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van der Heide, M; Hollenberg, CP; van der Klei, Ida; Veenhuis, M

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ORIGINAL PAPER

M. van der Heide · C. P. Hollenberg
I. J. van der Klei · M. Veenhuis

Overproduction of BiP negatively affects the secretion of *Aspergillus niger* glucose oxidase by the yeast *Hansenula polymorpha*

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Abstract We have cloned the *Hansenula polymorpha BIP* gene from genomic DNA using a PCR-based strategy. *H. polymorpha BIP* encodes a protein of 665 amino acids, which shows very high homology to *Saccharomyces cerevisiae* KAR2p. KAR2p belongs to the Hsp70 family of molecular chaperones and resides in the endoplasmic reticulum (ER)-lumen. *H. polymorpha* BiP contains a putative N-terminal signal sequence of 30 amino acids together with the conserved –HDEL sequence, the typical ER retention signal, at the extreme C-terminus. We have analysed the effect of *BIP* overexpression, placing the gene under control of the strong alcohol oxidase promoter (P_{MOX}), on the secretion of artificially produced *Aspergillus niger* glucose oxidase (GOX) by *H. polymorpha*. BiP overproduction did not lead to any growth defects of the cells; at the subcellular level, proliferation of ER-like vesicles was observed. However, artificially enhanced BiP levels strongly affected GOX secretion and led to accumulation of this protein in the ER-like vesicles. This was not simply due to the high BiP overproduction, because it was also observed under conditions of low P_{MOX} induction during growth of cells on glycerol. Vacuolar carboxypeptidase Y was properly sorted to its target organelle in the BiP overproducing strains.

Introduction

In eukaryotes, protein secretion involves a complex transport pathway, which initiates with translocation of the protein from the cytosol into the lumen of the endoplasmic reticulum (ER). Subsequently, the proteins are sorted by means of vesicular transport via the Golgi apparatus to the cell membrane. Fusion of the transport vesicle with the cell membrane results in release of its contents into the extracellular space (Corsi and Schekman 1996). Probably one of the best studied proteins within the secretory pathway is BiP, originally described as "the Heavy Chain Binding Protein" in Abelson virus transformed pre-B mouse cell lines (Haas and Wabl 1983). BiP belongs to the Hsp70 family of molecular chaperones and is an essential constituent of the ER. The *Saccharomyces cerevisiae* homologue of BiP is Kar2p (Rose et al. 1989). It has been demonstrated that BiP plays a key role in both co- and post-translational translocation of proteins into the ER (Brodsky et al. 1995). Furthermore, it plays a role in protein folding. During import of proteins into the ER, BiP binds to hydrophobic stretches of the polypeptide (Blond-Elguindi et al. 1993) thereby preventing aggregation. Recently it was shown that BiP also facilitates ER-associated degradation (ERAD) (Brodsky et al. 1999). This degradation process involves the retrograde transport of malfolded proteins out of the ER.

Simultaneous overproduction of BiP and foreign proteins has been studied as a mode to enhance protein secretion; in bakers yeast, BiP overproduction may have a positive effect on the secretion of artificially produced proteins (Harmsen et al. 1996). However, the opposite effect has been observed in certain mammalian cells (Dorner et al. 1992).

In the past few years, the methylotropic yeast *Hansenula polymorpha* has gained increasing interest as an attractive host for the production of biologically active proteins (Hollenberg and Gellissen 1997; Gellissen 2000; Gellissen and Veenhuis 2001). Examples of proteins successfully produced by *H. polymorpha* include, among others, hirudin (Weydemann et al. 1995), hepatitis B vaccines (Gellissen 2000) and *Aspergillus niger* glucose oxidase (GOX) (Hodgkins et al. 1993).

In our laboratory, *A. niger* GOX is used as a model protein to study the use of *H. polymorpha* for the secre-

M. van der Heide · I.J. van der Klei · M. Veenhuis (✉)
Eukaryotic Microbiology,
Groningen Biomolecular Sciences and
Biotechnology Institute (GBB), University of Groningen,
Postbus 14, 9750AA Haren, The Netherlands
e-mail: M.Veenhuis@biol.rug.nl
Tel.: +31-50-3632176, Fax: +31-50-3638280

C.P. Hollenberg
Heinrich-Heine-Universität Düsseldorf, Institut für Mikrobiologie,
Universitätsstrasse 1, 40225 Düsseldorf, Germany

tion of biologically active complex flavo-proteins. Here we report the cloning of the *H. polymorpha BIP* gene and present an analysis of the effect of modulating BiP levels on the secretion of *A. niger* GOX by *H. polymorpha*.

Materials and methods

Organisms and growth conditions

Hansenula polymorpha NCYC 495 *leu1.1*(WT), NCYC 495 *leu1.1*::(P_{MOX}-*BIP*)^{2c} (GMH5), NCYC 495 *leu1.1 ura3*::P_{MOX}-*Sc*MFα-*GOX* (GMH1) and GMH1:: (P_{MOX}-*BIP*)^{2c} (GMH6) were grown at 37°C in rich complex medium containing 1% yeast extract, 2% peptone and 1% glucose (YPD), in mineral medium as described (van Dijken et al. 1976), or on YNB without amino acids containing 0.67% yeast nitrogen base (Difco). Carbon sources used were 0.5% glucose, 0.5% methanol or 0.1% glycerol; as nitrogen source, ammonium sulphate at 0.25% was employed. Leucine or uracil were added to final concentrations of 30 µg/ml. *Escherichia coli* DH5α (Gibco-BRL, Gaithersburg, Md.) was grown at 37°C in LB medium, supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) when required.

Miscellaneous DNA techniques

Standard recombinant DNA techniques, *E. coli* transformation and plasmid isolation procedures were performed essentially as described (Sambrook et al. 1989). *H. polymorpha* was transformed by electroporation (Faber et al. 1994).

Cloning and characterisation of the *H. polymorpha BIP* gene

A 1.5 kb fragment was obtained by PCR using chromosomal DNA of wild type (WT) *H. polymorpha* (ATCC 34438) and two 18-mer synthetic oligonucleotides (5′-CGGTATCGATTTAGGTAC and 5′-CGGTAGACTTTTCGACGG) (Appligene, Heidelberg, Germany) homologous to highly conserved regions of Hsp70 genes of *S. cerevisiae* (Mullisch et al. 1986). A [α-^{32}P]d-ATP-labelled version of this 1.5 kb fragment was used to screen a genomic library of WT *H. polymorpha* in *E. coli*, by colony hybridisation. Southern blot analysis of plasmid DNA obtained from a positive clone revealed that a 3.8 kb *Xba*I fragment of the genomic insert contained the complete *BIP* gene. This fragment was subcloned into pBluescript (Stratagene, La Jolla, Calif.), resulting in plasmid pHSD. From this plasmid, a 1 kb *Eco*RV fragment was deleted. The resulting plasmid pHSDΔ*Eco*RV was sequenced. For analysis of the DNA and amino acid sequences the PC-Gene program release 6.70 (IntelliGenetics) was employed. The BlastP and BlastX algorithms (Altschul et al. 1990) were used to screen GenBank and the *Saccharomyces* Genome Database (SGD, Stanford University, Calif.). The nucleotide sequence of *H. polymorpha BIP* was deposited with GenBank and was assigned Accession no. AF245405.

Construction of a GOX-producing *H. polymorpha* strain

A 4.9 kb *Bam*HI fragment from plasmid pWG30 (a kind gift from Dr. P. Sudbery, University of Sheffield, UK), containing the *S. cerevisiae* mating factor α (MFα) leader sequence fused in-frame to the *GOX* gene of *A. niger* under the control of the *H. polymorpha* alcohol oxidase promoter (P_{MOX}), was cloned into pHI1. The resulting plasmid pMH16 was linearised with *Stu*I and integrated into the genome of *H. polymorpha* NCYC 495 *leu1.1 ura3*. Correct integration into the P_{MOX} locus as a single copy was confirmed by Southern blot analysis (data not shown). The resulting *H. polymorpha* strain was designated GMH1.

Construction of BiP-overproducing strains

Overexpression of *H. polymorpha BIP* was achieved as follows. First a *Bam*HI site was introduced upstream of the start codon of *BIP* by PCR using the BiP-start primer (5′-CCCAAGCTTG-GATCCATGTTAACTTTCAATAAGTC) and the M13/pUC reverse sequencing primer. The PCR product obtained was sequenced. A 2.4 kb *Bam*HI-*Xho*I fragment containing *BIP* was cloned into *Bam*HI-*Sal*I-digested pHIPX4-B (Komori et al. 1997). The resulting plasmid was linearised with *Stu*I and integrated into the genome of *H. polymorpha* NCYC 495 *leu1.1* and GMH1, resulting in strains GMH5 and GMH6, respectively. Southern blot analysis confirmed the correct integration as two copies in the P_{MOX} locus.

Biochemical methods

Total cell extracts were prepared as follows. Three OD_{660} units (volume × OD_{660}) of cells were harvested by centrifugation. The cell pellet was resuspended in a 12.5% (final concentration) TCA solution followed by freezing of the samples at –80°C. After thawing, the samples were centrifuged at 14,000 rpm at room temperature. Pellets were washed twice with ice-cold 80% acetone and subsequently resuspended in 50 µl 0.1N NaOH/1% SDS. An equal volume of SDS sample buffer was added prior to boiling the samples for 5 min. Samples of 10 µl were used for electrophoresis.

For the generation of protoplasts, whole cells were pre-incubated in a solution containing 100 mM Tris pH 8.0, 50 mM EDTA, 140 mM β-mercaptoethanol and 1.2 M sorbitol for 15 min at 37°C. The cells were harvested by centrifugation, washed once in 50 mM potassium phosphate buffer pH 7.2, containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mg ml^{-1} of Zymolyase 20 T at 37°C until the bulk of the cells were converted into protoplasts. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation and washed twice with potassium phosphate buffer pH 7.2 containing 1.3 M sorbitol to prevent osmotic lysis. Finally, aliquots of the protoplast suspension, corresponding to 3 OD units of the original culture, were precipitated as described.

GOX activity was assayed at 30°C essentially as described for alcohol oxidase (Verduyn et al. 1984), using 1 mM glucose as a substrate. Carboxypeptidase Y (CPY) activities were determined by established procedures (Jones 1991). SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Gels were used for Western blotting (Kyhse-Anderson 1984), and blots were decorated using the chromogenic or chemiluminescent Western blotting kit (Boehringer Mannheim, Germany) and specific polyclonal antibodies against *A. niger* GOX and *S. cerevisiae* Kar2p (a kind gift from Dr. R. Schekman, University of California, Berkeley, Calif.).

Electron microscopy

Whole cells were fixed and embedded in Epon 812 or unicryl as described previously (Waterham et al. 1994). Ultrathin unicryl sections were labelled using polyclonal antibodies raised in rabbit and goat-anti-rabbit antibodies conjugated to gold according to the instructions of the manufacturer (Amersham, UK).

Results

Cloning and characterisation of the *H. polymorpha BIP* gene

Using *H. polymorpha* genomic DNA and two 18-mer synthetic oligonucleotides homologous to highly conserved regions of *HSP70* genes from *S. cerevisiae*, a 1.5 kb frag-

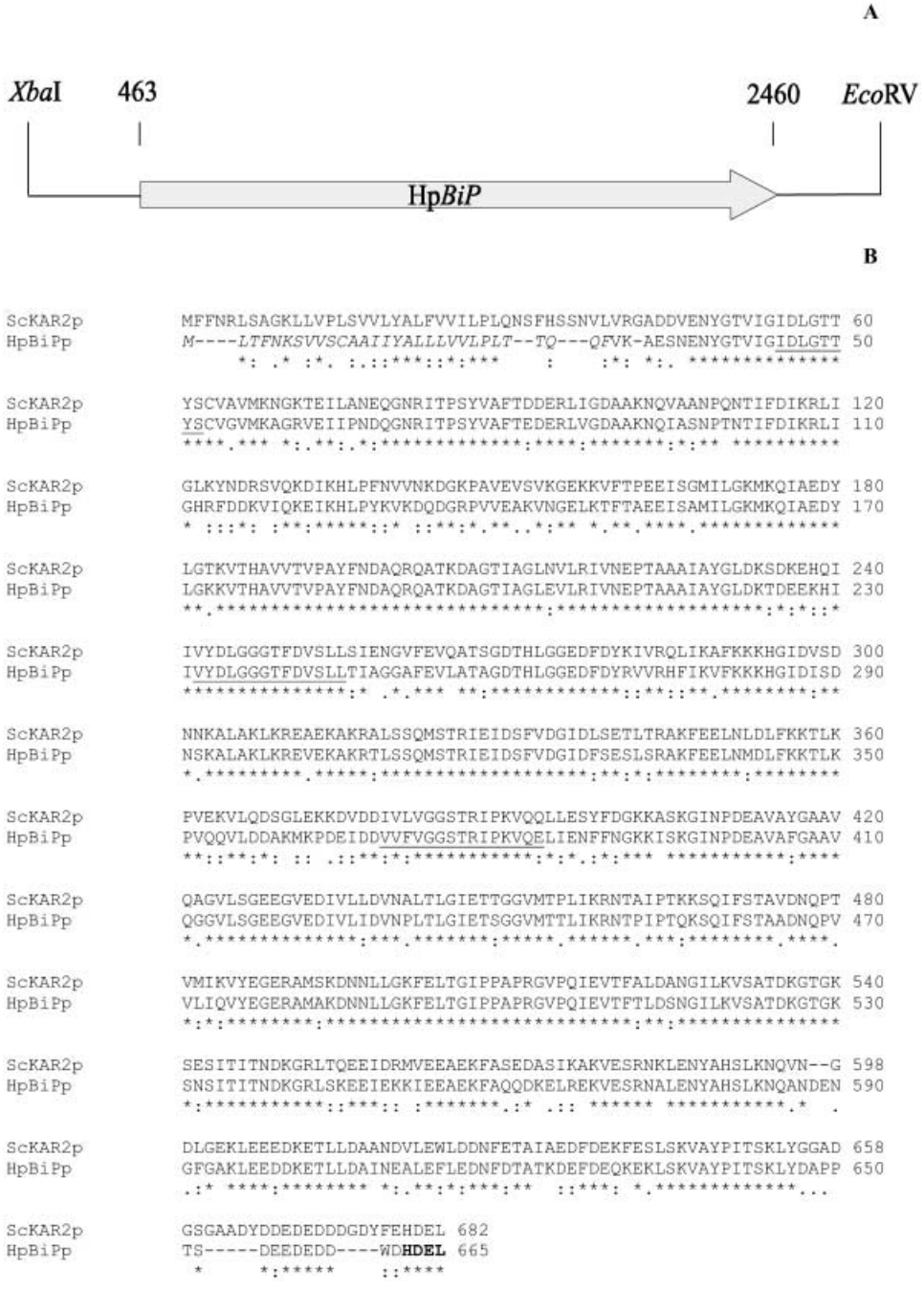

Fig. 1 **A** Schematic representation of the *BIP*-containing genomic DNA fragment. **B** Comparison of the deduced amino-acid sequences of *Hansenula polymorpha* BiP and *Saccharomyces cerevisiae* Kar2p. The two proteins are homologous over their entire length. Identical and similar amino acids are indicated by *asterisks* and *dots*, respectively. The putative signal sequence is indicated in *italics*, the –HDEL sequence in **bold** and the three Hsp70 signatures are *underlined*

ment was amplified by PCR. After hybridising this fragment to an *H. polymorpha* gene library, plasmid pHSDΔ*Eco*RV was obtained, which contained a full-length ORF (Fig. 1A). The deduced amino acid sequence of this ORF displayed a high sequence identity (73.8%) to *S. cerevisiae* Kar2p (Fig. 1B) and 48% identity to two cytosolic Hsp70ps of *H. polymorpha* (Titorenko et al. 1996). From this we concluded that the isolated gene most likely belongs to the Hsp70p family of molecular chaperones. Further analysis, using the PSORTII program (Horton and Nakai 1997), revealed that the N-terminal 30 amino acids were predicted to represent an ER targeting signal. Moreover, the protein contains a C-terminal –HDEL sequence, which is a typical ER-retention signal (Fig. 1B). Based on these characteristics, we concluded that the gene isolated encodes the *H. polymorpha* homologue of the ER-resident Hsp70p (BiP, Kar2p).

Overproduction of BiP in *H. polymorpha* leads to proliferation of the ER

In order to obtain an *H. polymorpha* BiP overproducing strain, the *BIP* gene was placed under the control of the strong alcohol oxidase promoter (P_{MOX}). The resulting plasmid was integrated in two copies into the genome of

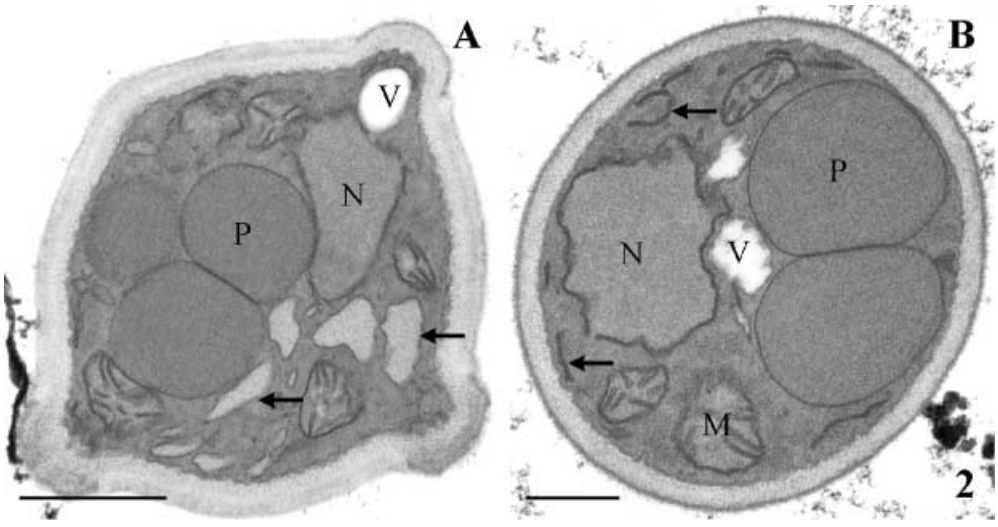

Fig. 2 Overall morphology of methanol-grown cells of both GMH5 (**A**) and wild type (**B**) showing the strongly proliferated endoplasmic reticulum (ER)-like vesicles in strain GMH5 (*arrows*). The electron micrographs are of $KMnO_4$-fixed cells *M* mitochondrion, *N* Nucleus, *P* peroxisome, *V* vacuole. Bar = 0.5 µm

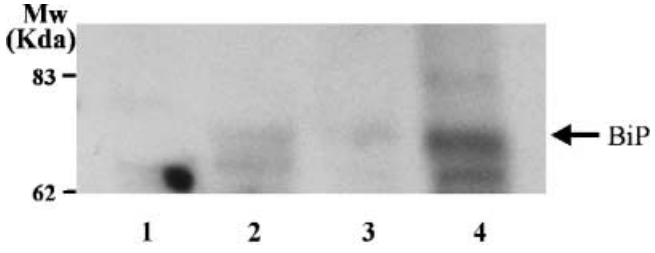

Fig. 3 Western blot analysis showing *BiP* protein levels in various constructed *H. polymorpha* strains grown for 24 h on methanol-containing medium. Gels were loaded with crude cell extracts. Lanes: *1* Wild-type, *2* GMH5, *3* GMH1, *4* GMH6. Blots were decorated using specific antibodies against *S. cerevisiae* Kar2p. Equal amounts of protein were loaded per lane. The protein band at approximately 62 kDa (lane 4) represents a BiP degradation product. *Mw* Molecular weight in *kDa*

WT *H. polymorpha* [the resulting strain *H. polymorpha* NCYC 495 *leu1.1*::$(P_{MOX}$-BiP$)^{2c}$ is further designated as GMH5]. GMH5 cells grew normally on glucose- or methanol-containing medium at WT rates (data not shown). Electron microscopy revealed that growth on methanol was associated with a distinct proliferation of ER-like vesicles in the cells, whereas the ultrastructure of other cell organelles was unchanged (compare Fig. 2A and B). Western blotting experiments confirmed that the level of BiP was strongly increased in these cells compared to WT cells (Fig. 3).

Construction of an *H. polymorpha* strain that produces *A. niger* GOX

To obtain an *H. polymorpha* strain able to produce and secrete *A. niger* GOX, a hybrid gene with the leader sequence of *S. cerevisiae* mating factor α (MFα) fused to mature *A. niger* GOX separated by a *KEX*2 cleavage site was constructed. This gene was placed under P_{MOX} control and integrated in one copy into the genome of WT *H. polymorpha*. The resulting strain, designated GMH1, grew on glucose, glycerol or methanol-containing medium at rates identical to the WT host strain (data not shown).

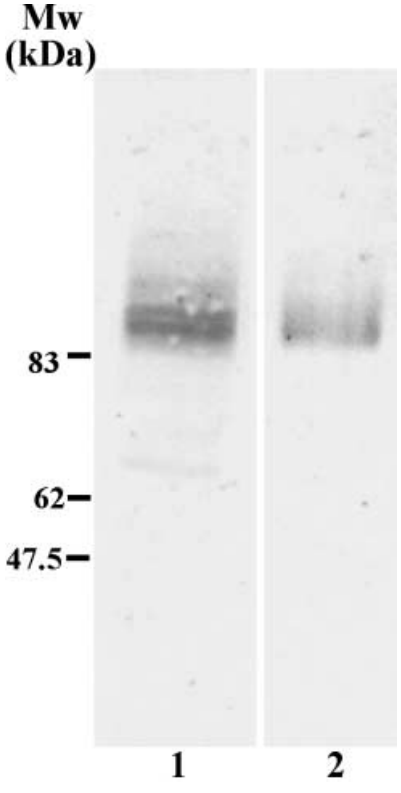

Fig. 4 Western blot analysis using α-glucose oxidase (GOX) antibodies of both crude cell extract (lane 1) and medium (lane 2) of strain GMH1 grown on methanol, showing GOX protein associated with the cells and present in the cultivation medium. *Mw* Molecular weight in *kDa*

As expected, GOX protein was not produced in glucose-grown cells. However, upon growth of cells on methanol, GOX protein was readily detectable, both in total cell lysates and in the culture fluid (Fig. 4). GOX

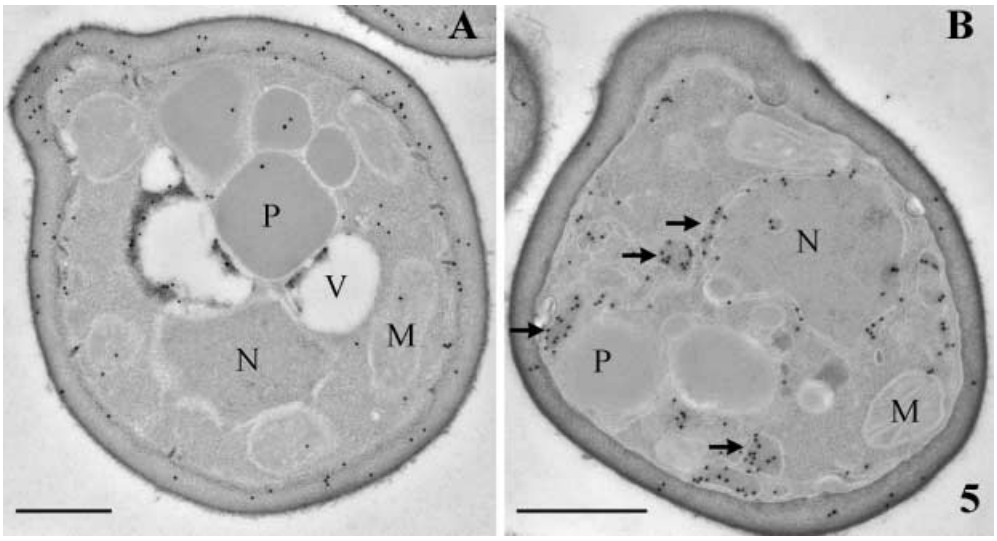

Fig. 5 Ultrathin section of glutaraldehyde-fixed, methanol-grown cells of GMH1 (**A**) and GMH6 (**B**), labelled with α-GOX antibodies and gold conjugated goat-anti-rabbit antibodies, showing cell wall labelling of strain GMH1. In strain GMH6, GOX label is found in the proliferated ER-like vesicles (*arrows*). *M* Mitochondrion, *N* nucleus, *P* peroxisome, *V* vacuole. Bar = 0.5 µm

enzyme activity measurements revealed that 7 mU/ml GOX activity was present in the culture medium, whereas in whole cell lysates the activity corresponded to 18 mU/ml culture. GOX activity was undetectable in protoplasts, indicating that the enzyme activity present in whole cell lysates was located in, or is associated with, the cell wall.

The location of GOX protein was subsequently studied by immunocytochemistry. Using α-GOX antibodies and ultrathin sections of methanol-grown GMH1 cells, labeling was found predominantly at the cell wall (Fig. 5A). Cytochemical experiments confirmed that the cell wall-bound GOX was enzymatically active. Moreover, using this technique, no GOX activity was detected in the cytosol (data not shown).

BiP overproduction reduces *A. niger* GOX secretion by *H. polymorpha*

To study the effects of enhanced levels of BiP on GOX secretion, the *BIP* overexpression cassette was integrated in two copies into the genome of strain GMH1. The resulting strain, designated GMH6, grew normally on glucose, glycerol or methanol at rates identical to the host strain GMH1 (data not shown). To analyse whether enhanced BiP levels stimulate GOX secretion, GMH1 and GMH6 cells were grown in batch cultures containing either glycerol (moderate overproduction) or methanol (high overproduction). Upon growth of cells on methanol, the secretion of GOX protein was severely reduced in strain GMH6 compared to strain GMH1 (Fig. 6). This reduction in secretion was also observed at low P_{MOX} induction during growth of cells on glycerol (Fig. 6).

In order to obtain further insight into the hampered secretion of GOX by GMH6, the levels and subcellular location of this protein was investigated biochemically by Western blotting using α-GOX antibodies. The results, shown in Fig. 7, indicate that in GMH1 and

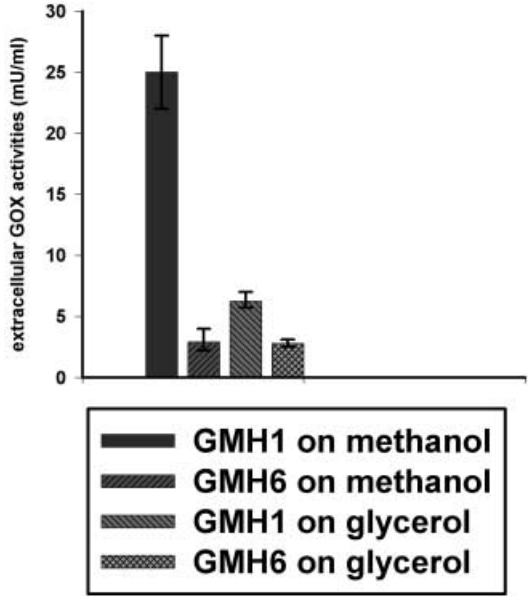

Fig. 6 Extracellular GOX activities of strain *GMH1* and *GMH6* after 24 h of growth on *methanol* or *glycerol*

GMH6, GOX protein is produced to approximately the same level (Fig. 7A; compare lanes 3 and 4). In protoplasts prepared from cells of strain GMH1, GOX protein was hardly detectable (Fig. 7B; lane 2). Strikingly, virtually all GOX protein produced by strain GMH6 was retained in the protoplasts (Fig. 7B; lane 3). These results indicate that GOX protein produced by strain GMH6 had accumulated intracellularly. Immunocytochemical experiments on ultrathin sections of GMH6 cells revealed that GOX protein accumulated in ER-like vesicles (Fig. 5B).

To test whether the overproduction of BiP specifically hampered the secretion of GOX, or if other processes that rely on the secretory pathway were also affected, we studied the fate of the vacuolar protease CPY in these cells. CPY enzyme assays on crude extracts of methanol-

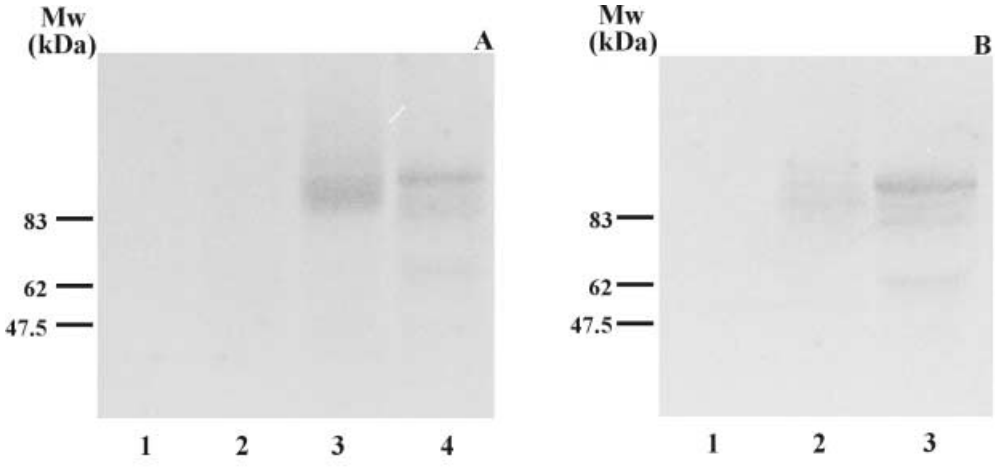

Fig. 7A, B Western blot analysis of different constructed *H. polymorpha* strains grown for 24 h on methanol-containing medium. Gels were loaded with **A** total crude cell extracts (lanes: *1* wild-type, *2* GMH5, *3* GMH1, *4* GMH6) or **B** protoplasts prepared from the cells (lanes: *1* GMH5, *2* GMH1, *3* GMH6). Blots were decorated using specific antibodies against GOX. Equal amounts of protein were loaded per lane. *Mw* Molecular weight in *kDa*

Table 1 Carboxypeptidase Y (CPY) activities of methanol-grown cells of *PDD1*, wild-type, GMH5, GMH1 and GMH6

Strain	PDD1/VPS34	Wild-type	GMH5	GMH1	GMH6
CPY activity (mU/mg protein)	1	3.8	3.5	3.5	3.3

grown cells revealed that the activities were comparable to WT levels (Table 1). Neither CPY activity nor protein could be detected under these conditions in the medium of methanol-grown cells (data not shown). These data are in contrast to observations on CPY sorting in the *H. polymorpha pdd*1 mutant (Kiel et al. 1999). The *H. polymorpha PDD*1 gene product, which is the functional homologue of *S. cerevisiae* Vps34p, is essential for selective peroxisome degradation. It was shown that sorting of CPY was impaired in the *H. polymorpha pdd*1 mutant. Besides reduced CPY enzyme activities (1 mU/mg protein), CPY was also mis-sorted to the culture fluid in this mutant.

Discussion

In this paper we describe the cloning of a *H. polymorpha* gene belonging to the *HSP*70 family. The presence of an N-terminal leader sequence, the high similarity to *S. cerevisiae* Kar2p (73.8% identity), and the C-terminal –HDEL sequence, which is a typical ER retention sequence, left little doubt that the gene in fact represents the *H. polymorpha BIP* homologue.

BiP is an essential protein in the eukaryotic cell. It fulfils crucial roles in the translocation of proteins across the ER membrane. During post-translational import of secretory proteins into the ER, BiP acts as a molecular ratchet, inhibiting the backward movement of the emerging polypeptide (Simon et al. 1992; Matlack et al. 1999). BiP is also essential for the opposite process, namely the export of malfunctional proteins from the ER prior to their degradation by the proteasome in the cytosol. The availability of BiP is infrequently observed to be one of the major bottlenecks in secretion of heterologous proteins. For instance, overproduction of BiP led to a 5-fold increase in secretion of human erythropoietin by yeast cells (Wittrup and Robinson 1994). Also, bovine prochymosine secretion increased 26-fold upon *BIP* overexpression in *S. cerevisiae* (Harmsen et al. 1996). In other cases, however, BiP overproduction did not result in higher secretion levels, as for example in the case of thaumatin secretion (Harmsen et al. 1996). Reducing the levels of BiP clearly reduced secretion of human granulocyte-colony-stimulating factor, *Schizosaccharomyces pombe* acid phosphatase and bovine trypsin inhibitor by *S. cerevisiae*. In these cases, secretion levels remained unaltered upon overproduction of BiP (Robinson et al. 1996). In *Aspergillus awamori*, elevated BiP levels did not show any significant effect on the secretion efficiency of different cutinase variants (van Gemeren et al. 1998). Similar results were observed in the case of secretion of heterologous proteins by mammalian cells (Dorner et al. 1992; Hsu et al. 1994).

To study the effect of enhanced levels of BiP on the secretion of heterologous *A. niger* GOX, we cloned the *H. polymorpha BIP* gene under control of the inducible *MOX* promoter and integrated this expression cassette into the genome of a strain that was able to produce and secrete GOX. Our results showed that in *H. polymorpha*, the upregulation of BiP levels clearly showed a negative effect on the secretion of GOX protein. The levels of secreted GOX activity in strain GMH6 were reduced 10-fold compared to the parental strain GMH1, which secreted 25 mU/ml. This reduction of secretion efficiency can be explained by the fact that GOX protein is accumulating intracellularly in ER-like vesicles, as was evident from both electron microscopical (Fig. 5) and Western blot analysis (Fig. 7).

At present, the molecular mechanisms underlying these observations are not clear. One explanation for this

phenomenon could be that *BiP* overexpression results in the retention of possible malfolded GOX in the ER. An important feature of BiP is that it assists in the proper folding of proteins that are translocated into the lumen of the ER. BiP appears to bind to hydrophobic sequences of polypeptides (Blond-Elguindi et al. 1993), thereby preventing premature and incorrect folding.

Malfolded proteins, accumulated in the ER lumen, are often eliminated by ERAD; BiP seems to be essential for this process (Brodsky et al. 1999). Associations between BiP and retained secretory proteins have often been observed, even as proteinaceous aggregates (Yang et al. 1998; Umebayashi et al. 1997; Sagt et al. 1998). From the electron microscopical analysis (Fig. 5B), it is clear that such aggregates are not present in strain GMH6. To test whether associations between BiP and GOX exist in this strain, co-immunoprecipitations were performed. The results from these experiments were not conclusive (data not shown).

Our data suggested that the block in secretion was not related to the extreme high overexpression of a single gene, but seems to be specific as it was also observed in glycerol-grown cells. During growth on glycerol, P_{MOX} is induced to levels up to approximately 25% of the values generally observed in methanol-grown cells. The inhibitory effect was apparently also not due to a general defect in ER protein import and passaging, since endogenous CPY, which is sorted via the ER and Golgi to vacuoles, was properly transported to the vacuole, as indicated by the processing of the protein to the mature, enzymatically active, form.

In order to gain more insight into the molecular mechanisms underlying the observation of reduced GOX secretion in the BiP overexpression strain, the effects of overproduction of other ER proteins on GOX secretion could be studied. In addition, the fate of homologous secretory proteins could be studied during overproduction of BiP. Currently, we are trying to identify and isolate such proteins from *H. polymorpha*.

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