**YlBMH1 encodes a 14-3-3 protein that promotes filamentous growth in the dimorphic yeast Yarrowia lipolytica**

Cleofe A. R. Hurtado and Richard A. Rachubinski

Most pathogenic fungi have the ability to alternate between a unicellular yeast form and different filamentous forms (hyphae and pseudohyphae). This attribute is generally regarded as an important virulence factor and has also attracted attention because of its implications in the study of eukaryotic cell differentiation. To identify genes that are involved in the regulation of these events, chemical mutagenesis of the dimorphic yeast *Yarrowia lipolytica* was performed and morphological mutants that were unable to form hyphal cells were isolated. Screening of a *Y. lipolytica* genomic DNA library for genes able to complement this defect led to the isolation of *YlBMH1*, a gene encoding a 14-3-3 protein and whose transcription levels are increased during the yeast-to-hypha transition. Remarkably, overexpression of *YlBMH1* was able to enhance pseudohyphae formation in a strain lacking functional *YlRAC1* but caused no visible effects in Δ*mhy1* and Δ*bem1* cells, thus suggesting that *YlBMH1* is involved in the regulation of both hyphal and pseudohyphal growth in *Y. lipolytica*. The identification of *YlBMH2*, a gene encoding a second 14-3-3 protein (YlBmh2p) that contains a 19 aa insertion absent in all other members of the 14-3-3 family, is also reported. Differently from *YlBMH1*, the transcription levels of *YlBMH2* do not show any apparent variation during the induction of hyphal growth, and its overexpression has no effects on cells lacking functional *MHY1*, *YIRAC1* or *YIBEM1*. Taken together, these observations suggest that, in spite of their high conservation, *YlBmh1p* and *YlBmh2p* have different cellular functions.

**Keywords:** hypha, pseudohypha, fungus, differentiation, transcription

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**INTRODUCTION**

The 14-3-3 protein family is a group of highly conserved, abundant, ubiquitous, dimeric proteins with a subunit molecular mass of approximately 30 kDa and an acidic pI of 4–5. Although their exact function is still not completely understood, 14-3-3 proteins are known to bind to more than 100 different cellular proteins and have been implicated in the regulation of biological processes as diverse as cell-cycle progression, cell differentiation, exocytosis, cytoskeletal organization, signal transduction, stress response, viral and bacterial pathogenesis, malignant transformation and apoptosis (Aitken et al., 1995; Baldin, 2000; Fu et al., 2000; van Hemert et al., 2001a).

In yeast, two genes encoding 14-3-3 proteins exist in *Schizosaccharomyces pombe* (rad24 and rad25) (Ford et al., 1994) and *Saccharomyces cerevisiae* (BMH1 and BMH2) (van Heusden et al., 1992, 1995), and one exists in *Candida albicans* (BMH1) (Cognetti et al., 2002). In spite of their high conservation, the proteins encoded by these genes do not appear to have identical functions. The *Schizosaccharomyces pombe* 14-3-3 proteins are required for DNA repair and cell-cycle control, and strains deleted for both rad24 and rad25 are not viable (Ford et al., 1994). Some strains of *Saccharomyces cerevisiae* deleted for both BMH1 and BMH2 are viable, but they exhibit increased sensitivity to stress and abnormal morphology (Roberts et al., 1997). The BMH1

The GenBank accession numbers for the *YlBMH1* and *YlBMH2* sequences reported in this study are AY090661 and AY090662, respectively.
gene from C. albicans is essential, and both wild-type alleles are necessary for optimal growth and morphogenesis of this organism (Cognetti et al., 2002).

Fungal dimorphism is a complex phenomenon triggered by a large variety of environmental factors and consists of a reversible alternating pattern of growth between different elliptical and filamentous forms of cells. Understanding the mechanisms that regulate these events is of major interest because of their implications in fungal pathogenesis and cell differentiation; studies performed in Saccharomyces cerevisiae, C. albicans and Ustilago maydis have revealed that important differences in how dimorphism is regulated exist among different yeast species. Accordingly, additional studies are required in other organisms to acquire a more complete understanding of the overall phenomenon of fungal dimorphism (Odds, 1988; Shepherd, 1988; Maresca & Kobayashi, 1989; Gow, 1994; Banuett, 1995; Lo et al., 1997; Madhani & Fink, 1998; Bolker, 2001). Due to its ability to alternate between a unicellular yeast form and different filamentous forms (hyphae and pseudohyphae), its amenability to genetic and molecular biological analyses and its ability to reproduce sexually, the yeast Yarrowia lipolytica has emerged as an excellent alternative model for the investigation of the molecular aspects of fungal morphogenesis (Enderlin & Ogrydziak, 1994; Barth & Gaillardin, 1996; Torres-Guzman & Dominguez, 1997; Hurtado & Rachubinski, 1999; Hurtado et al., 2000; Richard et al., 2001; Szabo, 2001).

In this study, we describe the isolation of two genes (YlBMH1 and YlBMH2) that encode 14-3-3 proteins of Y. lipolytica and show that they play different roles in the regulation of filamentous growth in this organism.

METHODS

Strains, culture conditions and microbial techniques. The Y. lipolytica strains used in this study are listed in Table 1. Strains were grown in complete (YPEP) or supplemented minimal (YNA, YNBGlc or YNBGlcNAc) media, as required. Media components were as follows: YEPD, 1% yeast extract, 2% peptone, 2% glucose; YNA, 0.67% yeast nitrogen base without amino acids, 2% sodium acetate; YNBGlc, 1.34% yeast nitrogen base without amino acids, 1% glucose; YNBGlcNAc, 1.34% yeast nitrogen base without amino acids, 1% N-acetylglucosamine, 50 mM citric acid, pH 6.0. YNA was supplemented with uracil, leucine, lysine and histidine each at 50 μg ml⁻¹, as required. YNBGlc and YNBGlcNAc were supplemented with Complete Supplement Mixture (Bio 101) at twice the manufacturer’s recommended concentration (2 × CSM) or with 2 × CSM minus leucine, as required. Media, growth conditions and transformation of Y. lipolytica have been described previously (Barth & Gaillardin, 1996; Barth & Weber, 1985; Nuttley et al., 1993).

DNA manipulation and growth of Escherichia coli were performed as described by Ausubel et al. (1989).

Isolation and characterization of the Y. lipolytica BMH1 gene. The Y. lipolytica BMH1 gene (YlBMH1) was isolated from a Y. lipolytica genomic DNA library contained in the replicative E. coli shuttle vector pINA445 (Nuttley et al., 1993) by functional complementation of the mutant strain CHY3350, which forms colonies with a smooth appearance. Plasmid DNA was introduced into yeast cells by electroporation and Leu⁺ transformants were screened on YNA agar plates for their ability to give rise to rough colonies similar to those formed by the wild-type strain E122.

Complementing plasmids were recovered by transformation of E. coli, and the smallest fragment capable of restoring hyphal growth was determined. Restriction fragments prepared from the genomic insert of one of these constructs (pBMH1) were subcloned into the vector pGEM-5Zf(+) or pGEM-7Zf(+) (Promega) for deoxyribonucleotide sequencing of both strands. The deduced polypeptide sequence, YBmh1p, was compared to other known protein sequences using the BLAST Network Service of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997).

Isolation and characterization of the Y. lipolytica BMH2 gene. A partial sequence (GenBank accession no. AL413320), obtained by a programme of random genomic sequencing (Casaregola et al., 2000), was used to isolate a 2.3 kbp fragment containing the complete coding region of the Y. lipolytica BMH2 gene (YlBMH2) by PCR of a Y. lipolytica genomic DNA library contained in the replicative E. coli shuttle vector pINA445 (Nuttley et al., 1993) with oligonucleotides BMH2U, BMH2D, pINA445-PrC and pINA445-PrD (Table 2). The entire YlBMH2 gene was subsequently isolated by PCR of the same genomic library with oligonucleotides 5’BMH2F, 5’BMH2R, 3’BMH2F and 3’BMH2R (Table 2) using the Expand High Fidelity PCR System (Roche). The 3/7 kbp BamHI–BamHI fragment thus obtained, which

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<td><strong>Strain</strong></td>
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<td>bem1KO157</td>
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RESULTS
Isolation of the Y. lipolytica CHY3350 mutant strain

The Y. lipolytica mutant strain CHY3350 (Fig. 1b) was isolated after chemical mutagenesis of Y. lipolytica E122 cells (Fig. 1a) with 1-methyl-3-nitro-1-nitrosoguanidine by its inability to form wild-type rough-surfaced colonies on YEPD-agar plates after 3 days incubation at 28 °C (Nuttley et al., 1993). Subsequent analysis showed that this attribute was lost at a rate of 3–5 × 10⁻² per generation and that a few of the CHY3350 cells were pseudohyphal (Fig. 1b). In addition, sequencing of genomic DNA isolated from this strain revealed that the smooth phenotype was not due to mutation of the YIBMH1 gene (data not shown).

Isolation and characterization of the YIBMH1 gene

The YIBMH1 gene was isolated from a Y. lipolytica genomic DNA library contained in the replicative E. coli shuttle vector pINA445 (Nuttley et al., 1993) by its ability to restore hyphal growth upon its introduction into CHY3350 cells. Of approximately 12000 transformants screened, 12 showed a wild-type filamentous phenotype, but only one contained a plasmid, pBMH1, capable of inducing the formation of rough-surfaced colonies upon re-introduction into CHY3350 cells (Fig. 1c). Restriction enzyme analysis revealed that this plasmid contained a 5.0 kbp BamHI–BamHI fragment capable of restoring filamentous growth to the CHY3350 strain. Sequencing of both strands of this fragment...
revealed an ORF of 1482 bp interrupted by one intron located between codons 4 and 5 (nucleotides +13 to +663 from the A residue of the potential initiating codon). Interestingly, the A nucleotide at the fifth position of the putative 5’-splice donor sequence (GTAAATPu) diverges from the G nucleotide normally found in eukaryotic genes (Newman, 1998), and an unusual 3’ intronic end (TAG, as opposed to the consensus sequence CAG) (Teem et al., 1984; Strick et al., 1992; Lopez et al., 1994; Hurtado et al., 2000) is found three nucleotides downstream of the 3’ internal consensus TACTAAC sequence.

The upstream regulatory region of the YlBMH1 gene lacks a putative TATA box but contains a CT/CA-rich region, which is thought to play a role in transcriptional regulation in Y. lipolytica (Xuan et al., 1990; Nuttley et al., 1994). Consensus sequences for the binding of several transcription factors implicated in the regulation of fungal development and in the response to specific environmental conditions in Saccharomyces cerevisiae, C. albicans and Aspergillus nidulans, are also found in the putative promoter of YlBMH1 (data not shown). Sequence analysis of YlBMH1 cDNA isolated from a Y. lipolytica cDNA library constructed in the ZAP Express vector (Stratagene) (data not shown) showed that transcription of the YlBMH1 gene preferentially begins at position +23 from the A nucleotide of the initiating codon and that polyadenylation occurs following the A nucleotide at position +1524.

The deduced protein product of YlBMH1, YlBmh1p, is 276 aa in length (Fig. 2), has a predicted molecular mass of 31166 Da and a typical acidic pI of 4.91 (Fu et al., 2000). Analysis of the predicted amino acid sequence revealed that YlBmh1p belongs to the 14-3-3 family of proteins and that it is most closely related to Ft2p from the filamentous fungus Trichoderma reesei (72.1% identity; Fig. 2). Additionally, a putative PEST region, which is commonly found in rapidly degraded proteins (Chevaillier, 1993; Rechsteiner & Rogers, 1996), is predicted at residues 234–263 of YlBmh1p.

Isolation and characterization of the YlBMH2 gene

A comprehensive database screening revealed the existence of a partial sequence (GenBank accession no. AL413320) encoding a potential 14-3-3 protein that was different from YlBmh1p. Based on this sequence, we isolated a 3.7 kbp BamHI–BamHI fragment by high-fidelity PCR using a Y. lipolytica genomic DNA library as template. Sequencing of this fragment (YlBMH2) revealed an ORF of 1583 bp, which was interrupted by one intron within codon 1 (nucleotides +1 to +813 from the A residue of the potential initiating codon) and flanked by 1.8 kbp and 340 bp of sequence upstream and downstream, respectively. A potential TATA box, TAATAT, is found at nucleotides −52 to −47 from the A nucleotide of the first ATG codon and, like YlBMH1, the upstream regulatory region of the YlBMH2 gene contains consensus sequences for the binding of several transcription factors involved in the response to environmental conditions and the regulation of fungal development (data not shown). Sequence analysis of YlBMH2 cDNA isolated from a Y. lipolytica cDNA library constructed in the ZAP Express vector (Stratagene) (data not shown) revealed that transcription of the YlBMH2 gene preferentially starts at position −14 from the A nucleotide of the initiating codon and that polyadenylation occurs following the A nucleotide at position +1719. The putative 5’-splice donor and 3’-splice acceptor sequences of YlBMH2 (GTGAGTPu and TACTAACNCAG, respectively) are identical to the motifs found in most Y. lipolytica genes (Teem et al., 1984; Strick et al., 1992; Lopez et al., 1994; Hurtado et al., 2000).

The deduced protein product of YlBMH2, YlBmh2p, is 256 aa in length (Fig. 2), has a predicted molecular mass

\[ \text{Molecular mass} = 256 \times 113 \text{Da} = 28672 \text{Da} \]

\[ \text{pI} = 5.5 \]

Fig. 1. Colony (upper panels) and cell (bottom panels) morphology of Y. lipolytica strains. (a) Wild-type strain £122; (b) mutant strain CHY3350; (c) strain CHY3350 transformed with pBMH1. Colonies and cells were photographed after 3 days incubation at 28 °C on YNA-agar plates. Colony magnification, × 100. Bars, 5 μm.
of 29435 Da and a typical acidic pI of 4.79 (Fu et al., 2000). Analysis of the predicted amino acid sequence of YlBmh2p showed that it also belongs to the 14-3-3 family of proteins, does not contain any predicted PEST sequence and is most closely related to Ftt1p from the filamentous fungus Schizophyllum commune (64–5% identity, data not shown). Interestingly, YlBmh2p contains a 19 aa insertion (PSAEATEAAKDGADPSEL, residues 112–130) (Fig. 2) that encompasses a putative N-myristoylation site not found in other known 14-3-3 proteins.

**YlBMH2** is unable to restore hyphal growth to the *Y. lipolytica* CHY3350 mutant strain

Because the *YlBMH1* gene was isolated by its ability to restore hyphal growth to the mutant strain CHY3350 and because sequencing of genomic DNA isolated from CHY3350 revealed that the mutation has been inactivated, further studies were carried out to test the ability of *YlBMH2* to restore hyphal growth to the CHY3350 mutant strain. The results showed that *YlBMH2* was unable to restore hyphal growth to the CHY3350 mutant strain, indicating that the mutation inactivates the *YlBMH1* gene.

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**Fig. 2.** Amino acid sequence alignment of Bmh1p and Bmh2p of *Y. lipolytica* (YlBmh1p and YlBmh2p) and 14-3-3 proteins from *T. reesei* (TrFtt1p and TrFtt2p), *C. albicans* (CaBmh1p), *S. cerevisiae* (ScBmh1p and ScBmh2p) and *S. pombe* (SpRad24p and SpRad25p). Percentages refer to the percentage identity of a given protein to YlBmh1p. GenBank accession nos: CAC20377 (TrFtt1p); CAC20378 (TrFtt2p); AAB96910 (CaBmh1p); CAA55795 (SpRad24p); CAA55796 (SpRad25p); CAA46959 (ScBmh1p); CAA59275 (ScBmh2p).
this strain revealed that its inability to form hyphae was not the result of mutation of the YlBMH1 gene (data not shown), the YlBMH2 gene was investigated as to its potential to suppress the mutation of CHY3350 and, consequently, to its possible role in the induction of hyphal growth in Y. lipolytica. Accordingly, a 3.5 kbp BamHI–BamHI fragment containing the entire YlBMH2 gene was cloned into the BamHI site of the shuttle vector pINA445 to obtain pBMH2; the ability of this plasmid to induce hyphal growth in Y. lipolytica was evaluated. Interestingly, no effect on hyphal formation was observed upon the introduction of pBMH2 into CHY3350 mutant cells.

**Transcription of YlBMH1 and YlBMH2 during the dimorphic transition**

The yeast-to-hypha transition was induced in exponentially growing E122 cells by a 15 min carbon-source starvation at 4 °C, followed by transfer to pre-warmed YNBGlCNAC medium and incubation at 28 °C (Guevara-Olvera et al., 1993). Under these conditions, more than 80% of the cells produced germ tubes after 10 h incubation, whereas cells transferred to fresh glucose-containing (YNBGlc) medium grew almost exclusively as the yeast form. Northern blot experiments performed with total RNA extracted from cells harvested at 3 and 10 h incubation showed that YlBMH1 mRNA was at levels undetectable by this procedure (data not shown). However, semi-quantitative RT-PCR carried out with YHIS1 as an endogenous internal standard revealed that transcription of the YlBMH1 gene increases during the formation of germ tubes (approx. twofold), while there is no apparent variation in the transcription levels of YlBMH2 (Fig. 3a).

**Overexpression of YlBMH1 enhances pseudohyphal growth in rac1 null-mutants of Y. lipolytica**

The genes MHY1, YIRAC1 and YLBEM1 are involved in regulating the yeast-to-hypha transition in Y. lipolytica. MHY1 is required for both hyphal and pseudohyphal growth (Hurtado & Rachubinski, 1999), while Δrac1 strains are able to form pseudohyphae but not hyphae (Hurtado et al., 2000) and Δbem1 cells exhibit severe morphological defects and cannot form hyphae (Hurtado & Rachubinski, 2002). To gain additional information regarding the involvement of YlBMH1 and YlBMH2 in filamentous growth in Y. lipolytica, we analysed the effects of introducing the multicopy plasmids pBMH1 and pBMH2 into the null-mutant strains mhy1KO9, rac1KO30 and bem1KO157 (Table 1). As expected, semi-quantitative RT-PCR analysis revealed that introduction of these plasmids resulted in a two- to fourfold increase in the levels of YlBMH1 and YlBMH2 mRNA in cells cultivated in liquid medium containing glucose as the sole carbon source (Fig. 3b).

Following 3 days incubation on YNA-agar plates at 28 °C, overexpression of the YlBMH1 gene induced the formation of peripheral extensions in rac1KO30 colonies (compare Fig. 4i to h), whereas no effect was observed when YlBMH2 was overexpressed in the same strain (compare Fig. 4j to h). Further analysis revealed that these extensions were produced by the enhancement of pseudohyphal growth by YlBMH1 overexpression in Δrac1 cells (compare Fig. 5i to h), and that no mor-

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**Fig. 3.** Semi-quantitative RT-PCR analysis of YlBMH1 and YlBMH2 mRNAs. (a) YlBMH1 and YlBMH2 mRNA levels are increased during the dimorphic transition. Total RNA was isolated from E122 cells incubated at 28 °C in YNBGlCNAC (induction of hyphal growth) or YNBGl (control culture, growth as the yeast form) for the times indicated and subjected to semi-quantitative RT-PCR analysis. (b) YlBMH1 and YlBMH2 mRNA levels are increased in cells of various backgrounds carrying pBMH1 and pBMH2. Total RNA was isolated from cells grown at 28 °C in YNBGl for 10 h and subjected to semi-quantitative RT-PCR. Lanes: 1, YlBMH1 mRNA levels in cells carrying pINA445; 2, YlBMH2 mRNA levels in cells carrying pINA445; 1’, YlBMH1 mRNA levels in cells carrying pBMH1; 2’, YlBMH2 mRNA levels in cells carrying pBMH2. The 600 and 400 bp RT-PCR products were resolved by electrophoresis on 2% agarose and visualized by staining with ethidium bromide. YlBMH1 and YlBMH2 mRNA expression was normalized to YHIS1 mRNA.
**DISCUSSION**

Here we report the isolation of the *Y. lipolytica* genes *YlBMH1* and *YlBMH2*, and show that *YlBMH1* is involved in the regulation of filamentous growth in this organism. *YlBMH1* and *YlBMH2* encode two different 14-3-3 proteins showing 88.2% identity, and their primary transcripts are interrupted by one intron near their 5’ ends. Interestingly, the A nucleotide at the fifth position of the putative 5’-splice donor sequence of *YlBMH1* (GTAAATPu) diverges from the G nucleotide normally found in *Y. lipolytica* genes, and an unusual 3’ intronic end (TAG, as opposed to the consensus sequence TACTAAC) is found three nucleotides downstream of the consensus sequence TACTAAC. These features, when compared to those previously found in other *Y. lipolytica* genes (Strick et al., 1992; Lopez et al., 1994; Hurtado et al., 2000; Smith et al., 2000), suggest that a higher degree of variation in the splice and branch sites is tolerated in *Y. lipolytica* than in Saccharomyces cerevisiae. Thus, as in higher eukaryotes (Newman, 1998), only the GT and AG dinucleotides at the intron termini are invariant, but an abbreviated version of the *Saccharomyces cerevisiae* consensus branchpoint sequence (CTAAC, as opposed to TACTAAC) (Teem et al., 1984; Kaufer & Potashkin, 2000) is still required in *Y. lipolytica*. Interestingly, the sequence TAG is also found in the putative 3’ intronic end of two other *Y. lipolytica* genes, *ACT1* and *SBH1* (GenBank accession nos AJ250347 and AJ277554, respectively), which suggests that PyAG is the consensus intron acceptor sequence in this yeast species.

Although the exact role of *YlBMH1* and *YlBMH2* in the induction of filamentous growth remains to be elucidated, analysis of the predicted amino acid sequences revealed that *YlBmh1p* and *YlBmh2p* are closely related to the *Saccharomyces cerevisiae* 14-3-3 proteins ScBmh1p and ScBmh2p and, thus, may have similar functions in *Y. lipolytica*. In *Saccharomyces cerevisiae*, BMH1 and BMH2 are required for Ras/MAPK signalling during pseudohyphal development (Roberts et al., 1997). More precisely, ScBmh1p and ScBmh2p have been found to be associated with Ste20p in a complex and to act downstream of Ras2p and Cdc42p to promote activation of the transcription factors Ste12p and Tec1p via the mitogen-activated protein kinases (MAPKs) Ste11p, Ste7p and Kss1p (Mösch et al., 1996; Roberts et al., 1997). Ste12p and Tec1p, in turn, have been shown to mediate pseudohyphal development through the cooperative activation of genes containing enhancer elements called filamentation and invasion response elements (FREs) (Madhani & Fink, 1997). However, it is noteworthy to mention that 14-3-3 proteins from different yeast species do not appear to have identical functions, and several other features of ScBmh1p and ScBmh2p suggest that the role of *YlBmh1p* in the regulation of filamentous growth may not be restricted to Ras/MAPK signalling. Thus, 14-3-3 proteins are known to bind to a wide variety of cellular proteins (more than 100 have been described so far), and it is currently believed that they function as direct regulators of enzyme activity and as localization anchors, adapters or scaffolds for numerous cellular processes (Fu et al., 2000; van Hemert et al., 2001a, b). In addition, ScBmh1p and ScBmh2p have been implicated in other processes connected with filamentous growth (such as vesicular transport and the RAS/PKA signalling pathway) (Gelperin et al., 1995), and ScBmh2p has been found to directly regulate the localization of Msn2p and Msn4p (Beck & Hall, 1999), two transcription factors that regulate the stress response via cis-acting DNA elements that are also recognized and bound by Mhy1p, a critical regulator of filamentous growth in *Y. lipolytica* (Hurtado & Rachubinski, 1999).

In *Y. lipolytica*, disruption of *MYH1* results in complete abolition of both hyphal and pseudohyphal growth (Hurtado & Rachubinski, 1999), deletion of *YIRAC1* results in impaired hyphal growth but retention of the ability to form pseudohyphae (Hurtado et al., 2000), and the lack of functional *YBEM1* causes severe morphological defects and only a slight formation of pseudohyphae on solid media (Hurtado & Rachubinski, 1999).
Fig. 5. Cell morphology of *Y. lipolytica* strains transformed with autonomously replicating plasmids carrying the *YlBMH1* and *YlBMH2* genes. (a) Wild-type strain E122; (b) mutant strain *bem1KO157*; (c) strain *bem1KO157* transformed with pBMH1; (d) strain *bem1KO157* transformed with pBMH2; (e) mutant strain *mhy1KO9*; (f) strain *mhy1KO9* transformed with pBMH1; (g) strain *mhy1KO9* transformed with pBMH2; (h) mutant strain *rac1KO30*; (i) strain *rac1KO30* transformed with pBMH1; (j) strain *rac1KO30* transformed with pBMH2. Cells were photographed after 3 days incubation at 28 °C on YNA-agar plates. Bars, 5 µm.
2002). Also, it has been hypothesized that, as in C. albicans (Lo et al., 1997), hyphal and pseudohyphal growth are controlled by at least two parallel signalling pathways in Y. lipolytica, each with a different and additive input, and that filamentous growth comprises a sequence of events that requires a quantitatively stronger regulatory input to produce hyphae rather than pseudohyphae (Hurtado et al., 2000). YIRAC1 is one of only two fungal Rac homologues described so far (Hurtado et al., 2000; Gorfer et al., 2001) and, although the mechanisms by which it regulates hyphal growth in Y. lipolytica have not yet been elucidated, Rac GTPases are known to act, in conjunction with Cdc42, Ras and Rho GTPases, in rather complex cascades that integrate the signals received from a variety of surface receptors to a network of pathways with multiple overlaps, feedback loops and uni- and bi-directional signals in higher eukaryotes (Symons, 1996; van Aelst & D'Souza-Schorey, 1997; Zohn et al., 1998; Scita et al., 2000).

Accordingly, our observation that overexpression of YIBMH1 enhances pseudohyphal growth in cells lacking YIRAC1, while producing no visible effect in Ambiy1 and Δbem1 cells, suggests that, in the absence of functional YIRac1p and, consequently, of the activation of effectors required for hyphal growth, increasing amounts of YBmnh1p would amplify the output of the remaining signalling pathways (perhaps the Ras/MAPK pathway among them) and, hence, increase the formation of pseudohyphae in the Arac1 strain.

In this work we also show that, in Y. lipolytica, the expression levels of YIBMH1 are increased during the yeast-to-hypha transition, whereas no variation is observed in the transcription of YIBMH2 during this event. Although no data on the variation of the expression levels of ScBMH1 or ScBMH2 during the dimorphic transition in Saccharomyces cerevisiae are currently available, it is known that overexpression of these genes stimulates cell elongation and agar invasion in this organism (Roberts et al., 1997). Similarly, disruption of one of the wild-type alleles of the essential C. albicans gene CaBMH1 results in a significant reduction of filamentation (Cognetti et al., 2002), thus suggesting the existence of a positive correlation between protein abundance and filamentous growth. This hypothesis is further supported by our observation that, in addition to enhanced pseudohyphae formation in cells lacking functional YIRAC1, overexpression of YIBMH1 is able to induce hyphal growth in CHY3350 mutant cells. However, the absence of variation in the transcription levels of YIBMH2, its inability to induce hyphal or pseudohyphal growth when overexpressed in the CHY3350, mby1KO9, rac1KO30 and bem1KO157 mutant strains and the presence of a 19 aa insertion that contains a potential N-myristoylation site in YBmnh2p, suggest that YIBMH2 may have functions different from those of YIBMH1 and, thus, may not be involved in the regulation of filamentous growth in Y. lipolytica. This is not entirely surprising, because it has been observed that in Schizosaccharomyces pombe, for instance, disruption of the rad24 gene results in serious morphological defects, while the absence of rad25, which encodes the other 14-3-3 protein of fission yeast, has little or no effect (Ford et al., 1994). Nevertheless, a role for YIBMH2 in the regulation of filamentous growth cannot be completely ruled out at this time.

Disruption of the YIBMH1 and YIBMH2 genes has so far proven elusive. Numerous attempts to delete these genes in the wild-type strain E122 have been unsuccessful, and no transformants with either of these genes correctly replaced by a disruption cassette containing the YLURA3 gene flanked by sequences from either YIBMH1 or YIBMH2 have been obtained. More precisely, 1280 Ura+ transformants (19 of which showed a smooth phenotype) were obtained after 60 independent transformations of E122 cells with a YIBMH1::YLURA3 disruption cassette, while 765 Ura+ transformants (11 of which showed a smooth phenotype) were obtained after 36 independent transformations of E122 cells with a YIBMH2::YLURA3 disruption cassette. PCR analysis of all of these transformants revealed that none had either the YIBMH1 or the YIBMH2 gene correctly replaced. A second approach to the isolation of disruptants of either the YIBMH1 or the YIBMH2 gene, involving the introduction of disruption cassettes in the wild-type strain carrying a second copy of the corresponding gene in a replicative plasmid, followed by screening of transformants by colony PCR for determination of correct integration and gene disruption, was also unsuccessful. A total of 600 independent transformants were analysed by this approach. However, it is interesting to note that disruption of one allele of YIBMH1 in a diploid strain was readily achieved, but the YIBMH1/Ybmmh1 diploids thus obtained were practically unable to sporulate (data not shown). This finding, added to the fact that Y. lipolytica diploid strains are generally not amenable to tetrad dissection, precluded the isolation of Ura+ haploid strains, while only three Ura− haploid strains were isolated (data not shown).

In conclusion, our results demonstrate that YIBMH1 is involved in the regulation of both hyphal and pseudohyphal growth in Y. lipolytica, while the participation of YIBMH2 or other, as yet, unidentified genes encoding 14-3-3 proteins in these events remains unclear. Experiments are under way to isolate genes encoding other 14-3-3 proteins and to identify proteins that interact with YBmnh1p and/or YBmnh2p, so as to further define the roles of these 14-3-3 proteins in regulating filamentous growth in Y. lipolytica.

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Dimorphism in Yarrowia lipolytica


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