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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 Δ*pex14* 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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[1] The abbreviations used are: AO, alcohol oxidase; WT, wild-type; PCR, polymerase chain reaction; aa, amino acid; PTS, peroxisome-targeting signal.

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EXPERIMENTAL PROCEDURES

Organisms and Growth Conditions—All *H. polymorpha* strains used in this study are derivatives of strain NCYC495 (*leu1.1 ura3*) (25): Δ*pex14*(*leu1.1*) (20), Δ*pex14*::P_{AOX}.*PEX5*^{mc} (4), and Δ*pex14* strains expressing full-length or truncated forms of *PEX14* under control of the *PEX14* promoter (P_{PEX14}) (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*) mineral 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 precultivated 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 than added to induce selective peroxisome degradation. *Escherichia coli* DH5α (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 *PEX14* in Δ*pex14* cells under control of its endogenous promoter, we first constructed the expression vector pHIPX10, a derivative of pHIPX4-B (20). The *PEX14* promoter was isolated by PCR using the P_{PEX14} primer (5′ GGG GAT CCG GTG AGG AAG AAA AAG AG 3′), the M13/pUC universal primer, and plasmid pBS3.2P14 (20) as template. The PCR fragment was digested with *Eco*RV and *Bam*HI and inserted between the *Not*I (blunted) and *Bam*HI sites of plasmid pHIPX4-B thus replacing the alcohol oxidase promoter. To obtain pX10-PEX14-WT we inserted the 1.5-kilobase *Bam*HI-*Eco*RV fragment of pP_{AOX}PEX14 (20) between the *Bam*HI and *Sac*I sites of pHIPX10. Plasmids expressing truncated versions of *PEX14* were constructed as follows: pX10-PEX14-ΔC58 was constructed by filling in the *Eco*RI site in the *PEX14* coding region followed by self-ligation. Similarly, pX10-PEX14-ΔC124 was obtained by blunting the *Pst*I 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 TTT C 3′) or the N64 primer (5′ AA GGA TCC **ATG** TCA CAG CAG TCC GTT GTA 3′) in combination with the M13/pUC sequencing primer and pBS1.3P14 as template. Subsequently, for pX10-PEX14-ΔN31, a *Bam*HI + *Hin*dIII-digested PCR fragment was cloned between the *Bam*HI and *Hin*dIII sites of pHIPX10. For pX10-PEX14-ΔN64 a *Bam*HI + *Eco*RV-digested PCR fragment was cloned between the *Bam*HI and *Sma*I sites of pHIPX10. Plasmids were transformed into *H. polymorpha* Δ*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 [^{32}P]orthophosphate for 16 h. ^{32}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 detailed before (32). Immunolabeling was performed on ultrathin section of unicryl-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 *pex14* deletion strain that overproduced Pex5p (Δ*pex14*::P_{AOX}.*PEX5*^{mc}), (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 (OD_{663}) of 1.5 before excess glucose (final concentration 0.5%) was added. As described before, glycerol-/methanol-grown Δ*pex14*::P_{AOX}.*PEX5*^{mc} cells contained several well developed peroxisomes (Fig. 1*A*) that lacked Pex14p but contained the bulk of two key enzymes in

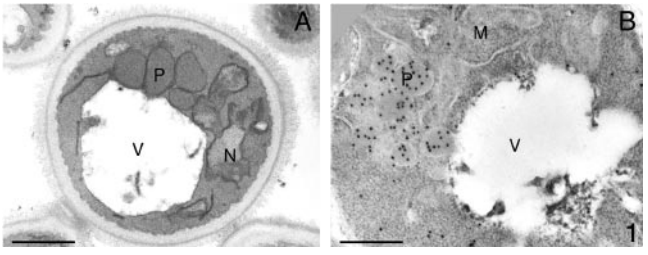

FIG. 1. **In Δ*pex14*::P_{AOX}.*PEX5*^{mc} selective peroxisome degradation is prevented.** Electron micrographs are taken of cells, fixed with glutaraldehyde, and embedded in unicryl for immunocytochemistry, unless otherwise indicated. *M*, mitochondrion; *N*, nucleus; *P*, peroxisome; *V*, vacuole. The marker represents 0.5 μm. *A*, overall morphology of $KMnO_4$-fixed mutant cells, grown in glycerol-/methanol-containing media. The cells contain several medium-sized peroxisomes. *B*, these organelles are not degraded upon a shift of cells to glucose-excess conditions. 4 h after the shift α-AO-dependent labeling was not detectable in the vacuole.

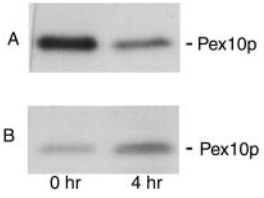

FIG. 2. **Western blots, prepared from crude extracts of glycerol-/methanol-grown WT and Δ*pex14*::P_{AOX}.*PEX5*^{mc} cells upon induction of selective peroxisome degradation by glucose.** Blots were decorated with antibodies against the peroxisomal membrane protein Pex10p. Equal volumes of samples were taken 0 and 4 h after the addition of glucose, and trichloroacetic acid was precipitated. For the WT (*A*) strain a strong reduction of the Pex10p level is observed whereas in the mutant (*B*) strain the Pex10p level has increased indicative for the inhibition of peroxisome degradation.

TABLE I
Phenotype of PEX14 mutants

+, normal; +/−, reduced; −, absent; perox., peroxisomal.

Strain	Pex14p level	Pex14p location	Growth on methanol	AO import	Phosphorylation	Pexophagy
WT	+	perox.	+	+	+	+
ΔN31	+	perox.	−	+/−	+	−
ΔN64	+	perox.	−	+/−	+	−
ΔC58	+	perox.	+	+	+	+
ΔC124	+	perox.	−	+/−	−	+

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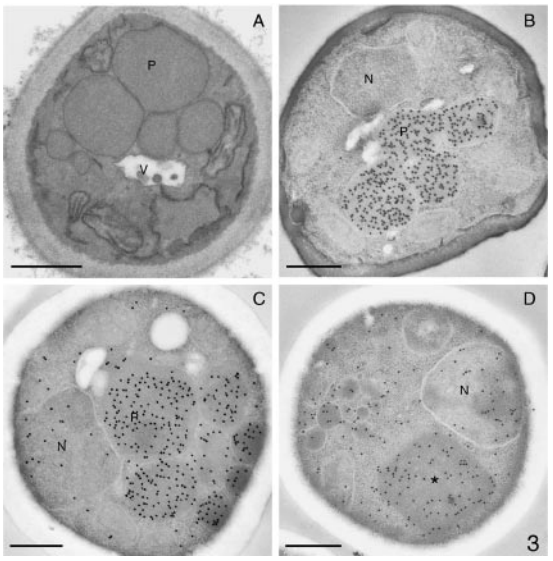

FIG. 3. **Electron micrographs of WT and Δ*pex14* cells expressing mutant *PEX14* genes, 16 h after induction of peroxisome biogenesis on glycerol-/methanol-containing media to show the morphology of peroxisomes and localization of AO protein.** *N*, nucleus; *P*, peroxisome; *V*, vacuole; *, alcohol oxidase crystalloid. The marker represents 0.5 μm. In cells expressing *PEX14*ΔC58 (*B*) peroxisomes were largely similar in size and number compared with WT (*A*) and were the sole sites of AO protein. In cells expressing *PEX14*ΔC124 (*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 *PEX14*ΔN31 (*D*) contained small peroxisomes that harbored a minor portion of AO protein judged by the presence of a cytosolic AO crystalloid.

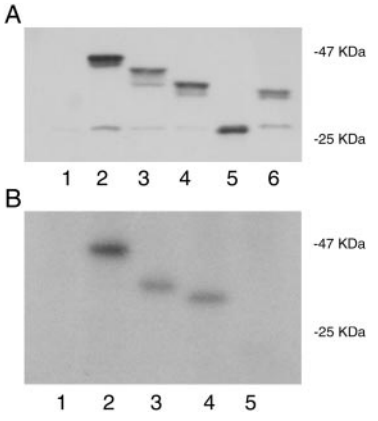

FIG. 4. *A*, synthesis of WT and mutant Pex14ps in glycerol-/methanol-grown cells of various constructed strains. Shown are Δ*pex14*, *lane 1*; WT, *lane 2*; ΔN31, *lane 3*; ΔN64, *lane 4*; ΔC124, line 5; and ΔC58, *lane 6*. Samples corresponding to three OD_{660} units were collected, and trichloroacetic 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* [^{32}P]orthophosphate labeling of WT and various mutant cells. Phosphate-depleted cells were incubated in glycerol/methanol media supplemented with [^{32}P]orthophosphate for 16 h. ^{32}P-labeled Pex14p was recovered from crude extracts by immunoprecipitation. A band corresponding to the ^{32}P-labeled *H. polymorpha* Pex14p was not detectable in the Δ*pex14* 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.

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

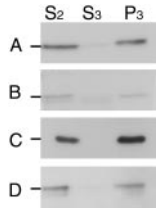

FIG. 5. **Biochemical localization of Pex14p in WT and various mutants cells.** Western blots were prepared from 30,000 × *g* organellar pellets (P_3) and the corresponding 30,000 × *g* supernatant fractions (S_3), which were obtained after differential centrifugation of homogenized protoplasts (S_2) 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 organellar pellet fractions of all the strains.

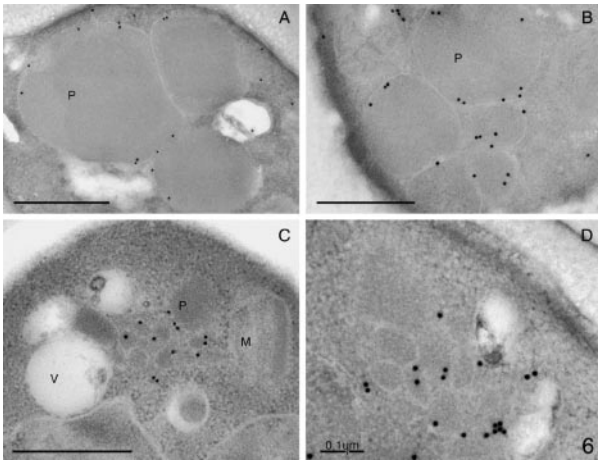

FIG. 6. **Immunocytochemical localization of Pex14p in WT and various mutant cells.** *M*, mitochondrion; *P*, peroxisome; *V*, vacuole. The marker represents 0.1 μm. Cells were grown in glycerol-/methanol-containing media for 16 h. *A*, WT control; *B*, ΔC58; *C*, ΔC124; and *D*, ΔN31. In all cases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

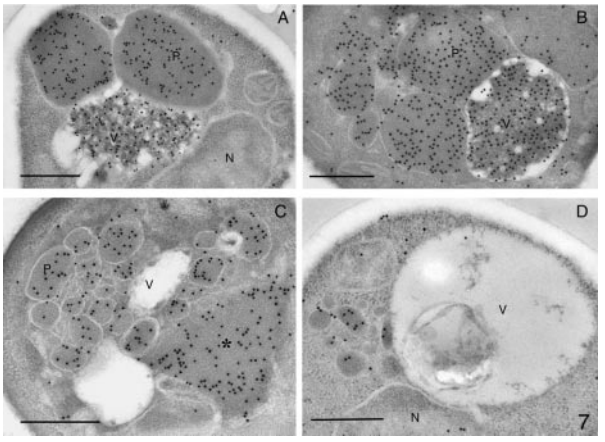

FIG. 7. **Immunocytochemistry of WT and *pex14* mutant cells expressing mutant *PEX14* genes upon glucose-induced selective peroxisome degradation.** *N*, nucleus; *P*, peroxisome; *V*, vacuole. *, alcohol oxidase crystalloid. The marker represents 0.5 μm. Ultrathin sections of unicryl-embedded cells 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.

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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. 1*B*). 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 Δ*pex14*::P_{AOX}.*PEX5*^{mc} 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 Δ*pex14*::P_{AOX}.*PEX5*^{mc} cells were not susceptible to glucose-induced pexophagy.

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 (Δ*pex14*). 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 Δ*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 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 Δ*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 ΔC124, ΔN31, and ΔN64 cells most likely explains the failure of the cells to grow on methanol.

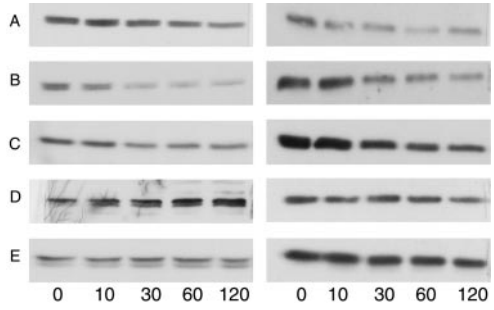

FIG. 8. **Western blots to demonstrate the fate of Pex14p and Pex10p upon a shift of cells from glycerol/methanol to glucose-excess conditions.** Shown are WT (*A*) and *pex14* mutant strains ΔC58 (*B*), ΔC124 (*C*), ΔN31 (*D*), and ΔN64 (*E*). Samples were takes at various time points (indicated in min) after the shift. To correct for growth, equal volumes of cell cultures were trichloroacetic acid-precipitated. Subsequently, equally volumes of crude extract were loaded per *lane*. Western blots were decorated with α-Pex14p antibodies (*left panel*) and α-Pex10p antibodies (*right panel*). The data show that, in contrast to WT and ΔC58- and ΔC124-producing cells, in ΔN31- and ΔN64-producing cells, the amounts of Pex14p and Pex10p did not diminish, indicating that degradation had not occurred.

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. 4*A*). 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. 4*B*, 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).

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

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HpPex14p : MSQQPATTSRAELVSSAVEFLLDQSLADSPLAKKVEFLESKGLTQQEIEEALQKARTG
PpPex14p : MS-----SIREEMVTSAVEFLKNPQLADSPLAKKIEFIESKGLNEAEVKEALLRSQGG
AnPex14p : -------MVREELISSAVTFLQDPSVASAPIEKRISFLQSKNLTKEEIDVALARAGED
NcPex14p : ------MAIREDLVASAAQFLGDPSVAASPIEKRIAFLQAKNLTQEEVSAALARAESG
ScPex14p : -MSDVVSKDRKALFDSAVSFLKDESIKDAPLLKKIEFLKSKGLTEKEIEIAMKEPKKD
SpPex14p : --------MREDLLRNSVEFLREKTVLDAPDVKKIEFLKSKGLTAEEIQEAFKLAKNP
CePex14p : MTDPSSSPIRPDMVEAARKFMLTPKVKETPFEEQRQFLLGKGVSEAEILEA--RASIP
HsPex14p : TPGSENVLPREPLIATAVKFLQNSRVRQSPLATRRAFLKKKGLLDEEIDMAFQQSGTA
DmPex14p : EAGVDEQLPRESLITTAVSFLQNTKVRHTTLIQKQQFLRSKGLLAHEIQLACERAGVF
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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*; GenBank™ accession number AAB40596, amino acid (aa) 1–58), *P. pastoris* (*Pp*; accession number AAG28574, aa 1–53), *Aspergillus nidulans* (*An*; translated EST, accession number AA966699, 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 AAC39843, 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.

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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 ΔN31 and ΔN64 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 methanol-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 cytosolic hydrogen peroxide metabolism (19).

In *H. polymorpha* pexophagy is a remarkable fast process; individual organelles can be degraded within a time interval of 15 min. Thus, a molecular switch controlling organelle homeostasis (both biogenesis and degradation) allows taking rapid decisions and consequently, may be of great value as a component in the control of cell viability. In surface plasmon resonance analyses Schliebs and colleagues (23) demonstrated that the N terminus of human Pex14p could efficiently bind to the PTS1 receptor, Pex5p . This indicates that the N terminus of the protein may be a prime target for the PTS1 receptor-cargo complex. These data are in line with our observations that N-terminal deletions of both 31 or 64 amino acids resulted in a partial AO protein-import defect. However, the same stretch of N-terminal amino acids also appears to control pexophagy. The mechanisms on how the discrimination is made between these functions of HpPex14p are still largely speculative. One possible 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 organellar matrix. On the topology of Pex14p in yeast nothing is known yet; only for human Pex14p has evidence been presented that the N terminus of the protein is (at least in part) exposed to the lumen of the organelle (24). Our experiments strongly suggest that modification of the protein by phosphorylation is most likely not discriminative in this respect.

Sequence comparison of the various Pex14ps currently available, reveals that the similarity predominantly resides in the N-terminal part of the protein (Fig. 9). This similarity becomes even stronger when only the proteins of the methylotrophic yeast species *H. polymorpha* and *P. pastoris* are compared. We have now initiated further studies to analyze the significance of the conserved residues in peroxisome homeostasis in depth.

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REFERENCES

1. Hansen, H., Didion, T., Thiemann, A., Veenhuis, M., and Roggenkamp, R. (1992) *Mol. Gen. Genet.* **235,** 269–278
2. Didion, T., and Roggenkamp, R. (1992) *FEBS Lett.* **303,** 113–116
3. Subramani, S., Koller, A., and Snyder, W. B. (2000) *Annu. Rev. Biochem.* **69,** 399–418
4. Salomons, F. A., Kiel, J. A. K. W., Faber, K. N., Veenhuis, M., and van der Klei, I. J. (2000) *J. Biol. Chem.* **275,** 12603–12611
5. Bellu, A. R., Kram, A. M., Kiel, J. A. K. W., Veenhuis, M., and van der Klei, I. J. (2001) *FEMS Yeast Res.* **1,** 1–9
6. Veenhuis, M., Douma, A. C., Harder, W., and Osumi, M. (1983) *Arch. Microbiol.* **134,** 193–203
7. Tuttle, D. L., Lewin, A. S., and Dunn, W. A., Jr. (1993) *Eur. J. Cell Biol.* **60,** 283–290
8. Tuttle, D. L., and Dunn, W. A., Jr. (1995) *J. Cell Sci.* **108,** 25–35
9. Gunkel, K., van der Klei, I. J., Barth, G., and Veenhuis, M. (1999) *FEBS Lett.* **451,** 1–4
10. Yuan, W., Tuttle, D. L., Shi, Y. J., Ralph, G. S., and Dunn, W. A., Jr. (1997) *J. Cell Sci.* **110,** 1935–1945
11. Yuan, W., Stromhaug, P. E., and Dunn, W. A., Jr. (1999) *Mol. Biol. Cell* **10,** 1353–1366
12. Sakai, Y., Koller, A., Rangell, L. K., Keller, G. A., and Subramani, S. (1998) *J. Cell Biol.* **141,** 625–636
13. Sakai, Y., and Subramani, S. (2000) *Cell Biochem. Biophys.* **32**, 51–61
14. Titorenko, V. I., Keizer, I., Harder, W., and Veenhuis, M. (1995) *J. Bacteriol.* **177,** 357–363
15. Kiel, J. A. K. W., Rechinger, K. B., van der Klei, I. J., Salomons, F. A., Titorenko, V. I., and Veenhuis, M. *(1999) Yeast* **15,** 741–754
16. Klionsky, D. J., and Ohsumi, Y. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 1–32., 1–32
17. Kim, J., and Klionsky, D. J. (2000) *Annu. Rev. Biochem.* **69 303–42,** 303–342
18. Veenhuis, M., Komori, M., Salomons, F. A., Hilbrands, R. E., Hut, H., Baerends, R. J. S., Kiel, J. A., and van der Klei, I. J. (1996) *FEBS Lett.* **383,** 114–118
19. van der Klei, I. J., Harder, W., and Veenhuis, M. (1991) *Arch. Microbiol.* **156,** 15–23
20. Komori, M., Rasmussen, S. W., Kiel, J. A., Baerends, R. J., Cregg, J. M., van der Klei, I. J., and Veenhuis, M. *(1997) EMBO J.* **16,** 44–53
21. van der Klei, I. J., Harder, W., and Veenhuis, M. (1991) *Yeast* **7,** 813–821
22. Sepulveda, S. J., van der Klei, I. J., Keizer, I., Lopez, A. P., Harder, W., and Veenhuis, M. (1992) *FEMS Microbiol. Lett.* **91,** 207–212
23. Schliebs, W., Saidowsky, J., Agianian, B., Dodt, G., Herberg, F. W., and Kunau, W. H. (1999) *J. Biol. Chem.* **274,** 5666–5673
24. Will, G. K., Soukupova, M., Hong, X., Erdmann, K. S., Kiel, J. A. K. W., Dodt, G., Kunau, W. H., and Erdmann, R. (1999) *Mol. Cell. Biol.* **19,** 2265–2277
25. Gleeson, M. A. G., and Sudbery, P. E. (1988) *Yeast* **4,** 293–303
26. van Dijken, J. P., Otto, R., and Harder, W. (1976) *Arch. Microbiol.* **111,** 137–144
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Pess, Cold Spring Harbor, New York
28. Faber, K. N., Haima, P., Harder, W., Veenhuis, M., and AB, G. (1994) *Curr. Genet.* **25,** 305–310
29. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25,** 4876–4882
30. Baerends, R. J. S., Faber, K. N., Kram, A. M., Kiel, J. A. K. W., van der Klei, I. J., and Veenhuis, M. (2000) *J. Biol. Chem.* **275,** 9986–9995
31. Komori, M., Kiel, J. A. K. W., and Veenhuis, M. (1999) *FEBS Lett.* **457,** 397–399
32. Waterham, H. R., Titorenko, V. I., Haima, P., Cregg, J. M., Harder, W., and Veenhuis, M. (1994) *J. Cell Biol.* **127,** 737–749

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