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 H. 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 H. 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),¹ 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 H. 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 H. 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 macropexophagy, is also observed in another methylotrophic yeast species, P. pastoris (7, 8). In this yeast, but not in H. 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 (gsa 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 H. 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 Pex14p or contained truncated forms of this peroxin. The results of this work are included in this paper.

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†‡ The abbreviations used are: AO, alcohol oxidase; WT, wild-type; PCR, polymerase chain reaction; aa, amino acid; PTS, peroxisome-targeting signal.
Peroxisome Biogenesis and Selective Degradation at Pex14p

Experimental Procedures

Organisms and Growth Conditions—All H. polymorpha strains used in this study are derivatives of strainNCYC495 (leu1.1 ura3) (25): \( \Delta \text{pex14} \) (20), \( \Delta \text{pex14}: P_{\text{AOX}}\text{PEX}^{5*} \) (4), and \( \Delta \text{pex14} \) strains expressing full-length or truncated forms of \( \text{PEX}^{14} \) under control of the \( \text{PEX}^{14} \) promoter (P\text{PEX}14) (this study).

\( H. \) polymorpha cells were grown in batch cultures at 37 °C on (a) selective minimal medium (YND) containing 0.67% (w/v) yeast nitrogen base without amino acids (DIFCO) supplemented with 1% (w/v) glucose, (b) rich medium (YPD) containing 1% (w/v) yeast extract, 1% (w/v) peptone, 1% (w/v) glucose, or (c) minimal medium (26) supplemented with 0.5% (w/v) glucose, 0.5% (v/v) methanol or a mixture of 0.1% (v/v) glycerol, 0.5% (v/v) methanol as a carbon source, together with 0.25% (w/v) ammonium sulfate as a nitrogen source. When required leucine (30 µg/ml) was added.

For analysis of peroxisome degradation, cells were extensively pre-cultivated to the mid-exponential phase on mineral medium with glucose and then shifted to mineral medium containing glycerol + methanol for a period of 16 h to induce peroxisome biogenesis. Subsequently 0.5% glucose was then added to induce selective peroxisome degradation. Escherichia coli DH5\( ^{\alpha} \) (27) was used for plasmid construction and was grown on LB medium supplemented with the appropriate antibiotics.

Molecular Techniques—Standard recombinant DNA techniques were carried out essentially according to Sambrook et al. (27). Transformation of \( H. \) polymorpha was performed by electroporation as described previously (28). Restriction enzymes and biochemicals were obtained from Roche Molecular Biochemicals and used as detailed by the manufacturer. Protein sequences were aligned with the program CLUSTALX (29).

Construction of Plasmids—To express WT and truncated forms of \( \text{PEX}^{14} \) cells under control of its endogenous promoter, we first constructed the expression vector pH1PX10, a derivative of pHIPX4-B (29). The \( \text{PEX}^{14} \) promoter was isolated by PCR using the \( \text{PEX}^{14} \) primer (5′ GGG GAT CCC TCT AGG AAG AAA AGG AGG 3′), the M13/pUC universal primer, and plasmid pBS3.2P14 (20) as template. The PCR fragment was digested with EcoRI and BamHI and inserted between the NotI (blunted) and BamHI sites of plasmid pHIPX4-B thus replacing the alcohol oxidase promoter. To obtain pX10-PEX14-WT we inserted the 1.5-kilobase BamHI-EcoRV fragment of p\(_{\text{AOX}}\text{PEX}^{14}\) (29) between the BamHI and SacI sites of pHIPX10. Plasmids expressing truncated versions of PEX14 were constructed as follows: pX10-PEX14-ΔC58 was constructed by PCR using the N31 primer (5′ AAC ATG ACC GGA TCC 3′) and pBS1.3P14 as template. The PCR fragment was digested with HinII and SmaI sites of plasmid pHIPX10. Plasmids expressing truncated versions of PEX14 were constructed as follows: pX10-PEX14-ΔC58 was constructed by filling in the EcoRI site in the PEX14 coding region followed by self-ligation. Similarly, pX10-PEX14-ΔC124 was obtained by blunting the PstI site in the PEX14 coding region followed by self-ligation. Plasmids pX10-PEX14-ΔN31 and pX10-PEX14-ΔN64 were constructed by PCR using the N31 primer (5′ AGA GGA TCC ATG GCC AAA AAG GTC GAA 3′) or the N64 primer (5′ AGA GGA TCC ATG TCA CAG CAC TCT GGG GTA 3′) in combination with the M13/pUC sequencing primer and pBS1.3P14 as template. Subsequently, for pX10-PEX14-ΔN31, a BamHI + HindIII-digested PCR fragment was cloned between the BamHI and HindIII sites of pHIPX10. For pX10-PEX14-ΔN64 a BamHI + EcoRV-digested PCR fragment was cloned between the BamHI and SmaI sites of pHIPX10. Plasmids were transformed into \( H. \) polymorpha \( \Delta \text{pex14} \) (leu1.1) (20) and produced Pex14p levels similar to those observed in WT cells.

Biochemical Methods—Crude extracts using trichloroacetic acid-precipitated \( H. \) polymorpha cells were prepared as described (30). SDS-polyacrylamide gel electrophoresis and Western blotting were performed by established procedures. Proteins on Western blots were detected using a chemiluminescent Western blotting kit (Roche Molecular Biochemicals) after decoration with polyclonal antibodies against various \( H. \) polymorpha proteins.

In vivo phosphorylation of HpPex14p was determined as described (31); cells were pregrown on YPD, depleted for phosphate, harvested, and subsequently suspended in the same volume of phosphate-depleted glycerol/methanol medium containing \([\beta^{32}\text{P}])\text{orthophosphate for 16 h.} \] \( 32\text{P}\)-labeled \( H. \) polymorpha Pex14p was recovered from crude extracts by immunoprecipitation, separated by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography.

Electron Microscopy—Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described before (32). Immunolabeling was performed on ultrathin section of unicyl-embedded cells, using specific antibodies against various proteins and gold-conjugated goat anti-rabbit antibodies according to the instructions of the manufacturer (Amersham Pharmacia Biotech).

Results

In an initial series of experiments we have analyzed the fate of peroxisomes that had developed in cells of a \( \text{pex14} \) deletion strain that overproduced Pex5p (\( \Delta \text{pex14}: P_{\text{AOX}}\text{PEX}^{5*} \)), 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 (OD\( _{600} \)) of 1.5 before excess glucose (final concentration 0.5%) was added. As described before, glycerol/methanol-grown \( \Delta \text{pex14}: P_{\text{AOX}}\text{PEX}^{5*} \) cells contained several well developed peroxisomes (Fig. 1A) that lacked Pex14p but contained the bulk of two key enzymes in

![FIG. 1. In \( \Delta \text{pex14}: P_{\text{AOX}}\text{PEX}^{5*} \) selective peroxisome degradation is prevented.](image)

![FIG. 2. Western blots, prepared from crude extracts of glycerol/methanol-grown WT and \( \Delta \text{pex14}: P_{\text{AOX}}\text{PEX}^{5*} \) cells upon induction of selective peroxisome degradation by glucose.](image)

Table I

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<th>Phenotype of PEX14 mutants</th>
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+, normal; +/−, reduced; −, absent; perox., peroxisomal.
double band. Pex14p is observed as a PEX14 and were the sole sites of AO protein. In cells expressing by the presence of a cytosolic AO crystalloid. small peroxisomes that harbored a minor portion of AO protein judged minor AO import defect. Cells expressing PEX14 AO protein was mislocalized to the cytosol in these cells indicative of a ( ).

Fig. 3. Electron micrographs of WT and Δpex14 cells expressing mutant PEX14 genes, 16 h after induction of peroxisome biogenesis on glycerol-methanol-containing media to show the morphology of peroxisomes and localization of AO protein. N, nucleus; P, peroxisome; V, vacuole; *, alcohol oxidase crystalloid. The marker represents 0.5 μm. In cells expressing PEX14ΔC58 (B) peroxisomes were largely similar in size and number compared with WT (A) and were the sole sites of AO protein. In cells expressing PEX14ΔC124 (C) peroxisomes were also of normal size. However, a small portion of AO protein was mislocalized to the cytosol in these cells indicative of a minor AO import defect. Cells expressing PEX14ΔN31 (D) contained small peroxisomes that harbored a minor portion of AO protein judged by the presence of a cytosolic AO crystalloid.

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, selec-

Fig. 4. A, synthesis of WT and mutant Pex14ps in glycerol-/methanol-grown cells of various constructed strains. Shown are Δpex14, lane 1; WT, lane 2; ΔN31, lane 3; ΔN64, lane 4; ΔC124, line 5; and ΔC58, lane 6. Samples corresponding to three OD 600 units were collected, and trichloroacetic acid was precipitated. Equal amounts of protein were loaded per lane. The Western blot was decorated with α-Pex14p antibodies. The data show that the mutant proteins are synthesized and migrate to the expected molecular mass. In all samples, except ΔC124, Pex14p is observed as a double band. The minor protein band at ~25 kDa is a degradation product of Pex14p. B, in vivo [32P]orthophosphate labeling of WT and various mutant cells. Phosphate-depleted cells were incubated in glycerol/methanol media supplemented with [32P]orthophosphate for 16 h. 32P-labeled Pex14p was recovered from crude extracts by immunoprecipitation. A band corresponding to the 32P-labeled H. polymorpha Pex14p was not detectable in the Δpex14 control (lane 1) and in ΔC124 mutant cells (lane 5) but were present in WT (lane 2) and ΔN64 (lane 3) and ΔC58 (lane 4) mutant cells.

Fig. 5. Biochemical localization of Pex14p in WT and various mutants cells. Western blots were prepared from 30,000 × g organellar pellets (P l.) and the corresponding 30,000 × g supernatant fractions (S l.), which were obtained after differential centrifugation of homogenized protoplasts (S 0) from WT cells (A) and cells from the mutants ΔC58 (B), ΔC124 (C), and ΔN64 (D). Cells were grown for 16 h in glycerol/methanol media. Pex14p was almost exclusively localized in the organelar pellet fractions of all the strains.

Fig. 6. Immunocytochemical localization of Pex14p in WT and various mutant cells. M, mitochondrion; P, peroxisome; V, vacuole. The marker represents 0.1 μm. Cells were grown in glycerol-methanol-containing media for 16 h. A, WT control; B, ΔC58; C, ΔC124; and D, ΔN31. In all cases α-Pex14p-dependent labeling was localized at the membranes of peroxisomal profiles.

Fig. 7. Immunocytochemistry of WT and pex14 mutant cells expressing mutant PEX14 genes upon glucose-induced selective peroxisome degradation. N, nucleus; P, peroxisome; V, vacuole. *, alcohol oxidase crystalloid. The marker represents 0.5 μm. Ultrathin sections of unicryl-embedded cells were labeled with α-AO antibodies. A, 4 h after supplementing glucose to WT cells growing on glycerol/methanol, α-AO-specific labeling was observed on peroxisomes and in the vacuole indicative of peroxisome degradation. Similar observations were made for ΔC124 mutant cells (B). α-AO-specific labeling was invariably not observed in the vacuole of ΔN64 (C) and ΔN31 (D) mutant cells indicating that the degradation of peroxisomes was impaired.
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 pexophagy, 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 ∆pex14::P_AOXPEX5mc 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 ∆pex14::P_AOXPEX5mc cells were not susceptible to glucose-induced pexophagy.

Mutational Analysis of Pex14p—To delineate the region of Pex14p controlling pexophagy turnover, we constructed mutant genes that encoded various truncated Pex14ps and transformed these into a PEX14 deletion strain (∆pex14). These proteins lacked either the initial 31 or 64 N-terminal amino acids (designated ∆N31 and ∆N64, respectively) or the extreme 58 or 124 C-terminal amino acids (designated ∆C58 and ∆C124, respectively). A ∆pex14 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 ∆C58 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 ∆C58 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 ∆pex14 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 ∆C124, ∆N31, and ∆N64 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 a-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 ∆N64 and ∆C58 cells but not in ∆C124 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 localization 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 a-Pex14p antibodies, the specific labeling was exclusively localized on the peroxisomal membrane (Fig. 6). From this we concluded that all truncated Pex14pss (WT, ∆C58, ∆C124, ∆N31, and ∆N64) were normally synthesized and sorted to the correct target membrane (summarized in Table I).

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 the cell producing full-length Pex14p peroxisomes were normally degraded. A similar phenomenon was observed for ∆C58 and ∆C124 cells. 30 min after addition of glucose the first cells were observed that contained AO protein in the cytosol but not in peroxisomes. 1 h after addition of glucose the WT control cells contained cells with AO protein both in peroxisomes and the cytosol, whereas in all other strains the AO protein was found to be totally excluded from the peroxisomes (Fig. 7). These results indicate that the primary structure of Pex14p is critical for selective degradation of peroxisomes.

The alignment of conserved amino acids at the N-terminus of Pex14p from different species to the human amino acid sequence is shown in Fig. 9. This alignment revealed that the N-terminus has important functional implications for the interaction of Pex14 and other proteins. For instance, the N-terminal domain of Pex14p is homologous to the N-terminal domain of the eukaryotic translation initiation factor 4G, which is involved in the regulation of translation initiation and stalls translation of certain mRNAs (24). The unique functional implications of the N-terminal domain of Pex14p await further investigation.

Fig. 9. Alignment of the primary structures of the conserved N terminus of Pex14p. The N-terminal sequences from (putative) Pex14 proteins from H. polymorpha (Hp; GenBankTM accession number AAB40596, amino acid (aa) 1–58), P. pastoris (Pp; accession number AAI28574, aa 1–53), Aspergillus nidulans (An; translated EST, accession number AA866689, aa 1–57), Neurospora crassa (Nc; translated from accession number AL356172, aa 1–52), S. cerevisiae (Sc; accession number P53112, aa 1–57), Schizosaccharomyces pombe (Sp; accession number T39404, aa 1–50), Caenorhabditis elegans (Ce; accession number T24035, aa 1–56), Homo sapiens (Hs; accession number AAC89433, aa 16–73), and Drosophila melanogaster (Dm; accession number AAF51637, aa 25–82) were aligned using the ClustalX program. The one-letter code was used. Gaps were introduced to maximize the similarity. Conserved residues have been shaded, and the intensity of the shading depending on the degree of conservation.
vacuole (Fig. 7), a characteristic feature for selective peroxisome degradation under these conditions (6). It should be stressed that the cytosolic portion of AO is not subject to degradation 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 glucose, a rapid decrease in the levels of Pex10p and mutant Pex14ps were observed (Fig. 8). Opposite results were obtained in identical experiments, using ∆N31 and ∆N64 cells. In these cells, electron microscopical analysis failed to resolve any sign of peroxisome turnover in the first 4 h after addition of glucose (Fig. 7, C and D), a time interval that is sufficient to remove the bulk of the peroxisomal population in WT control cells. Also, the levels of Pex10p and mutant Pex14p hardly diminished in this period (Fig. 8). From this we conclude that the N-terminal deletions of Pex14p prevent glucose-induced pexophagy in H. polymorpha.

DISCUSSION

In this paper we have provided evidence that the H. polymorpha PEX14 gene product (HpPex14p) plays a role in the selective degradation of peroxisomes (pexophagy). We showed that considerable portions of the C terminus of the protein could be deleted without affecting PTS1 protein import (ΔC58 cells) and pexophagy (ΔC58 and ΔC124 cells). In contrast, deletion of the first 31 N-terminal amino acids affected both peroxisome biogenesis (by reducing the rate of PTS1 protein import) and selective peroxisome degradation (pexophagy). The general function of Pex14p in PTS1 and PTS2 matrix protein import as a component of the docking site for the cytosolic receptors of the PTS1 and PTS2 targeting signals was demonstrated before (3). Very recently, we showed, however, that in H. polymorpha Pex14p most likely is not essential for PTS1 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 biogenesis versus degradation (homeostasis) in one “switch” has obvious 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 methylotrophic yeast species. The conserved residues in peroxisome homeostasis in depth.

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