Peroxisome Biogenesis and Selective Degradation Converge at Pex14p

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We have analyzed the function of Hansenula polymorpha Pex14p in selective peroxisome degradation. Previously, we showed that Pex14p was involved in peroxisome biogenesis and functions in peroxisome matrix protein import. Evidence for the additional function of HpPex14p in selective peroxisome degradation (pexophagy) came from cells defective in HpPex14p synthesis. The suggestion that the absence of HpPex14p interfered with pexophagy was further analyzed by mutational analysis. These studies indicated that deletions at the C terminus of up to 124 amino acids of HpPex14p did not affect peroxisome degradation. Conversely, short deletions of the N terminus (31 and 64 amino acids, respectively) of the protein fully impaired pexophagy. Peroxisomes present in these cells remained intact for at least 6 h of incubation in the presence of excess glucose, conditions that led to the rapid turnover of the organelles in wild-type control cells. We conclude that the N terminus of HpPex14p contains essential information to control pexophagy in Hansenula polymorpha and thus, that organelle development and turnover converge at Pex14p.

Hansenula polymorpha is a methylotrophic yeast that is used as a model organism in contemporary peroxisome research. Methylotrophic yeast species, also including Candida boidinii and Pichia pastoris, have the advantage that the ultrastructural changes accompanying peroxisome development and degradation are much more pronounced, relative to Saccharomyces cerevisiae. Also, enhanced numbers of growth substrate-dependent peroxisome functions can be induced compared with bakers’ yeast.

In Hansenula polymorpha highest peroxisome induction rates are observed during growth of cells on methanol. Under these conditions the organelles may occupy up to 80% of the cytoplasmic volume and are essential for growth as they contain the key enzymes of methanol metabolism, alcohol oxidase (AO), catalase, and dihydroxyacetone synthase. These enzymes all possess a C-terminal targeting signal and require the function of the PTS1 import machinery for sorting to peroxisomes (1, 2). In Hansenula polymorpha, Pex14p is involved in matrix protein import and functions (probably together with Pex13p and Pex17p) as the putative docking site for PTS1 receptor-cargo complexes at the peroxisomal membrane (3). Recent data, however, indicate that the protein is not essential for import but most likely enhances the efficiency of the process (4).

Peroxisome degradation may occur aselectively during general autophagy (5) or in a selective way, when the organelles have become dysfunctional or, alternatively, redundant for growth. In Hansenula polymorpha the selective degradation process is morphologically characterized by three subsequent steps, namely (i) tagging, followed by sequestration of the organelle to be degraded by multiple membranous layers, (ii) heterotypical fusion of the sequestering membranes with the vacuolar membrane, and (iii) hydrolysis of the organelle contents in the vacuole (6). This process, designated macroperoxophagy, is also observed in another methylotrophic yeast species, P. pastoris (7, 8). In this yeast, but not in Hansenula polymorpha, a second mode of selective degradation is described, specifically induced by glucose, which involves uptake of clusters of peroxisomes by engulfment by the vacuole (micropexophagy). Selective peroxisome degradation has also been described in the yeasts S. cerevisiae and Yarrowia lipolytica (9); however, the exact mode of degradation is as yet unknown.

Various mutants affected in pexophagy have been isolated from P. pastoris (paa and pag mutants) (10–13) and H. polymorpha (pdd mutants) (14, 15). The analysis of the corresponding genes has shown that pexophagy has several components in common with non-selective autophagy, vacuolar protein sorting, endocytosis, and the cytosol-to-vacuole transport pathway in bakers’ yeast (16, 17).

In Hansenula polymorpha pex mutants, peroxisomal remnants are normally susceptible to glucose-induced selective degradation, except for those present in Δpex14 cells (18). This raised the question whether Pex14p, besides being involved in matrix protein import, could have additional functions in the control of the susceptibility of individual organelles for selective degradation. This aspect was analyzed in cells of constructed mutants that contained peroxisomes that either completely lacked Pex14 or contained truncated forms of this peroxin. The results of this work are included in this paper.
In an initial series of experiments we have analyzed the fate of peroxisomes that had developed in cells of a pex14 deletion strain that overproduced Pex5p (Δpex14::pAOX.PEX5mc (4)), after a shift of cells from methanol to glucose-excess conditions. To this end cells were pre-grown on a mixture of glycerol and methanol until an optical density (OD660) of 1.5 before excess glucose (final concentration 0.5%) was added. As described before, glycerol-/methanol-grown Δpex14::pAOX.PEX5mc cells contained several well developed peroxisomes (Fig. 1A) that lacked Pex14p but contained the bulk of two key enzymes in peroxisome biogenesis and selective degradation at Pex14p.
double band Pex14p is observed as a and were the sole sites of AO protein. In cells expressing PEX14 by the presence of a cytosolic AO crystalloid. Minor AO import defect. Cells expressing PEX14 AO protein was mislocalized to the cytosol in these cells indicative of a (lane 2 WT, lane 3 N31, lane 4 C58, lane 5 C124; and lane 6). Cells were grown for 16 h in glycerol/methanol media. Pex14p was almost exclusively localized in the organellar pellet fractions of all the strains.

methanol utilization, AO and dihydroxyacetone synthase (data not shown), in conjunction with minor amounts of these proteins in the cytosol (4). Ultrastructural and biochemical analyses revealed that, upon exposure of such cells to glucose-excess conditions, selective peroxisome degradation (pexophagy) was inhibited. Electron microscopy revealed that after the addition of glucose the initial event in peroxisome degradation, namely sequestration
of the organelle to be degraded, was never observed (data not shown). Also, immunocytochemistry failed to demonstrate any AO protein in the vacuole, a typical morphological characteristic of peroxisomy, in the same time interval (Fig. 1B). In WT control cells both phenomena were frequently observed (data not shown; see Ref. 6). Biochemical experiments showed that the amount of Pex10p, an integral component of the peroxisomal membrane, had slightly increased 4 h after addition of glucose to Δpex14::Pex10pΔPEX5mt cells whereas in WT controls this marker protein markedly decreased during this time period (Fig. 2). Taken together these data suggest that the peroxisomes present in Δpex14::Pex10pΔPEX5mt cells were not susceptible to glucose-induced peroxisomy.

**Mutational Analysis of Pex14p**—To delineate the region of Pex14p controlling peroxisome turnover, we constructed mutant genes that encoded various truncated Pex14ps and transformed these into a PEX14 deletion strain (Δpex14). These proteins lacked either the initial 31 or 64 N-terminal amino acids (designated ΔN31 and ΔN64, respectively) or the extreme 58 or 124 C-terminal amino acids (designated ΔC58 and ΔC124, respectively). A Δpex14 strain expressing full-length PEX14 was taken as control (designated WT). Cells of these strains were subsequently analyzed for growth on methanol, Pex14p synthesis and location, peroxisome development, and the susceptibility of these organelles to selective degradation.

**The Mutant Pex14ps Are Normally Synthesized and Sorted to Peroxisomes**—Cells of the various constructed strains were analyzed for their capacity to grow on methanol as the sole source of carbon. As shown in Table I only the WT control and mutant ΔC58 grew normally on methanol at WT rates, whereas the other strains showed no or severely retarded growth. The reason for this became clear from electron microscopy, which revealed that WT and ΔC58 cells displayed normal peroxisomes that were the sole site of AO protein, judged from immunocytochemistry (Fig. 3, A and B). The three other strains contained several peroxisomes of smaller size (Table I). This most likely reflects the observation that only a portion of AO protein was present in peroxisomes, whereas the remaining portion resided in the cytosol (Fig. 3, C and D). However, in these three mutants the amount of AO imported into peroxisomes was substantially higher compared with the residual import in peroxisomal remnants in Δpex14 cells. Because AO is a PTS1 protein, these data indicate that the long (124 amino acids) C-terminal deletion, as well as N-terminal deletions of HpPex14p, affect Pex5p-dependent protein import. We showed before that a minor amount of active cytosolic AO prevents growth of *H. polymorpha* cells on methanol (19). Therefore, the cytosolic portion of AO protein in ΔC124, ΔN31, and ΔN64 cells most likely explains the failure of the cells to grow on methanol.

Subsequently, all constructed strains were analyzed for the presence of Pex14p by Western blotting, using α-Pex14p antibodies and crude extracts prepared from glycerol-/methanol-grown cells. These experiments revealed that all truncated Pex14ps were normally synthesized and were of the expected mass, judged from their migration pattern in the gel (Fig. 4A). In these blots Pex14 protein is observed as a double band at ~47 kDa, of which the upper band represents the phosphorylated state of the protein (20). As evident in Fig. 4B, normal phosphorylation of Pex14p can also be observed in ΔN64 and ΔC58 cells but not in ΔC124 cells, in which phosphorylation of Pex14p was hardly detectable. This suggests that the putative phosphorylation site(s) of the protein are present within the region of amino acids 228 to 293.

The subcellular location of these Pex14p variants was subsequently analyzed biochemically and immunocytochemically. Upon differential centrifugation of homogenized protoplasts, prepared from methanol-induced cells of the various strains, WT and mutant Pex14ps were predominantly found in the 30,000 × g organellar pellets (Fig. 5). This suggests that the proteins are indeed organelle-bound. Immunocytochemically, using α-Pex14p antibodies, the specific labeling was exclusively localized on the peroxisomal membrane (Fig. 6). From this we concluded that all truncated Pex14ps (WT, ΔC58, ΔC124, ΔN31, and ΔN64) were normally synthesized and sorted to the correct target membrane (summarized in Table I).

**An N-terminal Deletions of Pex14p Affect Selective Peroxisome Degradation**—Cells of the various strains were grown on glycerol/methanol mixtures and subsequently exposed to glucose-excess conditions. Electron microscopical analysis revealed that in the strain producing full-length Pex14p peroxisomes were normally degraded. A similar phenomenon was observed for ΔC58 and ΔC124 cells. 30 min after addition of glucose the first cells were observed that contained AO protein in the...
vacuole (Fig. 7), a characteristic feature for selective peroxi-
osome degradation under these conditions (6). It should be
stressed that the cytosolic portion of AO is not subject to degrada-
tion under these conditions (21) and therefore cannot be the source of vacuolar AO protein.

The above observations were confirmed by biochemical data. After Western blotting of crude extracts, prepared from the various strains at different time points after the addition of

The general function of Pex14p in PTS1 and PTS2 matrix protein import but enhances the efficiency of this process (4). Our current data imply that Pex14p may have multiple roles and functions in both the biogenesis and selective degradation of the organelle. This mode of controlling peroxisome biogene-
sis versus degradation (homeostasis) in one “switch” has obvi-
ous physiological advantages in that it enables the cell to
rapidly adapt peroxisome numbers and function upon changes in
the environment. This is particularly important in metha-
nol-grown H. polymorpha cells, in which specific organelles
have become dysfunctional through, e.g., chemically induced
damage of their membrane (22) resulting in the leakage of
matrix proteins into the cytosol. It has been shown before that
the presence of only minor amounts of enzymatically active AO protein in the cytosol prevents growth of cells on methanol because of the major energetic disadvantages related to cyto-
solic hydrogen peroxide metabolism (19).

In H. polymorpha pexophagy is a remarkable fast process; individual organelles can be degraded within a time interval of

Thus, a molecular switch controlling organelle homeo-

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functions of HpPex14p are still largely speculative. One possi-
bile option is that this may be related to conformational changes of HpPex14p, for instance by oligomerization from monomers to dimers or even by alteration of the location of the N terminus of

Pex14p from inside to outside the organelar matrix. On the
topology of Pex14p in yeast nothing is known yet; only for
human Pex14p has evidence been presented that the N ter-
minal of the protein is (at least in part) exposed to the lumen of

Our experiments strongly suggest that modi-

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