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

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The Ras1 signal transduction pathway controls the ability of the pathogenic fungus *Cryptococcus neoformans* to grow at high temperatures and to mate. A second *RAS* gene was identified in this organism. *RAS2* is expressed at a very low level compared to *RAS1*, and a *ras2* mutation caused no alterations in vegetative growth rate, differentiation or virulence factor expression. The *ras2* mutant strain was equally virulent to the wild-type strain in the murine inhalational model of cryptococcosis. Although a *ras1 ras2* double mutant strain is viable, mutation of both *RAS* genes results in a decreased growth rate at all temperatures compared to strains with either single mutation. Overexpression of the *RAS2* gene completely suppressed the *ras1* mutant mating defect and partially suppressed its high temperature growth defect. After prolonged incubation at a restrictive temperature, the *ras1* mutant demonstrated actin polarity defects that were also partially suppressed by *RAS2* overexpression. These studies indicate that the *C. neoformans* Ras1 and Ras2 proteins share overlapping functions, but also play distinct signalling roles. Our findings also suggest a mechanism by which Ras1 controls growth of this pathogenic fungus at 37 °C, supporting a conserved role for Ras homologues in microbial cellular differentiation, morphogenesis and virulence.

Keywords: microbial pathogenesis, fungi, yeast, actin, polarity

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

The GenBank accession number for the *RAS2* sequence of *C. neoformans* H99 is AF294349.

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 *ras1* 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 *ras1* 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 *ras1* mutant background suppressed *ras1* mutant phenotypes, indicating that the Ras1 and Ras2 proteins share some degree of functional redundancy. Analysis of the terminal phenotypes of the *ras1* 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 *ras1* 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 *ras1* mutant strain in which the wild-type *RAS1* gene has been reintroduced (Alspaugh *et al*., 2000). MWC12 and MWC13 are *ras2* mutant strains and MWC14, MWC15 and MWC16 are *ras1 ras2* double mutant strains all described in this study. Strain MWC17 was constructed by reintroducing the wild-type *RAS1* gene linked to the *hph* 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 *ras1* mutant strain LCC70 to create the *ras1*+*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 mating 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 1907 (5′-CTCGAGCTCGARTAYGAYACYATYG-3′) and primer 1908 (5′-CAGCTGCAGTAYTCYTCYTGRCCRGCRGTRTC-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 [^{32}P]dCTP (Amersham). A 2·6 kb *Sac*I fragment containing the entire *RAS2* genomic locus was isolated by screening a subgenomic library of *Sac*I 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′-CCTCAACCCACACCCACACC-3′) and 5067 (5′-GCCAACTTGATCTTCTTCACC-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 *ras2*Δ::*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′-CGAAGCCAACGTCCTCGCC-3′) and 4550 (5′-GCAGTAAGCGATCTTTGAACGATTCTACGAAGCGAGCGC-3′) (*URA5* sequence underlined); 3′ *RAS2* fragment primers 4551 (5′-CCCACCTCCTGGAGGCAAGCCGCGCAGTGCC-GTGTATTCC-3′) (*URA5* sequence underlined) and 4552 (5′-CCTCGAGATTCTCCACGCTGC-3′). *RAS2* sequence was added to the *URA5* gene by PCR using plasmid pCnTel as template (Edman, 1992) and the following primers: 4553 (5′-GCGCTCGCTTCGTAGAATCGTTCAAAGATCGCT-TACTGC-3′) and 4554 (5′-GGAATACACGGCAGTGCGCGGCTTGCCTCCAGGAGGTGGG-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, 50 °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 *ras2*Δ::*URA5* allele was obtained.

The linear, dideoxy-tailed *ras2*Δ::*URA5* 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 Toffaletti *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′-CCATCTCATCTCATCACAACAGG-3′) and the *URA5*-specific primer 5151 (5′-CGTCTTCTTCATCTAGTCGG-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 *ras2*Δ::*URA5* 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 *Eco*RI. 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 5×10^4 *C. neoformans* cells. Animals infected with pathogenic strains die of fulminant meningoencephalitis. Mice were sacrificed prior to death based on clinical end points previously correlated with mortality (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 10^8 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 GPD1* promoter linked to the *URA5* selectable marker, was obtained from Robert Davidson at Duke University. The *RAS2* gene was cloned into this vector and under control of the *GPD1* 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 *Sac*I 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 URA5* gene 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*::*URA5* 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

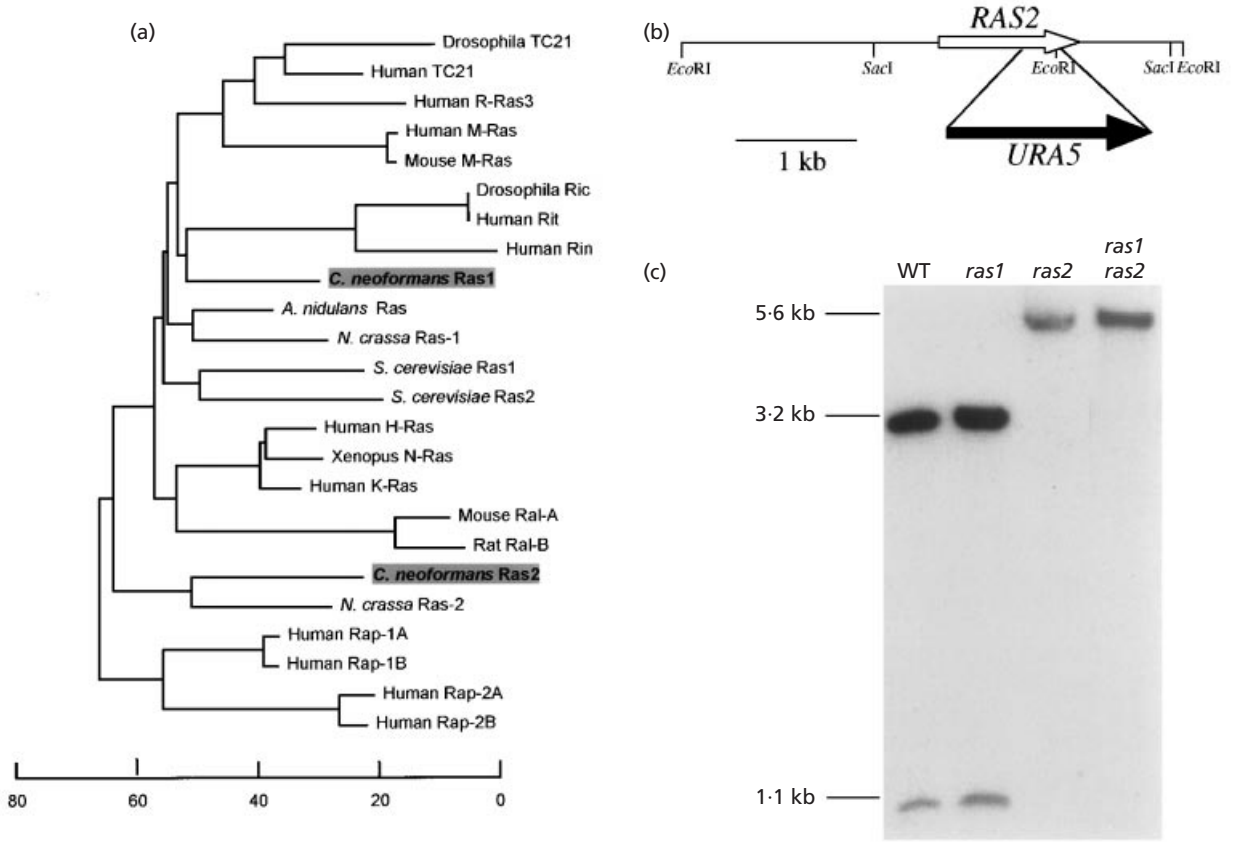

Fig. 1. Disruption of the *C. neoformans RAS2* gene. (a) The genetic relatedness of Ras superfamily proteins, including *C. neoformans* Ras1 and Ras2, is demonstrated by estimating evolutionary distance. (b) The *URA5* locus was cloned into the *RAS2* gene to create the *ras2*Δ::*URA5* disruption allele. (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 *Eco*RI 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 ($P = 0{\cdot}045$) 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

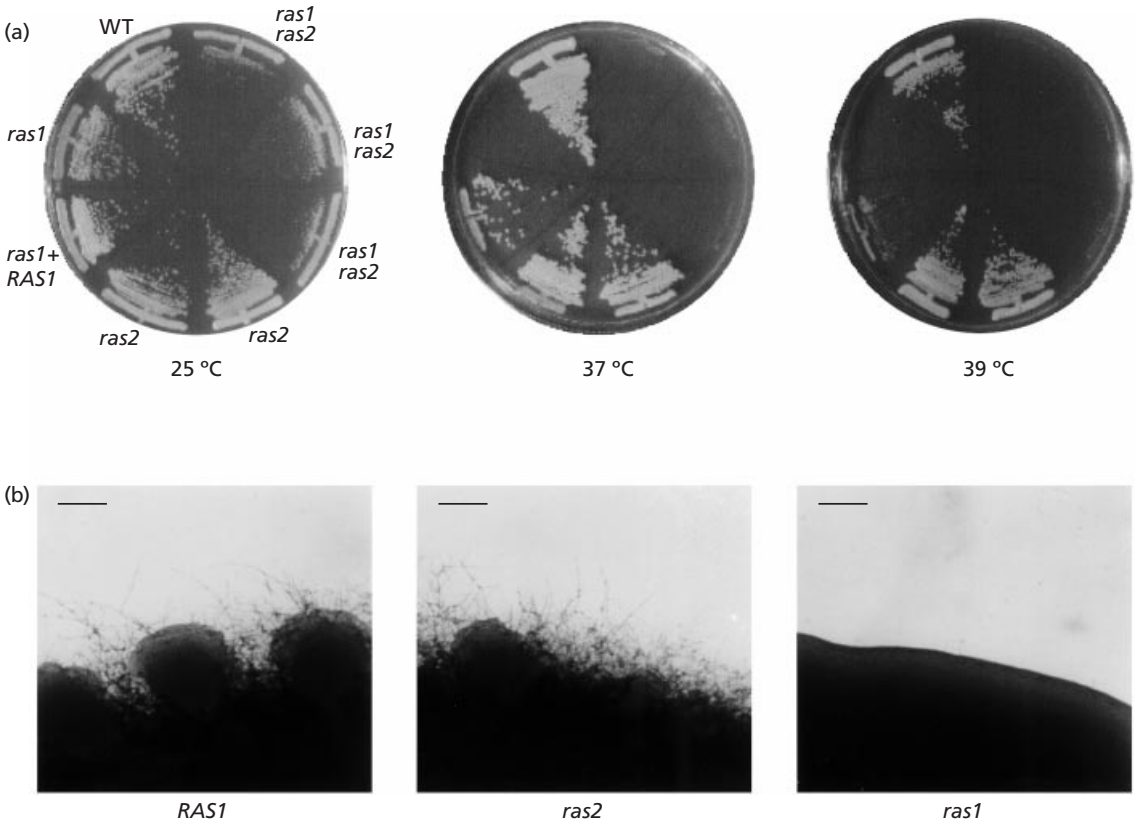

Fig. 2. Ras1 is the predominant Ras protein regulating growth at high temperature and mating of *C. neoformans*. (a) Wild-type (H99), *ras1* mutant (LCC1), *ras1*+*RAS1* reconstituted (LCC3), *ras2* mutant (MWC12, 13) and *ras1 ras2* double mutant (MWC14, 15, 16) strains were plated on YPD medium and 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.

synthetic effect of *ras1* and *ras2* mutation and not to a separate induced mutation.

Ras1 is the predominant Ras protein controlling *C. neoformans* mating and virulence

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 MAT**a** 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

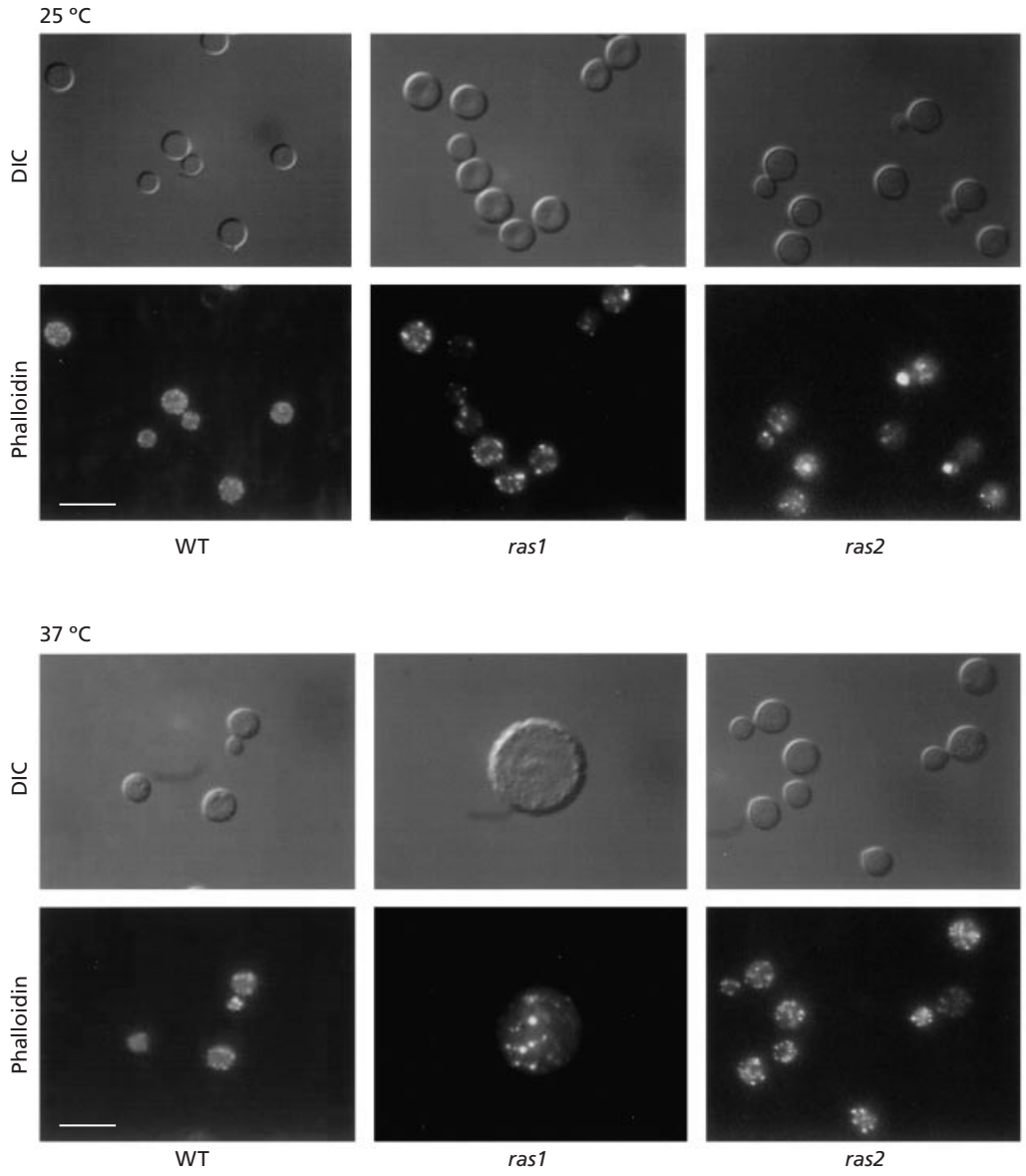

Fig. 3. Ras1 regulates actin polarization at 37 °C. Wild-type (H99), *ras1* mutant (LCC1) and *ras2* mutant (MWC12) strains were incubated at 25 and 37 °C in YPD medium for 48 h. Cells were examined by microscopy with Nomarski optics (DIC). F-actin localization was assessed by rhodamine-conjugated phalloidin staining. Bars, 20 μm.

the 60 d of the experiment ($P < 0{\cdot}001$). 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 ($P = 0{\cdot}113$). 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

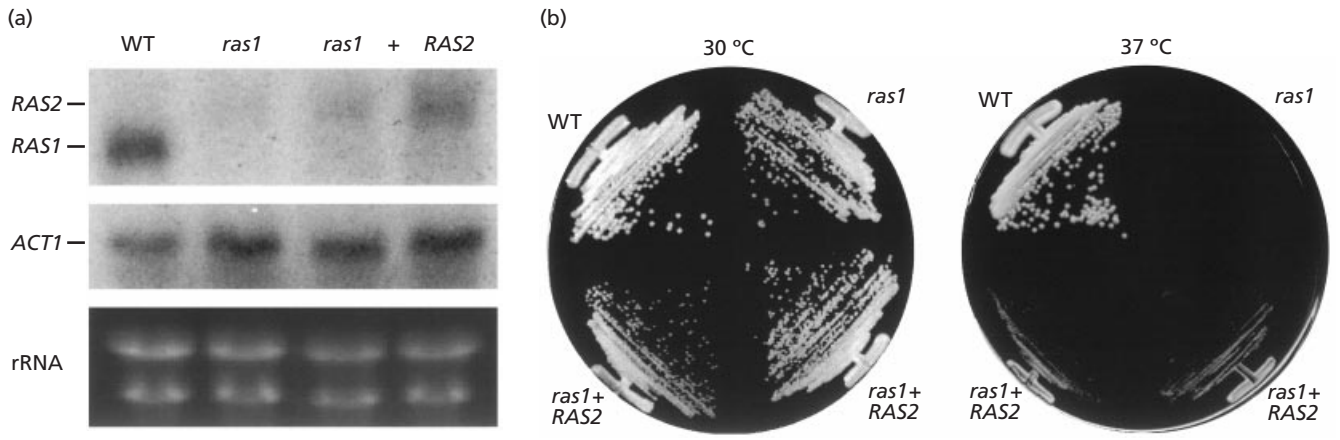

Fig. 4. *RAS2* overexpression partially suppresses the *ras1* mutant high-temperature growth defect. (a) Total RNA was extracted from exponential-phase cultures of the wild-type (H99), *ras1* mutant (LCC1) and *ras1*+*RAS2* mutant (MWC28, 29) strains incubated in YPD medium. Northern analysis was performed 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 were incubated on YPD medium for 48 h at 25 and 37 °C.

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 RAS*1 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 MAT**a** 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.

RAS2 overexpression partially suppressed the *ras1* mutant high temperature growth defect. The wild-type, *ras1* mutant and *ras1*+*RAS2* stains were incubated on YPD medium at 25 and 37 °C. In contrast to the *ras1* mutant strain in which no growth was apparent at

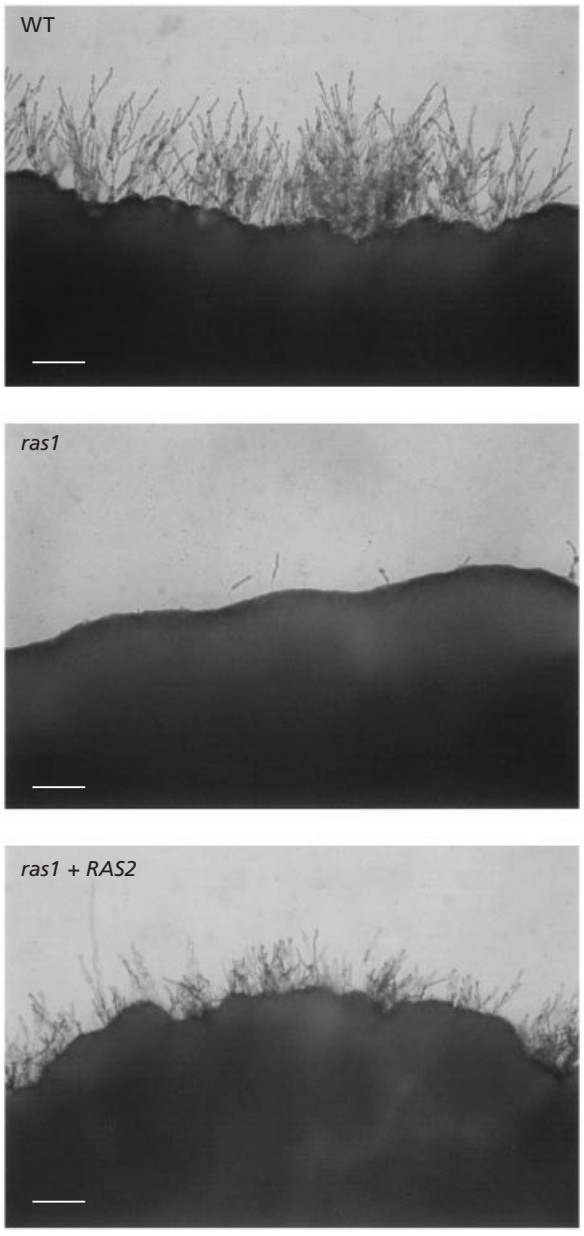

Fig. 5. *RAS2* overexpression suppresses the *ras1* mutant mating defect. Wild-type (H99), *ras1* mutant (LCC1) and *ras1*+*RAS2* strains (MWC28) were incubated in mating reactions with the serotype D MATa strain JEC20. The edges of the mating patches were microscopically assessed for mating filamentation after 2 d incubation. Bars 50 μm.

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 results 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.*

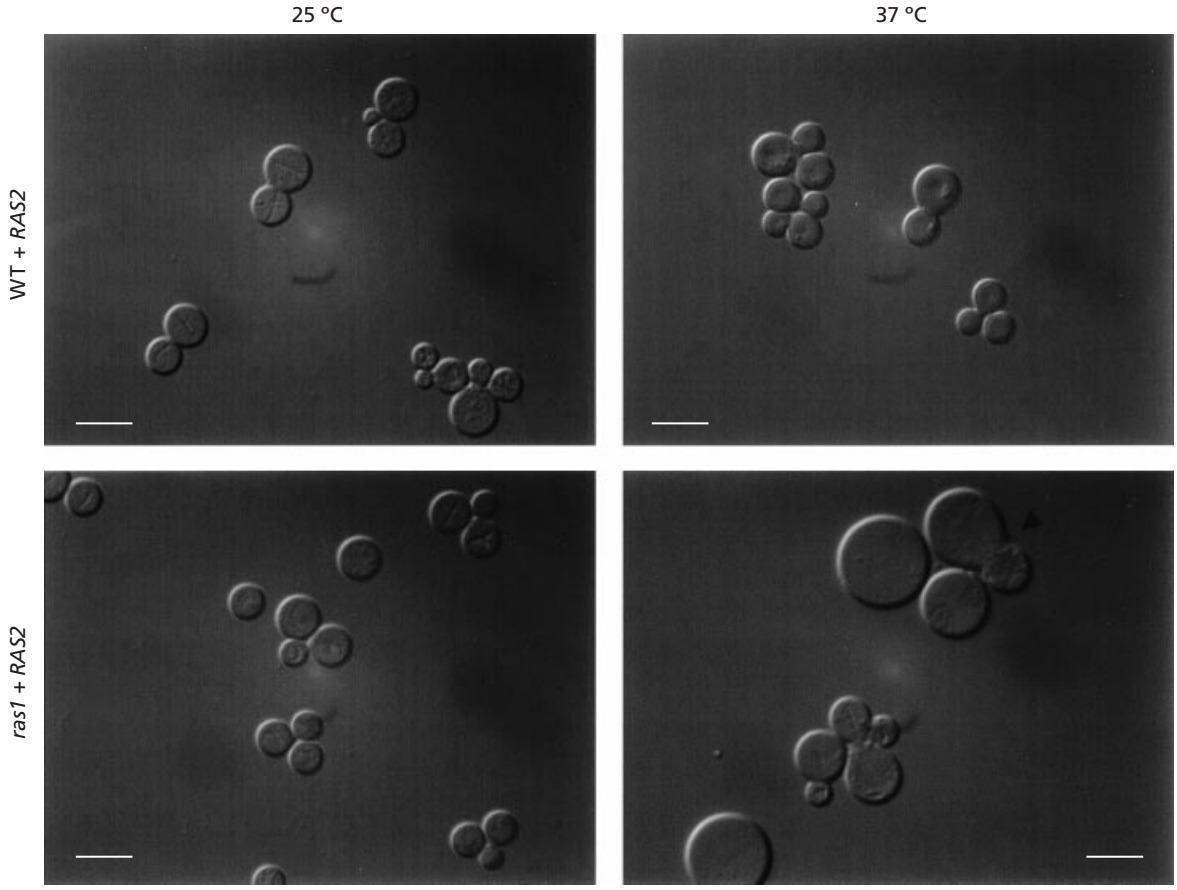

Fig. 6. *RAS2* overexpression partially suppresses the *ras1* mutant morphological defects. The *ras1* mutant strain overexpressing the *RAS2* gene (*ras1*+*RAS2*, MWC28) and the wild-type strain overexpressing the *RAS2* gene (WT+*RAS2*, MWC27) were incubated at 25 and 37 °C for 48 h in YPD medium.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

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 synteny 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 ρ-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.

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