Isolation and Characterization of YlBEM1, a Gene Required for Cell Polarization and Differentiation in the Dimorphic Yeast

Yarrowia lipolytica

Cleofe A. R. Hurtado and Richard A. Rachubinski*

Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Received 13 March 2002/Accepted 17 May 2002

The ability to switch between a unicellular yeast form and different filamentous forms (fungal dimorphism) is an important attribute of most pathogenic fungi. Dimorphism involves a series of events that ultimately result in dramatic changes in the polarity of cell growth in response to environmental factors. We have isolated and characterized YlBEM1, a gene encoding a protein of 639 amino acids that is essential for the yeast-to-hypha transition in the yeast Yarrowia lipolytica and whose transcription is significantly increased during this event. Cells with deletions of YlBEM1 are viable but show substantial alterations in morphology, disorganization of the actin cytoskeleton, delocalization of cortical actin and chitin deposition, multinucleation, and loss of mating ability, thus pointing to a major role for YlBEM1 in the regulation of cell polarity and morphogenesis in this fungus. This role is further supported by the localization of YlBem1p, which, like cortical actin, appears to be particularly abundant at sites of growth of yeast, hyphal, and pseudohyphal cells. In addition, the potential involvement of YlBem1p in septum formation and/or cytokinesis is suggested by the concentration of a green fluorescent protein-tagged version of this protein at the mother-bud neck during the last stages of cell division. Interestingly, overexpression of MHY1, YIRAC1, or YlSEC31, three genes involved in filamentous growth of Y. lipolytica, induced hyphal growth of bem1 null mutant cells.

The ability to switch between yeast and mycelial growth (dimorphism) is a property of most fungal species and is regarded as a subject of major interest due to its implications for the understanding of eukaryotic cell differentiation and fungal pathogenesis (4, 31, 37, 43).

Fungal dimorphism is a complex phenomenon that involves extensive modification of the cellular machinery in response to environmental signals (37, 53). This process consists primarily of a drastic change in the pattern of cell wall biosynthesis, which is driven by an increased polarization of the cytoskeleton that ultimately results in a steady concentration of the vesicles that carry cell wall-biosynthetic enzymes to the tip of the growing cell surface (22, 52).

Only a few fungal species have been systematically investigated with regard to their ability to undergo dimorphic transition, and these studies have concentrated mostly on the plant pathogen Ustilago maydis, the human pathogen Candida albicans, and baker’s yeast, Saccharomyces cerevisiae (4, 7, 27, 36, 43, 52, 53). However, although most aspects of dimorphism are common to all fungi, some features are more restricted, and further studies with other dimorphic species are necessary in order to obtain a more comprehensive understanding of this phenomenon. We have therefore chosen to investigate these events in Yarrowia lipolytica, a microorganism that, in recent years, has gained recognition as an excellent model organism with which to study fungal dimorphism. This is due mostly to its ability to alternate between a unicellular yeast form and different filamentous forms (hyphae and pseudohyphae), its amenability to genetic and molecular biological analysis (17, 42, 44), and its ability to reproduce sexually (60). Here, we report the isolation and characterization of YlBEM1, a gene whose product, YlBem1p, shows strong homology to conserved motifs of Bem1p and Scl2p, two proteins involved in the regulation of cell polarity and differentiation in the yeasts S. cerevisiae and Schizosaccharomyces pombe, respectively (11, 14, 15, 21, 29, 34, 55). Like its counterparts in other yeasts, YlBEM1 plays a crucial role in the regulation of cell polarity in Y. lipolytica, and most remarkably, its deletion results in the impairment of hyphal growth in this organism.

MATERIALS AND METHODS

Yeast strains and microbial techniques. The Y. lipolytica strains used in this study are listed in Table 1. Strain CHY33169 was isolated after chemical mutagenesis of Y. lipolytica E122 cells with 1-methyl-3-nitro-1-nitrosoguanidine, as previously described (42). Medium components were as follows: for YEPD, 1% yeast extract–2% peptone–2% glucose; for YNA, 0.67% yeast nitrogen base, 0.5% sodium acetate, 0.02% dextrose, 1% drop-out mixture, and 0.006% adenine sulfate.

TABLE 1. Y. lipolytica strains used in this study

| Strain   | Genotype          | Source or reference |
|----------|-------------------|---------------------|
| E122     | MATA ura3-302 leu2-270 lys8-11 | C. Gaillardin, Thiverval-Grignon, France |
| 22301-3  | MBT ura3-302 leu2-270 his1      | C. Gaillardin       |
| CHY33169 | MATA ura3-302 leu2-270 lys8-11 fil2 | This study          |
| bem1KO157| MATA ura3-302 leu2-270 lys8-11 bem1::URA3 | This study          |
| mly1KO9  | MATA ura3-302 leu2-270 lys8-11 mly1::URA3 | 24                  |
| rac1KO30 | MATA ura3-302 leu2-270 lys8-11 rac1::URA3 | 25                  |

* Corresponding author. Mailing address: Department of Cell Biology, University of Alberta, Medical Sciences Building 5-14, Edmonton, Alberta T6G 2H7, Canada. Phone: (780) 492-9868. Fax: (780) 492-9278. E-mail: rick.rachubinski@ualberta.ca.
without amino acids–2% sodium acetate; for YNBGlc, 1.34% yeast nitrogen base without amino acids–1% glucose; and for 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 at 50/μg/ml each, as required. YNBGlc and YNBGlcNAc were supplemented with Complete Supplement Mixture (Bio 101, Vista, Calif.) at twice the manufacturer’s recommended concentration (2× CSM) or with 2× CSM minus leucine, as required. Media, growth conditions, and the method of transformation of \( Y. \) lipolytica have been described previously (5, 42).

DNA manipulation and growth of \( E. \) coli were performed as described elsewhere (3).

Mycelial induction. Mycelial growth was induced as previously described (23). Cells were grown in YNBGlc for 12 h, harvested by centrifugation at room temperature, washed with sterile distilled water, kept on ice for 15 min in YNB medium without a carbon source, and inoculated at a final density of 10^7 cells/ml in YNBGlcNAc medium (for induction of the yeast-to-hypha transition) or YNBGlc medium (for growth as the yeast form).

Cloning and characterization of the \( Y. \) lipolytica \( BEM1 \) gene. The \( Y. \) lipolytica \( BEM1 \) (\( YlBEM1 \)) gene was isolated from a \( Y. \) lipolytica genomic DNA library contained in the replicative \( E. \) coli shuttle vector pINA445 (42) by functional complementation of strain CHY33169. Plasmid DNA was introduced into yeast cells by electroporation, and Leu^+ transformants were screened on YNA agar plates for the ability to give rise to rough colonies. 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 (\( pBEM1 \)) were subcloned into vector pGEM-5Zf(+) or pGEM-7Zf(+) (Promega, Madison, Wis.) for dideoxynucleotide sequencing of both strands. The deduced polypeptide sequence, \( YlBem1p \), was compared to other known protein sequences by using the BLAST Network Service of the National Center for Biotechnology Information (Bethesda, Md.).

Nucleic acid manipulation. Genomic DNA, plasmid DNA, and total RNA were prepared from \( Y. \) lipolytica as described elsewhere (3). Southern blot analyses were carried out with DNA probes prepared with the ECL direct nucleic acid detection system (Amersham Life Science, Arlington Heights, Ill.).

Table 2. Oligonucleotides used in this study

| Primer          | Sequence                      | Restriction site |
|----------------|-------------------------------|-----------------|
| BEMIF          | 5'-GTGGACACAGAGGTCTTAC-3'     |                 |
| BEMIR          | 5'-CTGGACCTTCGTTGAGC-3'       |                 |
| HISIF          | 5'-TCAGATTGGTGCGAGGCTC-3'     |                 |
| HISIR          | 5'-CCAGAATGTACAGAGCACC-3'     |                 |
| BEMITAG-5F     | 5'-GGGATCCCTCCTCAGACGG-3'     | ApaLI           |
| BEMITAG-5R     | 5'-GGCCGCGGCTACAGGTACA-3'     | ApaLI           |
| BEMITAG-3F     | 5'-GGATCCATCTCGGGCTATGTTGA-3' | BamHI           |
| BEMITAG-3R     | 5'-TTAGGCCCCTCTGGATAAGGAG-3'  | ApaLI           |
| GFP-ApaF       | 5'-TATAGGCTCAGGTTAAAGAGAC-3'  | ApaLI           |
| GFP-ApaR       | 5'-TATAGGCTCAGGTTAAAGAGAC-3'  | ApaLI           |

*Underlined nucleotides correspond to restriction sites.*
acid labeling and detection system (Amersham Biosciences, Oakville, Ontario, Canada). Electrophoresis conditions and transfer to nitrocellulose membranes were carried out as described elsewhere (3). Hybridization, stringency of washes, and signal generation and detection were performed as recommended by the manufacturer.

Semiquantitative RT-PCR. Relative abundances of *YlBEM1* mRNA under different conditions of cell growth were determined by semiquantitative reverse transcription-PCR (RT-PCR). Four micrograms of total RNA digested with RNase-free DNase I (Invitrogen, Carlsbad, Calif.) was used for cDNA synthesis with random primers and SUPERSCRIPT II RNase H− reverse transcriptase (Invitrogen). Several dilutions of cDNA were used as the template in the PCR performed for 5 cycles (60 s at 96 °C, 60 s at 52 °C, and 60 s at 72 °C) with Taq DNA polymerase (Invitrogen) and 10 pmol of primers BEM1F and BEM1R (Table 2) followed by 30 additional cycles after the addition of primers HIS1F and HIS1R (Table 2) at 10 pmol/µl. Aliquots from each reaction product were analyzed by electrophoresis on 2% agarose gels and stained with ethidium bromide. Results were documented with the VersaDoc Imaging System and analyzed with Quantity One quantitation software (both from Bio-Rad, Mississauga, Ontario, Canada). The solid background residues in at least two of the proteins; shaded background, similar residues in at least two of the proteins. The following amino acids are similar to each other: G, A, and S; V, I, L, and M; I, L, M, F, Y, and W; K, R, and H; D, E, Q, and N; and S, T, Q, and N. Percentages of identity between *YlBem1p* and *SpScd2p* and between *YlBem1p* and *ScBem1p* are given in parentheses at the end of the sequence. GenBank accession numbers are AAA50557 (SpScd2p) and CAA45320 (ScBem1p).

FIG. 3. Amino acid sequence alignment of *Bem1p* of *Y. lipolytica* (*YlBem1p*) and its homologs from *S. pombe* (*SpScd2p*) and *S. cerevisiae* (*ScBem1p*). Amino acid sequences were aligned by use of the ClustalW program (EMBL, Heidelberg, Germany). Solid background, identical residues in at least two of the proteins; shaded background, similar residues in at least two of the proteins. The following amino acids are similar to each other: G, A, and S; V, I, L, and M; I, L, M, F, Y, and W; K, R, and H; D, E, Q, and N; and S, T, Q, and N. Percentages of identity between *YlBem1p* and *SpScd2p* and between *YlBem1p* and *ScBem1p* are given in parentheses at the end of the sequence. GenBank accession numbers are AAA50557 (SpScd2p) and CAA45320 (ScBem1p).
Ontario, Canada). This information was used to establish a linear correlation between the amount of cDNA used and the level of product obtained (data not shown). Levels of YIBEM1 mRNA during dimorphic transition were subsequently determined by using data from reactions in which amplification was exponential.

**GFP tagging of YlBem1p.** An *ApaI* site was introduced before the stop codon of the YIBEM1 gene by replacement of the 2.2-kbp *KpnI-BamHI* fragment of YIBEM1 with a 2.2-kbp *KpnI-BamHI* fragment (obtained by ligation of a 0.8-kbp *KpnI-ApaI* fragment, amplified by PCR using oligonucleotides BEMITAG-5F and BEMITAG-5R [Table 2], and a 1.4-kbp *ApaI-BamHI* fragment, obtained by PCR using oligonucleotides BEMITAG-3F and BEMITAG-3R [Table 2]). A fragment with *ApaI* termini encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was generated by PCR using oligonucleotides GFP-ApaF and GFP-ApaR (Table 2) and was ligated into the newly introduced *ApaI* site of pBEM1 to produce plasmid pBEM1GFP, carrying YlBem1p tagged at its carboxyl terminus with GFP (YlBem1p-GFP). The integrity of the final construct was confirmed by sequencing, and pBEM1GFP was found to fully reproduce the phenotype produced by pBEM1 upon introduction into strain bemiKO157.

**Fluorescence microscopy.** F-actin was detected by incubating cells with 1.3 μM Oregon Green 488 phalloidin (Molecular Probes, Eugene, Ore.) as described previously (1). Chitin and bud scars were stained with 0.1 mg of Fluorescent Brightener 28 (Sigma, St. Louis, Mo.)/ml as described previously (48). Nuclei were detected by addition of 4',6'-diamidino-2-phenylindole (DAPI) to mounting medium at a final concentration of 1 μg/ml. Images were collected with SPOT software 1.2.1 (Diagnostic Instruments, Sterling Heights, Mich.), processed in Adobe Photoshop 4.0.1 (Adobe Systems, San Jose, Calif.), and printed on a Kodak DStix 650P PS color printer (Eastman Kodak, Rochester, N.Y.,).}

**Nucleotide sequence accession number.** The sequence data reported here are available from EMBL/GenBank/DDBJ under accession number AY084035.

## RESULTS

**Isolation and characterization of the YIBEM1 gene.** The *Y. lipolytica* mutant strain CHY33169 was initially isolated by its inability to form wild-type rough-surfaced colonies on YEPD agar plates after 3 days of incubation at 28°C (Fig. 1B), an attribute that was stably maintained through multiple generations.

The YIBEM1 gene was isolated from a *Y. lipolytica* genomic DNA library contained in the replicative *E. coli* shuttle vector pINA445 (42) by its ability to induce the formation of rough-surfaced colonies when introduced into CHY33169 cells. Of approximately 7,000 transformants screened, 3 showed a more enhanced filamentous phenotype (Fig. 1D). Restriction enzyme analysis revealed that all complementing plasmids shared a 4.7-kbp *BamHI-BamHI* fragment capable of restoring filamentous growth to CHY33169. Sequencing of this fragment revealed an open reading frame of 2,577 bp interrupted by one intron encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was generated by PCR using oligonucleotides BEMITAG-3F and BEMITAG-3R [Table 2]). A fragment with *ApaI* termini encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was generated by PCR using oligonucleotides GFP-ApaF and GFP-ApaR (Table 2) and was ligated into the newly introduced *ApaI* site of pBEM1 to produce plasmid pBEM1GFP, carrying YlBem1p tagged at its carboxyl terminus with GFP (YlBem1p-GFP). The integrity of the final construct was confirmed by sequencing, and pBEM1GFP was found to fully reproduce the phenotype produced by pBEM1 upon introduction into strain bemiKO157.

Fluorescence microscopy. F-actin was detected by incubating cells with 1.3 μM Oregon Green 488 phalloidin (Molecular Probes, Eugene, Ore.) as described previously (1). Chitin and bud scars were stained with 0.1 mg of Fluorescent Brightener 28 (Sigma, St. Louis, Mo.)/ml as described previously (48). Nuclei were detected by addition of 4',6'-diamidino-2-phenylindole (DAPI) to mounting medium at a final concentration of 1 μg/ml. Images were collected with SPOT software 1.2.1 (Diagnostic Instruments, Sterling Heights, Mich.), processed in Adobe Photoshop 4.0.1 (Adobe Systems, San Jose, Calif.), and printed on a Kodak DStix 650P PS color printer (Eastman Kodak, Rochester, N.Y.).

The upstream region of the YIBEM1 gene contains consensus sequences for the binding of several transcription factors of *S. cerevisiae*, *C. albicans*, and *Aspergillus nidulans* implicated in the regulation of fungal development and in the response to specific environmental conditions (data not shown), and a putative TATA box, TTATATATAA, is found between nucleotides −259 and −267 (numbering from the A nucleotide of the first ATG codon) (Fig. 2). Analysis of cDNA showed that transcription of the YIBEM1 gene preferentially starts at position −3 from the A nucleotide of the potential initiating codon and that polyadenylation occurs following the guanosine at position +2829.

The deduced protein product of YIBEM1, YlBem1p, comprises 639 amino acids and has a predicted molecular mass of 69,970 Da (Fig. 2). Analysis of the predicted amino acid sequence of YlBem1p suggests that its closest homologs are *S. pombe* Scd2p (SpScd2p; 38.4% identity) and *S. cerevisiae* Bem1p (ScBem1p; 32.5% identity) (Fig. 3). Notably, the regions of highest homology among the three proteins are segments corresponding to src homology region 3 (SH3; residues 34 to 95 and 178 to 239 of YlBem1p) and the PhoX and Bem1 domains of *S. pombe* Scd2p and *S. cerevisiae* Bem1p, respectively, while the second SH3 domain of YlBem1p exhibits 56.5 and 47.5% identity to the first SH3 domains of SpScd2p and ScBem1p, respectively, while the second SH3 domain of YlBem1p exhibits 70.0 and 57.4% identity to the second SH3 domains of SpScd2p and ScBem1p, respectively. The PB1 domain of YlBem1p shows 39.6 and 45.8% identity to the PB1 domains of SpScd2p and ScBem1p, respectively. In addition, three putative PEST regions, which are commonly found in rapidly degraded proteins (16, 50), are predicted at residues 98 to 113, 457 to 487, and 496 to 516 of YlBem1p.

**YIBEM1 mRNA levels are increased during the yeast-to-hypha transition.** The dimorphic transition was induced in exponentially growing E122 cells by a 15-min carbon source starvation period at 4°C, followed by transfer to YNBGlcNAc medium, as previously described (23). Under these conditions, more than 80% of the cells produced germ tubes after 10 h of incubation at 28°C, while cells transferred to fresh glucose-containing medium (YNBGlc) grew almost exclusively as the yeast form.

**RNA levels are increased during dimorphic transition.** Total RNA was isolated from E122 cells incubated at 28°C in YNBGlcNAc (induction of hyphal growth) or YNBGlc (control culture, growth as the yeast form) for the times indicated and subjected to semiquantitative RT-PCR analysis. The 600- and 400-bp RT-PCR products were resolved by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. YIBEM1 mRNA expression was normalized to that of *YHIS1* mRNA. Bars in micrographs, 5 μm.
yeast form (Fig. 4). Northern blot experiments carried out with total RNA extracted from cells harvested following 3 and 10 h of incubation showed that *YlBEM1* mRNA was at levels undetectable by this approach (data not shown). To circumvent this limitation, we performed semiquantitative RT-PCR experiments using *YlHIS1*, a gene whose expression is maintained at a constant level during the yeast-to-hypha transition (unpublished data), as an endogenous internal standard. RT-PCR analysis showed that *YlBEM1* mRNA levels are significantly augmented during dimorphic transition (four- to fivefold) but are only slightly increased during growth as the yeast form (Fig. 4).

**YlBem1p** is concentrated at sites of growth and at the mother-bud neck in *Y. lipolytica*. To determine the subcellular localization of *YlBem1p*, a pINA445 plasmid construct expressing *YlBem1p* fused at its carboxyl terminus to GFP was introduced into the *Y. lipolytica* strain bem1KO157. Expression from similar plasmid constructs has been shown to result in levels of protein two to three times that observed for the protein in wild-type cells (19, 28). After incubation of cells for 24 h in YNBGlc liquid medium at 28°C, the *YlBem1p*-GFP chimera was found to be localized in as yet unidentified vesicular structures dispersed throughout the cytosol and particularly concentrated at sites of growth of yeast, hyphal, and pseudohyphal cells (Fig. 5A, C, and D). Interestingly, the chimeric protein was also found to be concentrated at the mother-bud neck in yeast cells in the late stages of cell division (Fig. 5B).

*Y. lipolytica* cells with deletions of *YlBEM1* are viable but show severe morphological defects. A 2.7-kbp fragment of pBEM1, corresponding to nucleotides −3 to +2700 of *YlBEM1*, was replaced by a 1.6-kbp fragment containing the *Y. lipolytica* URA3 gene (Fig. 6A). This construct was digested with BamHI and ClaI to release a 3.2-kbp fragment containing the entire *YlURA3* gene flanked by 0.6 and 1.0 kbp of the 5’ and 3’ regions of *YlBEM1*, respectively, and this linear frag-

---

**FIG. 5.** Localization of GFP-tagged *YlBem1p* in yeast (A and B), pseudohyphal (C), and hyphal (D) bem1KO157 cells carrying plasmid pBEM1GFP. Arrows indicate sites at which the *YlBem1p*-GFP chimera concentrates. Bars, 5 μm.
ment was used to transform the wild-type *Y. lipolytica* strain E122 (Fig. 1A and Table 1) to uracil prototrophy. Of 199 Ura" transformants obtained, 5 showed a fully smooth phenotype after 3 days on YEPD agar. One of these five, bem1KO157 (Fig. 1C and Table 1), was selected for further studies following Southern blot analysis confirming the correct replacement of the YIBEM1 gene by YIURA3 (Fig. 6B).

Because the products of the *S. pombe* scd2 and *S. cerevisiae* BEM1 genes are required for cell polarization and morphogenesis (11, 14, 21), deletion of YIBEM1 was expected to result in morphological defects in *Y. lipolytica*. *bem1Δ* cells grown in liquid media were indeed unable to form hyphae or pseudohyphae, even after prolonged periods of incubation (Fig. 7). Also, *bem1Δ* cells were found to be spherical and considerably larger than their wild-type counterparts (Fig. 7). Furthermore, 30 to 40% of the cells were binucleate (Fig. 8). However, when *bem1Δ* cells were incubated on solid media, the defects observed were less severe, and a few pseudohyphal cells (less than 1%) could be observed (see Fig. 11B).

**Deletion of the YIBEM1 gene affects the localization of actin and chitin.** Since the organization of the actin cytoskeleton is directly involved in the definition of cell morphology, and since Bem1p is believed to be an actin cytoskeleton-associated protein that provides a cell surface scaffold for the localized concentration of signaling kinases in *S. cerevisiae*, we investigated the effects of deletion of the YIBEM1 gene on actin localization in *Y. lipolytica*. As previously described (25), actin-rich zones were observed at the apices of hyphal, pseudohyphal, and yeast forms of wild-type cells, combined with a background of diffuse staining and punctate actin patches (Fig. 9A to F). In the *bem1Δ* mutant strain, however, actin-rich zones were randomly distributed, and most of the actin was dispersed throughout the periphery of the cell (Fig. 9G to I). Furthermore, in contrast to what is observed in wild-type cells (Fig. 9E), *bem1Δ* cells appear to be unable to form organized actin cytoskeletal structures when cultivated in liquid media (Fig. 9G to I).

In a similar way, deposition of cell wall material was affected in the *bem1Δ* strain. While chitin was concentrated primarily at the bud scars and septa of wild-type cells, it was found over the entire surfaces of *bem1Δ* cells. In addition, bud scars of *bem1Δ* cells were considerably larger than those of wild-type cells, and the random selection of budding sites appears to be prevalent in *bem1Δ* cells, whereas budding in wild-type cells is preferentially bipolar, with rare occurrence of lateral budding events (Fig. 10).

**bem1 null mutants of *Y. lipolytica* are unable to mate.** Because mating is a phenomenon that involves extensive reorganization of the actin cytoskeleton and is closely connected to dimorphism in fungi (36), we investigated whether deletion of the YIBEM1 gene had an effect on the mating ability of *Y. lipolytica*. No diploid strains were obtained upon crossing strain bem1KO157 with the isogenic wild-type strain 22301-3 (Table 1; also data not shown), leading to the conclusion that YIBEM1 is essential for mating in *Y. lipolytica*.

**Overexpression of MHY1, YIRAC1, or YISEC31 partially restores hyphal growth to *bem1* null mutants of *Y. lipolytica*.** We have previously reported the isolation and characterization of the *Y. lipolytica* MHY1 and RAC1 (*YIRAC1*) genes and their involvement in the regulation of the yeast-to-hypha transition in this microorganism (24, 25). We have also isolated the *Y. lipolytica* homolog of *S. cerevisiae* SEC31 (YISEC31; EMBL/GenBank/DDBJ accession number AY084034) by its ability to enhance filamentous growth when introduced into the wild-type strain E122 (unpublished data). To gain some initial insight into the interactions between these genes, we introduced autonomously replicating plasmids carrying the YIBEM1 (pBEM1), YIRAC1 (pRAC1), YISEC31 (pSEC31), and MHY1 (pMHY1) genes into the null mutants bem1KO157, rac1KO30, and mhy1KO9 (Table 1). These plasmids were based on the shuttle vector pINA445, which is believed to be present in 2 to 5 copies per cell (20).

After 3 days of incubation on YNA agar plates at 28°C, overexpression of MHY1, YIRAC1, and YISEC31 appeared to partially suppress the morphological defects of the YIBEM1 gene deletion (Fig. 11A to E). Notably, MHY1 was the strongest inducer of filamentous growth in the *bem1Δ* background (Fig. 11C), and YIRAC1 was a stronger inducer of filamentation than YISEC31 (compare colonies in Fig. 11D and E, respectively). In addition, overexpression of MHY1 or YIRAC1 in *bem1Δ* cells resulted in hyphae with a higher degree of branching than hyphae of the wild-type strain or of *bem1Δ* cells in which YISEC31 was overexpressed (compare cells in Fig. 11C and D to cells in Fig. 11A and E).

Interestingly, YIBEM1 induced hyphal growth when overexpressed in *rac1Δ* cells (compare Fig. 11G to F), but to a much lesser degree than when YIRAC1 was overexpressed in the *bem1Δ* background (compare Fig. 11D to B). No apparent
effect was observed when YlBEM1 was overexpressed in mhy1Δ cells (compare Fig. 11H and I).

**DISCUSSION**

Here we report the isolation of the *Y. lipolytica* BEM1 gene and the initial characterization of its protein product, YlBem1p. YlBem1p shares a number of structural features with Bem1p from *S. cerevisiae* and Scd2p from *S. pombe*, is involved in the regulation of cell polarization, and is necessary for the yeast-to-hypha transition in the dimorphic yeast *Y. lipolytica*. Like its two closest homologs, YlBem1p contains two potential SH3 domains at its amino terminus and a potential PB1 domain at its carboxyl terminus. These domains are involved in the assembly of protein complexes via binding to proline-rich peptides (40) and tyrosine kinase-mediated signal transduction (26, 32), respectively.

In *S. cerevisiae* and *S. pombe*, Bem1p and Scd2p interact with several proteins involved in the activation of the small GTPase Cdc42p and are assumed to function as scaffolds for proteins involved in the development of cell polarity, pheromone signaling, and cytoskeletal organization (6, 10–13, 18, 26, 30, 35, 38, 41, 45, 47, 61). During vegetative growth of *S. cerevisiae*, cortical markers left by previous cell divisions result in recruitment and local activation of the Bud1p/Rsr1p GTPase (39), which is subsequently linked to Cdc42p via a Bem1p-mediated interaction with the guanine-nucleotide-exchange factor, Cdc24p (13, 45, 61). In addition, Bem1p interacts with actin and the p21-activated kinase (PAK) Ste20p (30), suggesting...
that Bem1p acts to concentrate active Cdc42p at a specific site of the cell membrane, thus promoting both the local activation of a mitogen-activated protein kinase (MAPK) cascade and reorganization of the actin cytoskeleton by Ste20p (8, 34, 41). Deletion of \textit{S. cerevisiae} \textit{BEM1} or \textit{S. pombe scd2} still yields viable cells, but the mutant cells display severe morphological defects and are unable to mate (12, 14, 21). Furthermore, \textit{S. cerevisiae bem1} null mutants are defective in butanol-induced cell elongation and filamentous growth, diploid pseudohyphal growth, and haploid invasive growth (34).

The \textit{Y. lipolytica} \textit{BEM1} gene is not essential, and its deletion results in a phenotype similar to that observed for \textit{S. cerevisiae bem1} and \textit{S. pombe scd2} null mutant cells, i.e., disorganized actin cytoskeleton, delocalized cortical actin and chitin deposition, multinucleation, round morphology, and inability to mate. In addition, \textit{Y. lipolytica bem1} null mutant cells show obvious defects in bud site selection. These characteristics clearly point to a role for YlBem1p in cell cycle control and the establishment of cell polarity in \textit{Y. lipolytica}. The latter role is further supported by the fact that, like actin, YlBem1p is concentrated at the growing tips of yeast, hyphal, and pseudohyphal cells. However, it is noteworthy that while YlBem1p levels are increased at the bud tip during early bud growth, YlBem1p appears to be concentrated at the mother-bud neck during the last stages of budding. Thus, although no role in cytokinesis or septum formation has been proposed for the homologs of YlBem1p, this hypothesis is compatible with the concept that localized deposition of cell wall material is required at the mother-bud neck during these events. We are currently investigating this possibility.

Interestingly, the levels of \textit{YlBEM1} mRNA are significantly increased during the yeast-to-hypha transition, and YlBem1p appears to be abundant during the entire cell cycle in actively growing hyphal and pseudohyphal cells. It is also noteworthy that although \textit{Y. lipolytica bem1} null mutant cells are unable to form hyphae in either liquid or solid media, their ability to form pseudohyphae is partially restored upon cultivation on agar plates. The causes of this behavior are unknown, but it has been demonstrated that filamentous growth in fungi is intimately linked to thigmotropism (directional growth response to physical contact) (46, 54), and it is generally proposed that pseudohyphae represent an intermediate state of cell polarization between yeast and hyphal growth (9). Thus, one may hypothesize that other polarity proteins exist in \textit{Y. lipolytica} and act in conjunction with YlBem1p to promote hyphal growth. In the absence of YlBem1p, these other factors would still be able to support pseudohyphal growth in response to thigmotropic stimuli, but this response would be insufficient to increase polarization to a level at which hyphal formation is possible.

We have previously reported the isolation and characterization of \textit{MHY1} and \textit{YIRAC1}, two genes involved in the regulation of filamentous growth in \textit{Y. lipolytica} (24, 25). \textit{MHY1} codes for a potential transcription factor that is necessary for both hyphal and pseudohyphal growth and binds in vitro to sequences containing putative stress response elements (STREs, or pentanucleotide CCCCT), while \textit{YIRAC1} is a nonessential gene that encodes a Rac GTPase whose deletion impairs hyphal growth but does not abolish the ability of \textit{Y. lipolytica} cells to polarize actin at the site of growth and to form pseudohy-
We have recently isolated YLSEC31 by its ability to enhance filamentous growth when introduced into the wild-type strain E122 (unpublished data). The S. cerevisiae SEC31 gene and its mammalian homologs encode an essential component of the COPII vesicle coat that is required for vesicular transport from the endoplasmic reticulum (51, 57).

Here we report that overexpression of MHY1 partially suppresses the morphological defects of Y. lipolytica bem1 null mutant cells, whereas YLBEM1 has no apparent effect when overexpressed in cells lacking functional MHY1. More remarkably, overexpression of YLRAC1 in bem1Δ cells was able to induce greater hyphal growth than overexpression of YLBEM1 in cells lacking functional YLRAC1. These observations, coupled with the observations that overexpression of MHY1 does not suppress the morphological defects of rac1Δ cells (25) and that bem1Δ and rac1Δ cells are still able to form pseudohyphae on solid media while MHY1 is essential for both hyphal and

FIG. 9. Distribution and organization of actin at different developmental stages of wild-type and bem1Δ cells. Actin was stained with Oregon Green 488 phallolidin and detected by fluorescence microscopy. (A to F) Yeast cells (A to C), pseudohyphal cells (D and E), and hyphal cells (F) of the wild-type strain E122. (G to I) Yeast-like cells of the bem1Δ strain bem1KO157. Arrows indicate actin-rich zones in E122 and bem1KO157 cells. Bars, 5 μm.

FIG. 10. Deletion of the YLBEM1 gene affects the budding pattern and chitin deposition in Y. lipolytica. Chitin was stained with Fluorescent Brightener 28 and detected by fluorescence microscopy. WT, wild-type strain E122; bem1Δ, strain bem1KO157.
pseudohyphal growth, suggest that MHY1 acts upstream of YlRAC1 and YlBEM1 and that YlRAC1 is a stronger regulator of hyphal growth than YlBEM1. Moreover, our results give further support to the proposition that these two morphologies in Y. lipolytica are controlled by at least two parallel signaling pathways, 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 than to produce pseudohyphae. Thus, increased production of polarity factors other than YlBem1p would support hyphal growth when either MHY1 or YlRAC1 is overexpressed in bem1Δ cells, but the lack of functional YlBem1p would

FIG. 11. Colony (upper panels) and cell (bottom panels) morphology of Y. lipolytica strains transformed with autonomously replicating plasmids carrying the MHY1, YlRAC1, YlSEC31, and YlBEM1 genes. (A) Wild-type strain E122; (B) mutant strain bem1KO157; (C through E) strain bem1KO157 transformed with plasmid pMHY1 (C), pRAC1 (D), or pSEC31 (E); (F) mutant strain rac1KO30; (G) strain rac1KO30 transformed with plasmid pBEM1; (H) mutant strain mhy1KO9; (I) strain mhy1KO9 transformed with plasmid pBEM1. Colonies and cells were photographed after 3 days of incubation at 28°C on YNA-agar plates. Colony magnification, ×100. Bars, 5 μm.
result in increased branching. Conversely, increased production of YIBm1p would result in partial induction of hyphal growth in rnc1Δ cells due to a partial increase in cell polarity, whereas in the absence of functional MHy1p this increase would be insufficient to induce any filamentation.

Vascular secretion in yeast involves the delivery of secretory vesicles along polarized actin cables (49), and it is generally proposed that increased transport of cell wall material to the growing tip is required for hyphal formation in fungi (22). Furthermore, a pivotal role for YlSec31p in the yeast-to-hypha transition in Y. lipolytica is supported by recent evidence showing that, in Plasmodium falciparum, Sec31p is attached to the cytokinetic (2) and by previous reports of the involvement of several components of the secretory pathway in the regulation of hyphal growth (33, 39). Our observation that YlSEC31 partially restores hyphal growth when overexpressed in bem1Δ cells further supports the hypothesis that polarity proteins other than YlBem1p exist in Y. lipolytica and suggests that the secretory apparatus is a limiting factor in the transport of these proteins during filamentous growth. Experiments addressing these issues are currently under way.

ACKNOWLEDGMENTS

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR) to R.A.R. R.A.R is a CIHR Senior Investigator and an International Research Scholar of the Howard Hughes Medical Institute. During this work, C.A.R.H. was the recipient of studentships from the Alberta Heritage Foundation for Medical Research and the Faculty of Graduate Studies and Research of the University of Alberta.

REFERENCES

1. Adams, A. E. M., and J. R. Pringle. 1991. Staining of actin with fluoro-chrome-conjugated phalloidin. Methods Enzymol. 194:729–731.
2. Adisa, A. F. R. Albano, J. Reeder, M. Foley, and L. Tilley. 2001. Evidence for a role for a Plasmodium falciparum homologue of Sec31p in the export of proteins to the surface of malaria parasite-infected erythrocytes. J. Cell Sci. 114:3377–3386.
3. Ausubel, F. J., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1999. Current protocols in molecular biology. Greene Publishing Associates, New York, N.Y.
4. Banuett, F. 1995. Genetics of Ustilago maydis, a fungal pathogen that induces tumors in maize. Annu. Rev. Genet. 29:179–208.
5. Barth, G., and H. Weber. 1986. Improvement of sporulation in the yeast Yarrowia lipolytica. Antonie Leeuwenhoek J. Microbiol. Serol. 51:167–177.
6. Bender, L., H. S. Lo, H. Lee, V. Kojohan, J. Peterson, and A. Bender. 1996. Associations among PH and SH3 domain-containing proteins and Rho-type GTPases in yeast. J. Cell Biol. 135:879–894.
7. Bolker, M. 2001. Ustilago maydis—a valuable model system for the study of fungal dimorphism and virulence. Microbiology 147:1395–1401.
8. Bose, I., E. J. Iraozqui, J. Moskov, E. S. Bardles, T. R. Zyla, and D. J. Lew. 2001. Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cdc24p, and the effector Cla4p required for cell cycle-regulated phosphorylation of Cdc24p. J. Biol. Chem. 276:1716–1726.
9. Brown, A. J. P., and N. A. R. Gow. 1999. Regulatory networks controlling Candida albicans morphogenesis. Trends Microbiol. 7:333–338.
10. Butty, A.-C., P. M. Przycaj, L. S. Huang, I. Herkowitz, and M. Peter. 1998. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. Science 282:1511–1516.
11. Chang, E. C., M. Barry, W. Wang, V. Jung, H. P. Xu, and M. H. Wigler. 1994. Cooperative interaction of S. pombe proteins required for mating and morphogenesis. Cell 79:131–141.
12. Chang, E. C., G. Bartholomeusz, R. Pimental, J. Chen, H. Lui, L. L. Wang, P. Yang, and S. Marcus. 1999. Direct binding and in vivo regulation of the fusion yeast p21-activated kinase Shk1 by the SH3 domain protein Sc2c. Mol. Cell. Biol. 19:8066–8074.
13. Chant, J. 1999. Cell polarity in yeast. Annu. Rev. Cell Dev. Biol. 15:365–391.
14. Chenoweth, J., J. Corrado, A. Bender, J. Pringle, and I. Herskowitz. 1992. A yeast gene (BEM1) necessary for cell polarization whose product contains two SH3 domains. Nature 356:77–79.
15. Chenoweth, J., N. Valtz, and I. Herskowitz. 1994. Identification of genes required for normal pheromone-induced cell polarization in Saccharomyces cerevisiae. Genetics 136:1279–1296.
suggested by mutational analysis and immunolocalization. Curr. Biol. 6:446–454.
40. Morton, C. J., and I. D. Campbell. 1994. SH3 domains. Molecular “Vekro.” Curr. Biol. 4:615–617.
41. Moscow, J. J., A. S. Gladfelter, R. E. Lamson, P. M. Pryciak, and D. J. Lew. 2000. Role of Cdc42p in pheromone-stimulated signal transduction in Saccharomyces cerevisiae. Mol. Cell. Biol. 20:7559–7571.
42. Nuttley, W. M., A. M. Brade, C. Gaillardin, G. A. Eitzen, J. R. Glover, J. D. Aitchison, and R. A. Rachubinski. 1993. Rapid identification and characterization of peroxisomal assembly mutants in Yarrowia lipolytica. Yeast 9:507–517.
43. Odds, F. C. 1988. Candida and candidosis. A review and bibliography. Baillei Tindal, London, United Kingdom.
44. Ogrydziak, D., J. Basell, R. Contopoulou, and R. Mortimer. 1978. Development of genetic techniques and the genetic map of the yeast Saccharomyces cerevisiae. In The Yeasts: A Comprehensive Treatise, Vol. 1, 2002 Dimorphism in Y. Lipolytica. K. W. A. Tindal, London, United Kingdom.
45. Peterson, J., Y. Zheng, L. Bender, A. Myers, R. Cerione, and A. Bender. 1994. Interactions between the bud emergence proteins Bem1p and Bem2p and Rho-type GTPases in yeast. J. Cell Biol. 127:1395–1406.
46. Pringle, J. R. 1991. Staining of bud scars and other cell wall chitin with calcofluor. Methods Enzymol. 194:732–735.
47. Pringle, J. R. 1997. Four distinct secretory pathways serve protein secretion, cell surface growth, and peroxisome biogenesis in the yeast Yarrowia lipolytica. Mol. Cell. Biol. 17:5210–5226.
48. Prunier, D., and A. Bretscher. 2000. Polarization of cell growth in yeast. J. Cell Sci. 113:571–585.
49. Prunier, D., and A. Bretscher. 2000. Polarization of cell growth in yeast. J. Cell Sci. 113:571–585.
50. Rechsteiner, M., and S. W. Rogers. 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. 21:267–271.
51. Salama, N. R., J. S. Chuang, and R. W. Schekman. 1997. SEC31 encodes an essential component of the COPII coat required for transport vesicle budding from the endoplasmic reticulum. Mol. Biol. Cell 8:205–217.
52. San-Blas, G., and F. San-Blas. 1984. Molecular aspects of fungal dimorphism. Crit. Rev. Microbiol. 11:101–127.
53. Shepherd, M. G. 1988. Morphogenetic transformation in fungi.Curr. Top. Med. Mycol. 2:279–304.
54. Sherwood, J., N. A. R. Gow, G. W. Goodway, D. W. Gregory, and D. Marshall. 1992. Contact sensing in Candida albicans: a possible aid to epithelial penetration. J. Med. Vet. Mycol. 30:461–469.
55. Snell, V., and P. Nurse. 1994. Genetic analysis of cell morphogenesis in fission yeast—a role for casein kinase II in the establishment of polarized growth. EMBO J. 13:2066–2074.
56. Strick, C. A., L. C. James, M. M. O’Donnell, M. G. Gollaher, and A. E. Farnke. 1992. The isolation and characterization of the pyruvate kinase-encoding gene from the yeast Yarrowia lipolytica. Gene 118:65–72.
57. Tang, B. L., T. Zhang, D. Y. Low, E. T. Wong, H. Horstmann, and W. Hong. 2000. Mammalian homologues of yeast Sec31p. An ubiquitously expressed form is localized to endoplasmic reticulum (ER) exit sites and is essential for ER-Golgi transport. J. Biol. Chem. 275:13597–13604.
58. Teem, J. L., N. Abovich, N. F. Kaufer, W. F. Schwindinger, J. R. Warner, A. Levy, J. Woolford, R. J. Leer, M. M. C. van Raamsdonk-Duin, W. H. Mager, R. J. Planta, L. Schultz, J. D. Friesen, H. Fried, and M. Rosbach. 1984. A comparison of yeast ribosomal protein gene DNA sequences. Nucleic Acids Res. 12:8295–8312.
59. Titorenko, V. I., D. M. Ogrydziak, and R. A. Rachubinski. 1997. Four distinct secretory pathways serve protein secretion, cell surface growth, and peroxisome biogenesis in the yeast Yarrowia lipolytica. Mol. Cell. Biol. 17:5210–5226.
60. Wickerham, L. J., C. P. Kurtzman, and A. I. Herman. 1970. Sexual reproduction in Candida lipolytica. Science 170:1141.
61. Zheng, Y., A. Bender, and R. A. Cerione. 1995. Interactions among proteins involved in bud-site selection and bud-site assembly in Saccharomyces cerevisiae. J. Biol. Chem. 270:626–630.