Mutant alleles of the essential 14-3-3 gene in *Candida albicans* distinguish between growth and filamentation

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The opportunistic fungal pathogen *Candida albicans* has the ability to exploit diverse host environments and can either reside commensally or cause disease. In order to adapt to its new environment it must respond to new physical conditions, nutrient sources, and the host immune response. This requires the co-regulation of multiple signalling networks. The 14-3-3 family of proteins is highly conserved in all eukaryotic species. These proteins regulate signalling pathways involved in cell survival, the cell cycle, and differentiation, and effect their functions via interactions with phosphorylated serines/threonines. In *C. albicans* there is only one 14-3-3 protein, Bmh1p, and it is required for vegetative growth and optimal filamentation. In order to dissect separate functions of Bmh1p in *C. albicans*, site-directed nucleotide substitutions were made in the *C. albicans BMH1* gene based on studies in other species. Putative temperature-sensitive, ligand-binding and dimerization mutants were constructed. In addition two mutant strains identified through random mutagenesis were analysed. All five mutant strains demonstrated varying defects in growth and filamentation. This paper begins to segregate functions of Bmh1p that are required for optimal growth and the different filamentation pathways. These mutant strains will allow the identification of 14-3-3 target interactions and correlate the individual functions of Bmh1p to cellular processes involved in pathogenesis.

INTRODUCTION

The opportunistic fungal pathogen *Candida albicans* usually resides as a commensal in the human host. In response to changes in environmental conditions, *C. albicans* can rapidly invade a variety of mammalian tissues. This transition requires regulatory networks that enable the cell to simultaneously respond to new nutrient sources, overcome the host’s immune defence and effect tissue invasion. Changes in the host environment are detected through multiple sensory networks which induce an adaptive response via signal transduction pathways to the target genes and proteins affecting multiple cellular processes (Berman & Sudbery, 2002; Brown, 2002). In intricate coordination of different signalling pathways permits each stimulus to be put into context and allows ‘fine-tuning’ of the adaptive response to optimize growth within a particular host site. Although many of the components of these signalling cascades have been identified, the proteins that modulate the cross-talk between these pathways are poorly described and may be important in deciphering pathogenesis.

The 14-3-3 family of proteins plays key functional roles in various pathways regulated by phosphorylation of serines and threonines. 14-3-3 proteins are highly conserved in all eukaryotic species, and domains are retained which appear to perform analogous functions (reviewed by Ferl et al., 2002; Tzivion & Avruch, 2002; van Hemert et al., 2001; Yaffe, 2002). They assemble as stable dimers and a phosphopeptide-binding pocket containing residues from both the amino and carboxy terminals resides in each monomer so that each can bind a peptide. There are at least five ways by which 14-3-3 proteins effect their function. These include altering the ability of the target protein to interact with other proteins, modifying the subcellular partitioning of a target, enhancing/inhibiting the catalytic activity of a target, protecting a target from dephosphorylation/proteolysis, and acting as a scaffold (Tzivion & Avruch,
2002). Over 200 targets have been identified for 14-3-3 proteins; they fall into four main categories: signalling components, primary metabolic enzymes, vesicular transport/trafficking, and chromatin function (Aitken, 2002; Aitken et al., 2002a,b; Milne et al., 2002; Pozuelo Rubio et al., 2004; Yaffe & Elia, 2001).

In both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* there are two 14-3-3 genes that are highly homologous except for the carboxy termini. Deletion of one 14-3-3 gene has little effect but disruption of both renders the cell inviable in most strains (Ford et al., 1994; Gelperin et al., 1995; van Heusden et al., 1992, 1995). In *S. cerevisiae*, the genes are designated BMH1 and BMH2, and in *Sch. pombe*, rad24 and rad25.

In *Sch. pombe*, the 14-3-3 gene, rad24, was first identified in a search for mutants sensitive to UV radiation (Ford et al., 1994). The interaction between the *Sch. pombe* 14-3-3 proteins and phosphorylated cyclin kinases is essential in checkpoint control (Chen et al., 1999, 2003). The 14-3-3 proteins in *Sch. pombe* have also been implicated in cell wall integrity, cytokinesis (Ishiguro et al., 2001) and sexual differentiation pathways (Kitamura et al., 2001).

In *S. cerevisiae*, the BMH genes also have multiple functions, including playing a role in the RAS/MAPK (Roberts et al., 1997), RAS/PKA (Gelperin et al., 1995), TOR (target of rapamycin) (Bertram et al., 1998; Beck & Hall 1999) and RTG (retrograde signalling due to mitochondrial dysfunction) signalling pathways (Liu et al., 2003; van Heusden & Steensma, 2001), exocytosis and vesicular transport steps (Gelperin et al., 1995; Mayordomo & Sanz, 2002; Roth et al., 1999), and inactivation of transcription factors (Beck & Hall, 1999; van Heusden & Steensma, 2001). In the Σ1278b genetic background, it was possible to delete both BMH genes in *S. cerevisiae* (Roberts et al., 1997). The deletion strain grows poorly, is extremely sensitive to environmental stress and unable to form pseudohyphae, yet mates normally, indicating that Bmh proteins are required for activation of MAPK signalling during pseudohyphal development. The 14-3-3 proteins also play a role in the RAS/cAMP pathway and this appears to be required for cell viability (Gelperin et al., 1995; Roberts et al., 1997). Thus, as in mammalian cells, the 14-3-3 proteins in *S. cerevisiae* are required for several downstream events of RAS-mediated pathways.

In yeast the two 14-3-3 isoforms act mostly redundantly to affect multiple cellular processes including mitotic checkpoint control and cellular differentiation in response to environmental signals. In *C. albicans*, there is only one 14-3-3 protein and it is required for vegetative growth and optimal filamentation (Cognetti et al., 2002). The ability of *C. albicans* to adapt is a key step in its pathogenesis and involves careful regulation of multiple cellular processes. Therefore, the 14-3-3 protein in *C. albicans* may play a key role in coordinating the regulatory pathways required for colonization and invasion. Since the gene is essential, it is not possible to construct conventional knock-out strains, and conditional mutants will not necessarily identify the multiple functions of Bmh1p. In this paper we describe the construction of three non-lethal BMH1 mutant strains and include the analysis of two mutant strains identified by random mutagenesis (G. E. Palmer & J. Sturtevant, unpublished results). These strains have allowed us to segregate Bmh1p’s functions in growth from its role in filamentation and tentatively assign functions in multiple signalling pathways.

## METHODS

### Strains

The *C. albicans* strains used in this study are listed in Table 1. Strain BWP17 was provided by Dr A. Mitchell (Columbia University). The prototrophic derivative of BWP17, YJB6284 was provided by Dr J. Berman (University of Minnesota). All strains constructed for this study are derivatives of BWP17.

### PCR disruption strains

The heterozygote strains BH1 and BA1 were constructed by transforming BWP17 with the PCR product *bmh1*: *HIS1* or *bmh1*: *ARG4* as described for BUM1 (Cognetti et al., 2002). The PCR products were derived by amplifying the *HIS1* gene or *ARG4* gene from pGEM-HIS1 or pRS-ARGASpel, respectively, with primers Bmh511FL and Bmh1300RL (Table 2). Correct integration was confirmed by using primer sets Bmh5amp/Bmh3amp and His5det/Bmh3amp and Southern hybridization. The auxotrophic marker replaces nucleotides 74 to 724 of the ORF.

### Conditional and site-directed mutant strains

For the MAL2 promoter conditional strain, BA1 was transformed with Stud-linearized pGP311 using conventional lithium acetate procedures to give strain BSM1 (Gietz et al., 1992). Integration of only one copy of the plasmid was confirmed by Southern analysis. The remaining wild-type allele was disrupted with the *bmh1*: *HIS1* PCR product as described above to yield strains BDM1–3. Transformants were first plated on YNB-maltose and then replicated on YNB-maltose and YNB-glucose. Colonies which grew significantly faster on YNB-maltose were screened for the correct integration as described above. The site-directed strains were made as described above except that BH1 was transformed with Nhel-linearized pLB, pLB-Ts, pLBK51E and pLBDim to give strains U-BMH, U-Ts, U-K51E and U-dim, respectively. Isogenic U-K51R and U-L231S strains were constructed in the same manner. Colonies were screened on YNB+Arg. Once correct integration was confirmed by Southern analysis, the remaining wild-type allele was transformed with the *bmh1*: *ARG4* PCR product to give Ud- strains. All Ud- strains are isogenic. *S. cerevisiae* strains were kindly supplied by Dr J. Heitman, Duke University. For complementation studies, the *bmh1Abmh2A* diploid strain JRY5a/s was transformed with approximately 2 µg of pRS316, pPRBts, pPRBKE, pPRBKets or pPRBdim using routine procedures (Burke et al., 2000). The growth of the transformed strains was compared to the control strain MLY61a/s.

### Plasmids

The plasmids used for templates in PCR amplifications, pGEM-HIS1 and pRS-ARGASpel, were kindly provided by Dr A. Mitchell (Columbia University) (Wilson et al., 1999). The yeast vector pRS316 was provided by Dr A. Rosenberg (Georgetown University). The plasmids constructed for this study are listed in Table 3.

### MAL2 promoter regulated construct

In order to construct the conditional mutant, the *BMH1* ORF with appropriate 3’ flanking sequence was cloned into pCp10-mal2 (provided by Dr M. Stark;
**Table 1.** Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Relevant genotype*</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJ6284</td>
<td>BWP17</td>
<td>BMH1/BMH1 his1Δ::HIS1/his1Δ arg4Δ::ARG4::URA3/arg4Δ</td>
<td>Bensen et al. (2002)</td>
</tr>
<tr>
<td>BA1</td>
<td>BWP17</td>
<td>BMH1/bmh1Δ::ARG4</td>
<td>This work</td>
</tr>
<tr>
<td>BH1</td>
<td>BWP17</td>
<td>BMH1/bmh1Δ::HIS1</td>
<td>This work</td>
</tr>
<tr>
<td>BH1P1</td>
<td>BH1</td>
<td>BMH1/bmh1Δ::HIS1 his1A/his1Δ arg4Δ::ARG4::URA3/arg4Δ</td>
<td>This work</td>
</tr>
<tr>
<td>BSM1</td>
<td>BA1</td>
<td>BMH1/ARG4 RP10/rp10Δ::URA3-MAL2proBMH1</td>
<td>This work</td>
</tr>
<tr>
<td>BDM1</td>
<td>BSM1</td>
<td>bmh1Δ::HIS1/bmh1Δ::ARG4 RP10/rp10Δ::URA3-MAL2proBMH1</td>
<td>This work</td>
</tr>
<tr>
<td>U-BMH</td>
<td>BH1</td>
<td>BMH1/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH1</td>
<td>This work</td>
</tr>
<tr>
<td>Ud-BMH</td>
<td>U-BMH</td>
<td>bmh1Δ::ARG4/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH1</td>
<td>This work</td>
</tr>
<tr>
<td>Ud-Ts</td>
<td>BH1</td>
<td>BMH1/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH1</td>
<td>This work</td>
</tr>
<tr>
<td>Ud-Ts</td>
<td>U-Ts</td>
<td>bmh1Δ::ARG4/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH1</td>
<td>This work</td>
</tr>
<tr>
<td>U-K51E</td>
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<td>This work</td>
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<tr>
<td>U-K51E</td>
<td>U-K51E</td>
<td>bmh1Δ::ARG4/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH1</td>
<td>This work</td>
</tr>
<tr>
<td>U-dim</td>
<td>U-dim</td>
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<td>This work</td>
</tr>
<tr>
<td>Ud-dim</td>
<td>U-dim</td>
<td>bmh1Δ::ARG4/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH1</td>
<td>This work</td>
</tr>
<tr>
<td>Ud-K51R</td>
<td>U-K51R</td>
<td>bmh1Δ::ARG4/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH</td>
<td>G. E. Palmer &amp; J. Sturtevant, unpublished results</td>
</tr>
<tr>
<td>Ud-L231S</td>
<td>U-L231S</td>
<td>bmh1Δ::ARG4/bmh1Δ::HIS1 ura3Δ/ura3Δ::URA3-BMH1</td>
<td>G. E. Palmer &amp; J. Sturtevant, unpublished results</td>
</tr>
</tbody>
</table>

*All strains are derived from BWP17 (ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG). Alterations to the genotype are noted above.

**Table 2.** Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>F/R</th>
<th>Position in BMH ORF</th>
<th>Sequence (5’–3’)*</th>
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<tbody>
<tr>
<td><strong>Cloning primers</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BMHMaliF</td>
<td>F</td>
<td>1</td>
<td>TTGATATCATGOCGAGCTCCGCCGTGAAGATT</td>
</tr>
<tr>
<td>BMHMRI</td>
<td>R</td>
<td>1092</td>
<td>TGACAGTCCGAGCTTGTTGAATTAACCCAG</td>
</tr>
<tr>
<td>316BFl</td>
<td>F</td>
<td>507</td>
<td>GCTCTAGAATCTAGGACATTTGTGCT</td>
</tr>
<tr>
<td>316BR1</td>
<td>R</td>
<td>1100</td>
<td>GCTGAGCGGGGCGATGCTCAAGCGCAGCCGGT</td>
</tr>
<tr>
<td>BMHBAM</td>
<td>F</td>
<td>3</td>
<td>GAGGGATCCGACCCTGCCGGT</td>
</tr>
<tr>
<td>BMHADHR</td>
<td>R</td>
<td>831</td>
<td>TGATCTCAGAGTTACACAAAGATATATAAC</td>
</tr>
<tr>
<td><strong>Mutagenesis primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tsmutF</td>
<td>F</td>
<td>549</td>
<td>ATGAAATTCTTGAACCCCCAGATAGCGTTGTCC</td>
</tr>
<tr>
<td>tsmutR</td>
<td>R</td>
<td>581</td>
<td>GACAAGCTCCTATCTGGGGGCGTCTAAATTTCAT</td>
</tr>
<tr>
<td>K51EF</td>
<td>F</td>
<td>140</td>
<td>CTGGTCGCTAGGAAGATGTGATGCT</td>
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<td>K51ER</td>
<td>R</td>
<td>165</td>
<td>GACCCATGCAATTTCGTTAAGGAAGC</td>
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<td>14mutF</td>
<td>F</td>
<td>24</td>
<td>CAGTTAACCTTTGCAAAGAACAGCGAAGAACCTTTG</td>
</tr>
<tr>
<td>14mutR</td>
<td>R</td>
<td>64</td>
<td>CATACGTTCTAGCTTGTTCTTGCTTAAAGGTAAC</td>
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<tr>
<td><strong>PCR disruption and detection primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmh511FL</td>
<td>F</td>
<td>3</td>
<td>GCCAGGCCCTCCGGTGAAGATCTCGGTTTACCTTCTGCTAAATTAGCAGAA-CAAGCCAGAAGCITATTGGAAGAAATTTTCCCCAGTCCGAGCGT</td>
</tr>
<tr>
<td>Bmh1300RL</td>
<td>R</td>
<td>795</td>
<td>ATTTCGTTAGCTGCCTTTACCTCTTCTGTGTTGAGCTGAGCTGAGCGT- GACTGGATTGTGTTGTTGTTGCTTCAGTTTGATAGTGGAGGGAAGTT</td>
</tr>
<tr>
<td>Bmh5amp</td>
<td>F</td>
<td>&lt;118</td>
<td>GTCGCGGAAGGAAATCTAAGAAGA</td>
</tr>
<tr>
<td>Bmh3amp</td>
<td>R</td>
<td>893</td>
<td>ACGGATAGAAAGCAGGCAATAGAAGG</td>
</tr>
<tr>
<td>His5det</td>
<td>F</td>
<td>in HIS1</td>
<td>GTCAAGGAAGATATGATCAATTC</td>
</tr>
<tr>
<td>Arg3det</td>
<td>F</td>
<td>in ARG4</td>
<td>GTCAAGGAAGATATGATCAATTC</td>
</tr>
</tbody>
</table>

*Italicized nucleotides denote residues that are not in BMH1; underlined nucleotides denote an introduced restriction site; bold, lower-case nucleotides denote sites of mutagenesis.
Table 3. Plasmids constructed for this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Parent plasmid(s)</th>
<th>Details (primers or restriction sites)</th>
<th>Location in BMH1 ORF (1–795 nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGP301</td>
<td>pGEM-TEz</td>
<td>BMHMAFL1–BMHM1R</td>
<td>1 to 1092</td>
</tr>
<tr>
<td>pGP311</td>
<td>pGP301 + pCLP10-Mal2</td>
<td>EcoRV/Mlu</td>
<td>1 to 1092</td>
</tr>
<tr>
<td>pKJ1</td>
<td>pGEM-TEz</td>
<td>316BF1–316BR1</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pKJ3</td>
<td>pKJ1 + pRS316</td>
<td>SalI/EcORl</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pLB</td>
<td>pKJ1 + pLUX</td>
<td>KpnI/SacI</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pLBKETs</td>
<td>pLB</td>
<td>TsmutF/R S188P</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pLBKETs</td>
<td>pLB</td>
<td>SalI/EcORl</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pLBdim</td>
<td>pLB</td>
<td>14mutF/R L14QA15QE16R</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pPRBKE</td>
<td>pLBKETs + pRS316</td>
<td>SalI/EcORl</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pPRBETs</td>
<td>pLBKETs + pRS316</td>
<td>SalI/EcORl</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pPRBKETs</td>
<td>pLBKETs + pRS316</td>
<td>SalI/EcORl</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pPRBdim</td>
<td>pLBdim + pRS316</td>
<td>SalI/EcORl</td>
<td>−507 to 1100</td>
</tr>
<tr>
<td>pDC1BB</td>
<td>pGEM-T</td>
<td>BMHBAM–BMHADHR</td>
<td>3 to 831</td>
</tr>
<tr>
<td>pGEX-BMH</td>
<td>pDC1BB + pGEX-4T3</td>
<td>BamHI/Xhol</td>
<td>3 to 831</td>
</tr>
</tbody>
</table>

Backen et al., 2000) which contains the Candida MAL2 promoter, URA3 gene and RP10 gene for site-specific integration. Genomic DNA from strain BW17 was amplified with primers BMHMAlF1 and BMHMHR and the 1-kb product was ligated into pGEM-T Easy (Promega) to give plasmid pGP301. Plasmid pGP301 was digested with EcoRI and MluI and ligated into pCLP10-mal2 restricted with EcoRI and MluI in order to place the entire BMH1 ORF including 294 bp of 3' flanking sequence adjacent to the MAL2 promoter. This plasmid was designated pGP311.

Cassettes containing mutated BMH1 alleles. The plasmid pLUX was used as a template vector for construction of BMH1 plasmids (Ramon & Fonzi, 2003). pLUX contains the URA3 gene, and when digested with Nhel allows complete reconstitution of the URA3 locus in strain BW17; it was kindly provided by Dr W. A. Fonzi (Georgetown University). The entire BMH1 ORF containing 507 nucleotides of 5' flanking sequence and 305 nucleotides of downstream flanking sequence was PCR-amplified from C. albicans strain BW17 genomic DNA with primers 316BF1 and 316BR1. The 1.6-kb product was ligated into pGEM-T Easy (Promega) to give plasmid pGP311. Plasmid pGP301 was digested with SalI and EcoRI and ligated into pLUX digested with the same enzymes to give pGEX-BMH. Sequencing confirmed the correct fusion between the GST tag and Bmh1p.

Growth media and conditions. Strains were routinely grown on YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose [dextrose]) at 30°C, supplemented with uridine (25 μg ml⁻¹) when necessary (Guthrie & Fink, 1991). Transformants were selected on minimal medium [67-5 g⁻¹ yeast nitrogen base plus ammonium sulfate and without amino acids, 2% glucose, 2% Bacto agar (YNB)] supplemented with the appropriate auxotrophic requirements, as described for S. cerevisiae (Burke et al., 2000) except for uridine, which was added at 25 μg ml⁻¹. In some instances, YP was supplemented with either 2% maltose (YPM) or 2% sucrose (YPs) and YNB was prepared with 2% maltose instead of glucose (YNB-M).

The growth of BMH1 mutant strains was compared with the prototrophic strain YJB6284 under a range of growth conditions described below. Strains were grown overnight in YPD at 30°C and pelleted, and resuspended in sterile distilled water at 1 x 10⁷ cells ml⁻¹, and serially diluted to give suspensions of 2 x 10⁶, 4 x 10⁶, 8 x 10⁶ and 1 x 10⁷ cells ml⁻¹. Each cell suspension was then transferred to a 96-well plate and applied to a solid agar test media using a sterile micropiprunged applicator. To test temperature sensitivity, the cells were plated onto YPD agar and incubated at 30, 37 or 42°C. To test sensitivity to rapamycin, the cells were plated onto YPD containing 2.0 μM rapamycin (Sigma R0395, 1 mM dissolved in 90% ethanol, 10% Tween 20) and incubated at 30 and 37°C. For liquid growth cultures, 2 ml cultures were grown overnight in YPD at 30°C and then reinoculated at 2 x 10⁶ cells ml⁻¹ in 25 ml fresh medium in 125 ml flasks. Cell density was assessed by counting on a haemocytometer or reading the OD₆₀₀. All experiments were repeated at least three times.

Colony and cell morphology assays. The yeast-to-hyphal transition was induced in either liquid or solid (inclusion of 2% agar) media. Transition media included 10% fetal calf serum (FCS) in YPD, M199 containing Earle’s salts and glutamine (Invitrogen) buffered with 150 mM HEPES adjusted to pH 7.5. Filamentation in liquid media was induced by inoculating 2.5 x 10⁶ cells ml⁻¹ and incubating with shaking at 37°C. Filamentation was assessed at various time points as noted below. For solid surfaces 5 x 10⁶ cells in 5 μl water were spotted on plates and incubated for 2–10 days at 37°C. Colonies on solid agar were observed with an Olympus SZX9 stereomicroscope at 40 x and photographed using an Olympus DP-12 digital system. For all experiments, strains were either made prototrophic for URA3 or uridine was included in the media.
For filamentation in matrix, studies were performed basically as reported by Brown and co-workers (Brown et al., 1999; Giussani et al., 2002). Briefly, 100 cells were plated onto YPS agar and then overlaid with a second layer of YPS agar, or approximately 100 cells were mixed with 25 ml molten YPS agar (2%) and poured into a Petri dish. The percentage of filamenting colonies was calculated by microscopically determining the number of colonies out of 100 which had at least 20 protruding filaments (Brown et al., 1999).

Chlamydospore formation was assessed by growth on cornmeal agar (17 g cornmeal agar $\pm$ 1, 0-33 % Tween 80). Approximately 100 cells in 100 µl were inoculated underneath a coverslip by capillary action. In some instances the Dalmau inoculation technique was used (Bebeshti et al., 1975). No significant differences were seen between the two inoculation methods. Plates were incubated in the dark at 25°C and followed for 21 days. Colonies were observed under 10 x and 40 x objectives with an Olympus CX31 (10 x oculars) and photographed using an Olympus DP-12 digital system. The borders of 100 colonies of each strain were monitored for filamentation and chlamydospore formation. All filamentation experiments were repeated at least three times.

**DNA manipulations.** Plasmid isolation and enzymic reactions were performed either by standard methods (Sambrook & Russell, 2001) or according to the manufacturer’s instructions. Bacterial transformations were performed using chemically competent *Escherichia coli* DH5α (Invitrogen). Genomic DNA from *C. albicans* was isolated using glass beads (Burke et al., 2000). Southern analysis was performed as previously described (Sturtevant et al., 1998). DNA sequence of selected clones was determined by MWG Biotech. Analysis of sequence data was performed using DSGene1.1.

**Western blot analysis.** An antibody directed against the *C. albicans* Bmh1 protein was produced by Cocalico Biologicals. The expression of GST-Bmh1p (from plasmid pGEX-BMH) was induced in *E. coli* BL21 cells with 1 mM IPTG after 3 h at 37°C. Protein was extracted by sonication in lysis buffer (50 mM Tris, pH 7-5, 5 mM MgCl$_2$, 50 mM NaCl, 2 mM PMSF), followed by centrifugation and collection of the supernatant. Bacterial extracts were loaded on Glutathione Sepharose 4B following the manufacturer’s directions (Pharmacia Biotech). GST-Bmh1p conjugated to glutathione agarose beads (1-6 µg µl$^{-1}$) was used as the antigen for antibody production. Dot-blot was used to assess specificity of anti-Bmh1p. Serial dilutions of pGEX4T3, pGEX-BMH (0-0075–1-0 µg) and BW17 cell extracts (0-75–100 µg) were spotted on membranes in triplicate and probed with prebleed antiserum and anti-Bmh1p from two animals and secondary antibody alone. Once reactivity was confirmed, anti-Bmh1p was tested against whole-cell *C. albicans* extracts and the *S. cerevisiae* bmh1Δbmh2A mutant transformed with *C. albicans* BMH1 or control plasmid to confirm that the antibody recognized a 29 kDa band and not other components in the cell extract. Since GST and Bmh1p migrate at approximately the same rate, blots were also probed with anti-GST (Amersham). No signal was seen in *Candida* protein extracts. For immunoblotting, whole-cell extracts of *C. albicans* were prepared by lysis with glass beads as described for *S. cerevisiae* (Roberts et al., 1997). Protein lysate from the equivalent of 4 x 10$^7$ cells were loaded per lane in 12 % SDS-PAGE gels (unless otherwise noted). Gels were transferred to PROTRAN (Schleicher and Schuell), blocked with TB (0-15 M NaCl, 0-05 M Tris, pH 8-0, 0-005 M EDTA, pH 8-0, 0-5 % Tween 20) plus 5 % non-fat milk for 1 h at room temperature, incubated with anti-Bmh1p antibody at 1:2000 dilution overnight at 4°C, washed in TB, and then incubated for 1 h at room temperature with horseradish-peroxidase-conjugated goat anti-rabbit IgG [H&L] (1:10 000) (Rockland). Blots were revealed by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

### RESULTS

**BMH1 is required for optimal growth and filamentation**

The 14-3-3 gene in *C. albicans*, BMH1, is essential (Cognetti et al., 2002) and thus it is not possible to identify its function using conventional knock-out strategies. Therefore, conditional mutants were constructed. A first attempt was to place the remaining BMH1 allele in a heterozygote strain under the control of the MET3 promoter (Care et al., 1999; Cognetti et al., 2002). However, all transformants retained an additional wild-type copy, even those that contained the desired integration (Cognetti et al., 2002). This suggested that levels of Bmh1p expressed from the exogenous MET3 promoter were insufficient for vegetative growth. Lethality was probably not due to overexpression of Bmh1p or expression of Bmh1p under inappropriate conditions since the protein is constitutively expressed. Additionally, integration of an extra copy of BMH1 under the MET3 promoter in the RP10 locus of a BMH1/BMH1 strain did not result in lethality. A second approach was to integrate the regulatable MAL2 promoter–BMH1 construct into an exogenous locus prior to disrupting both endogenous BMH1 alleles, to give strains BDM1–3 (Backen et al., 2000). Three separate transformants gave comparable phenotypic results. Strains grew significantly better and more rapidly on YPM (inducing conditions) compared to YPD. Growth on YPD was due to leakiness in the promoter as assessed by Western blot analysis. Bmh1p was detectable in the conditional strain under repressive conditions, though significantly less than seen under inducing conditions (Fig. 1a). However, Bmh1p expression was reduced in strains expressing one BMH1 allele compared to wild-type strains with two endogenous BMH1 alleles (Fig. 1a). Indeed, growth rate correlated with the expression of Bmh1p.

The conditional mutant was more severely affected, and filamented in the presence of glucose only after extensive incubation (Fig. 1b). Morphogenesis assays were not optimal since the inducing medium (maltose) is not conducive to filamentation. Although all strains eventually filamented on maltose, extensive incubation (7–10 days) was required and the difference between the strains was not significant (Fig. 1b).

### Strains with single-site mutations in BMH1 are viable

The conditional mutant strains were not favourable for several reasons. Expression was poorly regulated and many phenotypic studies cannot be performed under optimal conditions due to promoter-dependent nutritional constraints. It is likely that Bmh1p has a variety of distinct functions and these would be impossible to dissect if the gene is repressed. Therefore, strains were constructed with
site-specific mutations in BMH1. The specific amino acids that were substituted were based on their importance in other species and conservation in C. albicans. Three sites were chosen: Ts (S188P), ligand binding (K51E) and dimerization (L14QA15QE16R) mutations reported for S. cerevisiae (van Heusden & Steensma, 2001), human (Zhang et al., 1997) and Drosophila (Zhou et al., 2003), respectively. These mutant alleles were first tested to determine if they could complement the growth defects in S. cerevisiae. C. albicans BMH1 complemented the growth defects in the bmh1Δ/bmh2Δ deletion strain of S. cerevisiae on YPD at 30 and 37 °C (Fig. 2). The K51E and dimerization (dim) mutants partially complemented the deletion strain (Fig. 2). The Ts mutation in the CaBMH1 allele did not complement the defect at 37 °C (Fig. 2). Similar levels of complementation for control, native and mutant alleles was observed under a range of stressful conditions including 5 mM caffeine and 1 M sorbitol (data not shown). These results indicate that the CaBMH1 mutant alleles are functional and have varying abilities to support growth.

Two mutant alleles, L231S and K51R, were identified through random mutagenesis (G. E. Palmer & J. Sturtevant, unpublished results) and are included in the following analyses. C. albicans strains were constructed by introducing the BMH1 and mutant BMH1 alleles into the URA3 locus of the BMH1 heterozygous strain, BH1 (U strains, Table 1). Correct integrations of a single copy were confirmed by Southern analysis (data not shown). The remaining wild-type BMH1 allele was then deleted with ARG4 to give strains Ud-BMH, Ud-Ts, Ud-K51E, Ud-K51R, Ud-dim and Ud-L231S. Correct integration was confirmed by Southern hybridization (data not shown). In all Ud- strains the only BMH1 allele is expressed under its endogenous promoter in the URA3 locus.

**Growth is differentially inhibited dependent on the site of the bmh1 mutation**

The mutant strains were tested to determine if domains involved in growth could be identified. In liquid YPD at 30 °C, the mutant strains divided into two categories of

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**Fig. 1.** Expression levels of Bmh1p correlate with ability to filament. (a) Immunoblot of protein extracts of C. albicans strains probed with anti-Bmh1p antibody as described in Methods. Parental strain BWP17 (lane 1), heterozygous strain BA1 (lane 2) and maltose-regulatable strains BSM1 (lanes 3, 5) and BDM1 (lanes 4, 6) were grown for 40 h at 30 °C in YPD (glucose carbon source; lanes 1–4) or YPM (maltose carbon source; lanes 5, 6). BMH refers to native BMH1 genotype. MAL2p refers to the presence (+) absence (−) of MAL2 promoter. C source: G, glucose; M, maltose. (b) Filamentation is severely inhibited in MAL2p:BMH1 conditional mutant strain. Five microlitres of a 10⁸ cells ml⁻¹ suspension of YJB6284 (i, iv), BSM1 (ii, v) and BDM1 (iii, vi) were spotted on M199 (i–iii) or M199 containing 2 % maltose (iv–vi) and colonies were incubated for 7 days at 37 °C. +/− refers to native BMH1 allele genotype. This figure is representative of at least three separate experiments.

**Fig. 2.** The C. albicans BMH1 mutant alleles partially complement the S. cerevisiae bmh1Δ/bmh2Δ deletion mutant. S. cerevisiae strain JRY5a/α was transformed with the C. albicans BMH1 (CaBMH1) or the putative C. albicans bmh1 mutant alleles [ligand-binding mutation (K51E), temperature-sensitive mutation S188P (Ts), ligand-binding and Ts double mutation (K51E+Ts), or dimerization mutation L14QA15QE16R (Dim)] as described in Methods and incubated for 3 d at 30 or 37 °C on YPD. Control transformant was with empty vector, pRS316 (Vector). Growth phenotypes of transformed strains were compared to the BMH1/BMH2 diploid strain, MLY61a/α (Control).
growth (Fig. 3a). The Ud-Ts, Ud-L231S and Ud-K51E strains were inhibited in growth to a similar extent. At 30°C, generation times of the Ud-Ts, Ud-L231S and Ud-K51E strains were 200–300 min, compared to approximately 100 min for Ud-BMH, Ud-dim and Ud-K51R. Similar results were obtained on solid media. Serial dilutions of the strains were plated on YPD and allowed to grow at 30, 37 and 42°C (Fig. 3b). The Ts, K51E and L231S mutants showed similar levels of growth inhibition at 30 and 37°C at day 2 and no growth at 42°C (Fig. 3b, iii). However, by day 7 Ud-K51E and Ud-L231S but not Ud-Ts grew at 42°C (Fig. 3b, vi). Thus the Ts mutant demonstrated a genuine temperature-sensitive phenotype as well as a general growth defect as seen in S. cerevisiae (van Heusden & Steensma, 2001). Growth of Ud-K51R and Ud-dim on YPD was not significantly different from the control.

Since the BMH1/2 genes have been reported to play a role in the TOR pathway in S. cerevisiae (Beck & Hall, 1999; Bertram et al., 1998; Gelperin et al., 2002; van Heusden & Steensma, 2001), the growth of the mutant strains was tested on YPD with 10 nM rapamycin. Strains with only one BMH1 allele (BH1P1 and Ud-BMH) reproducibly demonstrated slightly more resistance to rapamycin than the BMH1 homozygote strains (Fig. 3b, iv, v). At 30°C the mutant strains except for Ud-L231S also grew slightly better than BMH1 homozygote strains in the presence of rapamycin (Fig. 3b, iv). Normally the growth of all strains is slightly increased at 37°C compared to 30°C but in the presence of rapamycin, Ts and K51E mutants were severely inhibited (Fig. 3b, v). Sensitivity to rapamycin appears temperature independent in all other strains. The L231S mutation confers increased sensitivity to rapamycin at both temperatures. Growth of Ud-K51R and Ud-dim in the presence of rapamycin was comparable to that of Ud-BMH.

**bmh1 mutants are differentially affected in filamentation**

**Filamentation in liquid media.** The ability of the mutant strains to form filaments was assessed in solid and liquid media. In liquid FCS medium the onset of filamentation was delayed and the percentage of Ud-Ts (15%), Ud-K51E (30%), Ud-dim (60%) and Ud-L231S (60%) cells that filamented after 5 h was consistently less than seen in controls (80% for Ud-BMH). After 16 h the percentage of Ud-Ts and Ud-K51E cells that formed extensions did not increase and the filaments were also shorter and more irregular than those of Ud-BMH (Fig. 4). The onset of germ tube formation in Ud-dim and Ud-L231S was

![Figure 4](image-url)
The mutant strains also filamented normally. The Ud-K51R strain filamented as Ud-BMH.

**Filamentation on solid agar.** On M199 and FCS agar, the onset of filaments, if seen at all, was severely retarded and tufts were only seen on Ud-Ts and Ud-L231S after prolonged incubation (Fig. 5a, b). Uniform filamentation was never seen. On M199 agar both Ud-K51E and Ud-K51R exhibited similar filamentation defects even though their growth rates were dissimilar (Fig. 5a). Although Ud-K51R was defective in filamentation on FCS, it was not as defective as Ud-K51E. Growth rates and filamentation also segregated on FCS since both Ud-K51R and Ud-dim filamented significantly less than Ud-BMH (Fig. 5b). Frequently, sectoring was observed in the Ud-Ts strain and filaments would protrude from the sector. Cells were taken from the sectors and centre of the colony, inoculated in fresh media and plated. No growth defect was observed for the sector-derived cells. Genomic DNA was isolated from the inoculated cells and sequenced. In the sectored portions, the S188P mutation had reverted to wild-type (P188S). DNA recovered from the centre section maintained the mutation. However, the sequencing trace revealed a large C peak with a smaller T peak indicating the reversion in progress.

**Filamentation in matrix.** The ability of the mutant strains to filament under embedded conditions was also tested. The mutant strains were either sandwiched between two layers of YPS agar or mixed with YPS agar. All strains, including the BMH1/bmh1Δ heterozygote strain, were severely retarded in onset and phenotype of filamentation. All the wild-type colonies were filamenting by day 4 and they appeared as homogeneous fluffy, furry colonies (Fig. 5c). In contrast, no filamentation was seen by day 4 in Ud-BMH. At later time points, Ud-BMH and mutant strains were heterogeneous in phenotype and colonies were always smaller than wild-type. Interestingly there was not a significant difference in filamentation between Ud-BMH and Ud-L231 strains (Fig. 5c). Ud-K51E, Ud-K51R and Ud-Ts were significantly defective in filamentation compared to Ud-BMH. By day 11, the percentage of Ud-K51E colonies that were filamenting was reproducibly more than Ud-K51R. The Ud-dim strain demonstrated a large variability between experiments; percentage filamentation ranged between 0% and 42% at day 11. This variability was not seen for any of the other strains. Thus, under embedment conditions there was not a direct correlation between growth rate and filamentation.

**Chlamydospore formation.** The mutant strains also demonstrated differential phenotypes on chlamydospore-inducing agar. In BMH1 homozygote control strains, filamentation was seen by day 4 in approximately 30% of the colonies. Almost all colonies that filamented also produced chlamydospores (Figs 5d and 6a). In contrast, no filament or chlamydospore formation was seen in BH1P1 or Ud-BMH strains even after 21d (Fig. 5d). Interestingly, several of the BMH1 mutant strains did filament, though the production of chlamydospores was sparse (Fig. 6). Two mutant strains were hyperfilamentous. The onset of filamentation in Ud-L231S was rapid and the filaments were significantly longer than in BMH1 homozygote strains. By day 6, Ud-L231S colonies had long branched filaments but very few chlamydospores were observed (<1% of filamenting colonies) (Fig. 6c). By day 9 filamentation was extensive and mats were visible by the naked eye (Figs 5d and 6d). The Ud-K51E strain demonstrated a similar phenotype but to a lesser degree. These results suggest that while Ud-L231S and Ud-K51E exhibit decreased filamentation under some solid inducing conditions they are competent to filament extensively. A small percentage of colonies filamented in the Ud-Ts strain. No filamentation or spore production was observed in Ud-K51R and Ud-dim strains (Figs 5d and 6b). Therefore, the hyperfilamentous phenotype was associated with charge mutations in the ligand-binding pocket. Although filament formation appeared to have an inverse relation with growth rate on YPD, all mutant strains and control strains grew equally well on cornmeal agar.

**Differences in growth and filamentation are not solely due to Bmh1p expression level**

The amount of Bmh1p expression correlated with growth and filamentation (Fig. 1a). Therefore, it was important to determine if the reduced filamentation and growth seen in the bmh1 mutants was due to decreased protein expression. Protein extracts from exponential phase cells were immunoblotted with anti- C. albicans Bmh1p antibody (Fig. 7). As demonstrated previously less protein was expressed in the heterozygote than the parental strain (Figs 1a and 7). Additionally, even less protein was expressed in the Ud-BMH strain than in BH1P1 (Fig. 7). This could explain the difference in filamentation between BH1P1 and Ud-BMH strains. However, in the mutant strains the protein expression did not always correlate with phenotype. Ud-Ts and Ud-K51E exhibited similar phenotypes, yet their Bmh1p expression levels were significantly different (Fig. 7). Ud-K51E grew significantly more slowly than Ud-K51R yet the protein expression levels in these two strains were comparable. Therefore, the phenotype of Ud-Ts could be explained by the low protein levels. On the other hand, the defective phenotypes of the putative ligand-binding mutants (Ud-K51E, Ud-K51R, Ud-L231S) could be explained by reduced activity of the mutant Bmh1p but not by reduced protein expression. Interestingly, in Ud-dim, whose phenotypic defects were much less severe than those of the other mutants, Bmh1p expression levels were similar to those in Ud-Ts (Fig. 7). This differential expression of Bmh1p in the bmh1 mutants was highly reproducible and the results were comparable in extracts from stationary- and exponential-phase cells. It cannot be ruled out, however, that the difference in detection is due to differential compartmentalization in the cell and that
Fig. 5. The C. albicans bmh1 mutants are defective in filamentation on solid agar. Colony morphology was assessed as in Fig. 1 and described in Methods. Control and bmh1 mutant strains were plated on M199 agar (a), FCS agar (b) or cornmeal agar (d), or mixed with molten YPS (c). Strains used are listed on the left. The length of filaments (mm) or percentage of colonies filamenting at different days is noted below the respective photograph. The days on which measurements were taken is stated at the bottom of the columns. Photos were taken after 9 days for (a), (b) and (d), and after 11 days for (c). Colonies were observed with an Olympus SZX9 stereomicroscope (a–c) or an Olympus CX31 (100 × magnification) (d). All photographs in a column are of the same magnification. This figure is representative of at least three separate experiments.
different extraction procedures could yield equivalent amounts of protein.

**DISCUSSION**

14-3-3 proteins play a role in a variety of signalling pathways in eukaryotic species, the majority being involved in cell survival, cell cycle and differentiation. In a pathogen, these cellular processes are essential for survival in the host and pathogenesis. In *C. albicans*, it is known that there is only one 14-3-3 protein which is required for vegetative growth and optimal filamentation (Cognetti *et al*., 2002). However, it was not clear if Bmh1p regulates these two processes independently. Since *BMH1* is essential, it was not possible to ascertain its function using knockout strains. Construction of conditional mutants using nutrition-dependent promoters was also not adequate for

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**Fig. 6.** The *C. albicans* bmh1 mutants are defective in chlamydospore formation: cell phenotypes on the outside edge of colonies on cornmeal agar of YJB6284 at day 3 (a), Ud-K51R at day 11 (b), Ud-L231S at day 3 (c) and Ud-L231S at day 9 (d) at 400 × magnification.

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**Fig. 7.** Bmh1p expression does not always correlate with phenotypic severity: immunoblot of control and bmh1 mutant strains incubated with anti-Bmh1p antibody as described in Methods. Strains were grown for 40 h and cytoplasmic extracts were prepared from an equivalent number of cells and protein from 4 × 10^7 cells was loaded per lane. Strains are depicted by site of mutation or B/B is YJB6284, B/b is BH1P1 and U-B/b is Ud-BMH. S designates slow-growing strains. The figure depicts a composite of two separate gels and is representative of at least five separate experiments.
phenotypic analysis. 14-3-3 proteins are highly homologous, sharing 76–89% identity. Therefore, specific amino acids were substituted in Bmh1p that were known to be important in other eukaryotic species. Several mutants were made in which the only functional BMH1 allele is under its endogenous promoter in the URA3 locus. In this manner, we were able to successfully construct viable strains with site-specific mutations in an essential gene. Two additional mutant strains were included in this analysis. Both of these strains had mutations in the ligand-binding pocket, K51R and L231S.

The strains with the amino acid substitutions exhibited phenotypic defects to varying degrees. Although the conditional mutant strains indicated that there is a correlation between quantity of Bmh1p expressed and ability to grow and filament, this could not entirely explain the different phenotypes seen in the bmh1 mutant strains. The Ts mutant demonstrated the most severe growth defect at all temperatures and did not grow at 42°C. The Ud-Ts strain also aggregated extensively. Thus it was very similar to the S. cerevisiae bmh2 Ts mutant (van Heusden & Steensma, 2001). The poor growth phenotype of C. albicans Ud-Ts may be explained by very low Bmh1p expression as demonstrated by immunoblotting and immunofluorescence (data not shown). Surprisingly, Ud-Ts reverted to a wild-type phenotype at a relatively high rate. This rate was not calculated but during the course of phenotypic testing, where the number of Ts colonies tested was low (≤10), we normally saw reversion in at least one out of three experiments. Not all revertants were sequenced, so it is not known if all revert to wild-type sequence, or if there are second-site mutations. The data suggest that the Ts mutation is certainly not favourable to the organism. The mechanism of reversion is mysterious since no other BMH1 sequence is present in this strain. This makes recombination or gene conversion unlikely mechanisms.

The crystal structure of 14-3-3 indicates a ligand-binding pocket lined with charged residues (K49, R56, R60) on one side and four hydrophobic leucine residues on the other (Liu et al., 1995). Based on the crystal structure, the first mutational studies in mammalian 14-3-3 proteins identified the requirement of K49 and to a lesser degree R56 (comparable to K51 and R58 in C. albicans) in binding to specific ligands (Zhang et al., 1997). The defective K49 mutation did not result in an overall change in the three-dimensional structure of the protein, and substitution of K49 with a panel of residues indicated that a positive charge is important for ligand binding (Zhang et al., 1997). Interestingly our K51E and K51R mutant strains demonstrated both same and different phenotypes depending on the assay. This suggests that a net positive charge at residue 51 may be important for some interactions (e.g. ligands involved with growth) but not other cellular processes (filamentation on M199). The C. albicans K51E, K51R and L231S mutations map within the ligand-binding groove and these mutant proteins are expressed to the same level as the control strain. Thus, it is likely that 14-3-3–ligand interactions are specifically perturbed by these changes.

In Drosophila the mutation L15AE to Q15QR abolished dimerization yet the monomer was still able to effect at least one function (Zhou et al., 2003). It is not yet known if dimerization is affected in C. albicans when L15AE is mutated to Q15QR; this is currently being addressed. The putative dimerization mutant Ud-dim had only a subtle phenotype except for its reduced ability to filament on FCS agar. It remains possible that this mutant form is unaffected in its ability to dimerize, or alternatively, some Bmh1p functions may not require dimerization. In mammalian cells, many ligands can still bind to the 14-3-3 monomer though either an inappropriate form of the protein binds to the monomer and/or the ensuing conformational changes do not occur so that the downstream functions are not activated (Shen et al., 2003; Tzivion et al., 2001).

In S. cerevisiae, BMH1 and BMH2 were the only strong suppressors of the rapamycin-sensitive phenotype of wild-type cells (Bertram et al., 1998). Studies with mammalian and yeast homologues determined that the phosphopeptide-binding pocket is required for rapamycin resistance though the overlap of the residues required for 14-3-3 binding with Raf-1 kinase is not complete. Interestingly, two yeast bmh1 alleles that were defective in filamentation (L232S, G55D) were able to confer rapamycin resistance (Bertram et al., 1998). Therefore the role 14-3-3 proteins play in the MAPK and TOR signalling pathways is distinct. The suggested function of Bmh1/2 proteins in TOR signalling is the sequestration in the cytoplasm of the stress-related transcription factors Msn2p and Msn4p (Beck & Hall, 1999). The overexpression of Bmh1/2p results in increased resistance to rapamycin while deletion of one or both BMH genes leads to hypersensitivity or no growth respectively (Bertram et al., 1998). The same scenario is not observed in C. albicans. The BMH1/bmh1Δ heterozygote and Ud-BMH strains that expressed reduced levels of Bmh1p always demonstrated an increased resistance to rapamycin. When Bmh1p levels are suboptimal, there may be preferential binding of targets. Stress response transcription factors may become inactivated by binding to the Candida 14-3-3 protein as reported in S. cerevisiae, but in C. albicans this binding may result in insufficient Bmh1p to mediate a second downstream signalling event. Alternatively, the TOR pathways or mechanism of resistance/sensitivity to rapamycin may not be the same in S. cerevisiae and C. albicans. Interestingly, the L231S mutant showed an increased sensitivity to rapamycin, which is also opposite to the effect seen in S. cerevisiae. Normally the Ts and K51E mutant strains grew better at 37°C than 30°C. However, in the presence of rapamycin, growth inhibition was more severe at 37°C than 30°C. This temperature-dependent effect of rapamycin was not observed in control, Ud-K51R, Ud-L231S or Ud-dim strains. Why the Ts and K51E mutants are more sensitive at 37°C is unknown but this
observation indicates that Bmh1p may play a role in pathways regulated by physiological temperatures.

In mammalian, yeast and plant cells, 14-3-3 proteins play a role in developmental regulation. In S. cerevisiae nitrogen starvation induces filament formation under the control of the RAS/MAPK and PKA pathways (Rupp et al., 1999). Bmh1/2p is required for filamentation at least in part via an interaction with Ste20p (Roberts et al., 1997). In C. albicans, filamentation is also induced under nitrogen-starvation conditions and by activation of MAPK and PKA pathways. However, unlike S. cerevisiae, in addition to nutrient starvation multiple in vitro conditions can induce filamentation, including pH, temperature, serum and embedment in agar (reviewed by Berman & Sudbery, 2002; Brown, 2002; Lengeler et al., 2000). In this study several induction methods were tested and Bmh1p was involved in all instances. Strains with one BMH1 allele exhibit reduced filamentation compared to strains carrying two BMH1 alleles (Cognetti et al., 2002). This suggests a gene dosage effect and that there is insufficient Bmh1p to carry out all required functions. Interestingly, the UdBmh control reproductively demonstrated a slight reduction in filamentous border on solid agar relative to the BMH1/ bmh1Δ heterozygous strains (data not shown). This may be due to promoter elements that are farther than 500 bp upstream of the ORF and therefore missing from the BMH1 gene in this UdBm strain or may indicate that the position of the BMH1 allele in the genome is important for full expression. The Ud-Ts, Ud-K51E, Ud-K51R and Ud-L231S strains were defective in filamentation on M199 and FCS solid agar. Low expression levels indicated that the Ts mutation renders the protein unstable but the other mutations probably disrupt ligand-binding sites in Bmh1p. We also tested filamentation under embedment conditions. In matrix, two BMH1 alleles are required for efficient filamentation. Ud-L231S but not Ud-K51E/R filaments like the isogenic control. This indicates the fine specificity and affinities of the ligand-binding pocket. Thus, the Czf1p-mediated pathway (Brown et al., 1999; Giusani et al., 2002) is likely to involve different interactions of Bmh1p with ligands than the MAPK/CAMP pathways.

Finally, filamentation in chlamydospore-inducing agar was analysed. The in vivo relevance of this pathway is unclear, though spores have been detected in vivo (Cole et al., 1991). Interestingly, strains expressing only one intact BMH1 allele were completely unable to filament or to produce spores. On the other hand, the ligand-binding mutants, Ud-K51E and Ud-L231S, were hyperfilamentous with very sparse, if any, production of chlamydospores. This phenotype is very similar to that described for the efg1/efg1 and phosphorylation EFG1(T206A) mutants (Sonneborn et al., 1999). Other pathways and genes have been implicated in chlamydospore formation, including the Hog1 oxidative stress response and alkaline pH response regulator mutants: mds3/mds3 and rim101/rim101 deletion strains (Nobile et al., 2003). These studies indicate that Bmh1p may interact more than once in the same filamentation pathway. This phenomenon has been demonstrated for 14-3-3 proteins in other species.

In this study C. albicans strains were constructed that express Bmh1p with mutations in different functional domains. As seen for other species, these domains may play integral roles in signalling cascades in C. albicans. The ligand-binding domain is important for both growth and filamentation but these two processes can be separated dependent on which amino acid is substituted. This demonstrates the sensitivity of the binding pocket and how one protein may modulate multiple cellular processes. It remains possible that sequestration of Bmh1p by a ligand of a particular pathway affects the activity of other 14-3-3-mediated pathways. This would represent a means of pathway cross-talk that permits coordination of multiple cellular processes. Ongoing studies will determine if the mutant Bmh1 proteins interact with a different subset of proteins or bind less effectively to targets. Even though Bmh1p is essential, it is not envisioned as a likely drug target. Instead, the identification of Bmh1p–ligand interactions in specific signalling cascades required for pathogenesis may identify targets for antifungal intervention. Moreover, we believe that through the study of proteins involved in the regulation of multiple signalling pathways, we will gain an insight into how C. albicans co-ordinates its cellular activities. This co-ordination is almost certainly critical for establishing infection.

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