Characterization of the \textit{Hansenula polymorpha} \textit{CPY} Gene Encoding Carboxypeptidase Y

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We have isolated the \textit{Hansenula polymorpha} \textit{CPY} gene encoding carboxypeptidase \textit{Y} (Hp-CPY). The deduced amino acid sequence revealed that Hp-CPY consists of 541 amino acids and has a calculated Mr of 60,793. The protein is highly similar to \textit{Saccharomyces cerevisiae} CPY (61.8\% identity). At the N-terminus of Hp-CPY signals for the entry into the secretory pathway and subsequent sorting to the vacuole were identified. Immunocytochemically, using monospecific antibodies raised against Hp-CPY, the protein was localized to the vacuole. On Western blots, a diffuse protein band was observed in extracts of \textit{H. polymorpha} cells, suggesting that the protein is glycosylated. This was confirmed by endoglycosidase H treatment, which resulted in a strong reduction of the apparent Mr of the protein. We have investigated the effect of \textit{CPY} deletion on the degradation of peroxisomes, an autophagous process that occurs when the organelles become redundant for growth. In \textit{Δcpy} cells peroxisomal proteins were degraded in the vacuole as efficiently as in wild-type \textit{H. polymorpha} cells, indicating that CPY is not a major proteinase in this pathway. Copyright © 1999 John Wiley & Sons, Ltd.

\textbf{KEY WORDS} — autophagy; glycosylation; peroxisome turnover; methylotrophic yeast; vacuolar proteinases

\section*{INTRODUCTION}

To date, the vacuolar proteolytic system in the methylotrophic yeast \textit{Hansenula polymorpha} has attracted little attention. In contrast, many proteases that occur in the \textit{Saccharomyces cerevisiae} vacuole have been studied in great detail. The bakers yeast vacuole was shown to contain several proteases: proteinase \textit{A} (pr\textit{A}); proteinase \textit{B} (pr\textit{B}); carboxypeptidase \textit{Y} (CPY); carboxypeptidase \textit{S}; aminopeptidase \textit{Y}, dipeptidyl aminopeptidase \textit{B} and aminopeptidase \textit{I} (for reviews, see Klionsky \textit{et al.}, 1990; Van den Hazel \textit{et al.}, 1996), proteins that play an important role in protein turnover. From these yeast proteases, CPY has been characterized in detail (see e.g., Valls \textit{et al.}, 1987, 1990). CPY is synthesized as a preprotein. It contains an N-terminal signal peptide, which is removed upon entry into the lumen of the endoplasmic reticulum (ER), where the resulting preprotein becomes Asn-glycosylated at four locations. Elongation of these carbohydrate chains occurs during transport of the protein through the Golgi complex. Finally, sorting of the proCPY to the vacuole occurs. This requires the Gln–Arg–Pro–Leu sequence in the pro-region of the protein. In the vacuole, a Pr\textit{A}/Pr\textit{B}-catalysed cleavage of the propeptide of proCPY occurs, a step that is required for proper folding and activation of the enzyme (Winther and Sorensen, 1991; Ramos \textit{et al.}, 1994). The proteases Pr\textit{A} and Pr\textit{B} have also been shown to be essential for the maturation and activation of other vacuolar enzymes (see Klionsky \textit{et al.}, 1990). Hence, \textit{pr\textit{A} pr\textit{B}} mutants are almost devoid of vacuolar proteolytic activity.

Vacular proteases play an important role in the degradation of peroxisomes (Tuttle and Dunn, 1995; Chiang \textit{et al.}, 1996). Peroxisomes are cell
organelles surrounded by a single membrane which are present in almost all eukaryotic cells. Characteristic features of these organelles are their functional versatility and their inducibility (Lazarow and Fujiki, 1985). In *H. polymorpha*, peroxisome proliferation is readily induced by a number of carbon and organic nitrogen sources used for growth. Under these conditions the organelles contain key enzymes involved in the metabolism of these substrates (Veenhuis and Harder, 1991). On the other hand, peroxisomes are rapidly degraded when cells are shifted to conditions in which the organelles become redundant for growth. In *H. polymorpha* peroxisomes are degraded individually by an autophagic process involving: (a) sequestration of the organelle to be degraded by a number of membranous layers, presumably derived from the ER; (b) fusion of the sequestered compartment with the vacuole or an autophagic vesicle; and (c) degradation of the peroxisome contents by vacuolar hydrolases (Veenhuis et al., 1983). It has been observed in *S. cerevisiae* and *P. pastoris* that mutants lacking PrA or PrA+PrB activity accumulate undegraded cytosolic proteins and organelles, including peroxisomes, in the vacuole under conditions that induce autophagy (Tuttle and Dunn, 1995; Chiang et al., 1996). Because prA prB mutants lack most vacuolar protease activity, the role of specific proteases in peroxisome degradation was not investigated in these studies. Here we focus on the role of carboxypeptidase Y (CPY) in peroxisome degradation in *H. polymorpha*. We have cloned the gene and studied the effect of CPY deletion on selective peroxisome degradation.

**MATERIALS AND METHODS**

**Organisms, media and growth conditions**

*Hansenula polymorpha* NCYC495 and auxotrophic derivatives thereof (Gleeson and Sudbery, 1988) were grown at 37°C in rich medium containing 1% yeast extract, 2% peptone and 1% glucose (YPD), or in selective minimal media containing 0·67% yeast nitrogen base without amino acids (DIFCO), supplemented with 1% glucose (YND) or 0·5% methanol (YNM). Amino acids and uracil were added to a final concentration of 30 µg/ml. *Escherichia coli* DH5α (Sambrook et al., 1989) was grown at 37°C in 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 described (*Sambrook et al.*, 1989). Southern blot analysis and colony hybridization were performed using the ECL direct nucleic acid labelling and detection system (Amersham Corp., Arlington Heights, IL). Biochemicals were obtained from Boehringer, Mannheim, Germany.

**Cloning and sequence analysis of the CPY gene**

To isolate the *CPY* gene, a 260 bp *H. polymorpha BglII* fragment was used as a probe to

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**Figure 2.** Alignment of yeast carboxypeptidase Y sequences. The primary sequences of carboxypeptidase Y from *H. polymorpha* (Hp-CPY), *P. pastoris* (Pp-CPY; *Ohi et al.*, 1996) and *S. cerevisiae* (Sc-CPY; *Valls et al.*, 1987) were aligned using the CLUSTAL program. Gaps were introduced to maximize the similarity. Identical residues are indicated by an asterisk below the sequences, conservative replacements by a dot. Putative pre-sequences, vacuolar targeting signals, N-linked glycosylation sites, and serine and histidine active site residues are indicated in white letters on a black background. The putative pro-sequences of the proteins are indicated by the black line above the sequences.
screen a *H. polymorpha* genomic DNA library constructed in vector pYT3 (Tan et al., 1995), using bacterial colony hybridization. Five positive clones were isolated. One of these clones, designated pYT3-CPY, which contained an insert of about 5 kb that hybridized to the probe, was analysed further. A *Bam*HI fragment of approximately 4 kb was subcloned in both orientations into *Bam*HI-digested pBluescript II SK*+* (Stratagene, San Diego, CA) to create plasmids pBS-CPYa and pBS-CPYb. Double-stranded DNA sequencing of a 2·5 kb region of the subclones was carried out on an ABI 313A automatic sequencer (Applied Biosystems Inc.) using the Taq Dye Deoxy Terminator Cycle Sequencing Kit. In addition, several selected oligonucleotides were synthesized to complete or confirm certain portions of the DNA sequence. For analysis of the DNA and amino acid sequences, the PC-GENE program release 6.70 (IntelliGenetic Inc., Mountain View, CA) was used. The TBLASTN algorithm (Altschul et al., 1990) was used to screen databases at the National Center for Biotechnology Information (NCBI, Bethesda, MD). The nucleotide sequence of *H. polymorpha* CPY was deposited at GenBank and was assigned Accession No. AF085063.

**Construction of a CPY deletion mutant**

To disrupt the wild-type CPY gene, a 2·3 kb *Bam*HI fragment containing the *H. polymorpha* URA3 gene (Merckelbach et al., 1993) was ligated into *Bgl*II-digested pBS-CPYa, resulting in a deletion of nt 639–904 of CPY encoding amino acids 195–282 (Figure 1A). The resulting plasmid was digested with *Bam*HI top yield a 6 kb linear fragment containing the *URA3* gene flanked by CPY sequences (Figure 1A), which was transformed to *H. polymorpha* NCYC495 (leu1.1 ura3). Uracil prototrophic transformants were examined for the proper integration of the *URA3* gene at the CPY locus by Southern blot analysis (Figure 1B). The resulting putative Δ*cpy* strains were also tested for CPY activity.

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Figure 3. Immunological detection of CPY protein. Western blot of whole-cell lysates of *H. polymorpha* WT (lane 1), Δ*cpy* (lane 2) and Δ*cpy*[PYT3-CPY] (slightly over-expressing CPY; lane 3) cells grown in batch culture on YND medium. The very weak, diffuse protein band reflects the low level of CPY protein in cells growing on glucose. Equal amounts of protein (20 μg) were loaded in each lane. Lanes 4 and 5 show whole cell lysates (30 μg of protein) of YND-grown *H. polymorpha* WT cells after SDS-denaturation (lane 4), followed by endoglycosidase H treatment (lane 5). The blots were decorated with α-CPY antibodies. Protein sizes of the marker are indicated in kDa. The arrow indicates deglycosylated CPY at approximately 47 kDa.
Generation of anti-CPY antibodies

The Protein Fusion and Purification System (New England Biolabs, Beverly, MA) was used for the production of a maltose-binding protein (MBP)-CPY fusion protein in E. coli. A 2·2 kb BglII–PstI fragment encoding amino acids 283–541 of CPY was cloned in frame behind the malE gene between the BamHI and PstI sites of the pMAL-c2 vector. Expression of the malE–CPY chimeric gene was induced by the addition of 0·3 mM IPTG to exponentially growing cultures. The fusion protein was recovered by centrifugation as inclusion bodies, which were used for immunization in rabbit (Harlow and Lane, 1988).

Biochemical methods

Crude extracts were prepared as described (Waterham et al., 1994), except that 1 mM phenylmethylsulphonylfluoride and 2·5 μg/ml of leupeptine were added. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as standard. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described (Laemmli, 1970). Western blotting was performed using either the Protoblot immunoblotting system (Promega Biotech) or the ECL system (Amersham Corp., Arlington Heights, IL). Alcohol oxidase (Verduyn et al., 1984), catalase (Lück, 1963) and CPY (Jones, 1991) activities were determined in cell extracts by established procedures. CPY activity in H. polymorpha colonies was detected using the FAST-Garnet overlay technique (Jones, 1991). Deglycosylation of CPY in cell extracts was performed using endoglycosidase H, as described (Elgersma et al., 1997). Degradation of peroxisomal proteins in batch cultures of H. polymorpha was followed by enzyme activity measurements and Western blotting using extracts of methanol-grown cells shifted to 0·5% glucose (Titorenko et al., 1995).

Electron microscopy

H. polymorpha cells were fixed in 3% formaldehyde in 50 mM Na-phosphate buffer (pH 7·2). Cryosections were prepared according to Tokuyasu (1973) and immunolabelled using α-CPY antibodies.

RESULTS AND DISCUSSION

Isolation and characterization of the H. polymorpha CPY gene

To clone the H. polymorpha CPY gene, we used a genomic 260 bp BglII H. polymorpha fragment, which showed high similarity to S. cerevisiae PRC1 encoding carboxypeptidase Y (Valls et al., 1987) as a probe (probe 1 in Figure 1A). This fragment had become available from an earlier screen for suitable H. polymorpha promoters (P. E. Sudbery, unpublished data). In Southern blot analyses this fragment strongly hybridized with H. polymorpha genomic DNA (data not shown). Subsequently, we screened a genomic H. polymorpha gene library with this probe. One positive clone, designated pYT3-CPY, containing a hybridizing insert of approximately 5 kb, was selected for further analysis. The nucleotide sequence of a 2509 bp region of this clone was determined. Sequence analysis revealed the presence of one complete and one incomplete open reading frame (ORF; see Figure 1A). The complete ORF encoded a protein of 541 amino acids with a calculated Mr of 60,793, which was highly similar to CPY from S. cerevisiae (Sc-CPY; Valls et al., 1987) and P. pastoris (Pp-CPY; Ohl et al., 1996) (62·4% and 60·4% identity, respectively; Figure 2).

Therefore, we concluded that ORF1 represents the H. polymorpha CPY gene. The incomplete ORF2, which showed similarity to the S. cerevisiae
Figure 5. (A) and (B).
RAD26 gene (Van Gool et al., 1994), and presumably represents its *H. polymorpha* orthologue, was not studied further.

The deduced primary sequence of *H. polymorpha* CPY (Hp-CPY) contains a putative pre-sequence involved in import of the protein into the ER (cf. Klionsky et al., 1990). This signal extends from amino acid 1 to the alanine at position 20 (Figure 2). A putative vacuolar targeting signal consisting of amino acids 30–33 (Gln–His–Pro–Leu) similar to that observed in Sc-CPY (Gln–Arg–Pro–Leu), was also evident. Additionally, a putative pro-region that shows only little conservation to Sc-CPY and Pp-CPY is located between amino acids 21 and 122. Thus we calculate that the mature Hp-CPY protein is presumably composed of 419 amino acids with a predicted Mr of 47,142. In contrast to Sc-CPY, which contains four N-glycosylation sites, Hp-CPY is predicted to contain only three sites. These sites correspond to three of the four locations in Sc-CPY. As expected, the active site Ser and His residues are conserved in Hp-CPY (Figure 2).

**Construction and characterization of a CPY disruption mutant**

To study the possible role of CPY in selective peroxisome degradation in *H. polymorpha*, a CPY disruption strain was constructed. We found that the CPY activity in the Δ*cpy* strain was reduced significantly as compared to the wild-type (0·2 vs 3·8 mU/mg protein). Polyclonal antibodies were raised against a MBP–CPY fusion protein in rabbit. In order to test the specificity of the antiserum (α-CPY), Western blots were prepared from crude extracts of YND-grown *H. polymorpha* WT, Δ*cpy* and Δ*cpy[pYT3-CPY]* cells (Figure 3, lanes 1–3). On the blots a diffuse protein band of approximately 60 kDa could be observed in WT and Δ*cpy[pYT3-CPY]* extracts, which was absent in Δ*cpy* extracts. From these results we conclude...
that the antiserum specifically recognizes *H. polymorpha* CPY. The larger size of the protein as compared to its calculated molecular mass (47 kDa), as well as its diffuse pattern on Western blots, indicates that Hp-CPY is presumably modified by N-linked glycosylation in the ER followed by mannosylation in the Golgi complex. This was confirmed by treatment of a WT cell extract with endoglucosidase H, which cleaves N-linked glycans from proteins. After deglycosylation, α-CPY antibodies recognized an approximately 47 kDa protein, instead of the 60 kDa protein (Figure 3, lanes 4 and 5).

The α-CPY antiserum was subsequently used for the localization of CPY by immunocytochemical methods. Labelling of cryosections of stationary YND-grown WT *H. polymorpha* cells with the antiserum resulted in the specific labelling of vacuolar profiles (Figure 4). Labelling of other cell compartments was not observed, suggesting that Hp-CPY is a constituent of the vacuole. No labelling was observed in Δcpy controls (data not shown).

Growth experiments indicated that deletion of the CPY gene did not significantly affect growth of *H. polymorpha* on glucose and methanol. Also, no major differences in the protein levels of the major peroxisomal enzymes alcohol oxidase (AO), catalase (CAT) and dihydroxyacetone synthase (DHAS) were observed between Δcpy and WT cell extracts (data not shown). Additionally, peroxisome proliferation in these cells was virtually unaffected, suggesting that deletion of CPY did not affect the synthesis of normal peroxisomes in *H. polymorpha*.

**Role of CPY in glucose-induced selective peroxisome degradation**

As described before, peroxisomal enzymes in methanol-grown *H. polymorpha* are susceptible to carbon catabolite inactivation. In order to study the role of CPY in the peroxisome degradation process, we analysed the fate of these organelles during the initial hours after the addition of 0.5% glucose to methanol-grown WT, Δcpy and Δcpy[pYT3-CPY] cells. This addition did not change the level of CPY protein in cell extracts of WT and Δcpy[pYT3-CPY] cells during subsequent growth (Figure 5A). Enzyme activity measurements revealed that the patterns of inactivation of the major peroxisomal enzymes AO and CAT after addition of glucose were largely identical for the three strains (Figure 5B). Western blot analysis of crude extracts prepared from these cells revealed that the inactivation of AO, DHAS and CAT was due to a decrease in the levels of AO, DHAS and CAT protein in the cells (Figure 5C). As demonstrated before, the data confirmed that inactivation of peroxisomal enzymes (most likely by modification) precedes the proteolytic degradation of these proteins (Bruinenberg et al., 1982). Identical degradation patterns were found for Pex3p, an integral component of the peroxisomal membrane, and Pex14p, a membrane-associated protein (Figure 5C, shown for Pex14p). These results suggest that in *H. polymorpha* CPY is not the major protease involved in the proteolytic degradation of peroxisomal constituents during selective degradation of the organelle.

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**REFERENCES**


