Ras1 and Ras2 contribute shared and unique roles in physiology and virulence of Cryptococcus neoformans

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INTRODUCTION

The mechanisms by which cells sense and adapt to changes in the environment are mediated by conserved signal transduction pathways that couple specific extracellular signals to defined cellular outputs. Ras proteins are conserved regulators of signal transduction pathways that mediate adaptive changes, such as cellular development and morphogenesis. In mammalian systems, Ras controls cell proliferation, and well-characterized mutations in these proteins are associated with malignant transformation and uncontrolled growth (Barbacid, 1987). In micro-organisms, Ras proteins are similarly involved in the regulation of growth and development. The Ras1 and Ras2 proteins in the budding yeast, Saccharomyces cerevisiae, play a central role in controlling cAMP levels (Toda et al., 1985) and MAP kinase signalling, and regulate both diploid pseudohyphal growth (Gimeno et al., 1992) and haploid invasive growth (Stanhill et al., 1999). More recently, Ras2 has also been shown to regulate polarization of the actin cytoskeleton (Ho & Bretscher, 2001). In the fission yeast, Schizosaccharomyces pombe, Ras does not appear to be involved in cAMP homeostasis. Instead, Sch. pombe Ras1 controls the activity of the pheromone response pathway and mating (Nielsen et al., 1992).

Pathogenic micro-organisms must be able to adapt to dramatic changes as they move from the environment into the infected host. In these organisms, the same signalling pathways that are used to detect changes in the environment may also be used to regulate the determinants of virulence and host infection. Therefore, pathogens can be excellent model systems to dissect the genetic control of cellular adaptation and development in addition to defining the mechanisms of pathogenesis. Cryptococcus neoformans is a human fungal pathogen that primarily causes a meningoencephalitis in hosts.
with impaired immune systems. We have used this basidiomycetous yeast as a model system to dissect signal transduction pathways that control fungal development and pathogenicity. Previously, we demonstrated that a conserved C. neoformans Ras protein was required for the growth of this yeast at 37 °C and thus for virulence (Alspaugh et al., 2000). The Ras1 protein is also required for mating and activation of a pheromone-sensing MAP kinase signalling cascade can suppress the rasI mutant mating defect (Alspaugh et al., 2000). This observation, and the elucidation of similar Ras signal transduction pathways in organisms such as budding yeast and fission yeast, suggest that C. neoformans Ras1 acts upstream of a pheromone-response/MAP kinase signalling pathway to control mating. In contrast, MAP kinase signalling elements do not suppress the rasI mutant high-temperature growth defect, indicating that Ras1 regulates vegetative growth at 37 °C via a second pathway (Alspaugh et al., 2000).

To further understand the molecular mechanisms of Ras signalling in C. neoformans, we identified a second Ras gene in this organism. The Ras2 gene is expressed at a very low level and was not induced under several different in vitro conditions or by disruption of the Ras1 gene. The Ras2 gene was disrupted by transformation and homologous recombination. ras2 mutant strains were viable and displayed no significant phenotypic alterations from wild-type in vitro or in an animal model of cryptococcal disease. Overexpression of the Ras2 gene in a rasI mutant background suppressed rasI mutant phenotypes, indicating that the Ras1 and Ras2 proteins share some degree of functional redundancy. Analysis of the terminal phenotypes of the rasI mutant strain incubated at 37 °C, as well as the complementation of these phenotypes by Ras2 overexpression, suggest a conserved role of Ras in the regulation of fungal growth, as well as a potential conservation of the downstream targets of Ras pathways in C. neoformans.

METHODS

Strains and media. All strains used in this study (except strain JEC20) are serotype A C. neoformans strains derived from the wild-type strain H99 (Perfect et al., 1980). LCC1 is a rasI mutant strain (Alspaugh et al., 2000) and H99-ura5 and LCC70 are spontaneous 5-fluoro-orotic acid (5-FOA)-resistant derivatives of H99 and LCC1, respectively, created by the method of Kwon-Chung et al. (1992a). LCC3 is a rasI mutant strain in which the wild-type rasI gene has been reintroduced (Alspaugh et al., 2000). MWC12 and MWC13 are ras2 mutant strains and MWC14, MWC15 and MWC16 are rasI ras2 double mutant strains all described in this study. Strain MWC17 was constructed by reintroducing the wild-type rasI gene linked to the bph gene conferring resistance to hygromycin B into strain MWC14 by biolistic transformation using the method described previously (Alspaugh et al., 2000). Biolistic transformation was used to integrate the Ras2 gene under control of the GPD1 promoter into the Ras1 wild-type strain H99-ura5 to create the Ras1 + Ras2 strain MWC27 and into the rasI mutant strain LCC70 to create the rasI + Ras2 strains MWC28 and MWC29. JEC20 is a serotype D wild-type strain used for the mating experiments (Kwon-Chung et al., 1992b). Standard yeast media were used for most experiments (Sherman, 1991). Niger seed agar and V8 matting medium (Kwon-Chung & Bennett, 1992), and no-iron medium (low-iron medium + 56 μM EDDHA) (Vartivarian et al., 1993) were prepared as described previously (Alspaugh et al., 2000).

PCR. Unless otherwise stated, all PCR reactions were performed in a Perkin Elmer GeneAmp 9600 thermocycler with 50 ng template DNA, 100 ng each oligonucleotide primer and standard reagents from the TaKaRa kit (Takara Shuzo).

Identification of the Ras2 gene. To identify the gene encoding a second Ras homologue (Ras2), degenerate primers were synthesized based on regions of homology among several fungal Ras genes: primer 1906 (5'-CTCGAGCTGARTAGYAYACATYG-3') and primer 1908 (5'-CAGCTGGAGTAYTCYTCYTGRCGRGTTRTC-3') (Y = pyrimidine, R = purine). A 115 nt fragment was amplified using these primers in a PCR reaction with cDNA from strain H99 as template using the following conditions: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 40 °C for 60 s, 72 °C for 30 s.

The 115 nt Ras2 fragment was used to probe a genomic Southern blot of strain H99 DNA. For all Southern hybridizations, electrophoresis, DNA transfer, prehybridization, hybridization and autoradiography were performed as described by Sambrook et al. (1989). The probes were labelled using the Random Primed DNA Labelling Kit (Boehringer Mannheim) and [32P]dCTP (Amersham). A 2.6 kb SacI fragment containing the entire Ras2 genomic locus was isolated by screening a subgenomic library of SacI fragments (2–3 kb) of H99 genomic DNA in the pBluescript SK plasmid (Stratagene) by colony hybridization using the 115 nt fragment as probe. Alignment with other Ras proteins was performed using the MEGALIGN program (DNAStar).

The Ras2 cDNA was amplified from a cDNA library of strain H99 using the following primers: 5064 (5'-CTCTCAACCCACACCAACCACACC-3') and 5067 (5'-GCCACATTTGATCTTTCC-3'). The resulting PCR fragment was sequenced and compared with the H99 genomic Ras2 sequence.

Disruption of the serotype A C. neoformans Ras2 gene. The ras2A::URA5 mutant allele was created by PCR overlap extension using the method of Ho et al. (1989). The 5' and 3' Ras2 fragments were amplified by PCR using the Ras2 gene as template and the following primers: 5' Ras2 fragment primers 4549 (5'-GAAGGCCACGTCCTCGGCC-3') and 4550 (5'-GCGATAGCCGATCTTTGAAACGCTTACGAGCGAGCC-3') (URA5 sequence underlined); 3' Ras2 fragment primers 4551 (5'-CCACACCTCGAGGACGAA- GCCGGCGAGTCG-3') and 5067 (5'-GCCACATTTGATCTTTCC-3') (URA5 sequence underlined) and 4552 (5'-CCCTGAGATTCCACGCGT-3'). Ras2 sequence was added to the URAS5 gene by PCR using plasmid pGnTel as template (Edman, 1992) and the following primers: 4553 (5'-GGGCTGCATTGCGTAAAGTBATGCT-C') and 4554 (5'-GAGATACCCGGATGCGCGGTTTGCGC-3') (RAS2 sequence underlined). The PCR conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min. The linear fragments obtained from each of the three PCR reactions were used together as template in a PCR reaction with oligonucleotide primers 4549 and 4552 and the following conditions: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 3 min. A 3.5 kb fragment representing the ras2A::URA5 allele was obtained.

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The linear, deoxy-ribo-tailed ras2A::UR5 disruption construct was transformed into strains H99-ura5 and LCC70 by biolistic DNA delivery, and transformants were selected on synthetic medium lacking uracil with 1 M sorbitol as described by Toffaleti et al. (1993). Transformants were screened using PCR to identify putative ras2 mutants. Genomic DNA was isolated from 36 transformants for each starting strain by the method of Pitkin et al. (1996) and used as the template for a PCR reaction using the RAS2-specific primer 5150 (5'-CCATCTCATCTCATCAACAGG-3') and the UR5-specific primer 5151 (5'-CGTCTTCTCTCACATAGTCAGG-3') to identify strains in which a targeted disruption of the wild-type RAS2 locus had occurred. The PCR conditions were 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. A 1.2 kb fragment was anticipated in those strains in which the wild-type RAS2 gene had been replaced by the ras2A::UR5 allele by homologous recombination. To evaluate the putative ras2 mutant and ras1 ras2 double mutant strains, genomic Southern hybridization was performed using genomic DNA isolated from strains H99, LCC1, MWC12, MWC13, MWC14, MWC15 and MWC16 and digested with EcoRI. The entire RAS2 locus from the start to termination codons was used as the probe.

Growth rate determination. Strains were pre-incubated in YPD medium for 48 h at 25 °C and subsequently inoculated into YPD medium. Exponential-phase doubling times were determined by quantitatively culturing these samples every 2 h for 18 h. Triplicate samples for each strain were analysed and statistical significance of differences between strains was calculated using a paired Student's t-test.

Virulence assay. Virulence of ras1 and ras2 mutant strains was assessed in the mouse inhalation model of cryptococcosis as described by Cox et al. (2000). Briefly, immunocompetent mice were anaesthetized and intranasally inoculated with 5 x 10⁴ C. neoformans cells. Animals infected with pathogenic strains die of fulminant meningoencephalitis. Mice were sacrificed prior to death based on clinical end points previously described by Cox et al., 2000. Survival was determined for 10 mice infected with the wild-type, ras1 mutant or ras2 mutant strains. The statistical significance of survival differences between mice infected with different strains was assessed using the Kruskal–Wallis algorithm.

Mating assay. All strains were initially grown for 48 h on YPD medium at 25 °C and suspended in water at 10⁶ cells ml⁻¹. For each strain to be tested, 5 µl of the cell suspension was mixed with 5 µl of the cell suspension of the MATa strain JEC20 and plated as a drop on V8 mating agar. The mating patches were incubated in the dark at 25 °C for 14 d and microscopically assessed daily for the appearance of hyphae and other mating structures.

RAS2 overexpression. Plasmid pRCD83, containing the C. neoformans GDP1 promoter linked to the UR5 selectable marker, was obtained from Robert Davidson at Duke University. The RAS2 gene was cloned into this vector and under control of the GDP1 promoter, and this new construct was biolistically transformed into the ras1 ura5 strain LCC70 and the RAS1 ura5 strain H99-ura5. For Northern analysis, total RNA was extracted from exponential-phase cells incubated in YPD medium as described previously (Alspaugh et al., 1997). Fifteen micrograms of RNA was loaded onto a formaldehyde RNA gel and electrophoresis, RNA transfer, hybridization and autoradiography were performed as described by Sambrook et al. (1989).

Microscopy and F-actin staining. All light and fluorescence microscopy was performed on a Nikon Optiphot-2 microscope using the appropriate filters and objectives. Images were captured and processed using the Spot RT digital camera (Diagnostic Instruments) with Adobe Photoshop software. C. neoformans cells were fixed by adding one-quarter volume of 37% formaldehyde for 20 min at room temperature. Cells were then washed three times with PBS, permeabilized by adding 1% Triton X-100 in PBS for 5 min and washed three times with PBS. To visualize F-actin, aliquots of fixed cells were incubated with rhodamine-conjugated phalloidin (1/10 dilution of a 10 µg ml⁻¹ stock) for 2 h.

RESULTS

Identification and disruption of the RAS2 gene

Two DNA fragments were amplified by PCR under low-stringency conditions using C. neoformans cDNA as template and primers designed against conserved sequences of RAS genes from diverse fungi. A 141 bp fragment was identical in sequence to the C. neoformans RAS1 gene (Alspaugh et al., 2000). A distinct 115 bp fragment represented a second RAS gene in C. neoformans that was homologous to fungal RAS genes, but distinct from RAS1. This smaller fragment was used to probe a Southern blot of genomic DNA from strain H99 and a 2.6 kb Sac1 fragment encompassing the entire RAS2 locus was isolated from a size-selected library of H99 genomic DNA by colony hybridization. Initiation and termination codons, as well as the intron boundaries of C. neoformans RAS2, were identified by alignment with other Ras homologues, defining a genomic locus of 1097 nt with four introns. The C. neoformans RAS2 cDNA was amplified from an H99 cDNA library using primers based on the predicted RAS2 ORF and sequenced to confirm the coding region. The RAS2 gene encodes a 238 aa protein that shares moderate sequence identity with C. neoformans Ras1 (37%). Its closest homologue is the Neurospora crassa Ras2 protein (46%) (Fig. 1a). Conserved GTP-binding sites and a C-terminal CAAX motif are present.

To determine the biological roles of this second RAS protein in C. neoformans, the RAS2 gene was disrupted by biolistic transformation and homologous recombination. The C. neoformans UR5 strain served as the selectable marker (Fig. 1b) and a ura5 derivative of the serotype A H99 strain as the recipient (H99-ura5). PCR amplification revealed that the RAS2 gene was replaced by the ras2::UR5 disruption allele in 15 of 36 (42%) Ura- transformants. Southern hybridization confirmed that the wild-type gene had been precisely replaced by the disruption allele with no ectopic integrations in two of these transformants (MWC12, 13) (Fig. 1c). The in vitro phenotypes were identical for both of these independent ras2 mutants.

In contrast to Sac. cerevisiae, in which ras1 and ras2 mutations are synthetically lethal (Kataoka et al., 1984; Tatchell et al., 1984), C. neoformans ras1 ras2 double mutants were found to be viable. Strains in which both the RAS1 and the RAS2 genes were disrupted were constructed in a similar manner to the ras2 single mutants by disrupting the RAS2 gene in a ura5 derivative.
Fig. 1. Disruption of the *C. neoformans* RAS2 gene. (a) The genetic relatedness of Ras superfamily proteins, including *C. neoformans* Ras1 and Ras2, is demonstrated by estimating evolutionary distance. (b) The *URA5* locus was cloned into the *RAS2* gene to create the *ras2*::*URA5* disruption allele. (c) Genomic DNA was isolated from the wild-type (WT) strain (H99), the *ras1* mutant strain (LCC1), one *ras2* mutant (MWC12) and one *ras1 ras2* double mutant strain (MWC14), digested with EcoRI and analysed by Southern hybridization using the RAS2 gene as probe.

of a *ras1* mutant strain (LCC70). Twenty-one of 36 (58%) *ras1* transformants were identified by PCR in which the endogenous RAS2 gene had been replaced by the *ras2*::*URA5* disruption allele. By Southern blot analysis, the RAS2 gene was disrupted and no ectopic copies of the disruption allele were present in three strains examined (MWC14, 15, 16) (Fig. 1c).

**Ras1, and not Ras2, primarily regulates *C. neoformans* growth at 37 °C**

The wild-type (H99), *ras1* mutant (LCC1), *ras1* + *RAS1* reconstituted (LCC3), *ras2* mutant (MWC12, 13) and *ras1 ras2* double mutant (MWC14, 15, 16) strains were incubated on YPD medium for 48 h at 25, 37 and 39 °C (Fig. 2). Wild-type, *ras1* mutant and *ras2* mutant strains all grow well at 25 °C. As previously reported, the serotype A *ras1* mutant strain was unable to grow at 37 or 39 °C (Alspaugh *et al.*, 2000). In contrast, the *ras2* mutant strain grew as well as wild-type at all temperatures, indicating that *RAS2* is not required for high temperature growth (Fig. 2a).

Although the *ras1 ras2* double mutants were viable, all three independent strains exhibited a growth defect at all temperatures tested (Fig. 2a). Microscopic analysis of the *ras1 ras2* double mutant strain revealed no discernible morphological changes compared to wild-type. All stages of budding were apparent and no mother–daughter neck abnormalities were observed. Cell size of the *ras1 ras2* strain was similar to that of wild-type.

To ascertain whether this phenotype was attributable to the *ras1* and *ras2* mutations, a complementation test was performed. The wild-type *RAS1* gene was reintroduced into a representative *ras1 ras2* double mutant strain (MWC14) and the exponential-phase growth rate in rich medium of the *ras1 ras2* + *RAS1* reconstituted strain (MWC17) was compared to that of the *ras1 ras2* double mutant. The doubling time of the *ras1 ras2* double mutant strain decreased from 4–25 to 2–47 h (*P* = 0.045) when the *RAS1* gene was reintroduced. Introduction of the *RAS1* gene into a wild-type strain did not affect the generation time of exponential-phase cells. This observation demonstrates that the decreased growth rate of the double mutant strain is due to a...
Fig. 2. Ras1 is the predominant Ras protein regulating growth at high temperature and mating of *C. neoformans*. (a) Wild-type (H99), ras1 mutant (LCC1), ras1+RAS1 reconstituted (LCC3), ras2 mutant (MWC12, 13) and ras1 ras2 double mutant (MWC14, 15, 16) strains were plated on YPD medium and incubated for 48 h at 25, 30 and 37 °C. (b) Wild-type (H99), ras1 mutant (LCC1) and ras2 mutant (MWC12) strains were incubated for 7 d in mating reactions with the MATa strain JEC20. The edges of the mating patches were assessed microscopically for mating filaments and photographed. Bars, 0.1 mm.

The wild-type, ras1 mutant, ras1+RAS1 reconstituted and ras2 mutant strains were incubated on V8 mating medium for 5 d in mating reactions with the MATa strain JEC20 (Fig. 2b). Among these strains, only the ras1 mutant strains displayed a significant mating defect, as noted previously (Alspaugh et al., 2000). In contrast, examination of the edge of the mating patches for mating filamentation revealed that the ras2 mutant strain mated normally. Therefore, Ras1, and not Ras2, is the predominant Ras element regulating *C. neoformans* mating.

Furthermore, ras2 mutants exhibited no defects in the *in vitro* expression of two well-characterized inducible virulence factors: capsule and melanin. The wild-type and ras2 mutant strains were incubated on Niger seed agar for 72 h to assess melanin production. The strains were also incubated in no-iron medium for 4 d at 30 °C and examined by India ink preparation to assess capsule production. There was no difference in melanin or capsule induction observed between the wild-type and ras2 mutant strains. Similarly, there was no difference between the ras2 mutant and wild-type strains in agar invasion or agar adhesion (Alspaugh et al., 2000). Microscopic analysis revealed no defects among the ras2 strains in cell morphology or budding.

Our previous studies revealed that a *C. neoformans* ras1 mutant strain was avirulent in an animal model of cryptococcal meningitis, likely due to its inability to grow at physiological temperature (Alspaugh et al., 2000). To test the pathogenicity of the ras2 mutant strain, immunocompetent mice were intranasally inoculated with wild-type (H99), ras1 mutant (LCC1) or ras2 mutant (MWC12) cells. In the murine inhalation model of cryptococcosis, animals infected with virulent *C. neoformans* strains develop fatal cryptococcal meningoencephalitis (Cox et al., 2000). In this experiment, all animals infected with the wild-type strain suffered a fatal outcome by 34 d (median survival 23.5 d). In accordance with our previous observations, the ras1 mutant strain was completely attenuated for virulence compared to the wild-type parental strain. All animals infected with the ras1 mutant strain survived throughout
Fig. 3. Ras1 regulates actin polarization at 37 °C. Wild-type (H99), ras1 mutant (LCC1) and ras2 mutant (MWC12) strains were incubated at 25 and 37 °C in YPD medium for 48 h. Cells were examined by microscopy with Nomarski optics (DIC). F-actin localization was assessed by rhodamine-conjugated phalloidin staining. Bars, 20 μm.

the 60 d of the experiment (P < 0.001). In contrast, there was no significant difference in the survival of mice infected with the ras2 mutant strain compared with those infected with the wild-type strain. The median survival of animals infected with the ras2 mutant strain was 25.5 d, with no animal surviving after 35 d (P = 0.113). Thus, Ras1, but not Ras2, is required for pathogenicity of C. neoformans.

Ras regulation of actin polarization in C. neoformans

The yeast form of C. neoformans is phenotypically similar to the budding yeast Sac. cerevisiae. The budding event in vegetative Sac. cerevisiae cells is well described and requires dramatic reorganization of the actin cytoskeleton (Pruyne & Bretscher, 2000). The organization and dynamics of the C. neoformans actin cytoskeleton is very similar to that in Sac. cerevisiae (Kopecká et al., 2001). F-actin is organized into patches, cables and rings, and cell morphogenesis is directed by actin reorganization and polarization.

Microscopic analysis of the wild-type (H99), ras1 mutant (LCC1) and ras2 mutant (MWC14) incubated for 48 h at 25 and 37 °C revealed striking morphological changes in the ras1 mutant incubated at the elevated temperature. When grown under either condition, the wild-type and ras2 strains appeared as elliptical yeast cells in all stages of budding. At the lower permissive
temperature, the ras1 mutant strain was indistinguishable from wild-type. In contrast, when the ras1 mutant strain was incubated at 37 °C, it arrested as large, unbudded cells (Fig. 3). Visualization of F-actin using rhodamine-conjugated phalloidin demonstrated that although actin was localized in the ras1 mutant at 37 °C, it was depolarized, indicative of a loss of the asymmetry of the actin cytoskeleton observed in budding wild-type cells (Fig. 3). Reintroduction of the wild-type RAS1 gene into the ras1 mutant complemented these ras1 mutant morphological and actin polarization defects. Therefore, the C. neoformans Ras1 protein controls proper actin polarization in a temperature-dependent manner.

Previously, we observed that ras1 mutant cells were growth-arrested but viable after 24 h incubation at 37 °C (Alspaugh et al., 2000). This finding was confirmed in the current experiments in which the wild-type and ras1 mutant strains incubated for 48 h at 37 °C were stained with the vital dye methylene blue. An identical proportion (5%) of wild-type and ras1 mutant cells incorporated dye, indicating cell death.

**RAS2 overexpression suppresses ras1 mutant phenotypes**

By Northern analysis, the C. neoformans RAS1 gene is expressed at low levels in rich medium and its expression is induced fivefold by nitrogen deprivation. In contrast, expression of the RAS2 gene under several conditions could not be detected by Northern analysis. cDNA corresponding to the C. neoformans RAS2 gene could be readily amplified by PCR from cDNA libraries, indicating that this gene is expressed at a very low level.

The RAS2 gene was overexpressed in the ras1 mutant background to differentiate between two possible models. In the first model, Ras1 and Ras2 share overlapping functions and Ras2 is unable to compensate for ras1 mutant phenotypes due to insufficient transcription. In this model, overexpression of the RAS2 gene should suppress ras1 mutant phenotypes. In the second model, Ras2 possesses functions completely distinct from Ras1 and overexpression of the RAS2 gene would not suppress ras1 mutant phenotypes. The RAS2 gene was cloned under the control of the constitutively active GPD1 promoter (Varma & Kwon-Chung, 1999) and integrated into the genome of the ras1 mutant strain (LCC70) by biolistic transformation. Two transformants were selected for further phenotypic testing (MWC28, 29) in which RAS2 expression was increased compared to wild-type based on Northern blot analysis (Fig. 4a). As a control, the RAS2 gene under control of the GPD1 promoter was similarly introduced into the RAS1 wild-type background to create the RAS1 + RAS2 strain (MWC27).

Overexpression of the RAS2 gene fully suppressed the ras1 mutant mating defect. When co-incubated with the MATa strain JEC20, the ras1 + RAS2 strains (MWC28, 29) mated as well as the wild-type strain (Fig. 5). After 48 h, vigorous mating filaments were observed in the mating reactions, including either the wild-type or ras1 + RAS2 strains. Rare, thin hyphae were visualized in ras1 mating reactions after 48 h of incubation (Fig. 5). After 7 d, all mating structures, including fused clamp connections, basidia and basidiospores, were observed in wild-type and ras1 + RAS2 mating reactions. No persistent mating filaments were present in the corresponding ras1 mating reactions after one week.

*Fig. 4.* RAS2 overexpression partially suppresses the ras1 mutant high-temperature growth defect. (a) Total RNA was extracted from exponential-phase cultures of the wild-type (H99), ras1 mutant (LCC1) and ras1 + RAS2 mutant (MWC28, 29) strains incubated in YPD medium. Northern analysis was performed using the RAS1, RAS2 and actin (ACT1) genes as probes. RNA loading is demonstrated by the ethidium-bromide-stained RNA gel (rRNA). (b) Wild-type (WT, H99), ras1 mutant (LCC1) and ras1 + RAS2 mutant (MWC28, 29) strains incubated on YPD medium for 48 h at 25 and 37 °C.
37 °C, the two ras1 + RAS2 strains were able to grow at the higher temperature, though not at the wild-type rate (Fig. 4b).

When examined microscopically, strains in which the RAS2 gene was overexpressed in the ras1 mutant background also resulted in partial suppression of the ras1 mutant morphological defects. In contrast to the ras1 mutant strain, fewer enlarged, unbudded cells were observed among the ras1 + RAS2 cells incubated at 37 °C. In fact, budding was restored among most of the ras1 mutant cells overexpressing the RAS2 gene, indicating that the Ras2 protein is able to partially suppress the morphogenesis and actin polarization defects of the ras1 mutant. No significant morphological changes were observed in the wild-type strain in which the RAS2 gene was overexpressed (Fig. 6).

**DISCUSSION**

Ras proteins belong to a highly conserved family of membrane-bound guanine nucleotide-binding proteins that regulate signal transduction pathways in diverse organisms. The ability to bind and hydrolyse GTP allows Ras proteins to exist in either an active GTP-bound form or an inactive GDP-bound form (Milburn et al., 1990). The controlled cycling between these two states is the basis by which Ras serves as a molecular switch in signalling pathways. For example, the p21 product of mammalian ras genes has clearly been demonstrated to control cell growth. Transforming mutations of these genes result in unregulated cell growth and tumorigenesis (Barbacid, 1987).

Ras proteins from mammals to micro-organisms are likely to share conserved roles in regulating cell growth in response to extracellular signals. In fact, divergent Ras proteins from simple eukaryotes such as fungi may substitute at some levels for mutated mammalian Ras proteins (DeFeo-Jones et al., 1985; Kataoka et al., 1985). However, the important biological functions of fungal Ras are often distinct from mammalian counterparts. The *Sch. pombe ras1* gene is predominately required for sexual differentiation (Fukui et al., 1986). In contrast, two RAS genes are present in the genome of the budding yeast *Sac. cerevisiae* and the fungal pathogen *C. neoformans*. Mutation of both RAS genes in *Sac. cerevisiae* results in growth arrest, suggesting that a certain basal level of Ras function is required for viability (Kataoka et al., 1984; Tatchell et al., 1984). Although these two RAS genes are quite homologous to each other, the encoded proteins serve overlapping but distinct functions. For example, the *Sac. cerevisiae* Ras2 protein plays a greater role than Ras1 in regulating differentiation and cAMP production (Toda et al., 1985). Such differences are likely to be a result of the differences in the level of transcription of these genes as well as actual protein functional divergence. Under most conditions, *Sac. cerevisiae* RAS1 is expressed at approximately one-tenth the level of RAS2. When the RAS1 gene was placed under control of the RAS2 promoter, overexpression of Ras1 restored haploid invasive growth of ras2 *Sac. cerevisiae* mutant strains (Mösch et al., 1999).

Our studies in *C. neoformans* underscore similarities among Ras pathways across divergent fungal species. However, these experiments also demonstrate the ways in which pathogenic micro-organisms have co-opted conserved signal transduction pathways to adapt to the environment of the infected host. Although neither of the two *C. neoformans* RAS genes is essential, the growth defect observed by *C. neoformans ras1 ras2* mutant strains is similar to the growth arrest of *Sac.
**C. neoformans** RAS1 and RAS2 genes

**Fig. 6.** RAS2 overexpression partially suppresses the ras1 mutant morphological defects. The ras1 mutant strain overexpressing the RAS2 gene (ras1 + RAS2, MWC28) and the wild-type strain overexpressing the RAS2 gene (WT + RAS2, MWC27) were incubated at 25 and 37 ºC for 48 h in YPD medium. Cells were microscopically analysed by Nomarski optics. An abnormal mother–daughter neck is indicated by an arrowhead. Bars, 20 µm.

C. cerevisiae ras1 ras2 double mutant strains. Together, these results suggest a conserved role of Ras proteins in regulating vegetative growth in fungi as diverse as basidiomycetes and ascomycetes. Inhibition of Ras protein function may offer novel targets for antifungal therapy. For example, farnesylation is required for proper function of Ras proteins. Therefore, drugs that block farnesyl transferase activity may be lethal to fungi.

We also observed a significant variation in transcriptional activity between the two C. neoformans RAS genes. The RAS2 gene encodes a functional protein, and spliced, polyadenylated RAS2 mRNA is detectable by reverse transcriptase-PCR. However, the transcriptional level of this gene is insufficient to be visualized by Northern analysis after incubation under several conditions. Additionally, mutation of the RAS2 gene results in no discernible mutant phenotype in vitro or in vivo in an animal model of cryptococcosis. Since even subtle defects in growth and differentiation often result in discernible effects on virulence, this result strongly argues that C. neoformans Ras2 does not play a major role in growth, morphological transitions or inducible virulence factor expression. When overexpressed, RAS2 is able to restore mating and high temperature growth of a ras1 mutant strain. These data are similar to Sac. cerevisiae which has two RAS genes that possess overlapping functions. In each organism, one of these genes is more highly transcribed and encodes the predominant Ras signalling element. Since overexpression of regulatory elements may result in non-physiological effects, we cannot yet establish whether the functions of Ras2 suggested in overexpression studies represents true shared activities of these two similar signalling molecules. However, the fact that all ras1 mutant phenotypes are suppressed at some level by RAS2 overexpression suggests that Ras2 is a functional Ras protein.

The two C. neoformans RAS genes may have arisen from individual gene duplication or by whole-genome duplication. The completed Sac. cerevisiae genome project has allowed detailed analysis of gene order and chromosomal organization. It is estimated that at least 15% of this yeast’s genome is composed of blocks of duplicated genes, and the patterns of duplication are consistent with an ancient genome duplication event followed by gene loss (Wolfe & Shields, 1997). The C.
neoformans genome project (Heitman et al., 1999) will help to determine if RAS1 and RAS2 lie in similar blocks of synten or if individual duplication of a RAS precursor gene is more likely. Other examples of potential gene duplication in C. neoformans include the cyclophilin A homologue genes CPA1 and CPA2 (Wang et al., 2001).

Although there are many shared features in Ras signalling in fungi as diverse as budding yeast and C. neoformans, there are also significant differences. The C. neoformans Ras1 protein plays a major role in allowing high temperature growth and is therefore required for this organism’s virulence potential. Although the RAS2 gene can partially suppress the ras1 mutant high temperature growth defect when overexpressed, we observed no evidence of a compensatory increase in RAS2 transcription after mutation of the RAS1 gene. Therefore, although these Ras proteins share a potentially redundant function, RAS2 does not appear to normally act in place of a dysfunctional Ras1. Ras2 may have evolved to simply provide a basal level of Ras function to allow efficient vegetative growth, but not an inducible Ras function sufficient for differentiation or growth at high temperature.

Our results do not support a model in which the two C. neoformans Ras proteins act in different steps of the same, linear signalling pathway since there is an additive effect on growth with mutation of both RAS1 and RAS2. Therefore, the physiological roles of these proteins are likely to be either in parallel signalling pathways or as redundant effectors of the same signalling event in a single pathway.

The morphological changes of the ras1 mutant strain are very similar to those observed in Sac. cerevisiae strains with temperature-sensitive mutations of the CDC42 gene (Adams et al., 1990). CDC42 encodes a p-like GTPase in both budding and fission yeasts that controls the activity of PAK kinases, playing a central role in determining cell polarity and morphogenesis. This protein appears to function downstream of Sac. cerevisiae Ras2 to regulate filamentous growth (Mösch et al., 1996). Recently, the Ras2 protein of Sac. cerevisiae was also demonstrated to be a primary regulator of actin cytoskeleton polarity. Similar to our findings in C. neoformans, mutation of the RAS2 gene in budding yeast resulted in a strain which was unable to grow at elevated temperatures and which displayed temperature-dependent depolarization of the actin cytoskeleton (Ho & Bretscher, 2001). Together, our observations and the findings in Sac. cerevisiae support a model of conserved Ras function in fungi to regulate cell polarity and actin localization.

In conclusion, the similarities in Ras function among divergent micro-organisms demonstrate that these signalling molecules play central roles in fungal growth and development. However, these pathways have also differentiated in pathogenic species to allow microbial adaptation and infection of mammalian hosts. Elucidation of the Ras activating signals and downstream effector molecules will provide further insights into the molecular mechanisms of microbial pathogenicity.

ACKNOWLEDGEMENTS

We thank P. Margolis and C. Yanofsky of Stanford University for providing the sequence of degenerate primers to amplify the RAS2 gene, and Tina J. Wilkins for assistance with manuscript preparation. This work was supported by NIAID K08 award AI01556 (J.A.A.), and NIAID R01 grant AI42159 (J.H.). This work was also supported by P01 award AI44975 from the NIAID to the Duke University Mycology Research Unit. A.A. is a Merck Young Investigator in Medical Mycology (IDSA). J.H. is a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology and an associate investigator of the Howard Hughes Medical Institute. G.C. and A.A. are Burroughs Wellcome Young Investigators in Molecular Pathogenic Mycology. M.W. is a Howard Hughes Medical Institute Medical Student Research Training Fellow.

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C. neoformans RAS1 and RAS2 genes


Received 22 June 2001; revised 4 September 2001; accepted 11 September 2001.