are consistent with the assumption that the unmodified protein is required for peroxisome biogenesis in receptor protein docking, whereas Pex14p functions in selective degradation. However, despite the observation that Pex14p is below the limit of detection in specific “mature” organelles, it cannot be excluded that very low amounts of unmodified Pex14p remain present that could account for the initiation of organelle degradation. Also, Agne et al. demonstrated that Pex14p may be present in different sub-complexes. In line with this, unmodified Pex14p and Pex14p may be located to different sub-complexes and the composition of such complexes may in fact specify the function of the protein in biogenesis or degradation. On the other hand, protein phosphorylation is a wide spread machinery involved in protein degradation although the amino acids that are involved in phosphorylation are sufficient to render the organelles susceptible for degradation and instant decision making. Clearly, much additional research is required to elucidate the molecular significance of Pex14p modification.

Our current data indicate that in fact very low Pex14p levels are sufficient to render the organelles susceptible for degradation and that the selective peroxisome degradation process by macroautophagy is not coupled to normal organelle assembly. It is still unknown which component Pex14p, after Pex3p removal, may react. A likely candidate may be Atg1, to promote interaction of the organelle with the preautophagosomal structure (PAS) to enter the organelle into the macroautophagy pathway. This aspect, together with the effects of Pex3p removal on the structure/stability of peroxisomal membrane protein complexes, is a topic of current investigations.

References