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Published in:
Yeast

DOI:
10.1002/(SICI)1097-0061(19990630)15:9<741::AID-YEA416>3.3.CO;2-F

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Publication date:
1999

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Citation for published version (APA):
Kiel, J. A. K. W., Rechinger, K. B., Van der Klei, I. J., Salomons, F. A., Titorenko, . N. V., Veenhuis, M., & Titorenko, V. I. (1999). The Hansenula polymorpha PDD1 gene product, essential for the selective degradation of peroxisomes, is a homologue of Saccharomyces cerevisiae Vps34p. Yeast, 15(9), 741-754. DOI: 3.3.CO;2-F" class="link">10.1002/(SICI)1097-0061(19990630)15:93.3.CO;2-F

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YEAST
Yeast **15**, 741–754 (1999)

The *Hansenula polymorpha PDD1* Gene Product, Essential for the Selective Degradation of Peroxisomes, is a Homologue of *Saccharomyces cerevisiae* Vps34p

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Via functional complementation we have isolated the *Hansenula polymorpha PDD1* gene essential for selective, macroautophagic peroxisome degradation. *HpPDD1* encodes a 116 kDa protein with high similarity (42% identity) to *Saccharomyces cerevisiae* Vps34p, which has been implicated in vacuolar protein sorting and endocytosis. Western blotting experiments revealed that *HpPDD1* is expressed constitutively. In a *H. polymorpha pdd1* disruption strain peroxisome degradation is fully impaired. Sequestered peroxisomes, typical for the first stage of peroxisome degradation in *H. polymorpha*, were never observed, suggesting that HpPdd1p plays a role in the tagging of redundant peroxisomes and/or sequestration of these organelles from the cytosol. Possibly, HpPdd1p is the functional homologue of ScVps34p, because—like *S. cerevisiae vps34* mutants—*H. polymorpha pdd1* mutants are temperature-sensitive for growth and are impaired in the sorting of vacuolar carboxypeptidase Y. Moreover, HpPdd1p is associated to membranes, as was also observed for ScVps34p. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — autophagosome; catabolite inactivation; methylotrophic yeast; microbody; phosphatidylinositol 3-kinase

INTRODUCTION

Yeasts can rapidly adapt to changes in the environment by regulating the amounts and repertory of endogenous proteins and organelles in a selective manner. The development of microbodies (peroxisomes) in yeasts is a typical example of a response to specific metabolic needs (Lazarow and Fujiki, 1985). In the methylotrophic yeast *Hansenula polymorpha*, the development of these organelles is strongly induced by methanol as well as by a number of organic nitrogen sources (Veenhuis and Harder, 1991). In contrast, methanol-induced peroxisomes are actively degraded after a shift of cells to conditions in which the organelles are redundant for growth (e.g. glucose or ethanol). In *H. polymorpha* peroxisome degradation occurs by means of a highly selective process resembling autophagy (Veenhuis *et al*., 1983). As an initial step, organelles to be degraded are sequestered from the cytosol by a number of membranous layers, resulting in the formation of autophagosomes. These membranes are thought to be derived from the endoplasmic reticulum (cf. e.g. Dunn, 1990; Ueno *et al*., 1991). Subsequently, the autophagosomes fuse with the vacuole or a vacuolar vesicle, which supplies the organelle with hydrolytic enzymes that degrade the microbody contents (Veenhuis *et al*., 1983). Also in *Saccharomyces cerevisiae* and in another

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CCC 0749–503X/99/090741–14 $17.50
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Received 3 September 1998
Accepted 31 January 1999

methylotrophic yeast, *Pichia pastoris*, selective degradation of peroxisomes has been demonstrated to occur via autophagic processes (Tuttle and Dunn, 1995; Chiang *et al.*, 1996; Sakai *et al.*, 1998).

Surprisingly, in *P. pastoris* peroxisome degradation may occur via morphologically distinct autophagic processes. During glucose adaptation of methanol-grown *P. pastoris* cells, peroxisomes were degraded via a process analogous to microautophagy, i.e. clusters of peroxisomes were engulfed by protrusions of the vacuole, incorporated and subsequently degraded. In contrast, adaptation of methanol-grown *P. pastoris* cells to ethanol resulted in degradation of peroxisomes via a process similar to that described for *H. polymorpha*. This process is analogous to macroautophagy observed in mammalian cells.

A number of mutants affected in the process of selective peroxisome degradation has been isolated from *H. polymorpha* (*pdd* mutants). So far, five complementation groups have been identified (Titorenko *et al.*, 1995). Recently, *P. pastoris* mutants defective in glucose-induced microautophagy (*gsa* and *pag* mutants) were also isolated (Tuttle and Dunn, 1995; Sakai *et al.*, 1998). Interestingly, most *gsa* and *pag* mutants are still capable of degrading peroxisomes during ethanol adaptation. Recently, Yuan *et al.* (1997) identified the *PpGSA1* gene product as the α-subunit of phosphofructokinase, which is apparently required for an early event in vacuolar microautophagy of peroxisomes in *P. pastoris*. Surprisingly, the activity of PpGsa1p in the degradation process was independent of its ability to metabolize glucose intermediates.

The *pdd* mutants isolated so far from *H. polymorpha* are deficient in the initial steps of peroxisome degradation (Titorenko *et al.*, 1995). The first type of mutant, exemplified by *pdd1*, is impaired in the sequestration process; in mutants of the second type (e.g. *pdd2*) the sequestered organelles fail to fuse to vacuolar compartments. Mutants impaired in the final step of peroxisome degradation—proteolytic degradation in the vacuole—were not selected.

Here we describe the characterization of the *H. polymorpha PDD1* gene. The gene product of *HpPDD1* is homologous to the phosphatidylinositol (PtdIns) 3-kinase encoded by the *S. cerevisiae VPS34* gene (reviewed by DeWald *et al.*, 1997). In wild-type (WT) *H. polymorpha* cells, HpPdd1p is localized on membranes. The specific function of the protein in selective peroxisome degradation is discussed.

MATERIALS AND METHODS

Micro-organisms and growth conditions

The following *Hansenula polymorpha* strains were used in this study: CBS4732 (CBS collection, The Netherlands), NCYC495 and auxotrophic derivatives thereof (Gleeson and Sudbery, 1988), *pdd1-201* (*leu1-1*) (Titorenko *et al.*, 1995) and *Δcpy* (*leu1.1*) (Bellu *et al.*, 1999). *H. polymorpha* strains were grown in batch cultures in rich medium containing 1% yeast extract, 2% peptone and 1% glucose (YPD), selective minimal medium containing 0·67% yeast nitrogen base without amino acids supplemented with 1% glucose (YND) or 0·5% methanol (YNM), or mineral medium using either glucose (0·5%) or methanol (0·5%) as carbon source (Van Dijken *et al.*, 1976). Leucine and uracil were added as required (final concentration 30 μg/ml). *Escherichia coli* DH5α (Sambrook *et al.*, 1989) was used for plasmid constructions and was grown on LB medium supplemented with the appropriate antibiotics.

DNA procedures

H. polymorpha cells were transformed using the electroporation method (Faber *et al.*, 1994). Recombinant DNA manipulations were as described (Sambrook *et al.*, 1989). Southern blot analysis was performed using the ECL direct nucleic acid labelling and detection system according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Polymerase chain reaction-mediated DNA amplification was performed with *Pwo*-polymerase according to the manufacturer's instructions (Boehringer–Mannheim, Germany). Oligonucleotides were obtained from Eurogentec, Seraing, Belgium. Biochemicals were obtained from Boehringer–Mannheim, Germany.

Cloning and sequence analysis of the HpPDD1 *gene*

To isolate the *HpPPD1* gene, the *H. polymorpha* mutant *pdd1-201* was transformed with an *H. polymorpha* genomic library in pYT3 (Tan *et al.*, 1995). Following selection of Leu^+ transformants on YND plates at 37°C, the colonies were replica-plated to YNM plates and screened for the ability to grow on methanol at 43°C. Plasmid DNA was

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recovered from six Mut$^+$ transformants in *E. coli* DH5α. After retransformation to *H. polymorpha pdd1-201*, three plasmids were found to be able to restore methylotrophic growth at 43°C, as well as the selective degradation of peroxisomes after a shift of cells to non-methylotrophic conditions, using the plate assay based on the activity of the peroxisomal matrix enzyme alcohol oxidase (AO) (Titorenko *et al.*, 1995). These plasmids contained overlapping chromosomal inserts. By subcloning, an approximately 4 kb *Bgl*II fragment was identified that still complemented *pdd1-201*. This fragment was inserted in both orientations in pBluescript II SK+ (Stratagene, San Diego, CA) and a series of nested deletions was generated by the limited exonuclease III digestion method (Sambrook *et al.*, 1989). Double-stranded DNA sequencing of the resulting subclones was carried out on a ABI PRISM 377 sequencer (Applied Biosystems Inc.) using the Dye Terminator Cycle Sequencing Ready Reactions (Perkin-Elmer). In addition, several selected oligonucleotides were synthesized to complete or confirm certain portions of the DNA sequence. Sequences were assembled with the aid of Microgene Sequence Software (Beckman). For analysis of the DNA sequence and the deduced amino acid sequence, the PC-GENE program (release 6.70, Intelligenetics Inc., Mountain View, CA) was used. To find protein sequences with similarity to HpPdd1p, the TBLASTN algorithm (Altschul *et al.*, 1990) was used to screen databases at the National Center for Biotechnology Information (Bethesda, MD).

Construction of a HpPDD1 *disruption strain*

To disrupt the WT *HpPDD1* gene, a 1·8 kb *Asp*718–*Bam*HI (up to nt 641 in GenBank Accession No. AF121671) and a 0·5 kb *Bam*HI–*Hin*dIII fragment (nt 3000–3527) of the region comprising *HpPDD1* were subcloned between the *Asp*718 and the *Hin*dIII sites of pUC19 (Yanisch-Perron *et al.*, 1985), resulting in plasmid pPDD1-1. Subsequently, a 1·8 kb *Bam*HI–*Bgl*II fragment containing the *H. polymorpha URA3* gene (Merckelbach *et al.*, 1993) was inserted into *Bam*HI-digested pPDD1-1. From the resulting plasmid, designated pPDD1-2, a 2·9 kb *Bgl*II–*Xho*I fragment was isolated and used to transform *H. polymorpha* NCYC495 (*leu1–1 ura3*). In *S. cerevisiae*, deletion of the putative *HpPDD1* homologue, *ScVPS34*, is not lethal but gives rise to a temperature-sensitive (ts) growth phenotype (Herman and Emr, 1990). Therefore, uracil-prototrophic transformants were selected at 30°C on YND agar plates supplemented with leucine. Randomly picked transformants were examined for correctly targeted genomic integration by Southern blot analysis. The resulting putative *Δpdd1* mutants were tested for their ability to grow on YNM plates at 43°C, and were also tested for complementation by the *pdd1-201* complementing clones.

Construction of a HpPDD1 *overexpression strain*

To construct an *H. polymorpha* strain overexpressing *HpPDD1*, the gene was placed behind the strong alcohol oxidase promoter (P_{AOX}). First, a 2·5 kb *Hin*dIII–*Xho*I fragment of *HpPDD1* (nt 880–3396) was cloned between the *Hin*dIII and *Xho*I sites of pBluescript II SK+, resulting in plasmid pPDD1-3. Then, a 1·3 kb fragment of *HpPDD1* was produced via PCR by which *Sac*I and *Bgl*II sites were introduced directly upstream of the ATG start codon of *HpPDD1* using the PDD1-ATG primer (5′ AGA GAG CTC AGA TCT ATG AAC TAC GAC TC 3′), the M13/pUC reverse sequencing primer and a subclone containing a 1·6 kb *Bgl*II–*Sal*I fragment of *HpPDD1* in pBluescript. The fragment was digested with *Sac*I and *Hin*dIII and a 0·6 kb fragment (nt 304–880) was inserted between the *Sac*I and *Hin*dIII sites of pPDD1-3. From the resulting plasmid, designated pPDD1-4, a *Bgl*II–*Xho*I fragment carrying the complete *HpPDD1* gene was isolated and inserted between the *Bam*HI and *Sal*I sites of pHIPX4–PAS3 (Kiel *et al.*, 1995), thus replacing the *S. cerevisiae PAS3* gene. Finally, the resulting plasmid, designated pHIPX4–PDD1, was linearized with *Stu*I and transformed into *H. polymorpha*. Selection on integration of the plasmid was performed as described (Faber *et al.*, 1993). Southern hybridization was used to detect single or multicopy integration at the P_{AOX} locus (data not shown).

Other plasmid construction

Plasmids pPDD1-5 and pPDD1-7 were used to determine whether the *pdd1*-complementing activity on the initially cloned fragment represented the authentic *HpPDD1* gene and not a suppressor. pPDD1-5 was constructed by inserting a 2·3 kb *Bam*HI–*Eco*RI (both blunted) fragment containing the *Candida albicans LEU2* gene (obtained from Dr E. Berardi, University of Ancona, Ancona, Italy) between the *Not*I and

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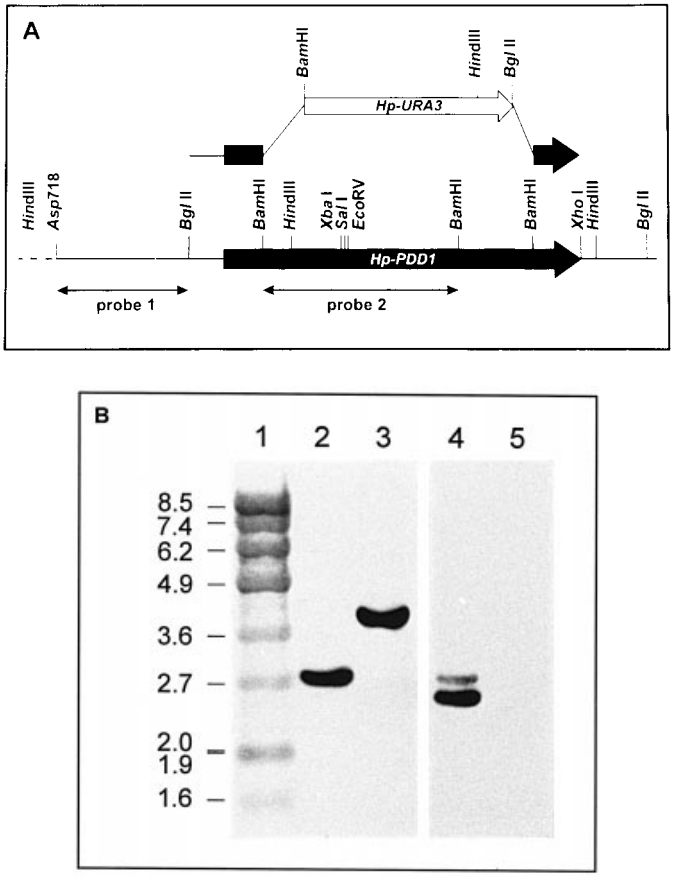

Figure 1. Disruption of the *H. polymorpha PDD1* gene. (A) Schematic representation of the genomic region comprising *HpPDD1*, and deletion of *HpPDD1* by homologous recombination with the *Bgl*II–*Xho*I fragment of plasmid pPDD1-2 (see Materials and Methods). Only relevant restriction sites are indicated. (B) Correct integration of the *Δpdd1* fragment was demonstrated by Southern blot analysis. Genomic DNA of WT *H. polymorpha* (lanes 2 and 4) and *Δpdd1* (lanes 3 and 5) cells was digested with *Hin*dIII, subjected to agarose gel electrophoresis and blotted. The filters were hybridized with a 1·2 kb *Asp*718–*Bgl*II fragment (probe 1 in A; lanes 2 and 3) or a 1·8 kb *Bam*HI fragment of the region comprising *HpPDD1* (probe 2 in A; lanes 4 and 5). Fragment lengths of the marker, phage SPPI DNA, restricted with *Eco*RI (lane 1), are given in kilobases (kb).

*Hin*dIII sites (both blunted) in the polylinker of plasmid pPDD1-3, which contains the 3′ end of *HpPDD1* (*Hin*dIII–*Xho*I fragment in Figure 1A, nt 880–3396, encoding amino acids 181–1016). pPDD1-7 was constructed as follows: first a 2·75 kb *Asp*718–*Sal*I fragment containing the 5′ end of *HpPDD1* (see Figure 1A, up to nt 1615, encoding amino acids 1–427) was inserted between the *Asp*718 and *Sal*I sites of pBluescript II SK+. The resulting plasmid, designated pPDD1-6, was digested with *Not*I and *Sal*I in the polylinker, blunted and ligated to the fragment containing *C. albicans LEU2*. This plasmid was designated pPDD1-7. Both pPDD1-5 and pPDD1-7 were linearized with *Xba*I in the *HpPDD1* region (nt 1317; see Figure 1A) and transformed into *H. polymorpha pdd1-201*. Because these plasmids cannot replicate in *H. polymorpha*, integration should occur at the *Xba*I site in the homologous *PDD1* region of the yeast genome. Correct

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integration was confirmed by Southern hybridization (data not shown).

Generation of polyclonal antibodies against HpPdd1p

A carboxy-terminal part of HpPdd1p comprising amino acids 481–1016 was produced in *E. coli* as part of a fusion protein with maltose-binding protein (MBP) using the Protein Fusion and Purification System (New England Biolabs, Beverly, MA). To this end, a 1·75 kb *Eco*RV–*Hin*dIII fragment (nt 1780–3527) was inserted between the *Xmn*I and *Hin*dIII sites of the pMal-c2 vector. Production and purification of the fusion protein was performed according to the instructions of the supplier of the system. Purified MBP/HpPdd1p fusion protein was used to immunize a rabbit.

Biochemical methods

Crude extracts were prepared according to Waterham *et al.* (1994). Preparation of *H. polymorpha* protoplasts was performed as described (Van der Klei *et al.*, 1998). Discontinuous sucrose density gradient centrifugation of post-nuclear supernatants obtained after homogenizing *H. polymorpha* protoplasts was performed according to Douma *et al.* (1985). Protein concentration determinations, SDS–PAGE and Western blotting was carried out using established procedures. Detection of proteins on Western blots was performed using either the Protoblot immunoblotting system (Promega Biotec) or the ECL system (Amersham, U.K.). The degradation of peroxisomes in batch cultures of *H. polymorpha* was determined by AO activity measurements and Western blotting using extracts of methanol-grown *H. polymorpha* cells shifted to 1% glucose or 0·5% ethanol (Titorenko *et al.*, 1995). The relative HpPex3p levels were determined by laser densitometric scanning of Western blots, decorated with specific antibodies against HpPex3p.

Carboxypeptidase Y secretion assay

The activity of the vacuolar proteinase carboxypeptidase Y (HpCPY) was determined in crude extracts of *H. polymorpha* cells according to Jones (1991)). To determine the presence of α-HpCPY specific protein in culture medium, *H. polymorpha* cells were grown overnight at 30°C in YPD, diluted in fresh YPD medium to an optical denisty at 663 nm of 0·1 and incubated for 20 h at 30°C. The cells were collected by centrifugation, and the culture medium was filtered through a 0·2 µm filter (Schleicher & Schull, Dassel, Germany). Proteins secreted into the culture medium were concentrated by TCA precipitation.

Electron microscopy

Cells were fixed and prepared for electron microscopy and immunocytochemistry as described by Keizer-Gunnink *et al.* (1992). Immunolabelling was performed on ultrathin sections of Unicryl-embedded cells, using specific antibodies against selected *H. polymorpha* proteins.

RESULTS

Isolation and characterization of the H. polymorpha PDD1 *gene*

The *H. polymorpha pdd1-201* mutant is one of a collection of 43 mutant strains impaired in the selective degradation of peroxisomes (Titorenko *et al.*, 1995). Methanol-grown *pdd1-201* cells contain normal peroxisomes, but are impaired in the initial stage of peroxisome degradation, the sequestration of organelles to be degraded from the cytosol. For the isolation of the *HpPDD1* gene we made use of the fact that the mutant is also temperature-sensitive (ts) for growth on methanol (Titorenko *et al.*, 1995). Thus, *H. polymorpha pdd1-201* was transformed with a genomic *H. polymorpha* library. Amongst approximately 10^4 Leu^+ transformants, six colonies were isolated that were able to grow on methanol at 43°C. Their plasmid content was rescued in *E. coli* and the plasmids were reintroduced in the *H. polymorpha pdd1-201* mutant to confirm their complementing ability. Three plasmids, which were able to complement both the ts growth phenotype and the Pdd phenotype of the *Pdd1-201* mutant, were selected for further analysis. These plasmids contained overlapping genomic *H. polymorpha* fragments, ranging from 6·5 to 12·5 hb. By subcloning, the complementing activity was found to reside in a 4·0 kb *Bgl*II fragment, which was sequenced. The sequence was deposited at GenBank and was assigned Accession No. AF121671. Sequence analysis revealed an open reading frame (ORF) with the potential to encode a protein of 1016 amino acids, which was designated *HpPDD1* (Figure 1A).

To determine whether the *pdd1*-complementing activity on the cloned fragment represented the authentic *HpPDD1* gene and not a suppressor, we

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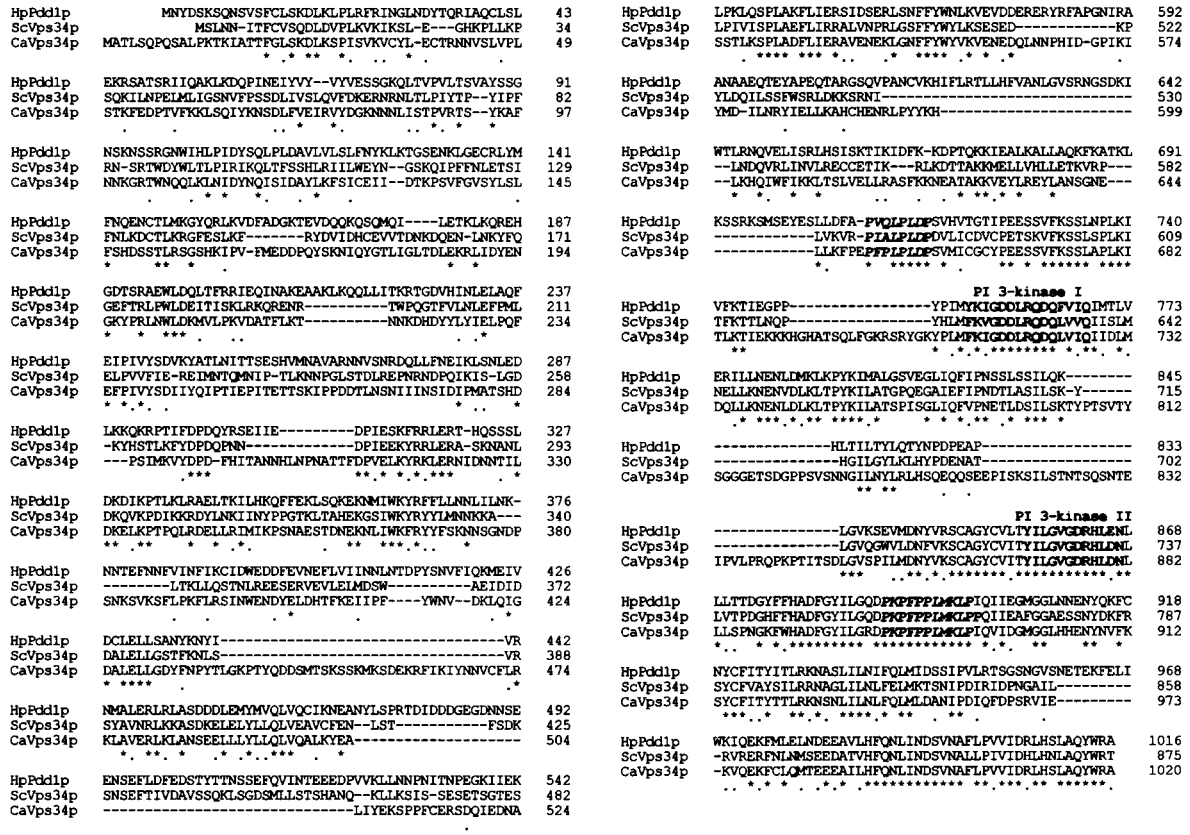

Figure 2. Alignment of the deduced primary sequence of *H. polymorpha* Pdd1p (HpPdd1p) and those of Vps34p of *S. cerevisiae* (ScVps34p) and *C. albicans* (CaVps34p) (Herman and Emr, 1990; Eck *et al.*, 1996). Gaps were introduced to maximize the similarity. The two PtdIns 3-kinase signatures are indicated in bold PI 3-kinase I and II, respectively. Two conserved proline-rich sequences similar to the SH3 ligand consensus are shown in bold italics. Identical residues are indicated by an asterisk below the sequences, conservative replacements by a dot.

tested whether plasmids pPDD1-5 and pPDD1-7, containing either the 3′ end or the 5′ end of the putative *HpPDD1* gene (see Materials and Methods) could complement the original *pdd1-201* mutant. To achieve single cross-over integration of the plasmids at the homologous locus, the plasmids were linearized with *Xba*I and transformed into *H. polymorpha pdd1-201*. Only transformants carrying the integrated pPDD1-7 plasmid were able to grow at elevated temperatures on methanol and also showed normal WT peroxisome degradation patterns (data not shown), indicating that the genomic region in this plasmid covered the mutation responsible for the phenotype of *pdd1-201*. From this we conclude that the genuine *HpPDD1* gene had been isolated, and that the chemically constructed mutant apparently carries its mutation in the 5′ end of the *HpPDD1* gene.

HpPDD1 encodes a protein with an M_r of 116 kDa. Sequence analysis using the PROSITE program revealed that Pdd1p is a member of the family of PtdIns 3-kinases (for review, see De Camilli *et al.*, 1996). A database search revealed highest similarity to the *S. cerevisiae* and *Candida albicans VPS34* gene products (42% and 37% identity over the entire length of the proteins, respectively; Figure 2) and their putative plant and mammalian homologues (33% identity; not shown). In *S. cerevisiae* ScVps34p plays a role in the sorting of soluble vacuolar proteinases to their target organelle (Herman and Emr, 1990) but also in endocytosis (Munn and Riezmann, 1994). Much weaker similarity was observed with other PtdIns kinases. The similarity of HpPdd1p to these proteins was confined to the C-terminus of the proteins, which contains the PtdIns 3-kinase signatures (cf. Figure 2). HpPdd1p lacked obvious

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organellar targeting sequences as well as putative membrane-spanning regions. However, two conserved proline-rich regions are present that are quire similar to SH3 ligand consensus sequences (Mayer and Eck, 1995; marked in Figure 2). The significance of these regions is unknown.

Construction and characterization of a HpPDD1 *deletion strain*

To examine the phenotypic consequences of a null allele of *HpPDD1*, we constructed a gene deletion in *H. polymorpha* by using plasmid pPDD1-2. In this plasmid the 2·4 kb *Bam*HI region of *HpPDD1* (nt 641–3000) encoding amino acids 103–887 was replaced by the *H. polymorpha URA3* gene (Figure 1A). Correct integration of the *URA3* gene in the *HpPDD1* locus was confirmed by Southern hybridization (Figure 1B).

Like the chemically induced *pdd1-201* mutant, the *Δpdd1* strain appeared to be ts for growth. However, the effect seemed much more pronounced in the null mutant, which could grow only reasonably well on glucose and methanol at 30°C (data not shown). At this temperature *Δpdd1* mutant cells were unable to degrade intact peroxisomes after a shift of cells from methanol to glucose- or ethanol-excess conditions. Degradation of peroxisomal proteins was not observed (shown for the matrix enzyme AO and the integral membrane protein HpPex3p in Figure 3A, B, respectively). Electron microscopy revealed that, like the original *pdd1-201* mutant, *H. polymorpha Δpdd1* cells were disturbed in the initial stage of selective peroxisome degradation. Frequently, small membranous structures were observed in the cytosol which were absent in WT controls; however, sequestration of individual peroxisomes—or initial stages of it—was never observed (Figure 4A). Immunocytochemistry confirmed that peroxisomal matrix enzymes were confined to the peroxisomes; a vacuolar localization was never observed (shown for AO in Figure 4B). When *HpPDD1* was reintroduced in *Δpdd1*, using either the *pdd1-201* complementing fragment or a *PDD1* overexpression cassette, the ability of the cells to grow at elevated temperatures was restored. Also, the transformants were able to degrade peroxisomes as efficiently as WT cells. Immunocytochemically, autophagic vacuoles (Figure 4C), characterized by the presence of peroxisomal matrix proteins, were readily detected (shown for AO in Figure 4D).

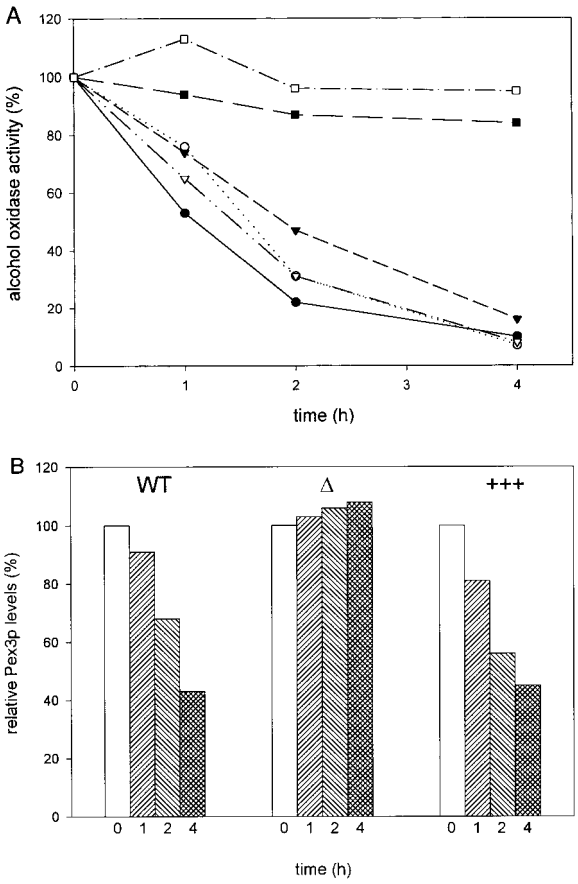

Figure 3. Selective inactivation of peroxisomes in *H. polymorpha*. (A) Kinetics of changes in the specific enzyme activity of alcohol oxidase, determined in crude extracts from *H. polymorpha* WT (circles), *Δpdd1* (squares) and *Δpdd1*::[P_{AOX}*PDD1*]^{4x} (triangles) cells after the addition of glucose (filled symbols) or ethanol (open symbols) to batch cultures growing exponentially at 30°C. The decrease in specific activities is expressed as a percentage of the initial value, which is set to 100%. Data are corrected for growth of the cells on glucose or ethanol. (B) Decrease in the level of the peroxisomal membrane protein Pex3p determined by laser densitometric scanning of Western blots, prepared from whole cell lysates of *H. polymorpha* WT, *Δpdd1* (*Δ*) and *Δpdd1*::[P_{AOX}*PDD1*]^{4x} (+++) cells (50 µg samples), using polyclonal antibodies against HpPex3p, in the initial hours after a shift of cells from methanol to ethanol. The initial value is set to 100%. Data are corrected for growth of the cells on ethanol. Identical results were obtained when cells from these strains were shifted from methanol to glucose (data not shown).

H. polymorpha pdd1 *mutants are affected in the sorting of HpCPY to the vacuole*

Recently, we have isolated the *H. polymorpha* gene encoding carboxypeptidase Y (HpCPY; Bellu *et al.*, 1999). We have shown that HpCPY is

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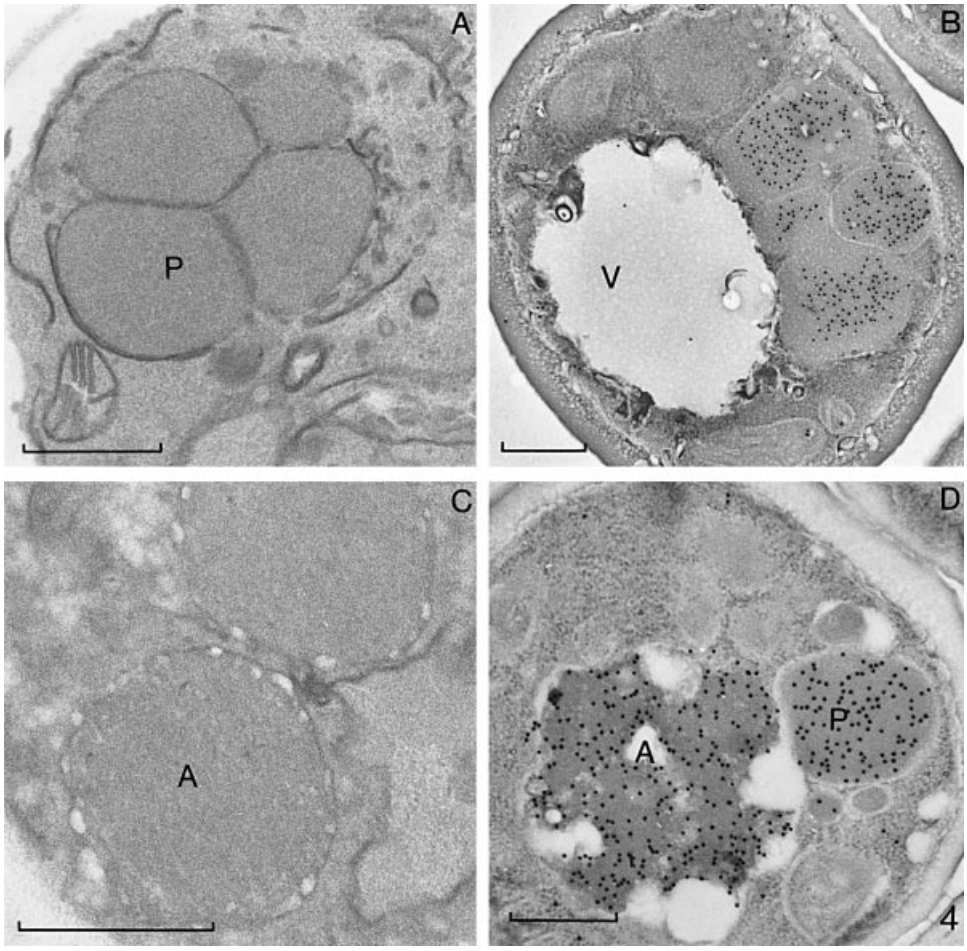

Figure 4. Electron micrographs of *H. polymorpha Δpdd1* cells (A, B), *Δpdd1* cells complemented with a plasmid carrying the 4 kb *Bgl*II fragment that specifies *H. polymorpha PDD1* (C) and *Δpdd1*::[P_{AOX}*PDD1*]^{4x} (D). Cells were grown on methanol and subsequently shifted to glucose for 1 h (D) or 2 h (A, B and C) to induce specific peroxisome degradation. In *H. polymorpha Δpdd1* cells, peroxisomes are not sequestered from the cytosol; note the membranous structures present in the vicinity of peroxisomes (A). When *H. polymorpha Δpdd1* is complemented with *HpPDD1*, the specific degradation of peroxisomes is restored; autophagosomes containing degrading peroxisomes are visible (C). Immunocytochemically, using α-AO antibodies, AO protein remains confined to peroxisomes in *H. polymorpha Δpdd1* cells; vacuolar labelling is not observed (B). In *Δpdd1* cells complemented with *HpPDD1* AO protein is also observed in autophagosomes (D). Abbreviations: A, autophagosome; P, peroxisome; V, vacuole. The bar represents 0·5 μm.

a vacuolar protein that is modified by N-linked glycosylation. Furthermore, deglycosylated HpCPY has an apparent M_r of 47 kDa, whereas the calculated M_r of the protein encoded by *HpCPY* is 61 kDa, suggesting that, like *S. cerevisiae* CPY, HpCPY undergoes maturation in the vacuole (Bellu *et al*., 1999). We have used HpCPY as a tool to determine whether, like *S. cerevisiae vps34* mutants, *H. polymorpha pdd1* mutants are affected in vacuolar protein sorting and missort vacuolar proteins to the culture medium. *H. polymorpha* WT, *pdd1-201, Δpdd1* and *Δcpy* cells were grown at 30°C in YPD medium to the late stationary growth phase. First, the specific activity of HpCPY was determined in crude cell extracts. Table 1 indicates that both *pdd1-201* and *Δpdd1* extracts showed reduced HpCPY activities compared to WT controls. To investigate whether this reduced activity in *pdd1* cells was due to missorting of the protein to the culture medium,

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Table 1. Specific enzyme activity of HpCPY in *H. polymorpha* strains.

Strain	Wild-type (WT)	*Δpdd1*	*pdd1-201*	*Δcpy*
Specific activity (mU/mg protein)	3·8	0·68	1·0	0·24

Carboxypeptidase Y activity was measured in crude cell extracts of *H. polymorpha* WT, *Δpdd1*, *pdd1-201* and *Δcpy* cells grown to the late stationary phase on YPD medium at 30°C.

we subsequently analysed whole-cell lysates and culture medium of the four different strains for the presence of HpCPY protein by Western Blotting. As expected, a single protein band corresponding to the mature form of HpCPY (Bellu *et al*., 1999) was detected in crude extracts of WT cells, which was absent in extracts of *Δcpy* cells used as a control (Figure 5). In the culture medium of both WT and *Δcpy* cells, HpCPY protein was not detectable. Hence, in WT cells HpCPY was normally sorted to the vacuole and processed to its mature size. In contrast, in crude extracts of *Δpdd1* and *pdd1-201* cells additional protein bands were observed, suggesting that normal maturation/processing of HpCPY was hampered. Moreover, in the medium of these cultures HpCPY protein was readily detected. These results indicate that similar to *S. cerevisiae Δvps34* mutants, sorting and maturation of HpCPY was affected in *H. polymorpha pdd1* mutants.

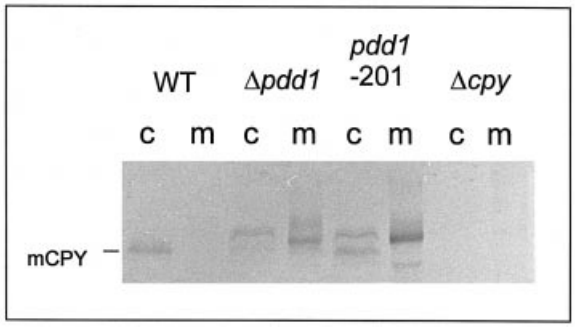

Figure 5. Analysis of secretion of carboxypeptidase Y (HpCPY) by various *H. polymorpha* strains. *H. polymorpha* WT, *Δpdd1*, *pdd1-201* and *Δcpy* cells were grown to the late stationary phase in YPD medium at 30°C. The presence of HpCPY protein in whole-cell lysates (c) and TCA-precipitates from the culture medium (m) was determined by Western blotting using α-HpCPY antibodies. The amount of protein loaded corresponds to 0·1 ml (cells) or 0·5 ml (medium) of culture. In WT cells only the mature protein (mCPY) is present, which is absent in the culture medium. Different forms of α-HpCPY specific protein can be observed in crude extracts and culture medium of *pdd1* mutants. In the *Δcpy* control strain, HpCPY-specific protein bands are absent.

Overproduction of HpPdd1p in H. polymorpha

In order to obtain an *HpPDD1* overexpression strain, *HpPDD1* was placed under the control of the strong *H. polymorpha* alcohol oxidase promoter (P_{AOX}). The resulting plasmid was integrated in multiple copies (approximately four) at the P_{AOX} locus of the *H. polymorpha Δpdd1* genome. *Δpdd1*::[P_{AOX}*PDD1*]^{4x} cells grew normally at 37°C and 43°C. Interestingly, overproduction of HpPdd1p did not induce degradation of peroxisomes during growth of cells on methanol (Figure 6A), suggesting that the regulation of the degradation process was not influenced by the increased level of HpPdd1p. Also, the kinetics of glucose or ethanol-induced peroxisome degradation in the HpPdd1p overproducing strain were indistinguishable from those of WT controls (shown for AO and HpPex3p in Figure 3A, B, respectively; see also Figure 4D).

Subcellular localization of HpPdd1p

To determine the subcellular location of HpPdd1p in *H. polymorpha*, antibodies were raised in rabbit against an MBP–HpPdd1p fusion protein (comprising amino acids 481–1016 of HpPdd1p). On Western blots, prepared from crude extracts of WT and HpPdd1p-overproducing cells, the antiserum recognized a single protein band with an apparent molecular weight of approximately 120 kDa, which is in good agreement with the calculated molecular weight of the *HpPDD1* gene product (116 kDa). Since this band was lacking in crude extracts from *Δpdd1* cells, we concluded that the antiserum specifically recognized HpPdd1p (Figure 7A). HpPdd1p seems to be a low abundant protein that is produced constitutively, since the amount of protein did not differ significantly in crude extracts prepared from variously grown

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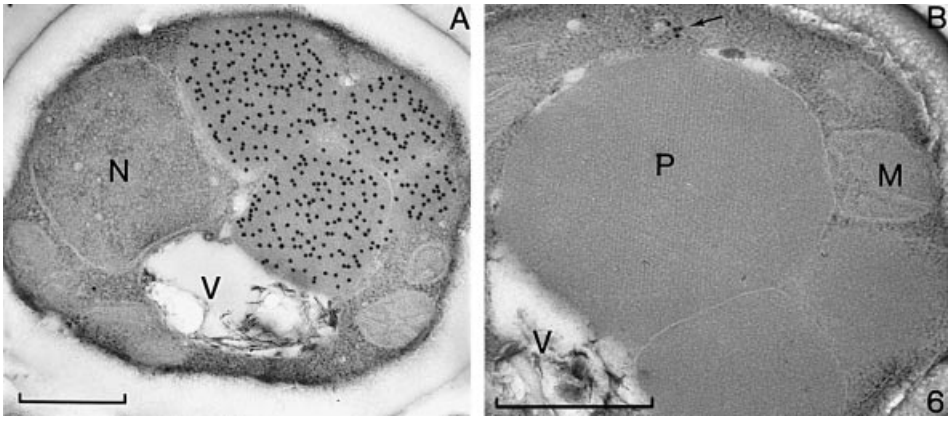

Figure 6. Immunocytochemical analysis of *H. polymorpha Δpdd1*::[P_{AOX}*PDD1*]^{4x} cells (A) and WT cells (B) grown on methanol. Panel A shows labelling with α-AO antibodies and indicates that AO protein remains confined to peroxisomes in *H. polymorpha* cells overexpressing *HpPDD1* during growth on methanol. Panel B shows the result of localization studies of HpPdd1p using α-HpPdd1p antibodies. In methylotrophically-grown WT cells, the protein is localized on membrane structures, distinct from mitochondria and peroxisomes (B). Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bars = 0·5 μm.

H. polymorpha WT cells (Figure 7B). Also, during adaptation of methanol-grown cells to ethanol-excess conditions, the relative amounts of HpPdd1p remained approximately constant (Figure 7B).

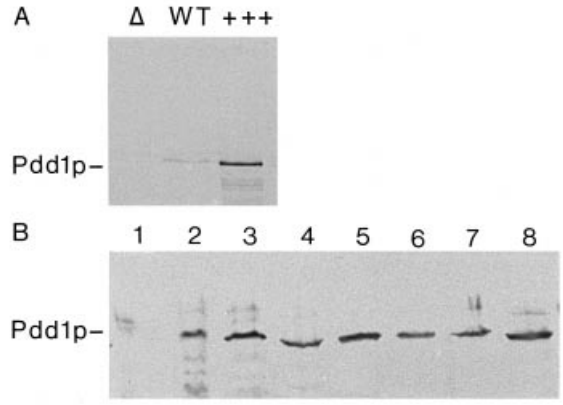

Figure 7. Immunological detection of HpPdd1p using α-HpPdd1p antibodies. Western blot of whole-cell lysates of *H. polymorpha Δpdd1* (Δ, 50 μg of protein), WT (50 μg of protein and *Δpdd1*::[P_{AOX}*PDD1*]^{4x} (+++, 10 μg of protein) cells grown in batch cultures on mineral medium containing methanol (detection using alkaline phosphatase)]. (B) HpPdd1p is a constitutively expressed protein. Western blot of whole cell lysates of *H. polymorpha Δpdd1* (lane 1) and WT (lanes 2–8) cells grown in batch culture on YPD (lanes 1 and 2), on mineral medium with glucose (lane 3), ethanol (lane 4) or methanol (lane 5), and after a shift of methanol-grown cells (lane 5) to ethanol (lanes 6–8, respectively, 1, 2·5 and 5 h after the shift). Equal amounts of protein (50 μg) were loaded per lane (detection with ECL).

The subcellular localization of HpPdd1p was studied immunocytochemically and biochemically, using conventional cell fractionation methods. Immunocytochemically, using ultrathin sections of methanol-grown WT cells and α-HpPdd1p antiserum, specific labelling was observed on small cytosolic vesicular structures (Figure 6B). In methanol-grown cells of a *HpPDD1*-overexpressing strain, the number of these HpPdd1p-containing vesicles was not significantly enhanced (data not shown). Biochemically, after sucrose density centrifugation of a post-nuclear supernatant of methanol-grown *H. polymorpha* WT cells (Figure 8A), HpPdd1p was only found in fraction 18 at 40% sucrose (Figure 8B). In this gradient the peroxisomal marker protein AO sedimented to fraction 6 at approximately 53% sucrose, the mitochondrial form of malate dehydrogenase to fraction 16 at 43% sucrose, and the endoplasmic reticulum marker protein HpSec63p to fraction 18 at 40% sucrose (Figure 8B). Soluble (cytosolic) proteins remained at the top of the gradient (fractions 19–21; data not shown). Thus, the *in situ* and fractionation data reveal that, like ScVps34p, HpPdd1p is not a soluble protein, but is apparently membrane-bound to structures distinct from mitochondria and peroxisomes. However, the nature of these structures is unknown.

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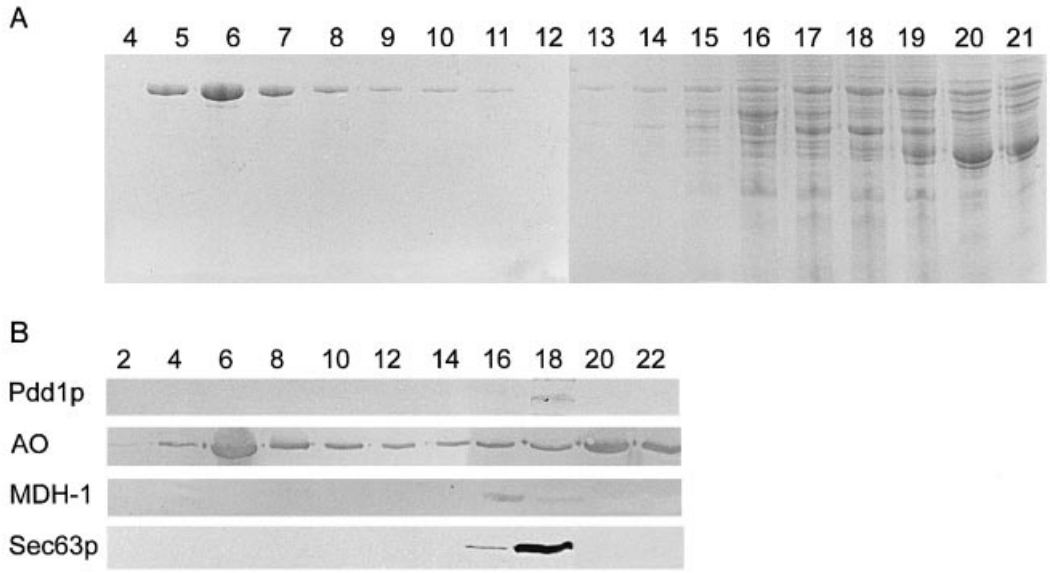

Figure 8. Subcellular localization of HpPdd1p in *H. polymorpha* (A) Coomassie Brilliant Blue staining of fractions of a sucrose gradient from a post-nuclear supernatant prepared from methanol-grown WT *H. polymorpha* cells. Only the relevant fractions are shown. A clear separation between peroxisomes (fractions 5–7), mitochondria (fraction 16), endomembranes (fraction 18) and the cytosol is shown (fraction 19 and higher) is shown. (B) Immunological detection of HpPdd1p in the even-numbered fractions of the sucrose gradient shown above. Equal volumes of each fraction were loaded per lane. Western blots were decorated with antibodies against HpPdd1p, the peroxisomal matrix protein alcohol oxidase (AO), the mitochondrial form of malate dehydrogenase (MDH-1) and the ER marker HpSec63p. HpPdd1p is present in fraction 18, indicating co-localization with endomembranes.

DISCUSSION

In this report we describe the cloning and characterization of the *H. polymorpha PDD1* gene which encodes a 116 kDa protein with strong similarity to the *S. cerevisiae VPS34* gene product. *H. polymorpha pdd1* mutants were affected in selective peroxisome degradation and also missorted the vacuolar proteinase HpCPY. Furthermore, using immunocytochemical and biochemical techniques, HpPdd1p was shown to be located on intracellular membranes in *H. polymorpha*.

Originally, *ScVPS34* was isolated as a gene involved in the sorting of the proteinase ScCPY to the vacuole. However, mutations in *ScVPS34* appear to result in a large number of phenotypes which include temperature-sensitive growth, defects in osmoregulation, disturbed vacuolar segregation and missorting of a number of soluble vacuolar proteinases (Herman and Emr, 1990). In addition, *S. cerevisiae vps34* mutants appear to be affected in endocytosis (Munn and Riezman, 1994). ScVps34p and its putative homologues from higher eukaryotes belong to a family of enzymes involved in the formation of various phosphoinositides (PI) (for review, see De Camilli *et al.*, 1996). Lipid kinases of the ScVps34p group specifically phosphorylate the D-3 position of the inositol ring of non-phosphorylated PtdIns molecules. In bakers' yeast, ScVps34p is located in a complex with a membrane-associated protein kinase, ScVps15p, which is essential for the activation of the lipid kinase (reviewed by Stack *et al.*, 1995; De Camilli *et al.*, 1996). An active ScVps34p–ScVps15p complex is required for vacuolar protein sorting. Also, in mammals, a complex containing orthologues of ScVps34p and ScVps15p is required for the efficient sorting and delivery of soluble proteins to the lysozome (Volinia *et al.*, 1995; Panaretou *et al.*, 1997).

Our data indicate that a number of phenotypical characteristics of baker's yeast *vps34* mutants can be observed for *H. polymorpha pdd1* mutants. These mutants are temperature-sensitive for growth and secrete HpCPY (Figure 5). In addition, we have indications that *pdd1* mutants are affected in the process of endocytosis as well (J. A. K. W. Kiel, unpublished results). These

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phenotypical characteristics combined with the high sequence similarity (42% identity) between the proteins suggest that HpPdd1p is the functional homologue of ScVps34p. However, we cannot exclude that HpPdd1p is merely a protein homologous to ScVps34p involved in peroxisome degradation. Recently, we isolated a putative Vps15p orthologue from the methylotrophic yeast *P. pastoris* and could show that disruption of the putative *P. pastoris VPS15* gene affected the degradation of peroxisomes after a shift of cells to non-methylotrophic conditions (O. V. Stasyk *et al.*, submitted). This finding suggests that a complex similar to ScVps34p–ScVps15p is apparently required for selective peroxisome degradation in methylotrophic yeasts.

Our finding that HpPdd1p is membrane-bound confirms data obtained in *S. cerevisiae*, which indicated that the ScVps34p–ScVps15p complex is located on the cytosolic face of a so-far uncharacterized membrane. DeWald *et al.* (1997) have suggested that this membrane might be a late Golgi compartment or an endosome. However, the nature of the membrane remains speculative. Our localization data also suggest that in *H. polymorpha*, HpPdd1p, which has no transmembrane spans, must be recruited to this membrane by another protein, which may well be HpVps15p. Future investigations will have to address this question.

The precise role of HpPdd1p in selective peroxisome degradation is not yet clear. The option that in *H. polymorpha* HpPdd1p is merely needed to sort proteases to the vacule to allow the degradation of peroxisomes is difficult to envisage, taking into account that *pdd1* mutants are specifically affected in the initial stage of peroxisome degradation and, hence, that these organelles do not enter the vacuole. One possibility is that in *H. polymorpha* HpPdd1p displays its presumed PtdIns 3-kinase activity at the peroxisomal membrane. Previously, we showed that the peroxisomal membrane is the prime target for the initiation of the degradation process (Van der Klei *et al.*, 1991; Veenhuis *et al.*, 1996). This suggests that one or more specific peroxisomal membrane components are essential to tag peroxisomes to be degraded. Also, we showed that from various *H. polymorpha* mutants defective in peroxisome biogenesis, solely the peroxisomal remnants present in *H. polymorpha Δpex14* cells are not susceptible to degradation, rendering HpPex14p a possible candidate essential in the tagging machinery (Veenhuis *et al.*, 1996). Moreover, in *H. polymorpha*, mature, import-incompetent peroxisomes particularly are subject to degradation, leaving the smaller ones virtually unaffected (Veenhuis *et al.*, 1983; Baerends *et al.*, 1997). The simplest explanation for these phenomena is that a proteinaceous factor or complex is present at the WT peroxisomal membrane that is absent or structurally modified in the peroxisomal remnants in *Δpex14* cells and in import-competent peroxisomes. We speculate that this factor may be important for recruiting a HpPdd1p–HpVps15p-containing structure during induction of catabolite inactivation. Analogous to the role of ScVps34p–ScVps15p in vacuolar protein sorting, this may allow the HpVps15p moiety of the complex to activate the PtdIns 3-kinase activity of HpPdd1p, resulting in phosphorylation of PtdIns molecules in the lipid bilayer. These may constitute the initiation site at which membrane components bind as a first step in the sequestration process. Whether the vesicles containing HpPdd1p also function as precursor membranes that sequester peroxisomes, has still to be investigated.

An alternative possibility is that the role of HpPdd1p in peroxisome turnover is an indirect one. HpPdd1p could be needed to sort proteins to the vacuole that are essential for recognition of organelles tagged for degradation by a mechanism independent from HpPdd1p functioning. However, this option is less likely, since peroxisome degradation in *H. polymorpha* is strictly dependent on a hetero-typical membrane fusion event between sequestered peroxisomes and vacuolar vesicles (Veenhuis *et al.*, 1983), whereas *H. polymorpha pdd1* mutants are already affected in a step prior to this fusion event. Furthermore, microautophagic peroxisome degradation events, as described for *P. pastoris* (Tuttle and Dunn, 1995; Sakai *et al.*, 1998), have never been observed in *H. polymorpha*. Further investigations, e.g. on the proteins complexed to HpPdd1p, are required to dissect the selective peroxisome degradation pathway in *H. polymorpha* in more detail and, associated with this, may also shed light on the mechanisms of organelle homeostasis.

ACKNOWLEDGEMENTS

We thank Anita Kram, Ineke Keizer-Gunnink, Anna Rita Bellu and Jan Zagers for expert assistance in different parts of this study.

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