Conserved Elements of the RAM Signaling Pathway Establish Cell Polarity in the Basidiomycete *Cryptococcus neoformans* in a Divergent Fashion from Other Fungi

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Submitted February 10, 2006; Revised May 16, 2006; Accepted June 7, 2006
Monitoring Editor: Charles Boone

In eukaryotes the complex processes of development, differentiation, and proliferation require carefully orchestrated changes in cellular morphology. Single-celled eukaryotes provide tractable models for the elucidation of signaling pathways involved in morphogenesis. Here we describe a pathway regulating cell polarization and separation in the human pathogenic fungus *Cryptococcus neoformans*. An insertional mutagenesis screen identified roles for the *ARF1*, *CAP60*, *NDH1*, *KIC1*, *CBK1*, *SOG2*, and *TAO3* genes in establishing normal colony morphology. *ARF1* and *CAP60* are also required for capsule production, a virulence factor, and *ARF1* confers resistance to the antifungal fluconazole. *KIC1*, *CBK1*, *SOG2*, and *TAO3* are homologues of genes conserved in other eukaryotes; in *Saccharomyces cerevisiae* they constitute components of the RAM (regulation of *Ace2p* activity and cellular morphogenesis) signaling pathway. A targeted deletion of a fifth component of RAM (*MOB2*) conferred identical phenotypes to *kic1*, *cbk1*, *sog2*, or *tao3* mutations. Characterization of these genes in *C. neoformans* revealed unique features of the RAM pathway in this organism. Loss of any of these genes caused constitutive hyperpolarization instead of the loss of polarity seen in *S. cerevisiae*. Furthermore, sensitivity to the drugs FK506 and cyclosporin A demonstrates that the RAM pathway acts in parallel with the protein phosphatase calcineurin in *C. neoformans* but not in *S. cerevisiae*. These results indicate that conserved signaling pathways serve both similar and divergent cellular roles in morphogenesis in these divergent organisms.

INTRODUCTION

Regulation of morphogenesis is a central feature of eukaryotic life. Organisms require coordinated signaling pathways to control polarized growth, asymmetric cell division, and specialized tissue development. For example, establishment and maintenance of proper cell polarity is critical to the function of neurons and epithelial cells (Wiggin *et al.*, 2005). Elucidating the mechanisms that generate cellular asymmetry has benefited from the study of this phenomena in phylogenetically diverse models including the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and cultured mammalian cells (Drubin and Nelson, 1996; Zallen *et al.*, 2000; Emoto *et al.*, 2004).

Morphogenesis also plays a critical role in the virulence of many pathogens including viruses, bacteria, and dimorphic fungi such as the human pathogen *Candida albicans* and the plant pathogen *Ustilago maydis* (Bölker, 2001; Liu, 2002). For example, the influenza virus has multiple morphological forms and altered virion morphology has been correlated with virulence (Mitnaul *et al.*, 1996). The sensitivity of *Staphylococcus* bacteria to penicillin has been attributed in part to changes in cellular morphology (Giesbrecht *et al.*, 1998). In *C. albicans*, conversion between the yeast and the hyphal form is associated with increased virulence (Lo *et al.*, 1997; Saville

et al., 2003; Noverr and Huffnagle, 2004). Similarly, signaling pathways that control morphology switches in *U. maydis* have been studied in order to provide insight into the stages of its pathogenesis on plants (Bölker, 2001).

Here, we utilize the model fungal pathogen, *Cryptococcus neoformans*, to identify novel genetic requirements for normal morphogenesis and polarized growth in this eukaryote. *C. neoformans*, a basidiomycete pathogen of world-wide distribution, is a major cause of life-threatening infections among immunocompromised persons, though infections in immunocompetent people also occur (Casadevall and Perfect, 1998; Hoang *et al.*, 2004). Concurrent with the rise in number of immunocompromised populations over the last several decades due to AIDS, cancer chemotherapy, and organ transplantation immunosuppression, the frequency of *Cryptococcus* infections has continued to mount (Mitchell and Perfect, 1995). Like *S. cerevisiae*, *C. neoformans* can undergo several regulated changes in morphology in response to environmental conditions or the presence of appropriate mating partners. During growth on rich medium under standard conditions, *C. neoformans* adopts a budding yeast morphology very similar to its ascomycete relatives, forming smooth round colonies on agar. During mating, a filamentous dikaryon is produced that terminates in a specialized structure (the basidium) where meiosis occurs to produce four chains of spores. In addition, pseudohyphal forms of *C. neoformans* are occasionally isolated from the wild or as phenotypic switch mutants (Fries *et al.*, 1999; Neilson *et al.*, 1978) and these forms may be better adapted to survival under some conditions. Visible morphological defects of yeast colonies, then, might indicate abnormal growth patterns or altered cell wall structure. These in turn could reflect underlying temperature and/or drug suscepti-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-02-0125>) on June 14, 2006.

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bilities, or deficiencies in the production of the polysaccharide capsule, a key virulence factor of this pathogen (Casadevall and Perfect, 1998). To explore the role of morphology in the virulence and drug susceptibility of *C. neoformans*, we performed a functional genomics screen to identify mutations which cause morphological defects.

Using *Agrobacterium*-mediated insertional mutagenesis, more than 50,000 mutants were screened for changes in colony morphology. This type of mutagenesis has been applied previously in *C. neoformans* to identify novel genes related to virulence (Idnurm *et al.*, 2004; Walton *et al.*, 2005). Through this approach, the genes *ARF1*, *CAP60*, *NDH1*, *KIC1*, *CBK1*, *SOG2*, and *TAO3* were found to be required for normal colony morphology of *C. neoformans*. Furthermore, the roles of each in capsule production, growth at high temperature, susceptibility to antifungal agents, and cell division were characterized. Interestingly, *KIC1*, *CBK1*, *SOG2*, and *TAO3* are homologues of the genes in *S. cerevisiae* that constitute part of the RAM (regulation of *Ace2p* activity and cellular morphogenesis) signaling pathway (Nelson *et al.*, 2003). Here this pathway is shown to play both conserved and divergent roles during the growth of these two fungi.

MATERIALS AND METHODS

Strains and Media

C. neoformans serotype A strains KN99a and KN99 α were used as wild-type strains (Nielsen *et al.*, 2003). *Agrobacterium tumefaciens* strains LBA4404 or EHA105 containing binary vectors able to confer drug resistance were used for transformation of *C. neoformans* KN99 α . Morphology was analyzed on standard yeast extract, peptone, and dextrose (YPD) medium. Strains exhibiting an altered colony morphology that were identified from insertional mutagenesis are listed in Table 1. Drug susceptibility was assessed on YPD media with the addition of rapamycin (100 ng/ml), caspofungin (10 μ g/ml), fluconazole (10 μ g/ml), FK506 (1 μ g/ml), or cyclosporin A (CsA) (100 μ g/ml). *C. neoformans* and *S. cerevisiae* strains are also listed in Table 2.

Insertional Mutagenesis and Genotypic Analysis

Insertional mutagenesis was performed using *Agrobacterium*-mediated T-DNA delivery of constructs bearing cassettes for drug resistance markers as described previously in a screen for melanin mutants (Idnurm *et al.*, 2004; Walton *et al.*, 2005). To determine if the altered colony morphology phenotypes were linked to the insertion of the nourseothricin resistance cassette, mutant strains were crossed with the congenic strain KN99a on V8 juice agar media (pH 5) in the dark to induce mating. After 2 to 4 wk, basidiospores were isolated using a micromanipulator, and the progeny of each cross were analyzed for nourseothricin drug resistance (by growth on YPD with 100 μ g/ml nourseothricin) and morphology. Regions flanking the T-DNA insertion were determined by inverse PCR, and the sequence was compared with the TIGR serotype D and Broad Institute/Duke University serotype A *C. neoformans* genome databases.

Targeted Deletion and Complementation of the *MOB2*, *CBK1*, and *KIC1* Genes

A *mob2* gene disruption allele was produced using overlap PCR (Fraser *et al.*, 2003). Primers (listed in Table 3) were used to amplify regions with ~1 kb homology on either side of the *MOB2* gene and the nourseothricin acetyltransferase (NAT) marker was amplified from plasmid pAI3 by PCR. Equimolar amounts of the three PCR products were amplified using the primers JOHE15969 and JOHE15972 to generate the disruption allele with a NAT marker flanked on either side by DNA homologous to the genomic region just upstream and downstream of the *MOB2* gene. The disruption allele, conferring nourseothricin resistance, was used to transform strain KN99 α with a biolistic apparatus (Bio-Rad Model PDS-1000/He Biolistic Particle Delivery System, Richmond, CA; Toffaletti *et al.*, 1993). Transformants were analyzed by PCR and Southern hybridization to confirm deletion of the *MOB2* gene. The same procedure was used to generate targeted deletions of the coding regions of the *KIC1* and *CBK1* genes to confirm that the hyperpolarized phenotype was the result of loss of gene function and not the production of truncated or otherwise aberrant proteins.

To complement the *mob2* Δ mutation, the *MOB2* coding region and ~1 kb of upstream sequence was amplified and subcloned into plasmid pZP-NEO1, which contains the selectable neomycin resistance marker (Walton *et al.*, 2005). *Agrobacterium* strain EHA105 was transformed with this plasmid, and the *mob2* Δ strain was transformed by coinoculation with that EHA105 strain.

Table 1. Gene identification in insertion mutants affected in morphology

Strain	Linkage	Disrupted gene-allele	GenBank accession
ST20S	Yes (14)	<i>ndh1</i>	CNA07650
ST20D	No (13)		
ST119	No (3)	<i>cbk1-2^a</i>	CNG00360
ST218	Yes (10)	<i>arf1</i>	CNI03960
ST219	No (17)		
ST224	No (9)		
ST239	No (8)		
ST258	Yes (14)	<i>kic1-1</i>	CNA03940
ST306	Yes (26)	<i>cap60</i>	CNA05830
ST308	No (6)		
SR22	No (8)	<i>kic1-2^a</i>	CNA03940
MS1	Yes (8)	<i>cbk1-1</i>	CNG00360
RM2	Yes (31)	<i>tao3-1</i>	CNB01240
AI109	No (10)	<i>sog2-2^a</i>	CNB04070
DR3	No (6)	<i>tao3-2^a</i>	CNB01240
DR8	Yes (12)	<i>cbk1-3</i>	CNG00360
DR17	Yes (12)	<i>kic1-3</i>	CNA03940
DR24	No (17)	<i>tao3-3^a</i>	CNB01240
AI131	Yes (28)	<i>sog2-1</i>	CNB04070
AI133	No (3)	<i>cbk1-4^a</i>	CNG00360
AI134	ND	<i>tao3-4^a</i>	CNB01240
AI135	Yes (23)	<i>kic1-4</i>	CNA03940
AI136	Yes (10)	<i>tao3-5</i>	CNB01240
FS1	Yes (9)	<i>kic1-5</i>	CNA03940

All strains are mating type α except for AI109, which is mating type a. STXXX refers to a unique 40-base pair signature tag adjacent to the NAT resistance cassette. Values in parentheses are number of progeny analyzed. ND, not determined.

^a Although these mutations are unlinked to the NAT insertion, the mutated gene has been identified through crossing with mutants of known genotype, followed by progeny analysis.

Transformants were selected on medium containing neomycin (200 μ g/ml) and cefotaxime (100 μ g/ml).

Light and Fluorescence Microscopy

To visualize the capsule, strains were grown for 4 d in liquid iron-deplete medium and then stained with India ink; stained cells were analyzed with a light microscope. A Zeiss Axioskop 2 Plus fluorescent microscope (Thornwood, NY) with an attached AxioCam MRM digital camera was used for microscopy. For calcofluor white and sytox green staining, cells were grown to midlog phase in liquid YPD medium. Calcofluor white (0.005% wt/vol) was added to the cells for 15 min before fixing. Cells were fixed with ice-cold 70% ethanol for 20 min, then washed in phosphate-buffered saline (PBS), permeabilized with 1% Triton-X100 (in PBS) for 5 min, and washed again and resuspended in PBS. One microliter of this suspension was mixed on a slide with 1 μ l of sytox green mixture (1 μ l/ml sytox green [Molecular Probes, Eugene, OR] in Vectashield). For actin staining, cells were grown to midlog and fixed with 37% EM grade formaldehyde for 20 min and then washed and permeabilized as above. To 200 μ l cell suspension was added 10 μ l rhodamine-conjugated phalloidin (10 μ l/ml; Molecular Probes), and this mix was incubated for one h before washing and resuspension in PBS. One μ l of the suspension was added to 1 μ l Vectashield (10 mg/ml; Vector Laboratories, Burlingame, CA; Nichols *et al.*, 2004).

Subcellular Localization with Protein-DsRED Fusions

To localize the RAM proteins, the coding regions of the genes were subcloned with the open reading frame adjacent to the N-terminal end of the red fluorescent protein, DsRed from the coral genus *Discosoma* (Invitrogen, Carlsbad, CA). Primers used for amplification are listed in Table 3. Expression of the construct was controlled by the *C. neoformans* histone H3 promoter. Strains KN99 α or *tao3*, *cbk1*, and *kic1* mutant strains were transformed with the plasmid containing the NAT-selectable marker and the RAM gene-DsRed fusion using *Agrobacterium*-mediated transformation. Transformants selected from several independent plasmid constructs were selected and analyzed for fluorescence.

Table 2. Strain list

Strain	Parent	Genotype	Reference
<i>C. neoformans</i>			
KN99 α		<i>MATα</i>	Nielsen <i>et al.</i> (2003)
KN99a		<i>MATa</i>	Nielsen <i>et al.</i> (2003)
KK8	KN99a	<i>MATa cna1Δ::NEO</i>	Kojima <i>et al.</i> (2006)
FJW8	KN99 α	<i>MATα kic1Δ::NAT</i>	This study
FJW9	KN99 α	<i>MATα cbk1Δ::NAT</i>	This study
FJW10	KN99 α	<i>MATα mob2Δ::NAT</i>	This study
FJW11	FJW9	<i>MATα cbk1Δ::NAT CBK1-NEO</i>	This study
FJW12	FJW10	<i>MATα mob2Δ::NAT MOB2-NEO</i>	This study
FJW13	KN99 α	<i>MATα P_{H3}-CBK1-DsRED-NAT</i>	This study
FJW14	KN99 α	<i>MATα P_{H3}-KIC1-DsRED-NAT</i>	This study
FJW15	DR24	<i>MATα tao3 P_{H3}-KIC1-DsRED-NAT</i>	This study
FJW16	FJW8	<i>MATα kic1Δ::NAT P_{H3}-KIC1-DsRED-NAT</i>	This study
FJW17	FJW9	<i>MATα cbk1Δ::NAT P_{H3}-CBK1-DsRED-NAT</i>	This study
ST258a	KN99a $\times$ ST258	<i>MATa kic1::T-DNA (NAT)</i>	This study
SR22a	KN99a $\times$ SR22	<i>MATa kic1</i>	This study
MS1a	KN99a $\times$ MS1	<i>MATa cbk1::T-DNA (NAT)</i>	This study
RM2a	KN99a $\times$ RM2	<i>MATa tao3::T-DNA (NAT)</i>	This study
DR3a	KN99a $\times$ DR3	<i>MATa tao3</i>	This study
DR8a	KN99a $\times$ DR8	<i>MATa cbk1::T-DNA (NAT)</i>	This study
DR17a	KN99a $\times$ DR17	<i>MATa kic1::T-DNA (NAT)</i>	This study
DR24a	KN99a $\times$ DR24	<i>MATa tao3</i>	This study
AI131a	KN99a $\times$ AI131	<i>MATa sog2::T-DNA (NAT)</i>	This study
AI135a	KN99a $\times$ AI135	<i>MATa kic1::T-DNA (NAT)</i>	This study
AI136a	KN99a $\times$ AI134	<i>MATa tao3::T-DNA (NAT)</i>	This study
FJW8a	KN99a $\times$ FJW8	<i>MATa kic1Δ::NAT</i>	This study
FJW9a	KN99a $\times$ FJW9	<i>MATa cbk1Δ::NAT</i>	This study
FJW10a	KN99a $\times$ FJW10	<i>MATa mob2Δ::NAT</i>	This study
AI139	FJW9 $\times$ ST20S	<i>MATa cbk1Δ::NAT ndh1::T-DNA (NAT)</i>	This study
<i>S. cerevisiae</i>			
PJ69-4a		<i>MATa trp1-901 leu2-3112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
Y1630	W303	<i>MATa ssd1-d2 ura3 leu2 his3 trp1 ade2 CAN1-100</i>	
BY4743	S288C	<i>MATaα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0</i>	Giaever <i>et al.</i> (2002)
FLY757	S288C	<i>MATa cbk1Δ::KANMX ura3-52 leu2-3112 trp1Δ1 his3Δ200 ssd1-d</i>	Nelson <i>et al.</i> (2003)
FLY1466	S288C	<i>MATa sog2Δ::KANMX ura3-52 leu2-3112 trp1Δ1 his3Δ200 ssd1-d</i>	Nelson <i>et al.</i> (2003)
FLY1001	W303	<i>MATa CBK1-13myc::HIS3 mob2Δ::KanR ura3-1 leu2-3112 his3-11,15 ade2-1 can1-100 lys2 trp1-1 ssd1-d</i>	Nelson <i>et al.</i> (2003)
FLY1002	W303	<i>MATa CBK1-13myc::HIS3 hym1Δ::URA3 ura3-1 leu2-3112 his3-11,15 ade2-1 can1-100 trp1-1 ssd1-d</i>	Nelson <i>et al.</i> (2003)
FLY1003	W303	<i>MATa CBK1-13myc::HIS3 kic1Δ::KANMX ura3-1 leu2-3112 his3-11,15 ade2-1 can1-100 lys2 trp1-1 ssd1-d</i>	Nelson <i>et al.</i> (2003)
FLY1004	W303	<i>MATa CBK1-13myc::HIS3 tao3Δ::KANMX ura3-1 leu2-3112 his3-11,15 ade2-1 can1-100 lys2 trp1-1 ssd1-d</i>	Nelson <i>et al.</i> (2003)

Protein Interactions

The cDNAs for the *CBK1*, *KIC1*, and *MOB2* genes were cloned into plasmids for yeast two-hybrid assays. cDNAs were amplified using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) with the polyT oligo nucleotide primer, followed by PCR with gene specific primers (Table 3) on mRNA isolated from total RNA using PolyAtract mRNA Isolation System IV (Promega, Madison, WI) according to the manufacturer's protocol. Products were cloned into plasmids pGAD.c1 and pGBD.c1 (James *et al.*, 1996). The *S. cerevisiae* reporter strain PJ69-4a was cotransformed with plasmids using the lithium acetate/heat shock method (James *et al.*, 1996). Transformants harboring both plasmids were selected on medium lacking leucine and tryptophan. Interactions were assessed by growth in the absence of adenine or histidine (+5 mM 3-aminotriazole) and by β -galactosidase assays (Cardenas *et al.*, 1994).

Complementation of *S. cerevisiae* RAM Mutants with *C. neoformans* Genes

S. cerevisiae RAM mutants were the generous gift of Dr. Francis Luca (Nelson *et al.*, 2003). These strains were transformed with plasmid constructs containing the corresponding *C. neoformans* homologues that were functional in the yeast two-hybrid assays. Strains harboring the pGAD.c1 or pGBD.c1 plasmid

with the appropriate *C. neoformans* homolog were selected on media lacking either leucine or tryptophan, respectively. Several transformants from each independent plasmid construct were analyzed with a light microscope for complementation of the morphology defect and on medium containing 50 μ g/ml calcofluor white.

RESULTS

Identification of Morphology Mutants

Agrobacterium-mediated insertional mutagenesis is a powerful tool for discovery of novel gene functions in fungi, especially in *C. neoformans* (Idnurm *et al.*, 2004; McClelland *et al.*, 2005; Michielse *et al.*, 2005; Walton *et al.*, 2005). *C. neoformans* wild-type strain KN99 α was previously transformed with *A. tumefaciens* cells harboring a Ti-plasmid containing a NAT cassette and molecular signature tag to isolate a collection of more than 12,000 mutant strains (Walton *et al.*, 2005). These strains were screened for abnormal colony morphology as an indicator of capsule production or cell wall

Table 3. Primer list

Primer name	Sequence (5'–3')
Inverse PCR	
JOHE8956	AACAGTTGCGCAGCCTGAATG
JOHE8957	AGAGGCGGTTTGGCTATTGG
Deletion of <i>MOB2</i>	
JOHE15969	TTGGGAGGTTGGTGAAGG
JOHE15970	GCTTATGTGAGTCTCCCTGACGAAG TGTATGGAG
JOHE15971	TCGTTTCTACATCTCTTCTGCATAGCTC TGCATTGG
JOHE15972	CTACTTCAGACGTGAGCC
Deletion of <i>KIC1</i>	
JOHE15337	GCTGAGACAGTCTTTGTGATACATG
JOHE14611	GCTTATGTGAGTCTCCCATCCAGT AAGGTGTGC
JOHE14306	TCGTTTCTACATCTCTTCAACAAGGAT CGACCAGCC
JOHE15338	GTCGAAGAGCTTGCCAAAGTTGCTG
Deletion of <i>CBK1</i>	
JOHE15898	AGGTATCATTCTGCCAAG
JOHE15899	GCTTATGTGAGTCTCCCTCCTCGCAA GCACACTCG
JOHE15900	TCGTTTCTACATCTCTTCTGTAAGCTG AACATGAGC
JOHE15901	CTTCCGAGCTAATCACTC
Complementation of <i>MOB2</i>	
JOHE15999	GGATCCTCCTCTGTGCCATCGAGC
JOHE16000	GGATCCTCCAGTTCTCTTACTGAC
Complementation of <i>CBK1</i>	
JOHE15902	GGATCCCTGCCACTGGTAATATCG
JOHE15903	GGATCCTCTAACGGTGAACGGTC
<i>KIC1</i> -DsRED fusion	
JOHE15626	GGATCCATGGCCGCTCGGAAGGGCG
JOHE15908	CCATGGCAGCATCTGGGAGTCTACCT
<i>CBK1</i> -DsRED fusion	
JOHE15904	GGATCCATGGCGTATCGCCCAATC
JOHE15905	CCATGGCCAGCATCTCGTATCGTCG
<i>CBK1</i> cDNA	
JOHE15904	GGATCCATGGCGTATCGCCCAATC
JOHE15906	GGATCCAGCATCTCGTATCGTCG
<i>KIC1</i> cDNA	
JOHE15626	GGATCCATGGCCGCTCGGAAGGGCG
JOHE15907	GGATCCAGCATCTGGGAGTCTACCT
<i>MOB2</i> cDNA	
JOHE15973	GGATCCATGGCAGGCTTCCTCAAC
JOHE15975	GGATCCTGACTCCAAAATGCCCAT

defects. Mutants with a dry or crinkled colony surface compared with the smooth, round surface of a wild-type yeast colony were selected for further analysis. In total, 10 mutants with altered colony morphology were isolated from this collection (~0.1%; Figure 1A). The strains were named according to the signature tag adjacent to the NAT cassette, with letters assigned following the signature tag number if multiple mutants were isolated from that background (i.e., ST20D and ST20S; Table 1). Two mutants, ST119 (*cbk1-2*) and ST258 (*kic1-1*), had a unique, crinkled colony morphology and a hyperpolarized, unseparated cellular morphology (Figures 1 and 5), a morphological defect so remarkable that at first they were considered to be contaminants. We therefore screened every subsequent *Agrobacterium* transformation event relating to other research in the laboratory for additional mutants with this hyperpolarized phenotype. An estimated 50,000 more *Agrobacterium*-insertional mutants

were screened for this hyperpolarized phenotype and 14 additional mutant strains were identified (Figure 1C).

Gene Identification in Mutants

To determine if the morphology phenotypes are linked to the insertion of the T-DNA, which confers resistance to nourseothricin, mutant strains were crossed with a congenic strain of opposite mating type, KN99a, and meiotic progeny were analyzed. Basidiospores were isolated from each mating reaction, germinated, and evaluated for cosegregation of the phenotypes and marker. Eight to 31 progeny were scored to confirm linkage (Table 1). Linkage was established for 12 of the 24 mutants, and the number that were linked (50%) was comparable to that previously observed in a similar screen for melanin-deficient *C. neoformans* mutants (Walton *et al.*, 2005).

In linked mutants, disruption of the *C. neoformans* genome by T-DNA insertion is the cause of the mutant phenotype rather than a spontaneous unlinked mutation; therefore, the mutated gene causing that phenotype can be identified by sequencing the flanking genomic DNA on either side of the inserted T-DNA. Inverse PCR was performed on each linked mutant, and the sequence of each product was compared with the *C. neoformans* genome databases at The Institute for Genome Research, the Broad Institute, and Duke University (Loftus, 2005). The putative gene sequences were compared using nucleotide BLAST searches against the NCBI GenBank database to identify homology with genes from other organisms to infer presumptive functions. The results of gene identification in the linked morphology mutants are listed in Table 1.

Two strains bore mutations in previously identified genes of *C. neoformans*. In the acapsular strain ST306, the T-DNA has inserted into the *CAP60* gene that is required for capsule production (Chang and Kwon-Chung, 1998). Acapsular mutant ST218 contains a T-DNA insertion into the *ARF1* gene, whose protein product is an ADP-ribosylating factor involved in vesicle formation (Orci *et al.*, 1986; Serafini *et al.*, 1991; Lodge *et al.*, 1994). Mutant ST20S contains a T-DNA insertion into an uncharacterized gene, *NDH1*, encoding a predicted NADH dehydrogenase.

Several mutants contained disruptions in genes homologous to those in the *S. cerevisiae* RAM (regulation of *Ace2p* activity and cellular morphogenesis) signaling pathway. In mutants ST258, DR17 and AI135, the T-DNA has disrupted the *KIC1* gene, a homolog of the *S. cerevisiae* *KIC1* gene that encodes a serine/threonine kinase similar to the Ste20-like kinases. Two other linked hyperpolarized mutants, MS1 and DR8, each contain a disruption in the *C. neoformans* homolog of *S. cerevisiae* *CBK1*, which encodes the second serine-threonine kinase in the RAM pathway (Nelson *et al.*, 2003). Mutants RM2 and AI136 are linked and contain insertions in the *TAO3* gene. Mutant AI131 contains a T-DNA insertion in the closest match to the *S. cerevisiae* *SOG2* gene.

The other unlinked hyperpolarized mutant strains were crossed with five representative mutant strains (*cbk1* [MS1], *kic1* [ST258], *mob2Δ* [FJW10], *tao3* [RM2], and *sog2* [AI131]) and each other in order to identify allelic mutations based on the segregation of haploid meiotic progeny. In a cross of two strains harboring mutations in the same gene, no meiotic progeny should be found with a wild-type phenotype. Alternatively, when two strains harboring mutations in different genes are crossed, both mutant (~75%) and wild-type (~25%) meiotic progeny are produced. *C. neoformans* RAM mutants were less robust in mating than other strains; mating reactions were regularly conducted for 4 to 6 wk before basidiospore dissection by micromanipulation. Nevertheless

Figure 1. Colony morphology and drug susceptibility of mutant strains. (A) Ten strains with altered colony morphology were identified in a screen of 12,000 insertional mutants. Dilutions of these strains were plated onto YPD rich medium at 30 or 37°C or YPD at 30°C with fluconazole (10 μ g/ml), cyclosporin A (CsA; 100 μ g/ml), FK506 (1 μ g/ml), caspofungin (10 μ g/ml), or rapamycin (100 ng/ml) and grown for 48 h. The *arf1* (ST218) mutant shows increased susceptibility to fluconazole. Strains *cbk1-2* (ST119) and *kic1-1* (ST258) show increased susceptibility to FK506 and CsA. (B) Enlarged photographs of wild-type and mutant growth patches. (C) Additional mutants, $n = 50,000$, were screened to identify 14 new mutants that share the hyperpolarization phenotype of isolates *cbk1-2* (ST119) and *kic1-1* (ST258). Tenfold serial dilutions of these strains were plated onto YPD or YPD with FK506 and CsA at 30 or 37°C and grown for 48 h.

filaments and viable basidiospores were produced that could be dissected for genetic analysis. Through this analysis, it was determined that strains ST258, SR22, DR17, AI135, and FS1 all contain mutations in the *KIC1* gene, whereas strains MS1, ST119, AI133, and DR8 contain mutations in the *CBK1* gene. Strains RM2, DR3, DR24, AI134, and AI136 all contain mutations in the *TAO3* gene. Strain AI109 harbors a RAM mutation that is allelic with the *sog2* mutation in strain AI131. Thus, by this approach we provide additional evidence that the NAT insertion in ST258, MS1, AI131, and RM2 in *KIC1*, *CBK1*, *SOG2*, and *TAO3* confer the phenotype and identify 12 additional mutations in the RAM genes.

Because Mob2 is the conserved binding partner required for Cbk1 kinase activity characterized in both *S. cerevisiae* and *S. pombe* (Weiss *et al.*, 2002) and no insertional mutant in this gene was identified in our screen, a targeted *mob2* Δ deletion strain was generated. This strain had identical phenotypes to the other *C. neoformans* RAM mutants and was complemented by reintroduction of the wild-type *MOB2* gene (Figure 2). One reason this gene may not have been found in the genomic insertional mutant screen is its relatively small size, with a coding sequence of only 964 nucleotides compared with the *KIC1*, *CBK1*, *SOG2*, and *TAO3* genes which are 3171, 2237, 3846, and 7622 nucleotides, respectively. Furthermore, although 16 independent RAM

mutants were identified with the same hyperpolarized phenotype, we wanted to confirm that this defect resulted specifically from the loss of a RAM gene and was not an unusual effect of the mutagenesis process or production of aberrant protein versions. Thus targeted deletions of the complete open reading frames of the *KIC1* and *CBK1* genes were generated in addition to the targeted *MOB2* deletion strain. These two disruption strains appeared identical to the insertion mutants, confirming that the hyperpolarization defect is a true loss of function phenotype of the *C. neoformans* RAM mutants.

Capsule Production Is Abolished in Five Mutants with Altered Colony Morphology

Capsule production is a key virulence factor in *C. neoformans*, and an altered colony morphology may serve as an indication of altered production of capsular polysaccharide. The collection of morphology mutants was analyzed for levels of encapsulation by microscopy of cultures grown in low iron medium and stained with India ink. Five acapsular strains, including ST306 (*cap60*) and ST218 (*arf1*), were identified (Figure 3). The *CAP60* gene was previously shown to be required for capsule production (Chang and Kwon-Chung, 1998); however, the requirement of the *ARF1* gene for capsule synthesis is a new discovery and implicates proper

Figure 2. Deletion of *C. neoformans* *MOB2*. The predicted *MOB2* homolog was replaced by a nourseothricin resistance cassette (*mob2Δ*) and displays the same phenotypes as other RAM mutants. These phenotypes were complemented by reintroduction of a wild-type copy of the *MOB2* gene (*mob2Δ* + *MOB2*).

vesicle transport in synthesizing and transporting appropriately modified capsular polysaccharides to the cell surface. Although loss of any of the RAM genes results in severe morphological defects at the colony and cellular levels, these mutants produce normal levels of capsule (Figure 3).

arf1 and RAM Mutants Exhibit Increased Drug Susceptibilities

Altered morphology may indicate abnormal cell wall composition. The hyperpolarized RAM mutants could not grow at 37°C; growth at human body temperature is required for virulence in *C. neoformans* (Figure 1). Neither this temperature sensitivity, nor the altered colony morphology of any of the mutants could be rescued by the addition of sorbitol, an osmotic stabilizer that can rescue the growth of cell wall mutants (unpublished data). We also screened these mutants for sensitivity to several drugs, including the immunosuppressive agents rapamycin, FK506, and cyclosporin A (CsA), and the antifungal agents fluconazole and caspofungin (Figure 1A). None of the mutants showed increased susceptibility to rapamycin or caspofungin, but ST118 (*arf1*) is hypersensitive to fluconazole. The *S. cerevisiae* *arf1* mutant is also hypersensitive to fluconazole, a phenotype of that mutant which had not, to our knowledge, been previously reported (Figure 4B). Additionally, *S. cerevisiae* *ARF2/arf2* and *ARF3/arf3* heterozygous mutants are hypersensitive to fluconazole (Giaever *et al.*, 2004). This link between ADP-ribosylation factors and azole sensitivity warrants further study given that the azoles are the most commonly used antifungal drugs.

All of the hyperpolarized RAM mutants are hypersensitive to the immunosuppressive agents FK506 and CsA. On media containing FK506 (1 μg/ml) or CsA (100 μg/ml), the RAM mutants did not grow, whereas the wild-type strain, KN99α, and the other morphology mutants grew as well as

Figure 3. Capsule production in altered morphology mutant strains. Strains were grown for 4 d at 30°C in liquid low-iron medium, and then exclusion of India ink was examined. Strain ST224 shows greatly diminished capsule production, but is not abolished. Scale bar, 10 μm.

Figure 4. Drug susceptibility of *S. cerevisiae* mutants. (A) Serial dilutions of *S. cerevisiae* RAM mutants were plated onto YPD alone or with cyclosporin A (CsA; 100 μ g/ml) or FK506 (1 μ g/ml) at 30°C for 48 h. (B) Serial dilutions of *S. cerevisiae* and *C. neoformans* wild-type and *arf1* mutant strains were plated onto YPD or YPD with fluconazole (10 μ g/ml).

on YPD-rich medium (Figure 1). We tested survival of the mutants over time when grown at high temperature or in FK506 supplemented media. Liquid cultures of each RAM mutant were grown in YPD at 30°C, YPD at 37°C, or YPD with 1 μ g/ml FK506 for 0.5, 1, 4, or 24 h. They were then plated on solid YPD medium at 30°C, and colony forming units were counted after 3 d. All mutants grown for a period of time up to 24 h at 37°C survived but did not replicate during that time. This implies that the RAM genes are not required for viability at 37°C, but rather are required for proliferation at that temperature. Growth in YPD with FK506, on the other hand, completely killed the mutant strains after as little as 0.5 h; no cells were viable when plated onto rich medium. Thus the mechanism of action of FK506 in a RAM mutant background is fungicidal, whereas the mechanism of temperature sensitivity is fungistatic.

Synthetic Lethality between RAM and Calcineurin Pathways

The increased sensitivity of RAM mutants to drugs that target the calcineurin pathway suggested that cells compromised for the two pathways are likely to be inviable. To test this, we crossed a RAM mutant (*cbk1::NAT*) with a calcineurin mutant (*cna1::NEO*) and investigated the possible synthetic lethality between the RAM pathway and calcineurin pathways. From the 75 basidiospores that germinated (175 total), 28 were wild-type ($NAT^s NEO^s$), 23 were *cbk1* ($NAT^r NEO^s$), 23 were *cna1* ($NAT^s NEO^r$), and only 1 was a candidate *cbk1 cna1* double mutant ($NAT^r NEO^r$). The cellular morphology of this rare $NAT^r NEO^r$ strain was that of the *cbk1* mutant and by Southern blot analysis the strain was heterozygous at the *CNA1* locus (*CNA1/cna1::NEO*). Aneuploid progeny are occasionally obtained from *C. neoformans* crosses, explaining this unusual segregant. The proportion of strains obtained from the cross is highly skewed from the predicted 1:1:1:1 ratio (χ^2 test $p < 0.001$). The *CBK1* and *CNA1* genes are located on separate large contigs in the serotype A genome sequencing project so there is no chance that this recombination distortion is due to physical linkage. We also determined the mating type in the 75 progeny and observed that *a* and α alleles segregate independently of the

Figure 5. Visualization of the cell walls and nuclei of hyperpolarized mutant strains. Wild-type and mutant cells were grown to midlog phase in liquid YPD medium and stained with calcofluor white and sytox green to visualize cell walls/septa and nuclei, respectively. Scale bar, 10 μ m.

other markers, further demonstrating that the population exhibits normal recombination. This data, together with the observation of cell death in RAM mutants in the presence of FK506, indicates that the RAM pathway and calcineurin pathways function in parallel and their simultaneous inhibition leads to inviability.

FK506 and CsA bind to FKBP12 and cyclophilin A, respectively, to inhibit calcineurin activity, and thereby exert their antifungal and immunosuppressive activities (Rusnak and Mertz, 2000). The synthetic lethality of calcineurin inhibition and RAM gene mutation in *C. neoformans* suggests that these two pathways share an overlapping essential function, indicating a novel connection between calcineurin and RAM

Figure 6. Actin localization in hyperpolarized mutant strains. Wild-type and mutant cells were grown to mid-log phase in liquid YPD medium and stained with rhodamine-conjugated phalloidin. Actin, visualized as punctate dots and occasional filaments, appears evenly distributed throughout the wild-type cells. Cells of the RAM mutants, however, often accumulate an increased amount of actin at the bud tips compared with wild-type, suggesting a role for Kic1 and Cbk1 in actin localization in *C. neoformans*. Scale bar, 10 μ m.

signaling. Interestingly, however, *S. cerevisiae* RAM mutants are not hypersensitive to FK506 or CsA (Figure 4A), providing further evidence of functional divergence of this pathway in these two species.

Hyperpolarization Mutants Exhibit Defective Cytokinesis But Normal Nuclear Partitioning

In *S. cerevisiae*, the RAM mutants are abnormally round and fail to separate, indicating a loss of polarity and an inability to complete cytokinesis (Nelson *et al.*, 2003). Fluorescence microscopy of *C. neoformans* RAM mutants reveals a similar lack of detachment between mother and daughter cells, but instead of a loss of polarity, these mutant cells are strikingly hyperpolarized and result in cell aggregates reminiscent of pseudohyphae (Figure 5). Staining nuclei and cell walls with sytox green and calcofluor white, respectively, showed proper nuclear segregation and the formation of septa between dividing cells, indicating that the loss of the RAM

genes leads to an inability to complete the last stages of cytokinesis that lead to full cell separation. Similarly, in *U. maydis* mutation of *DON3*, a gene encoding a product in the same family of serine/threonine kinases as that encoded by *KIC1*, does not inhibit primary septum formation, but leads to the loss of initiation of the secondary septum, which normally allows the daughter cell to separate from the mother (Weinzierl *et al.*, 2002). The hyperelongation of *C. neoformans* RAM mutants, however, is unique as the homologous *S. cerevisiae*, *S. pombe*, and *U. maydis* mutants lose polarization (Verde *et al.*, 1998; Durrenberger and Kronstad, 1999; Nelson *et al.*, 2003).

Polarized growth involves changes in actin structure and positioning; we therefore hypothesized that the hyperpolarization defect of the RAM mutants may be a result of perturbation of actin localization. Actin localization in wild-type *C. neoformans* strain KN99 α appeared evenly distributed throughout the cell, visualized as punctate patches and occasional fila-

Figure 7. Subcellular localization of Cbk1 and Kic1. Wild-type or *tao3* (strain DR24) cells were transformed with a construct containing Cbk1 or Kic1 tagged with the fluorescent DsRed protein. (A) Autofluorescence of untransformed strains. (B) In wild-type background, Kic1 shows punctate cytoplasmic localization in the majority of cells and temporal localization to the septa in ~1% (3/236) of dividing cells. (C) Similarly, Cbk1 localizes diffusely to the cytoplasm and to punctate spots resembling vesicles in the majority of cells. Notably, Cbk1 localizes to the septa in actively budding cells (lower panel) in ~2% of budding cells (4/188). (D) Kic1 localization in the *tao3-1* mutant DR24 differs from localization in a wild-type strain. Localization of Kic1 to the septa was never observed (0/>400), suggesting a role for Tao3 in the localization of Kic1. Scale bar, 10 μ m.

ments when stained with rhodamine-conjugated phalloidin. RAM mutants, on the other hand, show increased amounts of actin at the bud tips (Figure 6). Despite differing polarization defects in RAM mutants of *S. pombe* and *C. neoformans*, both mutant phenotypes appear to be correlated with actin mislocalization, suggesting that the RAM proteins may be conserved in regulating fundamental cellular phenomena including actin localization. The reason that

deletion of the homologous genes confers opposing polarization phenotypes in *S. pombe* or *S. cerevisiae* and *C. neoformans* is unclear.

C. neoformans Cbk1 and Kic1 Localize to Punctate Structures and the Septum during Cell Division

To delineate functional differences between *S. cerevisiae* and *C. neoformans* RAM mutants, we analyzed the subcellular

	+HIS +ADE	-HIS	-ADE	β -galactosidase activity
1 AD + BD				12.5 +/- 5.2
2 AD-Cbk1 + BD-Mob2				215.4 +/- 79.8
3 AD-Cbk1 + BD				11.9 +/- 0.9
4 AD + BD-Mob2				7.3 +/- 1.6
5 AD-Cbk1 + BD-Kic1				535.5 +/- 319.2
6 AD + BD-Kic1				10.0 +/- 6.7
7 AD-Kic1 + BD-Kic1				409.2 +/- 182.6
8 AD-Cbk1 + BD-Cbk1				5.5 +/- 2.1
9 AD-Kic1 + BD				10.0 +/- 4.6
10 AD + BD-Cbk1				6.4 +/- 3.9
11 AD-Mob2 + BD				13.9 +/- 2.1
12 AD-Mob2 + BD-Kic1				36.2 +/- 9.0
13 AD-Mob2 + BD-Mob2				16.6 +/- 14.8

Figure 8. Cbk1, Kic1, and Mob2 physically interact. The coding regions of the *CBK1*, *KIC1*, and *MOB2* genes were fused adjacent to the activating domain (AD) or binding domain (BD) of *S. cerevisiae* Gal4 and expressed in the *S. cerevisiae* strain PJ69-4a, which has the *ADE2*, *HIS3*, and *lacZ* genes under the control of Gal4. Protein-protein interactions were detected by growth of transformed strains on media lacking histidine (–HIS) or adenine (–ADE), and by increased β -galactosidase activity. Average β -galactosidase activities of three independent experiments with the SE of the mean (in Miller units) are reported.

localization of the Cbk1 and Kic1 proteins tagged with fluorescent DsRED protein in the wild-type KN99 α background. To confirm that these fusion proteins were functional in vivo, we transformed *kic1* and *cbk1* deletion mutants with the plasmids carrying the corresponding DsRED fusion protein. Transformants bearing an integrated transgene were analyzed and showed phenotypes equivalent to wild-type cells, thus confirming that the DsRED fusion proteins complement the deletion of *CBK1* or *KIC1*. As an additional control, the *kic1*+*KIC1*-DsRED transformants were crossed to wild-type KN99a, and meiotic progeny were isolated by dissection. The isolation of mutant meiotic progeny further confirmed that the DsRED fusion protein could complement mutant strains.

At present, there is no system available to synchronize *C. neoformans* cell cultures, hampering localization studies. In the majority of cells, Cbk1 is localized diffusely throughout the cytoplasm and to punctate spots resembling vesicles (Figure 7C). The biological significance of the localization of Cbk1 to potential vesicles is unknown. However, in *D. melanogaster*, Trc and Fry also localize to punctate spots, which are suggested to be vesicles; furthermore, the Trc/Fry pathway genetically interacts with Dral, the *D. melanogaster* homolog of Ral, which is involved in vesicle trafficking (Feig, 2003; He *et al.*, 2005). Importantly, in actively budding cells of *C. neoformans*, Cbk1 localizes briefly to the septum, corroborating its specific role in the separation of mother-daughter cells (Figure 7C). Septal localization was seen in ~2% of budding cells (4/188). *S. cerevisiae* RAM proteins, including Cbk1, also localize temporally in the cell cycle to the septum (Nelson *et al.*, 2003).

We also observed the subcellular localization of Kic1 tagged with the fluorescent DsRED protein in the KN99 α background. In a pattern similar to Cbk1, the Kic1 protein localizes diffusely throughout the cytoplasm in the majority of the cells; however at a brief interval during cell division, the protein localizes to the septum where we hypothesize it mediates the final stages of cytokinesis in conjunction with

other RAM proteins (Figure 7B). Septal localization was seen in ~1% of budding cells (3/236). We also analyzed the localization of Kic1 in a *tao3* background (strain DR24), and were no longer able to detect localization of Kic1 to the septa (0/>400), suggesting a role for Tao3 in the localization of Kic1 (Figure 7D). Mislocalization of Kic1 in the *tao3* mutant may be partially responsible for the morphology or cell separation defect.

Cbk1, Kic1, and Mob2 Physically Interact

The novel phenotypes conferred by mutation of the RAM genes in *C. neoformans* may indicate different protein-protein interactions between the components of this pathway compared with interactions previously studied in *S. cerevisiae*. To test for pairwise physical interactions we performed yeast two-hybrid assays using *C. neoformans* Cbk1, Kic1, and Mob2 proteins. Robust interactions were observed between the two kinases Cbk1 and Kic1, between Cbk1 and its binding partner Mob2, Kic1 and itself, and a weaker interaction between Kic1 and Mob2 (Figure 8). These results suggest that the protein-protein complex architecture is similar in the two divergent yeasts, despite differing output from the signaling pathway.

We also tested the ability of *C. neoformans* Cbk1, Kic1, and Mob2 to complement the corresponding *S. cerevisiae* RAM mutants. Although these constructs functioned in two-hybrid assays, they failed to complement the cell separation defects of *S. cerevisiae* mutants (unpublished data). This is not completely unexpected, as homologues from *S. pombe* also failed to complement *S. cerevisiae* mutants (Racki *et al.*, 2000). These findings provide further confirmation of functional divergence between the *C. neoformans* and *S. cerevisiae* RAM pathways and may suggest that differences in protein structure between species might mediate interactions with different upstream or downstream components of the pathway that lead to distinct phenotypes.

DISCUSSION

The regulation of cellular morphology is critical to all organisms. Fundamental processes from cell division to the development of specialized tissues and organs in higher eukaryotes require the coordinated generation of cell polarity. One would assume that because the generation of polarity is so fundamental for life that there would be many conserved proteins and functions. In yeast, the polarity of dividing cells is precisely regulated by a number of pathways, including the recently elucidated RAM signaling pathway, which comprises the proteins Cbk1, Mob2, Kic1, Hym1, Sog2, and Tao3 (Nelson *et al.*, 2003; Irazoqui and Lew, 2004). *S. cerevisiae* strains harboring mutations in any of these genes display cell separation defects and a loss of polarity or are lethal, depending on the genetic background.

In this study, we identified five components of the homologous RAM-signaling pathway in *C. neoformans*, a human pathogenic fungus that grows as a yeast, yet which lies within the phylum Basidiomycota and is highly divergent from ascomycete yeasts like *S. cerevisiae*. RAM in *C. neoformans* also controls cell separation and polarity. However, in this fungus disruption of this pathway leads to hyperpolarization instead of depolarization, indicating that the pathway functions to negatively regulate polarization in this organism rather than to positively regulate it as it does in *S. cerevisiae* and *S. pombe*. Furthermore, the RAM genes are not essential in the *C. neoformans* standard serotype A strain background (H99/KN99a/ α), whereas they are essential in *S. cerevisiae* (unless the *SSD1* gene has also been mutated [Kurischko *et al.*, 2005]) and in *S. pombe*. A third difference is that the RAM pathway acts in parallel with the Ca^{2+} -calmodulin-activated protein phosphatase calcineurin in *C. neoformans* but not in *S. cerevisiae*. Simultaneous inhibition of both pathways is lethal, as observed when *C. neoformans* RAM mutants fail to grow in the presence of the drugs FK506 or cyclosporin A, which each function to inhibit calcineurin, and by the inability to obtain *cbk1* *cna1* double mutants through genetic crosses.

The RAM pathway is an abbreviation referring to both morphology and localization of Ace2, a C2H2 zinc finger transcription factor that specifically localizes to daughter cells in *S. cerevisiae*. Some of the differences between *S. cerevisiae* and *C. neoformans* may be accounted for by unique Ace2 functions. One major question about the *C. neoformans* RAM pathway is the function it has in regulating the equivalent of Ace2 in this fungus. We searched the completed genome for matches to *S. cerevisiae* Ace2. The closest match (e-11), CNA01450, is predicted to contain two C2H2 Zn finger domains at its C-terminal end (like Ace2). However, a reciprocal BLAST search of *S. cerevisiae* revealed that this protein is more similar to Crz1 or Swi5 than to Ace2. Furthermore, CNA01450 is also the closest match when *S. cerevisiae* Crz1 is used to search the *C. neoformans* genome. Thus at present, no clear Ace2 homolog has been found in the *C. neoformans* genome and an equivalent functioning gene has not been identified, although it is the object of ongoing research.

Despite these differences in RAM pathway function in the basidiomycete *C. neoformans* compared with model ascomycete fungi, some similarities exist to their homologues in more distant relatives. Homologues of proteins in the RAM pathway have been identified in diverse species, including fungi, the fruit fly *D. melanogaster*, the nematode *C. elegans*, and humans (Table 4). The other fungi generally have phenotypes equivalent to the *S. cerevisiae* and *S. pombe* mutants. In *Neurospora crassa*, mutation of the Cbk1 homolog (Cot-1) results in defective hyphal elongation and increased branching

Table 4. Characterized RAM pathway homologs

<i>Cryptococcus neoformans</i>	Cbk1	Kic1	Mob2	Tao3		Sog2
<i>Saccharomyces cerevisiae</i>	Cbk1	Kic1	Mob2	Tao3	Hym1	Sog2
<i>Schizosaccharomyces pombe</i>	Orb6	Orb3/ Nak1	Mob2	Mor2	Pmo25	
<i>Aspergillus nidulans</i>						HymA
<i>Neurospora crassa</i>	Cot-1					
<i>Ustilago maydis</i>	Ukc1					
<i>Caenorhabditis elegans</i>	Sax-1					
<i>Drosophila melanogaster</i>	Trc		Dmob	Fry	Mo25 ^a	
<i>Homo sapiens</i>	Ndr1	Mst3	Mob			

Cbk1 is a serine/threonine kinase in the AGC (protein kinases A, G, and C) family of kinases, which includes the mammalian Ndr and WARTS/LATS kinases (Bidlemaier *et al.*, 2001). Kic1 is also a serine/threonine protein kinase that is related to the PAK/Ste20 family of proteins (Sullivan *et al.*, 1998). Mob2, Sog2, Hym1, and Tao3 interact with at least one of these kinases to mediate their functions in *S. cerevisiae* (Nelson *et al.*, 2003).

^a Mo25 is the closest *D. melanogaster* homolog of Hym1, but it does not appear to function in a pathway with other RAM homologs in *Drosophila* (He *et al.*, 2005).

(Yarden *et al.*, 1992). In *U. maydis*, mutation of the Cbk1 homolog (*ukc1*) causes a distorted, compact, highly branched morphology and defects in pathogenesis (Durrenberger and Kronstad, 1999). In *Aspergillus nidulans*, mutation of the *HYM1* homolog *hymA* blocks conidiophore development (Karos and Fischer, 1996). Also, homologues of *CBK1* have recently been identified in the human fungal pathogens *Pneumocystis carinii*, where its transcript level is regulated by pH, and *C. albicans*, where deletion of *CBK1* leads to an inability to produce hyphae and a consequent aggregation of round cells (McNemar and Fonzi, 2002; Kottom and Limper, 2004).

Homologues of the RAM pathway have been recently characterized in higher eukaryotes. The *D. melanogaster* homologues of Cbk1 and Tao3, *tricornered* (Trc) and *furry* (Fry), respectively, control wing hair development and the positioning and dendritic branching of neurons (Emoto *et al.*, 2004; He *et al.*, 2005). Similarly, the *C. elegans* Cbk1 homolog (Sax-1) regulates neuronal cell shape and appears to function as an inhibitor of neurite initiation and spreading (Zallen *et al.*, 2000). Interestingly, the neuronal cells of *sax-1* mutants are irregularly expanded and extended instead of displaying a normal compact, spherical morphology, a phenotype qualitatively more similar to that observed in RAM mutants of *C. neoformans* than in *S. cerevisiae*. Furthermore, neurite initiation in *C. elegans* is regulated in parallel by both the Sax-1 RAM pathway and a calcium/calmodulin-dependent protein kinase, Unc-43 (Zallen *et al.*, 2000), similar to the parallel regulation of viability in *C. neoformans* by RAM and calcineurin.

In mammals, the homolog of Cbk1 appears to be the NDR1 protein kinase, which is regulated by a Kic1 homolog and Ca^{2+} phosphatase activity. The NDR family of protein serine/threonine kinases is a subclass of the AGC kinases, which includes the mammalian, fly, and worm large tumor suppressor (LATS) kinases and *S. cerevisiae* Dbf2 and Dbf20 in addition to the Cbk1 homologues (Tamaskovic *et al.*, 2003a). LATS/WARTS, a close relative of NDR1, is required as a tumor suppressor in flies and mammals. NDR1 physically interacts with S100B, a Ca^{2+} -binding protein, which af-

fects its phosphorylation state and kinase activity (Millward *et al.*, 1998; Tamaskovic *et al.*, 2003b). S100B is overexpressed in more than 80% of metastatic melanomas, and up-regulated NDR1 transcription indicates a higher risk of invasive breast cancer (Hauschild *et al.*, 1999; Adeyinka *et al.*, 2002). A mammalian Kic1 homolog, Mst3, has been recently identified and it acts to regulate NDR through phosphorylation (Stegert *et al.*, 2005). Both Mst3 and Ndr activity appear to be regulated by the mammalian protein phosphatase PP2A (Stegert *et al.*, 2005). It will be important to elucidate how phosphatases like PP2A in mammals and calcineurin in *C. neoformans* fit into the RAM signaling pathway.

The NDR1 pathway in mammals and the homologous RAM pathway in other eukaryotes from fungi to flies are both key regulators of cellular morphogenesis and proliferation. Cell morphology and viability in *C. neoformans* appears to be regulated in parallel by the RAM and calcineurin pathways, indicating that cross-talk between the RAM signaling pathway and intracellular calcium signaling may be conserved with multicellular eukaryotes. Thus, a better understanding of the function of this pathway may result from studies in *C. neoformans* in addition to the ascomycete model fungi such as *S. cerevisiae* or *S. pombe*. Elucidation of the molecular mechanism of this regulation, its cross-talk with other signaling pathways, and its upstream and downstream components represent important future research avenues in elucidating the function of the RAM signaling pathway in the eukaryotic kingdom.

ACKNOWLEDGMENTS

We thank Francis Luca and Cornelia Kurischko for the generous gift of *S. cerevisiae* RAM mutants. We thank Connie Nichols for the DsRED plasmid and assistance with actin staining. We also thank Andrew Alspaugh, Daniel Lew, Connie Nichols, and Julian Rutherford for critical reading of the manuscript. Gene identification was greatly facilitated by the following *C. neoformans* genome sequencing projects: Stanford Genome Technology Center (<http://www-sequence.stanford.edu>), The Institute for Genomic Research (<http://www.tigr.org/tdb/e2k1/cna1>), Duke University (<http://cneo.genetics.duke.edu>), and the Broad Institute (http://broad.mit.edu/annotation/fungi/cryptococcus_neoformans/). Felicia Walton was supported by a research fellowship from the American Society for Microbiology. This work was supported by the National Institute of Allergy and Infectious Diseases R01 Grants AI042159, AI063443, and AI50438 to J.H.

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