The Hansenula polymorpha PER9 Gene Encodes a Peroxisomal Membrane Protein Essential for Peroxisome Assembly and Integrity

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We have cloned and characterized the Hansenula polymorpha PER9 gene by functional complementation of the per9-1 mutant of H. polymorpha, which is defective in peroxosome biogenesis. The predicted product, Per9p, is a polypeptide of 52 kDa with sequence similarity to Pas3p, a protein involved in peroxisome biogenesis in Saccharomyces cerevisiae. In a per9 disruption strain (∆per9), peroxisomal matrix and membrane proteins are present at wild-type levels. The matrix proteins accumulated in the cytoplasm. However, the location of the membrane proteins remained obscure; fully induced ∆per9 cells lacked residual peroxisomal vesicles ("ghosts"). Analysis of the activity of the PER9 promoter revealed that PER9 expression was low in cells grown on glucose, but was enhanced during growth of cells on peroxisome-inducing substrates. The highest expression levels were observed in cells grown on methanol. Localization studies revealed that Per9p is an integral membrane protein of the peroxisome. Targeting studies suggested that Per9p may be sorted to the peroxisome via the endoplasmic reticulum. Overexpression of PER9 induced a significant increase in the number of peroxisomes per cell, a result that suggests that Per9p may be involved in peroxisome proliferation and/or membrane biosynthesis. When PER9 expression was placed under the control of a strongly regulatable promoter and switched off, peroxisomes were observed to disintegrate over time in a manner that suggested that Per9p may be required for maintenance of the peroxisomal membrane.

Peroxisomes are cell organelles that are present in virtually all eukaryotic cells. They perform specific metabolic functions that are often related to the developmental stage and/or the organism in which they occur (1). The metabolic importance of peroxisomes in humans is demonstrated by the fact that the absence of the organelles leads to severe abnormalities, followed by an early death (e.g. Zellweger syndrome (2)). Consequently, many studies are now devoted to unravel the molecular mechanisms of peroxisome biogenesis and function. Yeasts are excellent model systems for such studies having the advantages that (i) the induction and protein composition of peroxisomes can readily be manipulated by varying growth conditions and (ii) in the absence of peroxisomes, yeasts are viable (3, 4). Hence, peroxisome-deficient mutants have been isolated from different yeast species (4), and the corresponding genes are being cloned and characterized.

In yeast, peroxisomes normally develop by growth and fission from pre-existing ones. Peroxisomal matrix proteins are nuclear-encoded, synthesized in the cytoplasm, and directed to the organelle by topogenic signals (PTSs).1 Two PTSs have been identified and are located either at the extreme C terminus (PTS1) or the N terminus of the protein (PTS2) (4). Our knowledge on the sorting of peroxisomal membrane proteins is still limited, and consensus topogenic sequences have yet to be identified (5).

In our laboratory, we use the methylotrophic yeast Hansenula polymorpha as the model organism for studies on peroxisome biogenesis and function. We have isolated a collection of peroxisome-deficient (per) mutants of this organism that comprises 28 different complementation groups and that includes constitutive and conditional (temperature-sensitive) mutants. In previous reports, we described the cloning and characterization of three H. polymorpha PER genes, namely PER1, PER3, and PER8 (6–8), the protein products of which are part of the protein import (Per1p and Per3p) (6, 7) or peroxisome proliferation machinery (Per8p) (8). Here, we describe the molecular cloning and characterization of the PER9 gene and its protein product (Per9p). Per9p shares sequence similarity with Saccharomyces cerevisiae Pas3p (9) and plays a key role in peroxisome biogenesis and maintenance.

EXPERIMENTAL PROCEDURES

Organisms and Growth Conditions—Escherichia coli strain DH5a was cultivated as described (10). H. polymorpha was grown in batch cultures at 37 °C on (a) selective minimal medium containing 0.67% yeast nitrogen base without amino acids supplemented with 1% glucose or 0.5% methanol (YNM); (b) medium containing 1% yeast extract, 2% peptone, and 1% glucose; or (c) mineral medium (11) supplemented with 0.5% carbon source and 0.25% nitrogen source. Carbon sources used were glucose, glycerol, ethanol, and methanol; as nitrogen sources, methionine, o-alanine, and ammonium sulfate were added. In addition, cells were grown in continuum cultures at 37 °C in mineral medium (11) on a mixture of 0.25% glucose and 0.25% methanol. When

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1 The abbreviations used are: PTS, peroxisomal topogenic signals; kb, kilobase; PADOX, alcohol oxidase promoter; Pamox, amine oxidase promoter; PPER9, PER9 promoter; AO, alcohol oxidase; CAT, catalase; ER, endoplasmic reticulum.
needed, amino acids and uracil were added to a final concentration of 40 μg ml⁻¹. For growth on agar plates, all media were supplemented with 1.5% granulated agar. The following H. polymorpha strains were used in this study: (i) per9-1 (Ecoli1.1 per9) (12), (ii) NNCYC495 (Ecoli1.1 ura3), (iii) NNCYC495 cat::LEU2 (ura3 Δcat), (iv) per9::LEU2 (ura3 Δper9), and (v) per9::URA3 (Ecoli1.1 Δper9) (this study).

Isolation and Characterization of the PER9 Gene—Genetic manipulations of H. polymorpha were performed as described previously (12–15). H. polymorpha was transformed as detailed by Faber et al. (16). Standard recombinant DNA techniques were carried out essentially as described (10).

To clone the PER9 gene, mutant per9-1 was transformed with an H. polymorpha genomic library constructed in vector pY3T (8). Leucine prototrophic transformants were screened on YMN plates for the ability to grow on methanol. The complementing plasmids of positive clones were rescued and transferred to E. coli DH5α (15). To facilitate sequencing, restriction analysis, and construction of subclones, a 2.7-kb complementing DNA fragment was subcloned as a SalI fragment into Sall-digested phagemid pBluescript II KS⁺ (Stratagene, La Jolla, CA) in both orientations. Sequencing of both strands was carried out on an Applied Biosystems 373A automatic sequencer using the Taq dye deoxy terminator cycle sequencing kit supplied by the manufacturer. For DNA and amino acid sequence analysis, the PC-GENE™ program (Research Genetics, Mountain View, CA) was used. The TBLASTN algorithm (17) was used to search the GenBank™ database (Release 91.0, October 15, 1995) for DNA and protein sequences showing similarity to the PER9 gene and its protein product.

PER9 Disruption—For disruption of the PER9 gene, either the Candida albicans LEU2 gene (obtained from Dr. E. Berardi, University of Ancona, Ancona, Italy) or the H. polymorpha URA3 gene (18) was used. The LEU2 gene was isolated as an EcoRI (blunt-ended)-BamHI fragment and ligated between the BglII and Stul sites of the 2.7-kb PER9 fragment in pBluescript II KS⁺, deleting half of the PER9 gene. The URA3 gene was isolated as a BamHI (blunt-ended) fragment and ligated between the same BglII (blunt-ended) and Stul sites. Disruption fragments (see Fig. 2B) were isolated by digestion with Tth111 (per9::LEU2) and XhoI (per9::URA3) and used to transform H. polymorpha NNCYC495 (Ecoli1.1 ura3). Methanol utilization-defective strains (Mut⁻) were selected, and correct integration of LEU2 and URA3 was determined by Southern blot analysis using the ECL direct nucleic acid labeling and detection system (Amersham Corp.). Selected per9 disruption strains (Δper9) were also tested for complementation by the per9-1 complementing fragment. To identify putatively peroxisomal membranes resident in Δper9 cells, a per9::URA3 disruption strain was transformed with pET4 (8), which contains the H. polymorpha PER8 gene under the control of the alcohol oxidase promoter (Perox). Construction of PER9-Catalase Fusions—Fusions were constructed between PER9 and a catalase gene (catalase A) from which the PTS1 function was destroyed by changing the last triplet coding for isoleucine to one coding for lysine (CAT(ΔPTS1)) (19). An HindII site (5-primer, 5'-GGAGGTCATTGCAACCCCTCCCTT-3') was introduced by primer PCR chain reaction directly 5' of the ATG start codon of the mutant CAT gene present on plasmid pHCAT-K (a gift from Dr. R. Roepenekamp, Heinrich Heine University, Düsseldorf, Germany). The CAT(ΔPTS1) gene was cloned in frame downstream fragments encoding selected portions of Per9p using (i) the HindIII N(NcPer9p-CAT(ΔPTS1)); HindIII site of CAT(ΔPTS1), (ii) NheI (blunt-ended) NcPer9p-CAT(ΔPTS1); HindIII site of CAT (blunt-ended), and (iii) HindIII NcPer9p-CAT(ΔPTS1); HindIII terminus of CAT made blunt-ended by mung bean exonuclease in the PER9 gene. Plasmid pHCAT-K, which had the H. polymorpha URA3 gene had been inserted was used to replace the chimeric PER9-CAT(ΔPTS1) genes behind Perox. The resulting plasmids were used to transform an H. polymorpha catalase disruption mutant (Δcat).

Deletion of the Membrane-spanning Region of Per9p—Deletion of DNA sequences encoding the predicted membrane-spanning region of Per9p (amino acids 16–36) was carried out in several steps. First, an HindII site in the 2.7-kb per9 complementing fragment (in pBluescript II KS⁺) was cut, followed by treatment with Klenow polymerase and religation, thereby generating a new Nhel site. Next, the resulting plasmid was Nhel-digested and religated. This resulted in the deletion of a Nhel fragment encoding 21 amino acids (amino acids 16–36). A fragment containing the PER9 promoter and the mutant PER9 gene was subcloned into pHIPX3 after digestion of both plasmids with BamHI and SalI. The pHIPX3 shuttle vector was constructed by cloning a 5.7-kb BamHI-Nrul (blunt-ended) fragment from pHIPX1 (20) into BamHI- and Stul-digested pOK12 (21). The final plasmid carrying the mutant PER9 gene was introduced into the per9::URA3 disruption mutant and analyzed for its ability to restore peroxisome formation. Expression of PER9—Functional Mutants of PER9—Overexpression of PER9, the plasmid pHIPX4-PER9 (22), containing PER9 behind Pperox was transformed into H. polymorpha leu1.1. To study the reapearence of peroxisomes in relation to the reintroduction of Per9p, the PER9 gene was placed under the control of the substrate-inducible H. polymorpha amine oxidase promoter (Pama).Pama is fully repressed by ammonium ions, but is induced by primary amines (23, 24). Plasmid pHIPX5-PER9 (22), containing PER9 under the control of Pama, was linearized with SacI and transformed to the per9::URA3 disruption mutant. Correct integration of the plasmid into the genome at the Pama locus was confirmed by Southern blot analysis.

Generation of Antiserum—For synthesis and purification of Per9p, the protein fusion and purification system supplied by New England Biolabs Inc. (Beverly, MA) was used. An Ahal-Sall PER9 fragment (in pBluescript II KS⁺) was subcloned in frame behind the malE gene in pMAL-C2 digested with EcoRI and treated with mung bean exonuclease and then deaved with Sall. Expression of the malE-PER9 chimeric gene under the control of the tac promoter was induced by addition of isopropyl-β-D-thiogalactopyranoside (1 mM) to exponentially growing cultures. The hybrid protein was purified according to the manufacturer (New England Biolabs Inc.) and used for immunization of a rabbit.

Expression Levels of the PER9 Gene—The PER9 expression levels were studied using β-lactamase as reporter protein in H. polymorpha wild-type cells. For this purpose, the E. coli β-lactamase gene was cloned in frame with part of the PER9 gene, encoding the first 7 amino acids (using the BglII site), expressed under the control of Pperox for details, see Ref. 6).

Biochemical Methods—Crude extracts were prepared according to Ref. 25. Cell fractionation was performed as described previously (26), except that 1 mM phenylmethylsulfonfluryl fluoride and 2.5 μg ml⁻¹ leupeptin were added to all solutions. Peroxosomal peak fractions were subjected to carbonatodextrization (27) or high salt treatment (incubation in 0.5 M sodium chloride in 50 mM potassium phosphate, pH 7.5, for 30 min at 4 °C, followed by centrifugation for 30 min at 100,000 × g and 4 °C). Enzyme activities of alcohol oxidase (28), catalase (29), and β-lactamase (6) were assayed by established procedures. Protein concentrations were determined using the Protoblot immunoblotting system (Promega) and specific polyclonal antibodies against selected H. polymorpha peroxisomal proteins.

Immunocytochemistry and Immunoelectron Microscopy—Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as detailed before (6). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against various peroxisomal proteins and gold-conjugated goat anti-rabbit antibodies (6).

RESULTS

Cloning of PER9 by Functional Complementation—The original nitrosoquinidine-induced per9-1 mutant of H. polymorpha was unable to grow on methanol (Mut⁻ phenotype) and lacked peroxisomes (Fig. 1A). The PER9 gene was cloned after transformation of the per9-1 mutant with an H. polymorpha genomic DNA library using restoration of the Mut⁺ phenotype as selection criterion.

From 10⁵ colonies, three Mut⁻ transformants were selected, each of which carried the library vector pH3T with a 7.5-kb insert. By subcloning, the complementing activity was found to reside within a 2.7-kb SalI fragment. Sequence analysis of the 2.7-kb complementing fragment revealed two open reading frames of 1374 and 621 base pairs (Fig. 2A). Further subcloning analysis demonstrated that the 1374-base pair open reading frame represented the complementing gene. This 1374-base pair open reading frame, hereafter referred to as the PER9 gene, encoded a putative protein (Per9p) of 457 amino acids with a calculated mass of 52 kDa. Hydrophy analysis (32) predicted one membrane-spanning region (amino acids 16–36)
H. polymorpha PER9 Gene

**Fig. 1.** Ultrathin sections of methanol-incubated cells of the original per9-1 mutant and the wild-type strain of H. polymorpha. The per9-1 mutant (A) lacks peroxisomes, which are evident in the wild-type cell (B) (KMnO₄). Electron micrographs are taken from aldehyde-fixed cells, unless otherwise indicated. Bar = 0.5 μm.

**Fig. 2.** Schematic representations of the 2.7-kb per9-1 complementing fragment containing open reading frame 2 (ORF2) and the PER9 gene (A) and the disruption of PER9 using C. albicans LEU2 or H. polymorpha URA3 (B). The SalI site to the left originates from vector pY73. The nucleotide sequence has been deposited in the GenBank Data Bank under accession number U37763.

and one membrane-associated region (amino acids 159–179). A database search revealed strong similarity to the integral peroxisomal membrane protein Pas3p of S. cerevisiae (Fig. 3) (9). H. polymorpha Per9p and S. cerevisiae Pas3p display a similar hydrophathy pattern, and both have one predicted membrane-spanning region and one membrane-associated region.

Construction and Characterization of a PER9 Disruption Mutant—To study the effect of the absence of Per9p on peroxisome biogenesis, an H. polymorpha per9 disruption strain was constructed in which 691 base pairs of H. polymorpha URA3 (amino acids 7–236) were deleted and replaced with a DNA fragment encoding the C. albicans LEU2 gene. This strain, Δper9, showed absolute cosegregation of the Mut- and Leu- phenotypes during random spore analysis of diploids obtained after mating the strain with auxotrophic H. polymorpha wild-type strains. Diploids obtained from crossing of the Δper9 strain with the per9-1 mutant were Mut+ as well. Together, these genetic results demonstrated that per9-1 and Δper9 were closely linked and most likely alleles of the same gene. Thus, the cloned DNA fragment encodes the gene that is defective in per9-1 and not a suppressor gene.

The Δper9 strain grew at a wild-type rate on rich medium (e.g. glucose/ammonium sulfate) and also on selected carbon (e.g. ethanol) and nitrogen (e.g. d-alanine and methyl- and ethylamine) sources known to require the activity of peroxisomal enzymes (33). However, growth on methanol as sole source of carbon and energy was totally defective (data not shown). Electron microscopic analysis of thin sections of Δper9 cells demonstrated that under all growth conditions employed, intact peroxisomes were invariably absent (data not shown). The subcellular morphology of Δper9 was studied in cells grown in a continuous culture on a glucose/methanol mixture. Enzyme activity measurements (data not shown) and Western blot analysis (Fig. 4) revealed that each peroxisomal protein examined (alcohol oxidase (AO), catalase (CAT), dihydroxyacetone synthase, malate synthase, Per8p (8), and Per10p) was present at normal wild-type levels. Ultrastructural analysis showed that peroxisomes were absent. As we have observed in other H. polymorpha per9 mutants (34), the Δper9 cells contained a large cytoplasmic crystalloid (Fig. 5A). Immunocytochemistry indicated that the other matrix proteins were also not in peroxisomes, but were located in the cytoplasm (shown for AO and CAT in Fig. 5, B and C, respectively; the wild-type CAT control is shown in Fig. 5F). Membranous remnants of peroxisomes (vesicles, ghosts) were not observed. In addition, overproduction of the H. polymorpha peroxisomal membrane protein Per8p (8) also failed to resolve such structures. Instead, Per8p was found in small aggregates that were often associated with a mitochondrial profile (Fig. 5E). Since peroxisomal vesicles are readily detected in other per disruption strains overexpressing

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3 M. Komori and M. Veenhuis, unpublished results.

**Fig. 3.** Amino acid sequence alignment of H. polymorpha Per9p (HpPer9p) and S. cerevisiae Pas3p (ScPas3p). The putative membrane-spanning region and membrane-associated region in both proteins are underlined. Identical residues are indicated by asterisks, and similar ones by dots. Gaps were introduced to maximize the similarity.

**Fig. 4.** Protein levels of peroxisomal matrix and membrane proteins in H. polymorpha Δper9 and wild-type cells. Western blots were prepared of crude extracts from methanol-grown wild-type (WT) or Δper9 cells and decorated with antibodies against H. polymorpha AO, CAT, dihydroxyacetone synthase (DHAS), malate synthase (MAS), Per8p, and Per10p. 30 (AO, CAT, dihydroxyacetone synthase, and MAS), 40 (Per8p and Mas), and 60 (Per8p) μg of protein were loaded on each lane.

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PER8 (e.g., \( \text{per} \)) (6, 35), we concluded that membranous remnants were either absent in \( \text{per} \) cells or present at a level below the limit of detection of the ultrastructural methods.

Subcellular Location of Per9p—The subcellular location of Per9p was studied by both conventional cell fractionation and ultrastructural techniques. Antibodies were raised against Per9p synthesized in \( \text{E. coli} \). On Western blots decorated with anti-Per9p antiserum, a dominant protein band of \( 59 \text{kDa} \) was found in crude extracts of \( \text{H. polymorpha} \) wild-type and \( \text{PER9} \)-overexpressing cells (Fig. 6A). Western blot analysis of organellar peak fractions, obtained after sucrose density centrifugation of organellar pellets prepared from homogenates of methanol-grown wild-type \( \text{H. polymorpha} \), showed that Per9p was pelletable and cosedimented with the peroxisomal peak fraction, characterized by the presence of Per8p (Fig. 6B). After high salt treatment of the peroxisome-enriched fractions and subsequent centrifugation, Per9p was present in the pellet. Similarly, after carbonate treatment, the major portion of Per9p was sedimentable (Fig. 6C). CAT, used as control, behaved like a soluble protein, while Per8p was pelletable (Fig. 6D). These data indicate that Per9p is an integral peroxisomal membrane protein. This location was confirmed immunocytochemically using antibodies against Per9p (Fig. 5D).

To gain information on the topogenic signals of Per9p, we expressed selected chimeric genes composed of \( \text{PER9} \) sequences encoding different N-terminal parts of Per9p fused to a CAT gene encoding CAT lacking a functional PTS1 (CAT(\( \text{PTS1} \)). In previous studies, we showed that this PTS1-mutated CAT gene product is not sorted to peroxisomes of \( \text{H. polymorpha} \), but instead accumulates in the cytosol (36). The fusion products were synthesized in \( \text{H. polymorpha} \) CAT disruption strain (\( \text{Dcat} \)). Immunocytochemical experiments performed on a strain producing the first 16 amino acids (N16) of Per9p fused to CAT(\( \text{PTS1} \)) revealed that the N16Per9p-CAT(\( \text{PTS1} \)) hybrid protein was located at membranous layers, most probably derived from the ER, since also the nuclear envelope often showed labeling (Fig. 7, A and B). In addition, low labeling intensities were found on the peroxisomal membrane (Fig. 7B). CAT protein was not, or to a very low extent as judged by the labeling patterns, in the peroxisomal matrix; it must be...
emphasized that the immunocytochemical methods allow us to discriminate between an intraperoxisomal and membrane-bound location in batch-cultured cells (37). From these results, we conclude that the first 16 amino acids of H. polymorpha Per9p contain topogenic information that is able to direct a reporter protein (CAT (−PTS1)) to the ER.

PER9 Is Induced under Peroxisome-Inducing Conditions—To gain insight in the regulation of PER9 expression, the activity of P<sub>PER9</sub> was studied in detail using P<sub>PER9</sub>-driven synthesis of bacterial β-lactamase. This method has been successfully used for analysis of the regulation of low abundant peroxisomal proteins (6). The results, summarized in Fig. 8, indicate that P<sub>PER9</sub> is active under all growth conditions tested. In glucose-and ethanol-grown cells, the activity of P<sub>PER9</sub> was low. Enhanced β-lactamase activities were observed when the cells were grown on nitrogen sources that require peroxisomal enzymes (methylamine or d-alanine) (33). The highest activities were observed in cells grown on methanol. As a control for our assay system, we used an identical plasmid containing the β-lactamase gene expressed under the control of P<sub>AOX</sub> (6). No β-lactamase activity was detected in cells grown on glucose or ethanol, conditions known to fully repress P<sub>AOX</sub> (33). As shown before (6), these results indicate that carbon catabolite repression of P<sub>AOX</sub> operates effectively on multicopy autonomously replicating plasmids. As expected, high activities were observed in methanol-grown cells. In methanol-grown cells, P<sub>AOX</sub>-driven β-lactamase activity was ∼10-fold higher than the activity observed under the control of P<sub>PER9</sub>. This indicates that PER9 is expressed at moderate levels in methanol-grown cells of H. polymorpha.

Functional Properties of Per9p—To examine the specificity of the anti-Per9p antiserum, Western blots were prepared from crude extracts of H. polymorpha wild-type cells (WT; lanes 1 and 4), Δper9 cells (lane 2), and cells overexpressing the PER9 gene (lane 3). No signal was observed when the preimmune serum (lane 4) was used. Cells were grown/incubated in batch cultures on 0.5% methanol. Equal amounts of protein were loaded per lane. B, shown is the localization of Per9p. The 30,000 × g supernatant (S3; lane 2) and organellar pellet (P3; lane 1), obtained after differential centrifugation of homogenates prepared from methanol-grown wild-type cells of H. polymorpha, as well as the peroxisomal (Per; lane 3) and mitochondrial (Mit; lane 4) fractions, obtained after subsequent sucrose density centrifugation of the P3 organellar pellet, were subjected to Western blotting. The data show that Per9p is a peroxisomal protein. As a control, the H. polymorpha integral peroxisomal membrane protein Per8p was used. C, shown are Western blots demonstrating the distribution of Per8p over the soluble (S; lanes 1 and 2) and pelletable (P; lanes 2 and 4) fractions after high salt treatment (NaCl; lanes 1 and 2) and sodium carbonate extraction (CO<sub>3</sub><sup>−</sup>; lanes 3 and 4) of the peroxisome-enriched peak fractions. D, as controls for the location of Per9p (see C), the distribution of the matrix protein CAT and Per8p (an integral component of the peroxisomal membrane of H. polymorpha) was studied. Per8p was pelletable after both high salt treatment (NaCl; lanes 1 and 2) and sodium carbonate extraction (CO<sub>3</sub><sup>−</sup>; lanes 3 and 4) of the peroxisomal peak fractions; instead, catalase was partially solubilized after NaCl treatment (lanes 1 and 2), but completely soluble after carbonate treatment of these fractions (lanes 3 and 4). These data confirm that Per9p is an integral membrane protein of peroxisomes of H. polymorpha.

We examined the kinetics of the biogenesis of peroxisomes by electron microscopy in P<sub>AMO</sub>-PER9-containing Δper9 cells after a shift from ammonium sulfate- to methanol-containing medium. These experiments indicated that new peroxisomes were first detected ∼30 min after the shift. Typically, only one small organelle per cell initially developed, which was characterized by the presence of AO and Per9p (Fig. 9, A and B). These organelles subsequently increased in size and multiplied during further cultivation, as described before for a shift of wild-type cells from glucose to methanol (38). The mechanisms of peroxisome reintroduction in these cells are currently being studied in depth and will be detailed in a separate paper.

Subsequently, cells grown on methanol/methylamine were shifted to methanol/ammonium sulfate-containing medium, thus repressing Per9p synthesis, and the effect on peroxisome morphology was followed by electron microscopy. In the initial hours after the shift, morphological alterations of existing organelles were not detectable. After ∼8 h of incubation, a partial disintegration of the peroxisomal membranes was observed (Fig. 9C). This result was highly reproducible with regard to both the time interval (∼8 h) and the deterioration effect on the membrane. At this stage, soluble AO protein was also first observed in the cytosol (Fig. 9D). At later stages, AO crystalloids that lacked a surrounding membrane appeared in the cells; a number of these crystalloids were subsequently degraded in the vacuole (data not shown). These results indicate that Per9p plays a role in maintenance of the peroxisomal membrane in vivo.

Per9p may also play a role in matrix protein assembly. This aspect was further investigated in Δper9 cells transformed with an expression plasmid carrying a truncated PER9 lacking the region coding for the predicted membrane-spanning region (amino acids 16–36). These transformants could not grow on methanol and lacked peroxisomes, suggesting that the peroxisomal location of Per9p is essential for its functioning. Surprisingly, AO was not assembled and active in these cells, as it is in Δper9 cells, but instead, AO was present in cytoplasmic aggregates in which the truncated Per9p protein was also located.

Fig. 6. A, shown is the specificity of the anti-Per9p antiserum. Western blots were prepared from crude extracts of H. polymorpha wild-type cells (WT; lanes 1 and 4), Δper9 cells (lane 2), and cells overexpressing the PER9 gene (lane 3). No signal was observed when the preimmune serum (lane 4) was used. Cells were grown/incubated in batch cultures on 0.5% methanol. Equal amounts of protein were loaded per lane. B, shown is the localization of Per9p. The 30,000 × g supernatant (S3; lane 2) and organellar pellet (P3; lane 1), obtained after differential centrifugation of homogenates prepared from methanol-grown wild-type cells of H. polymorpha, as well as the peroxisomal (Per; lane 3) and mitochondrial (Mit; lane 4) fractions, obtained after subsequent sucrose density centrifugation of the P3 organellar pellet, were subjected to Western blotting. The data show that Per9p is a peroxisomal protein. As a control, the H. polymorpha integral peroxisomal membrane protein Per8p was used. C, shown are Western blots demonstrating the distribution of Per8p over the soluble (S; lanes 1 and 2) and pelletable (P; lanes 2 and 4) fractions after high salt treatment (NaCl; lanes 1 and 2) and sodium carbonate extraction (CO<sub>3</sub><sup>−</sup>; lanes 3 and 4) of the peroxisome-enriched peak fractions. D, as controls for the location of Per9p (see C), the distribution of the matrix protein CAT and Per8p (an integral component of the peroxisomal membrane of H. polymorpha) was studied. Per8p was pelletable after both high salt treatment (NaCl; lanes 1 and 2) and sodium carbonate extraction (CO<sub>3</sub><sup>−</sup>; lanes 3 and 4) of the peroxisomal peak fractions; instead, catalase was partly solubilized after NaCl treatment (lanes 1 and 2), but completely soluble after carbonate treatment of these fractions (lanes 3 and 4). These data confirm that Per9p is an integral membrane protein of peroxisomes of H. polymorpha.
ultrathin sections of beling patterns after incubation of tein of the methylotrophic yeast gene encoding an integral peroxisomal membrane pro-
We have identified and characterized the protein.
peroxisome biogenesis. An H. polymorpha (Fig. 9, b 30% sequence identity with the struct-
strain was con-
FIG. 8. Activity of the PER9 promoter determined by Perg−
driven β-lactamase synthesis under selected growth conditions. β-Lactamase enzyme activities are expressed as units/milligram of protein.
osition in a constructed conditional mutant. Probably, the integ-
gerness of the Per3p-PTS1 protein complex reaches the per-
Our results indicate that Per9p is a key component of the peroxisome assembly machinery and in fact may have multiple functions. One of these is that the protein plays a role in maintenance of the peroxisomal membrane, as was evident after the repression of PER9 expression in a constructed conditional mutant. Probably, the integ-
ity of peroxisomal membranes in H. polymorpha requires a continuous addition of newly synthesized Per9p.
Moreover, Per9p seems to be involved in peroxisome proliferation. The multiplication of organelles, observed under conditions of PER9 overexpression, could simply reflect a Per9p-mediated enhanced synthesis of peroxisomal membranes. On the other hand, it is tempting to speculate that the mechanisms controlling peroxisome proliferation and membrane biogenesis are functionally related in that the proteins involved in these processes are associated (or only can function) in one complex. Such functional interactions were already predicted from a classical genetic study in which various H. polymorpha genes, including PER9, were shown to be functionally linked (12).
H. polymorpha Per3p may also play a role in this putative protein complex. Recently, Per3p was identified as the H. polymorpha PTS1 receptor (7), showing both functional and structural similarities to the Pichia pastoris PTS1 receptor, Pas8p (42, 43). We postulated a model in which Per3p acts as the cytosolic receptor of newly formed PTS1 proteins that shuttles these polypeptides from the cytoplasm into the organellar matrix. One of the missing links in this model is that it does not explain how the Per3p-PTS1 protein complex reaches the peroxisomal membrane. Per3p lacks any known PTS and does not enter the peroxisome after overexpression of PER3 in H. polymorpha wild-type cells (7). Hence, we proposed that Per3p undergoes a conformational change due to or after binding to disruption strains; peroxisomal membrane proteins (Per8p (8) and Per10p3) were also present at approximately normal levels in Δper9 cells. However, vesicular structures (“ghosts”) (39) were never observed. The method used (overproduction of the H. polymorpha peroxisomal membrane protein Per8p as marker protein) enabled such vesicles in other H. polymorpha Δper strains to be readily discerned (35). By a comparable method, Purde and Lazarow (40) demonstrated the presence of peroxisomal membrane vesicles in S. cerevisiae peb mutants. The apparent absence of peroxisomal vesicles in Δper9 cells makes this mutant attractive for molecular studies on the reintroduction of peroxisomes as occurs in transformants that synthesize Per9p under the control of a substrate-inducible promoter. This reintroduction is in line with earlier observations that peroxisomes in H. polymorpha do not necessarily derive from pre-existing peroxisomes (41). The mechanisms of the reintroduction of peroxisomes in per disruption strains, including Δper9, are currently being studied in our laboratory.
What Is the Function of PER9?—Our results indicate that Per9p is a key component of the peroxisome assembly machinery and in fact may have multiple functions. One of these is that the protein plays a role in maintenance of the peroxisomal membrane, as was evident after the repression of PER9 expression in a constructed conditional mutant. Probably, the integ-
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the PTS1 protein. The modified Per3p is subsequently recognized by a second protein that mediates sorting to the peroxisome. We speculate that Per9p could represent this protein; the involvement of Per9p in this process is also suggested by the simultaneous aggregation of AO and truncated Per9p lacking its membrane-spanning region. This hypothesis is in line with genetic studies by Titorenko et al. (12), who predict a functional interaction between Per9p and Per3p; it also does not conflict with the proposed function of Pas3p of *S. cerevisiae* (9), which was suggested to act as a protein receptor.

Unexpectedly, the first 16 amino acids of *PER9* were already sufficient to sort CAT, lacking its functional PTS, to the ER and nuclear membrane. One possible but unlikely explanation for this result is the cryptic nuclear targeting signal present in the 16 N-terminal amino acids of Per9p. The low labeling of CAT on peroxisomes can then be explained as a result of “piggybacking” (44, 45) in that the N16-Per9p-CAT fusion protein associates with authentic Per9p, which is also synthesized in these cells. The alternative is that the ER is directly involved in peroxisome biogenesis and that protein import and membrane biosynthesis are coupled processes. This can be envisaged in the view that peroxisomes are compartments, filled with proteins, that can only incorporate additional protein when the internal volume is simultaneously increased by recruiting phospholipids from the ER. The view that the ER may be involved in the biogenesis of peroxisomes is further supported by the finding that brefeldin A prevents peroxisome formation, resulting in the accumulation of peroxisomal matrix proteins at the ER. 4

It is interesting to note in this context that Bodnar and Rachubinski (46) described a 50-kDa integral membrane protein of mammalian peroxisomes that was synthesized on membrane-bound polysomes. It is still unclear, however, whether Per9p has properties similar to this protein.

The concept of protein import coupled to membrane insertion, e.g. via vesicle formation (45), of course leaves many questions unsolved, but takes its attractiveness from the fact that it unites a number of yet unexplained observations in various organisms in one model. Among these are the observations of Bellion and Goodman (47) on the import of AO in *Candida boidinii*. They showed that import and subsequent octamerization of AO was prevented in the presence of ionophores and resulted in the formation of peroxisomal membrane-associated protein complexes consisting of AO, dihydroxyacetone synthase, and several other unknown proteins (47). It can be envisaged that these proteins are not imported because membrane vesicle formation and/or fusion is prevented due to the lowered ATP levels in the cell. This mode of import could also explain how mature complex proteins or even gold particles enter the peroxisome (44, 45, 48). Further studies on the putative role of the ER in peroxisome biogenesis are currently in progress.

4 F. A. Salomons, I. J. van der Klei, and M. Veenhuis, manuscript in preparation.
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