A Rac Homolog Functions Downstream of Ras1 To Control Hyphal Differentiation and High-Temperature Growth in the Pathogenic Fungus Cryptococcus neoformans

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The Cryptococcus neoformans Ras1 protein serves as a central regulator for several signaling pathways. Ras1 controls the induction of the mating pheromone response cascade as well as a distinct signaling pathway that allows this pathogenic fungus to grow at human physiological temperature. To characterize elements of the Ras1-dependent high-temperature growth pathway, we performed a multicopy suppressor screen, identifying genes whose overexpression allows the ras1 mutant to grow at 37°C. Using this genetic technique, we identified a C. neoformans gene encoding a Rac homolog that suppresses multiple ras1 mutant phenotypes. Deletion of the RAC1 gene does not affect high-temperature growth. However, a rac1 mutant strain demonstrates a profound defect in haploid filamentation as well as attenuated mating. In a yeast two-hybrid assay, Rac1 physically interacts with the PAK kinase Ste20, which similarly regulates hyphal formation in this fungus. Similar to Rac1, overexpression of the STE20α gene also restores high-temperature growth to the ras1 mutant. These results support a model in which the small G protein Rac1 acts downstream of Ras proteins and coordinately with Ste20 to control high-temperature growth and cellular differentiation in this human fungal pathogen.

Ras proteins are highly conserved, small guanine-nucleotide binding proteins that control the activation of diverse signaling pathways. Among microorganisms, Ras proteins regulate fundamental and varied cellular processes such as morphological transitions, mating, cyclic AMP (cAMP) metabolism, and microbial pathogenesis (1, 12, 25, 40). In mammalian systems, Ras mutations are associated with a large percentage of malignancies (4), confirming the central roles that Ras proteins play in cellular differentiation.

Cryptococcus neoformans is an important opportunistic fungal pathogen in humans, most frequently causing disease in the lungs and central nervous systems of immunocompromised patients (29). This organism typically grows as a haploid yeast, but it can undergo hyphal differentiation in response to environmental stresses or when confronted with an appropriate mating partner (22, 46). Such differentiation events likely allow survival in harsh environmental conditions. Also, the spores produced during hyphal phases of growth may be the most important infectious propagule of this fungus (39).

Ras proteins are required for C. neoformans hyphal development. Activation of Ras signaling in C. neoformans results in an accelerated hyphal response. Additionally, strains with null mutations of the RAS1 gene are sterile, failing to undergo a hyphal transition when coincubated with a mating partner (1). The mating defects of the ras1 mutant are multifactorial. First, ras1 mutant strains fail to appropriately activate the components of the pheromone response pathway when incubated with a mating partner (44). Additionally, the ras1 mutants demonstrate a primary defect in filamentation, unable to form hyphae even when the pheromone response pathway is genetically activated (44). The morphological defects associated with a ras1 mutation are also evident in this strain’s inability to undergo haploid fruiting, a form of hyphal differentiation distinct from mating that occurs in response to starvation and desiccation (1, 44).

The C. neoformans Ras1 signaling pathway also controls the ability of this fungus to grow at 37°C. Strains with ras1 mutations are unable to grow at mammalian body temperatures and are accordingly avirulent in animal models of cryptococcosis (1, 43, 44). Additionally, the ras1 mutant strain demonstrates alterations in actin polarization at these higher temperatures; when incubated at 37°C, this strain arrests as large, unbudded cells with depolarized actin (43).

Similar temperature-dependent alterations of growth and actin polarization have also been observed in Saccharomyces cerevisiae ras2 mutants (15). At 35°C, these mutant yeast strains demonstrate temperature-dependent morphological changes as well as altered cellular localization of the polarity-directing proteins Myo2p and Cdc42p. Although Ras proteins are major activators of the cAMP pathway in yeast, activating cAMP/PKA signaling did not complement the ras2 mutant defects in actin polarization and high-temperature growth. These data suggest a cAMP-independent pathway by which Ras proteins control fungal cytoskeletal integrity and growth at elevated temperatures (15).

In both S. cerevisiae and C. neoformans, the cAMP-independent targets of Ras proteins that control these cellular processes are poorly defined. The recent availability of a completed and annotated genome for C. neoformans, as well as the increased molecular pliability of this pathogenic microorganism, allowed us to pursue multicopy suppressor analysis to...
**MATERIALS AND METHODS**

**Strains and media.** The strains used in this study are listed in Table 1 and are derived from the *C. neoformans* var. *neofor mans* (serotype D) congenic mating pair JEC20 and JEC21. The *MATa rac1* mutant (CBN1) was isolated by spore micromanipulation from a cross between MVC47 and JEC20. Standard yeast media was prepared as previously described (38). 5-Fluoroorotic acid (5-FOA) was added to media (1 g/liter) as indicated. V8 medium was used for all mating experiments (23). Filament agar was prepared as described previously (46). When indicated, 3-amino-1,2,4-triazole (3AT) was added to media at a concentration of 5 mM.

**Multicopy suppressor library.** The multicopy suppressor library was made as previously described (6, 11). Briefly, genomic DNA from the serotype D strain C21F2 (11) was partially digested with Sau3A, and genomic DNA was added to media (1 g/liter) as indicated. V8 medium was used for all mating experiments (23). Filament agar was prepared as described previously (46).

**Identification of downstream effectors of Ras1 involved in fungal growth at 37°C and cellular differentiation.**

**Overexpression of RAC1.** The *RAC1* gene was amplified by PCR using primers AA0489 (5’-CTGAGGATCTGATGGTACTTAAAGAATCT-3’), BamHI sequence underlined) and AA0572 (5’-CGTTAGCCCTGGTATGCGCTGCAAATC-3’) to generate a PCR product containing the BglII cloning site (12). The amplified product was cloned into the pGPD vector, pGPD-RAC1 (12), a plasmid vector containing a transcriptional control element corresponding to the GPD promoter. The resulting construct was validated by restriction analysis and verified by sequencing.

**Disruption of the RAC1 gene.** A rac1::URA5 mutant allele was constructed by PCR overlap extension as previously described (7), replacing the entire *RAC1* coding region with the *URA5* selectable marker. This allele was transformed into the *MATa una5* strain JEC34 by biolistic transformation (28). Transformants were selected on synthetic medium lacking uracil. Mutation of the *RAC1* locus was confirmed by PCR and Southern hybridization (see Results).

**Fusion and recombination assays.** Fusion assays were performed as described previously (1) with the following modifications. Mixtures of wild-type (JEC43 × JEC30) and rac1 (MVC39 × CBN5) mutant strains were cocultured on V8 mating medium. Equal numbers of cells were used for each cross. Each mating partner contained a complementing auxotrophic mutation (lys1 and una5) to allow selection of prototrophic fusion products. After 48 h, the mating mixes were excised, resuspended in 1 ml of H2O, and plated in serial dilutions onto minimal medium. Colonies were counted after 3 days of incubation.

**Microscopy.** Bright-field, differential interference microscopy (DIC), and fluorescent images were captured with a Zeiss Axioskop 2 Plus fluorescent microscope equipped with an AxiosCam MRM digital camera. Additional bright-field images were captured with a Nikon Eclipse E400 microscope equipped with a Nikon Coolpix 4500 digital camera. For filament staining, slide mounts were prepared as previously described (33). Briefly, wild-type and *rac1* crosses were prepared on V8 agar-coated slides and incubated for 3 days prior to processing. To visualize septa and DNA, the slide mounts were fixed in 70% ethanol, washed, and incubated in a 10-mM preparation of calcofluor (fluorescent brightener 28 F-3397; Sigma) at 40 μg/ml and a 5,000× dilution of a 0.5-mM solution of Sytox Green ( Molecular Probes) in phosphate-buffered saline for 20 min. Agar sections containing filaments were excised from each slide and mounted on a fresh microscope slide with 5 μl of antifade mixture containing 8 μg/ml DAPI (4',6-diamidino-2-phenylindole) (D-21490; Molecular Probes).

**Yeast two-hybrid experiments.** This yeast two-hybrid technique was used to explore physical interactions between *C. neoformans* proteins. *C. neoformans* cDNA sequences were PCR amplified and cloned into the yeast two-hybrid vectors pGAD424 and pGBT9 (Clontech). These plasmids were cotransformed into the *S. cerevisiae* strain P369-4A, and transformants were selected on synthetic medium lacking leucine and tryptophan to confirm the presence of both plasmids. Physical interaction between proteins encoded in these plasmid was tested by assessing restoration of adenine prototrophy, restoration of histidine prototrophy, and increased β-galactosidase activity. Yeast transformations and determination of β-galactosidase activity were performed as previously described (5).

**Molecular methods.** Genomic DNA was isolated from mycelial *C. neoformans* cells (36). For genomic Southern hybridizations, 20 μg of genomic DNA was digested with PvuI, electrophoresed, DNA transfer, hybridization, and autoradiography were performed as previously described (37). The cDNA sequence of the *RAC1* gene was determined using the Marathon cDNA amplification kit (Clontech). All PCRs were performed using a Technne Genius thermocycler with 50 ng of template DNA, 100 ng of each oligonucleotide primer, and standard reagents from a Takara kit (Takara Shuzo Co.). The PCR conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min for each kb of DNA amplified in the reaction.

**Nucleotide sequence accession number.** The *RAC1* gene sequence has been submitted to the NCBI database under accession number ATY78054.
growth at 37°C to the ras1 mutant strain. Ten thousand transformants in the MWC1 strain background were initially analyzed, representing approximately 4 to 5× genome equivalents, given an estimated genome size of 20 Mb (28).

In this initial screen, four transformants were isolated with a restored ability to grow at 37°C. When the strains were cured of the URA5-containing plasmid on a medium containing 5-FOA, they were no longer able to grow at this elevated temperature. Three of the plasmids contained overlapping C. neoformans genomic fragments, with 7,729 nucleotides in common. This genomic sequence contained two putative genes: a theoretical gene of unknown function and a gene encoding a Rac protein homolog. Rac proteins are highly conserved, Ras-like G proteins initially identified as substrates of C. botulinum Rac protein homolog. Rac proteins are highly conserved, Ras-like G proteins initially identified as substrates of C. botulinum toxin (9). In mammalian systems, Rac proteins are involved in maintaining the assembly and organization of the actin cytoskeleton (13, 34).

The C. neoformans RAC1 gene contains eight introns within the coding sequence and one intron upstream of the start codon, encoding a predicted protein of 198 amino acids. Similar to many of the proteins in the Ras superfamily, the Rac1 peptide sequence has a C-terminal CAAX motif (Cys-Leu-Val-Met), suggesting that this protein is prenylated and thereby associated with the plasma membrane (10).

In order to confirm that overexpression of the RAC1 gene was responsible for the suppression of ras1 mutant phenotypes, C. neoformans RAC1 was subcloned under control of the constitutive GPD promoter and retransformed into the ras1 mutant strain. The ras1+pGPD-RAC1 strain (CBN2) grew well at 25°C and 30°C and displayed no altered colony or cellular morphology on YPD medium. However, in contrast to the ras1 mutant strain, CBN2 was able to grow at 37°C (Fig. 1). Therefore, in accordance with the suppressor screen, specific overexpression of the RAC1 gene suppresses the ras1 high-temperature growth defect.

**RAC1 overexpression complements ras1 mutant hyphal defects.** When confronted with a mating partner, C. neoformans cells initiate a mating process involving cell fusion and hyphal differentiation in response to pheromone and nutrient signals. In the absence of a mating partner, this fungus can undergo a similar yeast-to-hyphal transition in response to extreme nutrient deprivation and desiccation, a process known as haploid fruiting, which is characterized by the formation of hyphae with unfused clamp connections, basidia, and mitotic spores (46). The ras1 mutant strain displays striking defects in both of these types of hyphal differentiation (1, 44).

Overexpression of the RAC1 gene suppresses the ras1 mutant mating defect. Microscopic inspection of JEC20 (MATα wild type) × CBN2 (MATα ras1+pGPD-RAC1) crosses revealed that mating filamentation was restored to wild-type levels (Fig. 1). These hyphae contained all of the characteristic structures of wild-type C. neoformans mating reactions, including fused clamp connections, basidia, and basidiospores. Thus, in addition to restoring growth at 37°C, RAC1 overexpression suppresses the mating defect of the ras1 mutant. These data support a model in which Rac1 functions downstream of Ras1 in the C. neoformans mating process.

**Deletion of the RAC1 gene results in altered hyphal differentiation.** To further investigate the biological roles of the Rac1 protein, we generated a strain with a rac1 null allele (Fig. 2). By using biolistic transformation, we replaced the native RAC1 locus with a rac1:URA5 mutant allele. Of 40 transformants, 24 were rac1 mutants. Each of these demonstrated identical phenotypes, and one of these strains (MVC47) was selected as a representative rac1 mutant for subsequent experiments.

In contrast to the ras1 mutant strain, the rac1 mutant demonstrated no temperature-sensitive growth defects at 30°C, 37°C, or 39°C. Also, the rac1 mutant strain’s cellular morphology was similar to that of the wild-type strain at all temperatures tested. The rac1 mutant was able to grow well on minimal media, indicating no auxotrophies associated with the gene mutation.

However, mutation of the rac1 gene was associated with a dramatic defect in hyphal differentiation. When incubated on the nutrient-poor filament agar, C. neoformans strains typically undergo a form of hyphal differentiation known as haploid fruiting (46). After 3 weeks of incubation on filament agar, no haploid fruiting was observed among the rac1 mutants. When incubated under identical conditions, wild-type strains displayed a consistent and reproducible hyphal transition (Fig. 3A). To ensure that the hyphal defect was due to the rac1 mutation, a wild-type copy of the RAC1 gene was ectopically introduced into the rac1 mutant to create a rac1+RAC1 reconstituted strain (MVC48). This strain underwent haploid fruiting in a manner indistinguishable from the wild type (Fig. 3A).

In addition to reduced haploid fruiting, mutation of the RAC1 gene also resulted in defective mating. Mating reactions in which MATα or MATα rac1 mutants were crossed with a wild-type strain demonstrated absolutely normal hyphal mating structures and basidiospores. However, bilateral mutant crosses, in which both partners were rac1 mutants, displayed markedly reduced mating filamentation (Fig. 3B). Mating was restored to wild-type levels in these crosses by reintroducing a wild-type RAC1 allele into either one of the rac1 mutant mating partners (Fig. 3B). These observations indicate that Rac1 is involved in the C. neoformans mating process and that the requirement for Rac1 activity is dose dependent; one copy of the RAC1 gene is sufficient for mating. These data contrast with the mating defects observed in the ras1 mutant strains in which a ras1 mutation in either of the parental strains results in profound reductions in mating (44).

**The rac1 mutation affects mating at several steps by altering hyphal development. (i) Pheromone production and response.** To determine the precise cellular mechanisms by which Rac1 influences mating, we divided the C. neoformans mating process into distinct steps. The first step involves the production of mating pheromone in response to nutrient deprivation and to a mating partner. This process was assessed using a confrontation assay, the microscopic analysis of the initial cellular response of MATα and MATα cells to strains of the opposite mating type (44). In response to an appropriate mating partner, MATα cells produce hyphal structures known as conjugation tubes. Unlike MATα cells, MATα cells produce few hyphae in confrontation assays, but they undergo a distinct morphological change in which they become larger and more refractile (44).

In contrast to wild-type MATα cells, MATα rac1 mutant cells did not produce conjugation tubes in response to either MATα
wild-type or rac1 strains (Fig. 3C and data not shown). However, MATa rac1 cells were able to induce cellular changes in MATa cells in confrontation, suggesting that this rac1 mutant produces α-pheromone. In contrast, the MATa rac1 mutant strain responded normally to either MATa wild-type or MATa rac1 mutant cells (Fig. 3C). These observations indicate that Rac1 does not regulate the production of pheromone in either MATa or MATa cells; however, Rac1 is required for the initial hyphal response of MATa cells to the MATa pheromone.

(ii) Fusion. After the initial pheromone response, MATa and MATa cells fuse to form a dikaryon. We hypothesized that the MATa rac1 mutant defect in the morphological response to pheromone would influence its ability to find and fuse with its mating partner. To determine if rac1 mutants have a defect in the cell fusion process, rac1 auxotrophic strains were generated to allow for the selection of prototrophic isolates formed by cell fusion. Crosses were incubated on mating medium for 48 h prior to plating onto selective medium. In this assay, the wild-type and the rac1 crosses produced equal numbers of prototrophic isolates, indicating that Rac1 is not required for cell fusion. However, the overall colony size of these diploid rac1 isolates was reduced compared to those resulting from wild-
type crosses. Therefore, Rac1 is not required for the vegetative growth of haploid C. neoformans yeast cells, but it may be required for the growth of the transient diploid phase. The normal cell fusion observed among rac1 mutants contrasts with ras1 mutant strains, which display a marked defect in cell fusion, even in unilateral crosses (1).

(iii) Dikaryotic hyphal development. After mating cell fusion, the dikaryotic cells undergo dramatic hyphal differentiation. Wild-type C. neoformans mating filaments consist of a series of hyphal cells separated by septa and containing fused clamp cells. These hyphal structures are markedly reduced in bilateral rac1 mutant crosses. Additionally, the few filaments that are produced in these crosses exhibit morphological defects. Initial microscopic observation of the filaments resulting from a bilateral rac1 cross demonstrated that the hyphal cells are shorter and thicker than those resulting from a wild-type cross (Fig. 4A). The filaments from the bilateral rac1 cross also contained more protrusions than wild-type filaments (Fig. 4A).

To further assess the morphological defect of rac1 filaments, wild-type and rac1 filament samples were costained with Sytox Green and calcofluor to visualize DNA and septa, respectively. C. neoformans mating filaments are dikaryotic (two nuclei per hyphal cell). Additionally, the associated clamp cell functions in mitosis to ensure that each resulting hyphal cell receives two nuclei: one MATα nucleus and one MATa nucleus. Sytox Green staining demonstrated that, for the most part, rac1 mating filament cells contained two nuclei (Fig. 4B, first panel). Calcofluor staining and DIC microscopy revealed that the clamp cells were abnormal in shape and did not appropriately fuse with the subapical filament cell (Fig. 4B, first and second panels).

In wild-type mating filaments, polarized (new growth) occurs primarily at the tip of the leading cell and clamp cell. However, new growth can also occur from subapical clamp cells to generate secondary filaments or branches (21). Polarized growth is characterized by the accumulation of cortical actin patches. In C. neoformans, cortical actin patches localize throughout the filament but concentrate at the growing tip of the filament and at the tips of clamp cells (33). To determine if actin polarization is abnormal in the rac1 filaments, wild-type and rac1 mating hyphae were visualized with rhodamine-conjugated phalloidin. In both wild-type and rac1 filaments, actin patches concentrated in areas of polarized growth (Fig. 4B, third panel). Thus, the defects in clamp cell morphogenesis observed in rac1 mating filaments are not due to improper actin localization.

(iv) Basidium formation, meiosis, and sporulation. The tips of maturing mating hyphae differentiate into flask-shaped basidia, the site of meiosis and spore formation. Microscopic examination revealed that the abnormal rac1 mating filaments produced wild-type-appearing basidia and basidiospores, suggesting that the predominant defect in the rac1 mating process is in hyphal differentiation (Fig. 4A, insets).

To confirm that the basidiospores produced by rac1 bilateral crosses were viable, we measured the production of recombinant meiotic progeny in rac1 bilateral crosses (1). Wild-type and rac1 bilateral crosses were incubated for 7 days on mating medium, excised, and plated onto selective medium. Each strain was genetically marked to allow for the selection of recombinant progeny. We were able to recover recombinant progeny from both the wild-type and from the bilateral rac1 crosses. Therefore, although the overall production of rac1 filaments was reduced and the filaments exhibited morphological abnormalities, the Rac1 protein is not required for a complete sexual cycle in C. neoformans. These results contrast greatly to crosses involving ras1 mutants, which result in profound decreases in mating competence and recombinant spore production (44). Ras1 is required both for mating pheromone production and for hyphal differentiation. In contrast, the Rac1 protein plays its major role in the C. neoformans mating process by influencing hyphal formation but not in regulating pheromone activity.

Rac1 interacts with the Ste20 PAK kinase in the yeast two-hybrid system. PAK kinases are signaling molecules that often function downstream of Ras proteins and Rho-like GTPases. In C. neoformans, three PAK kinases have been identified and play distinct roles in mating and cellular differentiation (33). The genes encoding two of these proteins, Ste20α and Ste20α, are present in the mating type loci of MATa and MATα strains, respectively. The Ste20 proteins play an important role in haploid fruiting and mating. Additionally, these PAK kinases are required for high-temperature growth and for full virulence of serotype A strains of C. neoformans (42). These proteins also

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**FIG. 2.** Southern hybridization confirms RAC1 gene deletion. (A) A rac1::URA5 mutant allele was made by replacing the entire RAC1 coding sequence with the URA5 selectable marker. (B) Genomic DNA was obtained from the wild-type (WT) (JEC43), rac1 mutant (MVC47), and rac1::URA5 reconstituted strain (MVC48), digested with Pvul, and analyzed by Southern hybridization using two probes as indicated in panel A.
FIG. 3. Rac1 is required for hyphal differentiation. (A) Haploid fruiting. The RAC1 wild-type, rac1 mutant, and rac1+RAC1 reconstituted strains were incubated for 3 weeks on filament agar. The edges of the culture patches were photographed to assess for filamentation due to haploid fruiting (40×). (B) Mating. The effect of a rac1 mutation on the mating process was assessed by coculturing MATα and MATa strains on V8 mating medium. Each strain possessed either a wild-type (RAC1) or a mutant (rac1) allele. The edges of the mating patches were assessed at 7 days for mating filamentation (100×). (C) Pheromone-induced hyphal formation. MATα and MATa strains were streaked on filament agar in close proximity to one another without physically touching. The cellular response to diffusible pheromone was assessed in this confrontation assay at 48 h (100×).
contain an N-terminal consensus motif for the binding of Rac and Cdc42 proteins. This CRIB (Cdc42/Rac-interacting and binding) domain exerts an autoinhibitory effect on kinase activity, which is released by the binding of the Rac or Cdc42 proteins (3).

To determine whether Rac1 binds Ste20α in C. neoformans, we cloned the cDNAs corresponding to these and other related signaling molecules into the yeast two-hybrid vectors pGAD424 and pGBT9 (Clontech). Yeast strains cotransformed with C. neoformans CDC42 and STE20α demonstrated physical interaction between these two signaling molecules by restoration of adenine and histidine prototrophy and by induction of β-galactosidase activity, as previously described (42). C. neoformans Rac1 and Ste20α demonstrated no evidence of physical interaction when the wild-type alleles were tested. However, the introduction of a dominant activating mutation into the RAC1 gene resulted in physical interaction between the Rac1-G15V mutant protein and Ste20α, as assessed by this technique (Fig. 5). This result is consistent with a similar observation in the basidiomycete fungus Ustilago maydis, in which a dominant active Rac protein demonstrated evidence of interaction with PAK kinases in a two-hybrid assay (26). The finding that Ste20α interacts with a dominant active form of Rac1 and not the native protein suggests two things. First, the Ste20α PAK kinase acts directly downstream of Rac1 in a signal transduction pathway. Second, these proteins likely only interact when Rac1 is activated by upstream signals.

**Genetic interactions between RAC1 and STE20.** Similar to Rac1, the Ste20 PAK kinase is required for proper filament morphogenesis during C. neoformans mating. Ste20 is required to maintain polarity during hyphal development, and dikaryotic filaments lacking Ste20 become highly branched due to a tip-splitting defect (33).

To further analyze the genetic relationship between STE20 and RAC1, we examined the morphology of filaments produced from crosses of strains with mutations in one or both of these genes. Similar to unilateral rac1 crosses, ste20 mutants crossed with wild-type strains produce mating hyphae with wild-type morphology. However, due to a defect in maintaining tip polarity, the filaments produced in bilateral ste20 crosses are short and highly branched (33). The mating hyphae in crosses between two rac1 mutant strains are also shorter, thicker, and more highly branched than those of wild-type strains. However, they do not exhibit the tip-splitting defect of the ste20 mutant hyphae. Instead, the additional branches and protrusions of the rac1 hyphae appear to be the result of deformed clamp cells (Fig. 4).

A single copy of STE20 and RAC1 among either of the crossed strains is sufficient to support wild-type mating. Mating reactions involving MATa ste20α × MATa rac1, MATα ste20α rac1 × MATa, and MATα ste20α rac1 × MATa produced completely wild-type mating filaments (Fig. 6 and data not shown). Moreover, the filaments produced in a MATα ste20α rac1 × MATa rac1 cross exhibit a rac1 phenotype, with reduced numbers of hyphae that were shorter and thicker than those of

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**FIG. 4.** Altered morphology of the rac1 mutant mating hyphae. (A) Wild-type and bilateral rac1 crosses were incubated on V8 mating medium for 7 days. Areas at the edge of the mating reaction containing filaments were photographed at 200× magnification with DIC optics to visualize filaments, basidia (inset), and basidiospores (inset). Bar, 10 μm. (B) Bilateral wild-type and rac1 crosses were grown on V8 agar-coated glass slides. After incubation, filaments were fixed and costained with calcofluor and Sytox Green to visualize septa, clamp cell junctions, and DNA (first panel). Filaments and clamp cells were visualized using DIC optics (second panel). Wild-type clamp cells are fused (arrow) while rac1 clamp cells are unfused (*). Wild-type and bilateral rac1 filaments were also costained with rhodamine-conjugated phalloidin and DAPI to visualize actin and DNA (third panel). The fourth panel depicts the corresponding DIC images. Magnification, 1,000×. Bar, 10 μm.

| Interaction | Medium                  | Interaction | Medium                  |
|-------------|-------------------------|-------------|-------------------------|
|             | SD−Leu−Trp              | SD−Leu−Trp−Ade                   |
| Ste20 + Cdc42 | +                      | SD−Leu−Trp−Ade                   |
| Ste20 + Rac1  | +                      | SD−Leu−Trp−Ade                   |
| Ste20 + Rac1 G15V | +                  | SD−Leu−Trp−Ade                   |
| Cdc42 + Rac1 | +                      | SD−Leu−Trp−Ade                   |
| Cdc42 + Rac1 G15V | +                  | SD−Leu−Trp−Ade                   |
| pGAD424 + pGBT9 | +                      | SD−Leu−Trp−Ade                   |

Leu = leucine, Trp = tryptophan, Ade = adenine, His = histidine, 3AT = 3-amino-1,2,4-triazole, n/a = not evaluated.
the wild type. Therefore, a single copy of the \textit{STE20} gene in one of the mating partners was able to provide adequate Ste20 protein function for its role in mating hyphal development. Similarly, the filaments produced by a \textit{MATa ste20a rac1} \times \textit{MATa ste20a} cross exhibit a \textit{ste20} phenotype, with short and highly branched hyphae (Fig. 6). When both mating partners were \textit{rac1 ste20} double mutants, the resulting hyphae were indistinguishable from those produced in a bilateral \textit{ste20} mutant cross (Fig. 6). Therefore, the \textit{ste20} mutation is epistatic to \textit{rac1} in mating filament morphogenesis.

Overexpression of the \textit{STE20} gene suppresses the \textit{ras1}, but not the \textit{rac1}, mutant phenotypes. \textit{STE20} overexpression did not suppress the mutant mating defect of the \textit{rac1} mutant. Plasmid pCBN19, which contains the \textit{STE20} gene under control of the constitutive \textit{GPD} promoter and the \textit{URA5} selectable marker (33), was transformed into a \textit{MATa rac1 ura5} mutant strain. When several independent transformants were crossed with a \textit{MATa rac1} strain, the resulting mating hyphal morphology was identical to that from bilateral \textit{rac1} mutant crosses. Similarly, introduction of the \textit{pGPD-RAC1} overexpression transgene (pCN3) into a \textit{ste20} mutant strain failed to restore normal mating morphology in a \textit{ste20} bilateral cross.

In contrast, overexpression of the \textit{STE20} gene suppressed multiple \textit{ras1} mutant phenotypes. Plasmid pCBN19 (33) was transformed with the \textit{ras1 ura5} mutant strain MWC1. Unlike \textit{ras1} strains transformed with empty vector, the \textit{ras1} strains overexpressing \textit{STE20} grew well at 37°C (Fig. 7). Similarly, \textit{STE20} overexpression suppressed the \textit{ras1} mutant mating defect (Fig. 7).

**DISCUSSION**

In mammalian cells, the Rho family of G proteins controls the organization of the actin cytoskeleton (14). For example, Rho, Rac, and Cdc42 proteins play complementary roles in adhesion to extracellular surfaces and in cellular migration. Rac proteins, in particular, regulate the polymerization of actin at the cell periphery, which is important for mammalian cell protrusion and migration (14).

Proteins that control mammalian cell morphology, motility, and malignant transformation by regulating cytoskeletal architecture may serve analogous roles in microorganisms. Major reorganization of actin polymerization must occur for microbial cells to undergo morphological transitions. Additionally, altered actin cytoskeleton function can result in dysfunctional growth and cell division among microorganisms. In fungi, the transition from the yeast to hyphal state requires actin reorientation and represents an important way in which these cells adapt to new environments. For example, \textit{S. cerevisiae} changes its morphology from a yeast to a pseudohyphal form in response to extreme nutrient deprivation, perhaps as a way to forage for a better growth environment (12). Pathogenic fungi such as \textit{Candida albicans} must be able to switch between yeast and hyphal forms to survive in the various tissues of the infected host. Mutant \textit{C. albicans} strains that are unable to alter their cellular morphology in this way are not virulent in animal models of candidiasis (27). Therefore, those proteins that control cytoskeletal organization likely play major roles in microbial adaptation to new environments and in pathogenicity.

We have previously demonstrated that Ras proteins are required for actin polarization and normal cell division in the pathogenic yeast \textit{C. neoformans}, especially at elevated temperatures (1, 43). In these studies, we have identified the gene encoding a Rac protein in a genetic screen for downstream effectors of \textit{C. neoformans} \textit{Ras1}. Overexpression of the \textit{RAC1} gene restores mating competence and 37°C growth to a \textit{ras1} mutant strain. Since Rac proteins play similar roles in mammalian cells, they are plausible downstream effectors of Ras to control fungal cytoskeletal architecture.

Several lines of evidence, however, suggest that \textit{C. neoformans} \textit{Rac1} works with other, similar proteins to coordinate...
normal cytoskeletal function. For example, even though \textit{RAC1} overexpression suppresses the high-temperature growth defect in a \textit{ras1} mutant background, \textit{rac1} mutant strains grow well at elevated temperatures. Additionally, the yeast forms of the \textit{rac1} mutant cells display no obvious defects in actin polarization. Lastly, even though the hyphal transition in \textit{C. neoformans rac1} mutant strains is greatly reduced, those hyphal cells that are observed also have normal actin staining patterns. These results suggest that other proteins are able to play complementary or redundant roles with Rac1 as a downstream target of Ras.

Two genes encoding homologs of the Cdc42 GTPase have been identified in \textit{C. neoformans}. These proteins are highly similar to Rac1. Interestingly, both \textit{C. neoformans} Rac1 and one of the Cdc42 homologs interact with Ste20\textalpha in the yeast two-hybrid system; therefore, these two G proteins may perform similar functions to regulate Ste20\textalpha function. If so, residual Cdc42 activity in a \textit{rac1} mutant may explain why this strain does not itself have a defect in growth at 37°C. Our preliminary studies suggest that the CDC42 homolog genes may be essential in \textit{C. neoformans}. Therefore, alternative strategies other than gene disruption may be required to further

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\centering
\includegraphics[width=\textwidth]{figure7}
\caption{\textit{STE20a} overexpression complements the \textit{ras1} mutant high-temperature growth and mating defects. (A) The wild-type (WT) strain (JEC21), \textit{ras1} mutant (MWC8), \textit{ras1} strain containing the empty pRCD83 plasmid (\textit{ras1}+ vector), and \textit{ras1} mutant overexpressing the \textit{STE20a} gene (\textit{ras1}+ \textit{STE20a}) were incubated on YPD medium at 30°C or 37°C for 48 h. (B) The wild-type strain (JEC21), \textit{ras1} strain containing the empty pRCD83 plasmid (\textit{ras1}+ vector), and \textit{ras1} mutant overexpressing the \textit{STE20a} gene (\textit{ras1}+ \textit{STE20a}) were co-incubated with the MAT\textalpha strain JEC20 in mating reactions on V8 medium. After 7 days of incubation, the edges of the mating patches were assessed for mating filamentation and photographed (40×).}
\end{figure*}
elucidate functional interactions among this family of G proteins. However, these related proteins are likely to play interacting, and potentially redundant, roles in cytoskeletal organization.

Many fungal species that grow primarily as yeasts, such as S. cerevisiae, do not possess RAC genes, suggesting that Rac proteins play a primary role in fungi to control hyphal development. In contrast, the dimorphic fungus Yarrowia lipolytica has a RAC gene, YLRAC1, that is required for hyphal growth (16). Additionally, as this fungus transitions from the yeast to hyphal form, YLRAC1 transcript levels increase. Interestingly, strains with mutations in this gene are able to mate, and they demonstrate normal actin localization and polarization (16).

A gene encoding a Rac homolog has also been identified and disrupted in the dimorphic human fungal pathogen Penicillium marneffei (2). Deletion of the cflB (rac) gene results in severe disruption of proper actin polarization such that growth is altered in both the conidiophore and hyphal states (2). Together these results suggest that Rac proteins have been specifically adapted for dramatic morphological changes, such as those required for mammalian cell motility or for the yeast-hyphal transition in fungi. However, the precise mechanisms by which Rac proteins regulate cytoskeletal organization and actin polarization may differ between species.

Ras and Rac function coordinately in mammalian cells. Several lines of investigation demonstrate that Ras and Rac proteins act together to coordinate cellular functions. For example, the Ras-mediated oncogenic transformation of cultured mammalian cells requires the activation of Rac and Rho proteins. Dominant negative mutations in either Rac1 or RhoA reduce the ability of oncogenic Ras mutations to induce the transformation of cultured NIH 3T3 fibroblasts. Moreover, dominant activating mutations in Rac1 and RhoA enhance the transforming ability of Ras (20).

Many investigators have suggested potential mechanisms by which Ras and Rac proteins might mediate oncogenic transformation. Ras and Rac increase the production of reactive oxygen intermediates such as superoxide. These molecular species can in turn damage DNA and induce mutations leading to Ras-mediated transformation (20). Even though the Rac1 and Ste20 proteins may physically interact, our results are not consistent with these proteins merely acting in a simple, linear signaling pathway to control cytoskeletal organization in C. neoformans. The phenotypes of mating hyphae resulting from bilateral rac1 crosses and bilateral ste20 crosses are quite distinct. Additionally, the ste20-like hyphae resulting from a bilateral cross of rac1 ste20 double mutants suggests that the Ste20 PAK kinase plays a more substantial role in mating hyphal morphology than Rac1. The apparent complexity of these molecular and phenotypic interactions further suggests that the Rho-like GTPases (Rac, Cdc42) and PAK kinases ( Pak1, Ste20a, Ste20c) play distinct, coordinated roles to regulate cell morphology.

One of the main ways in which Rac proteins may act is by recruiting other proteins to cell membranes. Therefore, simply overexpressing downstream targets of Rac may not result in the proper localization of these proteins required for activation of downstream effectors. This may explain our observation that overexpression of Ste20a fails to restore filamentous differentiation to a rac1 mutant. We are currently defining the cellular localization patterns of the Rac and PAK kinase proteins to explore this possibility.

In conclusion, these studies further define the complex ways in which microbial cells respond and adapt to their surroundings. Fungi such as C. neoformans must be able to precisely regulate their morphology, whether in a specific host tissue or in the external environment. Many of these morphological...
events are driven by cytoskeleton changes controlled by Ras, Rho-like G proteins, and PAK kinases. In addition to elucidating mechanisms of microbial morphogenesis, a better understanding of the molecular interaction of these conserved signaling proteins will also demonstrate the central roles that they play in mammalian cellular adherence, motility, and malignant transformation.

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