Selective Peroxisome Degradation in Hansenula polymorpha

Transcriptional Down-regulation of Peroxisome Numbers Affects Selective Peroxisome Degradation in Hansenula polymorpha*

Received for publication, April 17, 2003, and in revised form, July 22, 2003
Published, JBC Papers in Press, August 5, 2003, DOI 10.1074/jbc.M304029200

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We have isolated and characterized a novel transcription factor of Hansenula polymorpha that is involved in the regulation of peroxisomal protein levels. This protein, designated Mpp1p, belongs to the family of Zn(II)$_2$Cys$_6$ proteins. In cells deleted for the function of Mpp1p the levels of various proteins involved in peroxisome biogenesis (peroxins) and function (enzymes) are reduced compared with wild type or, in the case of the matrix protein dihydroxyacetone synthase, fully absent. Also, upon induction of mpp1 cells on methanol, the number of peroxisomes was strongly reduced relative to wild type cells and generally amounted to one organelle per cell. Remarkably, this single organelle was not susceptible to selective peroxisome degradation (pexophagy) and remained unaffected during exposure of methanol-induced cells to excess glucose conditions. We show that this mechanism is a general phenomenon in H. polymorpha in the case of cells that contain only a single peroxisome.

Eukaryotic cells are thought to have evolved ~1.5 billion years ago. The development of cell organelles allowed primitive eukaryotes to compartmentalize specific cellular functions. Concomitantly, genetic mechanisms that control the biogenesis and function of these compartments had to be developed. Obviously, the separate classes of organelles are characterized by their specific function, as in energy metabolism (mitochondrion), degradation processes (vacuole, lysosome), or protein transport (Golgi system, endoplasmatic reticulum).

Among the organelles, peroxisomes are remarkable because of their highly versatile functions most of which are related to specific metabolic pathways in the organism in which they occur. This functional flexibility is not reflected in their morphology. The organelles are invariably very simple of construction and consist of a proteinaceous matrix, surrounded by a single membrane. Nevertheless, their function varies from the oxidation of very long chain fatty acids in man, germination of oil-bearing seed and photorespiration in green plants, to the metabolism of unusual carbon and/or nitrogen sources in fungi (1). In the methylotrophic yeast Hansenula polymorpha peroxisomes are essential to support growth of cells on media containing methanol as the sole source of carbon and energy. Under these conditions many organelles that contain the key enzymes involved in methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS), and catalase, develop in the cells. Conversely, when methanol-grown wild type (WT) cells are shifted to conditions in which the organelles are redundant for growth (e.g. glucose), they are rapidly and sequentially degraded by a process designated pexophagy (reviewed in Ref. 2). Morphological data suggest that in each cell generally a single (or few) small peroxisome(s) escape(s) the degradation process. The resistance of these organelles to degradation is thought to be of physiological advantage in that it allows the cells to quickly adapt to new environments that require new peroxisome functions.

In the present work, we report the identification of a novel H. polymorpha transcription factor, Mpp1p, which is involved in the regulation of peroxisomal proteins. In H. polymorpha mpp1 cells, various peroxisomal matrix enzymes involved in methanol metabolism and proteins essential for peroxisome biogenesis (peroxins) are present at reduced levels. As a result, mpp1 cells cannot grow on methanol as the sole source of carbon and energy. Interestingly, methanol-induced mpp1 cells generally contained a single enlarged peroxisome. Remarkably, these single organelles are protected from selective degradations upon exposure of cells to excess glucose.

MATERIALS AND METHODS

Micro-organisms and Growth—The H. polymorpha strains used in this study are listed in Table I. H. polymorpha cells were grown at 37 °C in YPD media (1% yeast extract, 1% peptone, 1% glucose), selective minimal media containing 0.67% yeast nitrogen base without amino acids (Difco) supplemented with 1% glucose (YND) or 0.5% methanol (YNM) or mineral media (3) supplemented with 0.5% glucose, 0.5% methanol, or 0.5% methanol + 0.1% glycerol. Wherever necessary, media were supplemented with 30 μg/ml leucine or 100 μg/ml zeocin. For growth on plates, 2% granulated agar was added to the media. For cloning purposes, Escherichia coli DH5α (Invitrogen) was used and grown at 37 °C in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl), supplemented with 100 μg/ml ampicillin, 25 μg/ml kanamycin, or 25 μg/ml zeocin when required.

Gene Tagging Mutagenesis and Isolation of Mut* Mutants—The RALF (random integration of linear DNA fragments) method (4) was used to generate yeast mutants. H. polymorpha HP246 was transformed with BamHI-linearized pEMI-Z plasmid (Table II) in the presence of 1 unit of BamHI restriction enzyme. Transformants were initially selected on YPD plates supplemented with zeocin and
subsequently replica-plated to YND and YMN plates, respectively. Colonies unable to grow on YMN plates (methanol utilization-defective, Mut− colonies) were further analyzed. Two Mut− mutants, designated mpp1−1 and mpp1−2 (previously designated ARJ-59; see Ref. 4), were studied further.

**Cloning of the MPP1 Gene**—To identify the gene(s) disrupted by pREMI-Z in mutants mpp1−1 and mpp1−2, the chromosomal DNA of the cells was digested with either EcoRI or SphI, self-ligated, and transformed to E. coli, giving rise to the following plasmids: pANL7, pANL15, pREMI-59, and pANL22 (Table II). The genomic regions of pANL7 and pREMI-59 were initially sequenced using vector-based primers (4). Sequence analysis showed that the pREMI-Z vector had integrated in mutants mpp1−1 and mpp1−2 at two different locations in the same gene that was designated MPP1. Subsequently, the entire nucleotide sequence of the MPP1 gene was determined by primer walking on the rescued pREMI-Z plasmids. The nucleotide sequence of the MPP1 gene was deposited in GenBank™ (accession number AT180621).

To clone the MPP1 open reading frame, mutant mpp1−1 was transformed with a *H. polymorpha* genomic library constructed in the pYT3 vector (5). Leucine prototrophic transformants were screened on YMN plates for the ability to grow on methanol. From a complementing plasmid, a sub-clone containing a 3.4-kb BglII fragment within the entire MPP1 gene was obtained, designated pANL26, that was used for complementation studies.

**Construction of an H. polymorpha MPP1 Null Mutant**—A strain deleted for MPP1 was constructed by replacing the region of MPP1 comprising nucleotides +1 to +1042 by an auxotrophic marker. To this end, a deletion cassette was constructed as follows. First, two DNA fragments comprising the regions −916 to −1 and +1042 to +1841 of the *MPP1* genomic region were obtained by PCR, using primers MPP1del-1 + MPP1del-2 and MPP1del-3 + MPP1del-4, respectively (see Table III). After restriction with NotI + BglII and PstI + Asp718I, respectively, the resulting fragments were inserted upstream and downstream of the *H. polymorpha* URA3 gene (6) in pBSK-URA3. From the resulting plasmid, designated pANL17, a 2650-bp BamHI-PvuI fragment was used to transform *H. polymorpha*NCYC495 leu1.1 ura3-1. Uracil prototrophic transformants were selected by their inability to grow on YMN plates. Proper deletion of MPP1 was confirmed by Southern blotting (data not shown). The resulting strain was designated mpp1−1.

To enable visualization of peroxisomes by fluorescence microscopy, the eGFP-SKL reporter gene was introduced in the resulting mpp1−1 strain. First, we constructed a *H. polymorpha* integrative plasmid containing the *p*anno-eGFP-SKL cassette and the zeocin-resistance gene by inserting the 1.2-kb SphI-SalI fragment of pEHM34 (7) in pPH24 (8). Subsequently, the resulting plasmid, designated pANL29, was linearized with SphI and integrated in the *mpp1* genome. Zeocin-resistant transformants were analyzed for correct integration in the *MPP1* region by Southern blotting (data not shown). A strain containing a single copy of the integrated plasmid, designated mpp1.eGFP.SKL, was used for further studies.

**Construction of a Strain Expressing an MPP1.eGFP Fusion Gene**—To enable replacement of the genomic *MPP1* gene by a *MPP1.eGFP* fusion gene, we first constructed a plasmid pANL22 containing the *MPP1* region from *H. polymorpha* URA3 gene (6) in *E. coli* transformed to *MPP1* gene was deposited in GenBank™ (accession number AF316407) and was inserted in *HindIII/BglII*-digested pANL31. The resulting plasmid, designated pANL32, was linearized with EcoRI in the *MPP1* region and transformed to WT *H. polymorpha*NCYC495 leu1.1. Zeocin-resistant colonies were analyzed by Southern blotting to confirm correct integration in the *MPP1* region (data not shown).

**Biochemical Assays**—Crude cell extracts were prepared as described (15). SDS-PAGE (16) and Western blot analysis (17) were performed by established methods. The degradation of peroxisomes in batch cultures of *H. polymorpha* was determined as described (18). Relative AO levels were determined by densitometric scanning of Western blots decorated with specific antibodies against AO. The decrease in AO levels during peroxisome degradation is expressed as a percentage of the initial value, which is arbitrarily set to 100%. β-Lactamase activities were assayed by established methods (19).

**RESULTS**

**Mpp1p Is a Member of the Zn(II)/Cys4 Cluster Protein Family and Is Induced during Growth of H. polymorpha on Methanol**—From a collection of 5,000 RALF transformants, mutants were selected that were impaired in growth on methanol as sole carbon source (Mut− phenotype). Two mutants, designated mpp1−1 and mpp1−2, were identified of identical morphological phenotype. Characteristically, upon growth in glycerol/methanol-containing media, conditions that lead to a strong peroxisome development in WT cells, generally a single peroxisome was observed in the vast majority of the mpp1−1 and mpp1−2 cells (Fig. 1). Sequencing of the plasmids recovered from these mutants revealed that the flanking regions were overlapping. Hence, both were apparently disturbed in the function of the same gene, termed *MPP1* (methylotrophic peroxisomal protein regulator 1). Further sequencing of the plasmids recovered from mutants mpp1−1 and mpp1−2 allowed the determination of the remainder of the *MPP1* open reading frame (ORF). Sequence analysis indicated that in case of mutant mpp1−1, the integration of the pREMI-Z plasmid had occurred 456 bp downstream from the initiation of the *MPP1* ORF. In mutant mpp1−2, pREMI-Z had inserted at the promoter region of the same ORF (nucleotide −195). The sequence upstream the 5′ region of this ORF comprised the 3′ end of the *H. polymorpha* dihydroxyacetone synthase gene (DAS; see Fig. 2A).

The *MPP1* gene encodes a protein of 684 amino acids. A BLASTP search revealed that the N-terminal region of Mpp1p is similar to that of many DNA-binding proteins. However, a true homologue was not found in the available databases. Further analysis using the ScanPROSITE program revealed that

| Table I | H._polymorpha strains used in this study |
|---------|------------------------------------------|
| Strain | Genotype and characteristics | Source |
| NCYC495 | leu1.1 derivative | Ref. 44 |
| NCYC495 | leu1.1 ura3 derivative | Ref. 44 |
| HP246 | eGFP-SKL | Ref. 4 |
| mpp1−1 | HP246:[pREMI-Z], leu1.1, Mut−, zeo6 | This study |
| mpp1−2 | HP246:[pREMI-Z], leu1.1, Mut−, zeo6 | Ref. 4 |
| mpp1 | NNCYC495 Δmpp1::HplURA3, leu1.1 | This study |
| mpp1.eGFP.SKL | Δmpp1::(pAnlGFP-SKL)IΔ, leu1.1, zeo6 | This study |
| MPP1.eGFP | NNCYC495-MPP1.eGFP, leu1.1, zeo6 | This study |

obtained using primers MPP1-Or12 and MPP1-InsStop (Table III), was inserted in *HindIII/BglII*-digested pANL31. The resulting plasmid, designated pANL32, was linearized with EcoRI in the *MPP1* region and transformed to WT *H. polymorpha*NCYC495 leu1.1. Zeocin-resistant colonies were analyzed by Southern blotting to confirm correct integration in the *MPP1* region (data not shown).
Mpp1p is a putative member of the Zn(II)2Cys6 family of transcription factors. These transcription regulators are exclusively detected in fungi and contain a well conserved DNA binding domain (20). This domain consists of a cystein-rich motif (CysX2-CysX2-CysX2-CysX2-CysX2) that complexes two Zn2+ ions and in most cases recognizes a pair of 5′-CGG-3′ triplets in the promoters of target genes (20, 21). A primary sequence comparison of the region comprising the DNA binding domain of characteristic Zn(II)2Cys6 proteins of Saccharomyces cerevisiae and the putative DNA binding domain of Mpp1p is depicted in Fig. 2B.

The subcellular location of Mpp1p in *H. polymorpha* was studied in a strain in which the endogenous MPP1 gene was replaced by a MPP1eGFP fusion gene. The presence of the C-terminal tag most likely did not interfere with Mpp1p function, because the resulting strain, MPP1eGFP, grew normally on glucose and methanol (data not shown). In MPP1eGFP cells grown on glucose, GFP fluorescence was invariably undetectable, independent of the growth stage. However, upon a shift of glucose-grown cell to fresh methanol-containing media, GFP fluorescence was readily observed as a single dot at all growth phases. This spot was observed in the same region of the cells as Hoechst 33258, which is a nuclear stain. These findings therefore indicate that Mpp1p is associated with the nucleus (Fig. 3).

Cells of the MPP1 Deletion Strain Generally Contain a Single Peroxisome—For construction of a MPP1 deletion strain, we replaced a 1042-bp fragment from the MPP1 open reading frame by the *H. polymorpha* URA3 gene (Fig. 2A). Cells of the mpp1 strain and the original RALF mutants, mpp1-1 and mpp1-2, showed identical phenotypes. Mpp1 cells were unable to grow on methanol as sole carbon source and, when grown on glycerol/methanol mixtures, characteristically contained a single peroxisome (Fig. 4B). Identification of the MPP1 gene in an autonomously replicating plasmid (pANL26) restored normal growth of mpp1 cells on methanol, as well as normal peroxisome proliferation (Fig. 4C).

In crude extracts of glycerol/methanol-grown WT and mpp1 cells, the levels of the peroxisomal matrix protein AO were strongly reduced, whereas DHAS, another peroxisomal matrix enzyme, could not be detected (Fig. 5A). However, catalase was present at approximately WT levels. Also the levels of malate synthase, a peroxisomal key enzyme of glyoxylate (C2) metabolism (22), did not change significantly under the conditions tested (Fig. 5A). In addition, the level of various peroxins that play a role in peroxisome formation was analyzed. All were present at significantly reduced levels under the conditions tested (Fig. 5A). In addition, the level of various peroxins that play a role in peroxisome formation was analyzed. All were present at significantly reduced levels under the conditions tested (Fig. 5A).

Despite the low peroxisome numbers we did not observe a defect in matrix protein import in mpp1 cells. GFP.SKL was solely observed in spots (compare Fig. 4B), and immunocytochemistry revealed that anti-alcohol oxidase and anti-catalase-dependent-specific labeling was confined to the peroxisomal profiles (Fig. 4D), indicating that these proteins were incorporated into their correct target organelle. As expected, using antidi-hydroxyacetone synthase antibodies, specific labeling was not detected (not shown).

**Single Peroxisomes in *H. polymorpha* Cells Are Not Susceptible to Glucose-induced Selective Peroxisome Degradation (Pexophagy)—**In methanol-grown WT *H. polymorpha* cells peroxisomes are rapidly degraded when they have become redund-

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**TABLE II**

**Plasmids used in this study**

| Plasmid                  | Characteristics                                      | Source                  |
|-------------------------|------------------------------------------------------|-------------------------|
| pBlueScript II SK+      | Cloning vector, amp<sup>R</sup>                      | Stratagene, La Jolla, CA|
| pBK-URA3                | pBlueScript SK<sup>+</sup> containing the 2.3-kb *H. polymorpha* URA3 fragment, amp<sup>K</sup> | This study              |
| pREMI-Z                 | Used for gene-tagging mutagenesis, zeo<sup>B</sup>, amp<sup>R</sup> | Ref. 4                  |
| pANL7                   | Rescued plasmid of mutant mpp1–1, obtained by digestion of chromosomal DNA with EcoRI followed by selfligation, zeo<sup>R</sup> | This study              |
| pANL15                  | Rescued plasmid of mutant mpp1–1, obtained by digestion of chromosomal DNA with Spel followed by selfligation, zeo<sup>B</sup> | This study              |
| pANL17                  | Plasmid containing the cassette for the deletion of the MPP1 gene, amp<sup>Y</sup>, *H. polymorpha* URA3 gene | This study              |
| pREMI-59                | Rescued plasmid of mutant mpp1–2, obtained by digestion of chromosomal DNA with EcoRI followed by selfligation, zeo<sup>R</sup> | This study              |
| pANL22                  | Rescued plasmid of mutant mpp1–2, obtained by digestion of chromosomal DNA with Spel followed by selfligation, zeo<sup>B</sup> | This study              |
| pANL26                  | *E. coli/H. polymorpha* shuttle plasmid containing the uninterrupted MPP1 gene, amp<sup>Y</sup>, *S. cerevisiae* LEU2 gene, HARS1 | This study              |
| pANL29                  | pHIPZ4 containing eGFP.SKL, zeo<sup>B</sup>, amp<sup>R</sup> | This study              |
| pANL31                  | pBlueScript derivative containing the eGFP gene without a startcodon, zeo<sup>R</sup>, amp<sup>K</sup> | This study              |
| pANL32                  | pANL31 with a 748-bp fragment containing the 3′ end of the *MPP1* gene fused in-frame to the eGFP gene, zeo<sup>R</sup>, amp<sup>R</sup> | This study              |
| pFEM34                  | Plasmid containing the P<sub>AOX</sub>,eGFP.SKL cassette and the kan<sup>R</sup>, *S. cerevisiae* LEU2 gene | Ref. 7                  |
| pHIPZ4                  | Integrative plasmid for *H. polymorpha*, contains the *H. polymorpha* AOX promoter, zeo<sup>R</sup>, amp<sup>R</sup> | Ref. 8                  |
| pX3-F<sub>pAOX</sub>βlac | Plasmid expressing the β-lactamase gene under control of the AOX promoter | Ref. 19                 |
| pX3-F<sub>pEEX3</sub>βlac | Plasmid expressing the β-lactamase gene under control of the PEX3 promoter | Ref. 23                 |

**TABLE III**

**Primers used in this study**

| Name         | Sequence           |
|--------------|--------------------|
| MPP1-del1    | 5′-AGAGAGGGCCGCCGCCGCTATAGACGCTC G-3′ NotI |
| MPP1-del2    | 5′-AGAGATCTTTGGACAAACCTGACG-3′ BglII |
| MPP1-del3    | 5′-AGCGCGATGGCTCTCCCGACACCTTG-3′ PstI |
| MPP1-del4    | 5′-AGAGGATCCACCGATCTGCTGTCG-3′ Asp718I |
| MPP1-Orig2   | 5′-GACACCTTGGAGAAATGTCAGATC-3′ |
| MPP1w/stop   | 5′-AGAGATCCGATCCCGGTGGGCG-3′ BamHI |
To analyze the fate of the single organelles in mpp1 cells, glycerol/methanol-grown cells of this strain were exposed to excess glucose conditions. In the first 2 h after the shift of cells, no significant AO protein degradation had occurred in mpp1 cells, as judged from Western blots (Fig. 6, A and B). In mpp1-eGFP.SKL cells, fluorescence microscopy studies failed to demonstrate the uptake of the fluorescent reporter protein in vacuoles, a phenomenon that was readily observed in WT controls (Fig. 6C). Also, electron microscopically we were never able to detect peroxisome sequestration, the typical initial event of pexophagy in H. polymorpha (not shown).

To further substantiate the possibility that also in WT cells specific organelles escape the pexophagy process, we have grown H. polymorpha WT cells on methanol to a phase that they generally contain only one or two organelles per cell (early exponential growth; see Ref. 25) and studied pexophagy relative to cells that were in the mid-exponential growth phase and contained several peroxisomes. The results clearly show that the single peroxisome present in early exponential WT cells is not degraded within a period of 4 h after exposure of the cells to excess glucose (Fig. 7C). In contrast, in mid-exponential cells a rapid reduction of peroxisome numbers was observed. Nevertheless, in these cells generally a single fluorescent spot...
remained indicating that not all peroxisomes were degraded (Fig. 7C). Remarkably, the morphological phenotypes of early and mid-exponential WT cells after 4 h of exposure of cells to glucose was indistinguishable in that they generally contained a single fluorescent spot. Most likely, solely small organelles remain unaffected as was evident after careful electron microscopical observations (Fig. 4E).

These observations were confirmed biochemically by Western blot analysis of samples taken from the same cultures using specific antibodies against AO (Fig. 7, A and B). When WT cells in the early exponential growth phase were subjected to pexophagy conditions, AO protein levels only slightly decreased during the first hour after the shift. However, at later time points the AO levels remained constant, indicating that the peroxisomes in these cells were not degraded. The initial decrease most likely is because of the presence of a minor fraction of the cells that contains more than one peroxisome at the time of the shift. Mid-exponentially grown cells showed the continuous decrease in AO protein characteristic for WT cells subjected to pexophagy (18).

**DISCUSSION**

We have cloned and characterized a novel member of the family of zinc cluster proteins of the yeast *H. polymorpha*, designated Mpp1p. Mpp1p plays a role in the control of peroxisomal protein synthesis (see below) and represents the third *H. polymorpha* zinc cluster protein reported so far. The other two proteins, Yna1p and Yna2p, activate genes involved in nitrate assimilation (26, 27). Zinc cluster proteins are a class of transcription factors involved in the modulation of various cell activities in fungi, among which are transcriptional control of genes required for growth on various carbon sources, synthesis of specific metabolites (e.g. amino acids, pyrimidines), and the expression of ABC transporters that mediate multi-drug resistance (reviewed in Refs. 28 and 29). A well known example of these proteins is Gal4p (Fig. 2B), responsible for the activation of genes of galactose metabolism. Gal4p function involves binding of the N-terminal zinc cluster to CGG triplets present in the promoter of target genes and the formation of a dimer through one or more very short coiled-coils present C-terminal from the zinc cluster motif (20). Most members of this family bind to
DNA as a homodimer, although formation of heterodimers or activation by only one monomer has also been reported (30–32).

We can now add methyotrophic growth to the list of Zn(II)$_2$Cys$_6$ transcription factor-modulating activities. Our data clearly demonstrate that Mpp1p is necessary to sustain growth of H. polymorpha cells on media containing methanol as the sole carbon source. Interestingly, this is yet the only growth condition where the role of Mpp1p is essential as mpp1 cells grow normally on several other carbon and nitrogen sources (e.g. glucose, ethanol, glycerol, dihydroxyacetone, and ethylamine; data not shown), some of which require peroxisome functions. These alternative functions are presumably not regulated by MPP1 but require different transcription factors to induce the required enzyme repertoire.

The Mut$^-$ phenotype of mpp1 cells is readily explained by the observation that the levels of the two major enzymes of methanol metabolism, AO and DHAS, are strongly reduced (AO) or absent (DHAS). Also, in mpp1 cells peroxisomes do not proliferate in response to the presence of methanol. This inhibitory effect is most probably not related to the reduced amounts of AO and DHAS protein. In fact, several earlier observations argue against a direct relation between matrix protein levels and peroxisome numbers in H. polymorpha. For instance, H. polymorpha pim mutants that are affected in matrix protein import contain enhanced numbers of small-sized peroxisomes relative to wild type organelles (33). On the other hand, overproduction of AO protein in glucose-grown cells resulted in enlarged organelles but not an increase in organelle numbers (34).

It has been proposed that peroxisomes may divide (multiply) through a constitutive or a regulated mechanism (35, 36). In this view the constitutive mechanism is responsible for the partitioning of peroxisomes between mother and bud in non-induced (glucose-grown) cells, a phenomenon that may be re-

![Fig. 6. Induction of pexophagy in H. polymorpha WT and mpp1 cells.](image)

WT and mpp1 cells were grown for 15 h in glycerol/methanol-containing media and subsequently supplemented with 0.5% (w/v) glucose. Samples were taken at the indicated time points. For biochemical analysis (A and B), cells were trichloroacetic acid precipitated and subjected to Western blot analysis. Equal volumes of cultures were loaded per lane. A shows Western blots decorated with specific antibodies against the indicated peroxins. C, activities of the AOX and PEX3 promoters in WT and mpp1 cells analyzed by measuring the enzyme activities of the reporter enzyme β-lactamase produced under control of the AOX or PEX3 promoter. Enzyme activities were measured in crude extracts of glycerol/methanol-grown cells and expressed as units/mg protein.
lateral to the cell cycle. The regulated division occurs in induced cells, and this mechanism is responsible for the formation and partitioning of the characteristic peroxisome cluster during growth of cells on methanol. In line with this hypothesis we speculate that the absence of Mpp1p prevents the regulated peroxisome division in *H. polymorpha* during C1 metabolism but not the constitutive mechanism as developing buds are generally administered with a single organelle. In *H. polymorpha* mpp1 cells the effect on peroxisome proliferation is therefore likely to be either because of the down-regulation of a yet unknown initial (signaling) compound of the proliferation machinery or directly related to the strongly reduced level(s) of protein(s) (e.g. Pex3p) involved in biogenesis and/or fission. It has been shown before (37) that in *H. polymorpha* a direct relation exists between the levels of the peroxin Pex3p and the number of peroxisomes. Possible additional candidates besides Pex3p are *H. polymorpha* homologues of *S. cerevisiae* Vps1p and Pex11p (35, 38, 39). In bakers’ yeast, oleate-induced peroxisome proliferation was shown to be dependent on Oaf1p and Pip2p, both Zn(II)2Cys6 transcription factors (29, 31). Extensive studies showed that Oaf1p is constitutively synthesized and is the receptor for the oleate signal, whereas in the absence of the inducer, Pip2p is produced at low levels, and its activity is inhibited by Oaf1p. The current model (40) proposes that upon oleate induction, Oaf1p becomes active thereby abolishing its inhibitory effect on Pip2p, which, in turn, becomes able to up-regulate is own synthesis. As a consequence, maximal amounts of the heterodimer Oaf1p-Pip2p are produced that efficiently bind to the oleate response element, thereby activating transcription of genes involved in oleate metabolism. Mpp1p is not the homologue of *S. cerevisiae* Pip2p or Oaf1p. First, *H. polymorpha* Mpp1p only shares similarity with these proteins in the Zn(II)2Cys6 region. Moreover, a search in the *H. polymorpha* genome database revealed that *H. polymorpha* does contain genes that encode proteins with significant overall similarity to *S. cerevisiae* Pip2p and Oaf1p.2

It is possible that a mechanism similar to the *S. cerevisiae* Oaf1p-Pip2p activation complex functions during methylotrophic growth of *H. polymorpha* and that Mpp1p behaves analogous to Pip2p, because the protein was only detected in cells grown on methanol. The identification of a putative Mpp1p partner and the definition of a DNA-response element are issues of current investigations.

We observed that peroxisomal matrix proteins were not mislocalized in *mpp1* cells; both AO and catalase, as well as GFP-SKL, were normally imported into the single organelles of *mpp1* cells. Apparently, the strongly reduced levels of specific peroxins (Pex3p, Pex5p) in methanol-induced *mpp1* cells still suffice to allow quantitative matrix protein import into peroxisomes. Hence, the efficient matrix protein import in *mpp1* cells may be related to the normal levels of Pex14p that showed no decrease in methanol-induced cultures.

We were particularly interested in the fate of the single

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2 G. Gellissen, personal communication.
peroxisome of mpp1 cells upon the induction of pexophagy. We observed that these organelles were protected from degradation after a shift to conditions that led to a rapid degradation of peroxisomes in identically grown WT cells. Subsequent studies demonstrated that also the single organelles present in shorty induced WT cells were resistant to pexophagy. Possibly, these single organelles are protected from degradation thus enabling the cell to rapidly respond to changes in the environment that require new peroxisome (2). Previously, we demonstrated that no longer import matrix proteins are degraded during pexophagy. Indeed, we observed that these organelles were protected from degradation thus enabling the cell to rapidly respond to changes in the environment that require new peroxisome (2). An attractive possibility is that exclusively the peroxisomes that no longer import matrix proteins by a yet unknown mechanism (41).

Acknowledgments—We thank Klaas Sjollem, Marcel Lunenborg, Michel Meijer, and Patricia Stevens for expert assistance on different parts of this work.

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