A Ric8/Synembryn Homolog Promotes Gpa1 and Gpa2 Activation To Respectively Regulate Cyclic AMP and Pheromone Signaling in Cryptococcus neoformans

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The G protein α subunits Gpa1, Gpa2, and Gpa3 mediate signal transduction and are important in the growth and virulence of Cryptococcus neoformans. To understand how Gpa1 functions without a conventional Gβ subunit, we characterized a resistance to inhibitors of cholinesterase 8 (Ric8) homolog from C. neoformans, which shares amino acid sequence homology with other Ric8 proteins that exhibit guanine nucleotide exchange factor (GEF) activity toward Ga. We found that the ric8 mutant was reduced in capsule size and melanin formation, which could be suppressed by cyclic AMP (cAMP) supplementation or by introducing the activated GPA1Q284L allele. Consistent with the fact that Ric8 participates in cAMP signaling to regulate virulence, the ric8 mutant was attenuated in virulence toward mice. Interestingly, disruption of RIC8 also resulted in opposing effects on pheromone signaling, as the ric8 mutant showed reduced mating but an enhanced ability to induce the pheromone response in the mating partner. To identify Ric8 functional mechanisms, we examined the interactions between Ric8 and the three Gα proteins. Ric8 interacted with Gpa1 and Gpa2, but not Gpa3. The presence of Gpa1Q284L negatively affected its interaction with Ric8, whereas the activated Gpa2Q203L allele abolished the interaction. Collectively, these findings suggest that Ric8 functions as a GEF to facilitate the activation of Gpa1-cAMP signaling and to promote Gpa2, affecting mating efficiency. Our study highlights the distinct and conserved characteristics associated with G protein signaling and contributes to our overall understanding of how G protein α subunits function with or without a canonical Gβ partner in C. neoformans.

Cryptococcus neoformans is an opportunistic fungal pathogen of global importance, because cryptococcosis annually afflicts nearly 1 million people, resulting in approximately 600,000 deaths worldwide (1). C. neoformans is normally soilborne, and desiccated yeast cells or spores produced by sexual reproduction are thought to be the main propagules of infection through pulmonary inhalation (2, 3). Although many of the disease mechanisms remain obscure, the abilities of C. neoformans to resist harsh environmental conditions, grow at body temperature, and produce the polysaccharide capsule and the melanin pigment are thought to be the main contributors to virulence (4, 5).

Signal transduction pathways play critical roles in the adaptation of fungi such as C. neoformans to different living conditions, including those in a mammalian host, and proliferation. Canonical heterotrimeric GTP-binding proteins, consisting of Ga, Gβ, and Gγ, regulate a myriad of cellular functions, such as growth, sexual and asexual reproduction, development, and virulence, in pathogenic fungi (6–8). In C. neoformans, there are at least two distinct G protein signal transduction pathways. The cyclic AMP (cAMP)-dependent signaling pathway consists of the Gα protein Gpa1, the adenyl cyclase Cac1, the protein kinase A catalytic subunit Pka1 and regulatory subunit Pkr1, the adenyl cyclase-associated protein Acal, and the phosphodiesterases Pde1 and Pde2 (9–12). The second pathway consists of the Gβ protein Gpb1, which couples with either Gpg1 or Gpg2 as a heterodimer to complex with the Gα protein Gpa2 as the typical heterotrimeric complex to govern the pheromone-responsive mating pathway (13, 14). In C. neoformans, cAMP signaling is also closely linked to the production of virulence factors, such as the melanin pigment and the capsule. On the other hand, mating is generally not considered to be closely tied to virulence despite the observation that the basidiospores from sexual reproduction might be more infectious (3).

The G protein signaling pathway is thought to be triggered by the binding of a ligand to the specific G protein-coupled receptor (GPCR) that results in conformational changes of Ga and the exchange of GDP for GTP. The GTP-bound Ga then dissociates from the Gβγ heterodimeric complex. The activated Ga will then resume the inactive GDP-bound state upon GTP hydrolysis, and further signaling is blocked by Ga reassociation with Gβγ (15). There are varieties of accessory proteins that can regulate the activities of heterotrimeric G proteins. Regulators of G protein signaling (RGS) can stimulate the GTPase activity of Ga to inactivate Ga. Guanine nucleotide dissociation inhibitors (GDI) can stabilize GDP-Ga to inhibit the nucleotide exchange for GTP binding, whereas guanine nucleotide exchange factors (GEF) can facilitate the GDP-to-GTP nucleotide exchange to promote Ga activation.

The Ric8 (resistance to inhibitors of cholinesterase 8) protein, also known as synembryn, was first identified in the nematode Caenorhabditis elegans as a noncanonical GEF that exhibits GEF activity toward Ga in either a receptor-dependent or -independent manner (16). Ric8 is also involved in asymmetric cell division...
During early embryogenesis of *C. elegans* and in the neural progenitor of the fruit fly *Drosophila melanogaster* (17, 18). Two Ric8 isofoms are found in humans: Ric8A and Ric8B. Ric8A, identified using the Goα protein as bait to look for interactive partners through a yeast two-hybrid (Y2H) screen (19), preferentially interacts with GDP-bound Goα to facilitate GDP release and forms a complex with Goα until Goα binds to GTP (20). Ric8A also functions as a chaperone protein to fold nascent Goα subunits (21), and it prevents ubiquitination and degradation of Goα2 and Gqα (22). Ric8B was found to be involved in receptor-dependent signaling processes, because it was able to stimulate cAMP production only after GPCR-Goα/olf was stimulated first (23), and translocation of Ric8B to the plasma membrane is observed only after GPCR stimulation (19). Ric8 was also identified in *Dictyostelium discoideum*, where it functions as a receptor-independent GEF for Goα to amplify signaling amplitude (24). Ric8 homologs have also been found to perform various regulatory functions in several fungal organisms. In *Neurospora crassa*, disruption of the *Ric8* gene results in a phenotype similar to that of Goα *gna1* and Goα *gna3* mutants (25), and expression of a dominant-active GNA3 in the *ric8* deletion mutant leads to a significant increase in conidial germination (26). Deletion of the *ric8* gene (a *Ric8* homolog) leads to impaired colony growth and total or partial loss of asexual sporulation in *Aspergillus nidulans* and *Aspergillus fumigatus*, respectively (27). In the rice blast fungus *Magnaporthe oryzae*, MoRic8, a Ric8 homolog, interacts with the Goα protein MoMAGB to regulate appressorial differentiation (28). Interestingly, no Ric8 homologs have been found in any plant species or in the budding yeast *Saccharomyces cerevisiae*.

Previous studies identified three Goα proteins (Gpa1, Gpa2, and Gpa3), one Goβ protein (Gpb1), and two Gγ proteins (Gpg1 and Gpg2) in *C. neoformans* (13, 14, 29, 30). Several RGS proteins, including Crg1 and Crg2, have also been described (31, 32). While Gpb1 couples only with Gpa2, no conventional Goβ protein that couples with Gpa1 has been found. The identification of Ggb2 as a noncanonical Goβ coupling with Gpa1 reveals one of the activation mechanisms for Gpa1. In a search through the Y2H screen for additional proteins that interact with Gpa1 and function in Gpa1 signaling, we examined four candidates, including a Ric8 homolog and three members of the Rho family GTPases (Cdc42, Cdc42o, and a Ran1 homolog). In this study, we characterized the Ric8 protein as a potential GEF of Goα proteins in either cAMP signaling that controls virulence or pheromone signaling affecting mating in *C. neoformans*.

**MATERIALS AND METHODS**

**Strains and media.** The *C. neoformans* var. *grubii* archetype H99 (MATα) and the near-congeneric KN99a (MATα) strains were used as parental strains in this study (Table 1) (33, 34).

**Yeast extract-peptone-dextrose (YPD), synthetic medium (SD), 10% V8 agar (pH 5.0) for mating, synthetic low ammonium dextrose (SLAD) medium for the confrontation assay, Nigereagard for melamin production, and Dulbecco’s modified Eagle’s medium (DMEM) with calcium bicarbonate for capsule formation were prepared following the standard protocols previously described (30, 35).

**cDNA synthesis and plasmid construction.** Total RNA was isolated with the TRIzol reagent (Invitrogen, CA), and cDNA was synthesized using an oligo(dT) primer and reverse transcriptase as previously described (35). Rapid amplification of cDNA ends (RACE) was performed to verify the *Ric8* open reading frame (ORF), and full-length cDNA was generated by reverse transcription (RT)-PCR using primers PW1624 and PW1625. The cDNA was inserted into the pGADT7 and pgBDT7 plasmids (BD Biosciences) at EcoRI and BamHI sites following the standard protocols (36). cDNAs for Gpa1, Gpa2, and Gpa3 were synthesized using primers PW1654/PW1659 and PW1633/PW1634, respectively, and the fragments were inserted into pgBDT7. Gpa1 was inserted at the NcoI and PstI sites, whereas Gpa2 and Gpa3 were inserted at the BamHI and PstI sites.

To generate the *Gpa1::GFP*, dominant-active allele, primer pairs PW1654/PW1659 and PW1633/PW1634 were used to create two overlapping PCR fragments. These fragments were mixed as the template for a new round of PCR amplification to create the *Gpa1::GFP* allele using primers PW1654 and PW1680. Using a similar method, primer pairs PW1633/PW1657 and PW1656/PW1632 were used to generate the *Gpa2::GFP* allele. Similarly, primer pairs PW1663/PW1663 and PW1662/PW1664 were used to generate the *Gpa3::GFP* allele. These constructs were inserted into pgBDT7 using the same restriction sites as their wild-type alleles.

For constructs in pgADT7, the primer pair PW1664/PW1685 was used to produce the *Gpa1* and *Gpa1::GFP* alleles, and the DNA fragments were inserted into pgADT7 at the Xmal and Xhol sites. For Gpa3 and *Gpa3::GFP*, primers PW1682 and PW1866 were used for PCR amplification, and DNA was digested by BamHI and Xhol and inserted into pgADT7. The oligonucleotide primers for PCR amplification are listed in Table 2. All plasmid constructs were verified by DNA sequencing.

**Semi-quantitative RT-PCR.** Cells grown overnight in liquid YPD medium were harvested, washed with sterile distilled water, and divided into three portions. One portion was kept in ice, the second portion was suspended in yeast nitrogen base (YNB), and the third portion was suspended in liquid V8 medium in the presence of the mating partner (KN99a; 1 × 10⁷ cells). Cells in liquid YNB and V8 media were returned to the 30°C shaker (2,500 rpm) for incubation for four additional hours. All the cells were then lyophilized, and RNA was isolated with TRIzol reagent (Invitrogen, CA). Following digestion with RNase-free DNase (RQ1; Promega), RNA was quantified using a NanoVue Plus spectrophotometer (GE Life Sciences). One microgram RNA was used for reverse transcription using random hexamers (SuperScript First Strand; Invitrogen). An equal amount of cDNA (1 µl) was used in the PCR with gene-specific primers. Primer pairs JH7278/JH7279, PW116/PW315, PW54/PW140, PW986/PW1086, and PW1614/PW1615 were used to amplify the partial fragments of Gpa1, Gpa2, Gpa3, Ric8, and ACT transcripts, respectively.

**Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a Kodak Gel Logic 2200 imaging system, and bands were quantified using Carestream MI software (Carestream).** The band intensity was expressed as relative absorbance units using the constitutively expressed actin gene (ACT) as a control for normalization of initial vari-

### Table 1 Major strains (C. neoformans var. grubii) used in this study

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| H99    | Wild-type MATα | 33 |
| KN99a  | Congenic to H99 MATα | 34 |
| CDX6   | gpr4::NEO MATα | 32 |
| JG3    | gpa1::URA5 P insurgents; GPA1::rict8::NAT MATα | This study |
| JG23   | rict8::NAT MATα | This study |
| JG33   | gpa1::ADE2 rict8::NAT MATα | This study |
| JG41   | rict8::NAT RIC 8-NEO MATα | This study |
| JG51   | gpr4::NEO rict8::NAT MATα | This study |
| JG258  | gpa1::ADE2 MATα | This study |
| JG259  | gpa2::URA5 MATα | This study |
| JG260  | gpa3::URA5 MATα | This study |
| JG261  | rict8::NAT GPA1::GFP, NEO MATα | This study |
TABLE 2 DNA primers used in this study

| Primer | Sequence |
|--------|----------|
| PW50   | CCACCGGAAAATGTTGGAAAAATG |
| PW116  | CCACTGAAAGCTCCACATCAAGG |
| PW315  | GTCAACAGTCCTCAAGCAGAT |
| PW140  | ATGCGGCGGATATGATTCCTCG |
| PW986  | AAGGATCGACTGACCATCTGGCAGG |
| PW1086 | ATCCGCTGCAACGCAAGCTGCGT |
| PW1614 | GCAGCGTATGCTGCAAAACT |
| PW1615 | ACTTTCGACGTAAGAG |
| PW638  | TGGCAACAGGATCCTGGAAAG |
| PW812  | AGTATCGGAGCTAGCAGTATG |
| PW859  | TAGAAGCTTGTGGGCCACCGAGCAG |
| PW983  | TTATCCCTGCTTCTAGTGA |
| PW984  | AAAGGATCTGCGGCTCTAGCG |
| PW1082 | GCCTCAACGTGACGAGCATTAC |
| PW1085 | TTAATCCATAGCGCTAGTTTTC |
| PW1602 | GAATACCGACGAGAGG |
| PW1609 | TCGAGTAAAACCCCAATAGGCA |
| PW1610 | GTCAATGCCTTTTCTGTGATCATTTACGCAAGG |
| PW1611 | CTGGCGCGGTTTTCTACTTCTACATATCTCACAAACAG |
| PW1612 | TCGAAGATCGATGAGTTGAAAG |
| PW1624 | AAGATTCCTCGTAAGGCTTATTAG |
| PW1625 | AAGATACGCTGCACTCAGGAGG |
| PW1626 | TAAATCGACTCATATAGGAC |
| PW1627 | AGATGTGTCGAGATGAGAG |
| PW1628 | TAAGATGCTTCTAAATTTCTT |
| PW1631 | AAGGATCGCTGCGTCACTAGCCAG |
| PW1632 | AACTCGACGAGGAAAGCAGCAGTCCGAG |
| PW1636 | AACTCGAATGAAATGGCATAATC |
| PW1645 | AACATCGCCGGGCTCTGATGTGCTACCTCCAGAGCC |
| PW1656 | GATGTTGAGCCGCTACCTGGCTAGAG |
| PW1657 | CGTCTGGCAGCTGGAGCCACATTC |
| PW1658 | GATGTCGGTGGACTGAGAAGGAGG |
| PW1659 | CTCTGGCTTTTCAGCTGACCAGC |
| PW1660 | CGAACCGCACAGATGAGCA |
| PW1661 | C1GATCGTGGGCGGCGGAG |
| PW1663 | CTTTCGACCTGAGCCGACCTACTAC |
| PW1668 | AAGGATCTGATGGCCTACATCTACC |
| PW1669 | AACTCGATGATGAAGACGAGCCAGTCTG |
| PW1670 | AAGGATCGCCGGCAAGTATGCTTCG |
| PW1671 | AACTCGATGATGAAGACGAGTATAGCT |
| PW1680 | AACTCGAGTTAATAGATACAGAGTGACG |
| PW1681 | AAGGATCTGATGGCCTACATCTACC |
| PW1682 | AAGGATCTGATGGCCTACATCTACC |
| PW1684 | AACTCGACCACAGATGATGCAAGCAG |
| PW1685 | AACTCGAGCTTATAGAAGTCCAGACGGAGGAG |
| PW1686 | AACTCGAGCTTATAGAAGTCCAGACGGAGGAG |
| PW1688 | AAGGTACCATGGGCGTGTAGTCTACTCCAGAAGCCCC |
| PW1716 | TATAAGCTACTATAGG |
| PW1717 | GCTAGTATGATGACTGAGG |
| PW1720 | AACTCGAAGATGGCTGACGGTATATTAG |
| PW1721 | AACCATTGTTGAAATCTCAGCTGAGG |
| PW1755 | AACCCTGGGATATAGATAGTACGGAGG |
| PW1771 | ATGTTCTGATGTTGGACTGAGAAGGAGAAGAAGG |
| PW1772 | GTTATCTTCTACGTCCTGACCGAGATCAGAT |
| HI7278 | GTACATCAAAAGGACAGT |
| HI7279 | GACAGATGGATGCTGAGTAG |

**Generation and complementation of ric8 mutants.** The ric8 gene was identified from the *C. neoformans* var. *grubii* H99 database ([www.broadinstitute.org](http://www.broadinstitute.org)) by BLAST (Basic Local Alignment Search Tool) search and confirmed by PCR amplification. The full-length cDNA sequence was also obtained by RT-PCR amplification. Both sequences were verified by DNA sequencing. To generate the ric8 mutants, overlap PCR was used. Primers PW638 and PW1603 were used to obtain the 3′ partial fragment of the *NEO* gene cassette for neomycin resistance, and primers PW812 and PW1602 were used to obtain the 5′ partial fragment of the *NEO* gene. The *ric8* upstream partial fragment was obtained with primers PW1609 and PW1610, and the downstream fragment was obtained by PCR amplification with primers PW1611 and PW1612. The two overlap PCR products amplified by primer pairs PW1609/PW638 and PW812/PW1612 were transformed into H99 or related strains through biolistic transformation. Neomycin-resistant colonies were screened by PCR amplification using primers PW983 and PW638. A ric8::NAT mutant was also generated using a similar strategy that allows verification of phenotype changes associated with *RIC8* gene disruption.

The ric8::NAT mutant strain was complemented by reintroduction of a 3.652-bp PCR fragment containing the full-length *RIC8* ORF, a 702-bp region preceding the start codon, and a 300-bp fragment downstream of the termination codon. A 4,320-bp fragment was amplified using primers PW983 and PW984 and digested with SacII and NotI, and the cutoff band of 3,652 bp was inserted into pGS200, which contains a NEO selection marker ([37](#)). The insert was verified by DNA sequencing before transformation into the ric8::NAT mutant strain. G418-resistant colonies were selected, screened by PCR amplification, and verified by observation for restoration of phenotypes such as capsule and melanin formation.

**Heterologous protein expression.** The GPA1 and GPA1224L (gene alleles were PCR amplified using primers PW1688 and PW1680, digested with KpnI and PstI, and ligated to pET41a (+) (Novagen). Primer pairs PW1688/PW638 and PW1670/PW1671 were used to obtain GPA2-GPA25032, respectively. DNA was digested with BamHI and PstI before ligation to pET41a (+). The primer pair PW1720/PW1721 was used to obtain *RIC8* cDNA by PCR amplification, followed by restriction by Xhol and NcoI and ligation into pRSET-B. All insert sequences were verified by DNA sequencing.

Fusion protein induction and purification were performed following the standard protocols or as previously described ([14, 38](#)). BL21–CodonPlus (DE3)–RIPL cells were used to express pET41a (+)-based protein constructs, while BL21(DE3)pLysS cells were used to express Ric8 from pRSET B. The glutathione S-transferase (GST)–Go fusion proteins were purified using Glutathione Sepharose 4B medium (GE Healthcare), while the 6His-Xpress Ric8 fusion protein was purified using a HIS-Select Nickel affinity gel (Sigma–Aldrich).

**Coimmunoprecipitation (co-IP) and Western blotting.** Purified GST-Go and 6His-Xpress Ric8 fusion proteins were buffer exchanged using PD-10 columns (GE Healthcare) with phosphate-buffered saline (PBS) (10 mM phosphate buffer, pH 7.4, 150 mM NaCl containing 5 mM MgCl₂) and 100 μM guanosine 5′-O-(2-thiodiphosphate) trilithium salt. Equimolar amounts of the Go and Ric8 proteins were mixed and loaded onto glutathione-agarose columns (Sigma–Aldrich). After incubation for 3 h at 4°C, the columns were washed with the same buffer 10 times, and the bound proteins were eluted by 10 mM glutathione in 50 mM Tris-HCl (pH 9.2). The proteins were separated by gel electrophoresis (NuPAGE; Life Technologies), transferred to an Immun-Blot polyvinylidene difluoride (PVDF) membrane (Bio–Rad), and incubated with the mouse anti-Xpress antibody (1:5,000; Invitrogen) or the mouse anti-GST antibody (1:5,000; Santa Cruz Biotechnology). An anti-mouse IgG horseradish peroxidase (HRP) conjugate (1:7,500; Promega) was used as the secondary antibody.
Phenotypic characterization and virulence assessment. Cells from each strain were inoculated into 5 ml YPD medium for overnight growth in a 30°C shaker (225 rpm), centrifuged, and washed with sterile distilled water. Cells were counted under a microscope following dilution, and the cell density was adjusted to 1 × 10⁶ per ml. Ten microliters of cell suspension containing 10⁶ cells (1 × 10⁶ cells/ml) was pipetted on 1.3-4-dihydroxyphenylalanine (L-DOPA) and Nigerseed agar plates for melanin induction at 30°C and 37°C and into 2 ml liquid DMEM for capsule induction at 30°C with shaking (225 rpm). Cells for capsule formation were mixed with India ink and observed under a Zeiss Axioskop microscope.

Virulence was assessed using the intranasal inoculation (inhalation) route in 4- to 6-week-old female A/JCr mice (Jackson Laboratory). Ten mice per group were each inoculated with 10⁶ cells, and the animals were monitored twice daily. Animals that appeared sick or in pain were sacrificed via CO₂ asphyxiation. Mouse survival was analyzed by the Kaplan-Meier method using Prism 4.0 software (Graphpad Software, Inc.) as previously described (35). Animal use was approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee (IACUC).

RESULTS

Identification and expression of C. neoformans RIC8. C. neoformans is a haploid basidiomycetous fungus with a bipolar mating system composed of two mating types, MATα and MATa. The pheromone response and mating can be observed in the laboratory environment, from which early knowledge regarding signal transduction in the organism was generated. Studies by Tolkacheva et al. and Alspaugh et al. identified the first G protein subunit (Gpa1), which mediates a cAMP-dependent signaling pathway important in fungal virulence (29, 39). Curiously, a classic G protein β subunit capable of coupling with Gpa1 was not found, leading to propositions that Gpa1 may function as a monomeric G protein whose activation depends on GEF or that certain proteins may function as a bona fide Gβ. We have since characterized a Gpa1 binding protein (Gib2) as a noncanonical G protein forming a heterotrimeric G protein complex with Gpa1 (38, 40). To identify additional proteins capable of binding and potentially activating Gpa1, we used the Y2H screen to examine candidate proteins that included a Ric8 homolog and three members of the Rho family GTPTases (Cdc42, Cdc420, and Ran1) (unpublished observations). We sought to characterize Ric8 functions first, as they have yet to be studied in this fungus.

A BLAST search of the C. neoformans H99 (var. grubii) database revealed that the Ric8 gene has a 2,397-bp open reading frame encoding a 692-amino-acid protein. The C. neoformans Ric8 protein shares relatively low amino acid sequence homology (identity and similarity) with the Ric8 proteins of nematodes and humans (23% and 26%, respectively). It shares moderate sequence homology with the Ric8 protein of the basidiomycetous jelly fungus T. mesenterica and is most distant from the Ric8 of C. elegans. The phylogenetic tree was drawn using ClustalW (v1.83) multiple-sequence alignment with the following parameters: Open Gap Penalty, 10.0; Extend Gap Penalty, 0.2; Delay Divergent, 30%; Gap Distance, 4; Similarity Matrix, gonnet. The accession numbers for the Ric8 proteins are NP_001023561 (C. elegans), EAA34708 (N. crassa), XP_000706336 (T. mesenterica), XP_003716129 (M. oryzae), and AFR98639 (C. neoformans). The expression of the RIC8 gene is highly inducible by nutrient-limiting medium and by conditions favoring mating. The induction time for cells in YNB and V8 media was 4 h. RT-PCR was repeated twice, and the mean values were used to calculate the expression rate relative to that of the ACT gene encoding actin (averages ± standard deviations are shown). The numbers of PCR cycles were 30 for GPA1 and GPA2, 35 for GPA3 and RIC8, and 25 for ACT.

Ric8 regulates capsule and melanin formation. To characterize Ric8 functional roles, the RIC8 gene was disrupted in the archetypical C. neoformans H99 strain (var. grubii) using the ric8::NEO allele. A ric8 mutation linked to the NAT marker was also obtained using the ric8::NAT allele. The complemented strain was obtained in the ric8::NAT strain (ric8 RIC8).

Given that C. neoformans Gpa1 functioning in the cAMP signaling pathway lacks a canonical Gβ protein, Ric8 could exert a conserved GEF function toward Gpa1 by promoting the activation of Gpa1. We sought to test whether this is the case. We first assessed capsule production and melanin formation, traits that are regulated by Gpa1-cAMP signaling. The polysaccharide capsule is regarded as one of the most important virulence factors for C. neoformans, as it protects cells from phagocytosis and killing by macrophages. The ric8 mutant lacked the capsule, indicating that Ric8 is required for capsule formation. This is similar to the gpa1 and ric8 gpa1 mutants that also lacked the capsule (Fig. 2A). Caps-

![FIG 1](image-url)
sule formation was restored in the complemented ric8 RIC8 strain, which was similar to the wild type; the ric8 mutant expressing the constitutively activated Gpa1 allele (ric8 GPA1 Q284L); and the gpa2 and gpa3 mutants, all producing normal-size capsules (Fig. 2A). This observation is consistent with a model in which Ric8 and Gpa1 could function in a linear pathway to regulate capsule formation.

The melanin pigment also protects cells from external damage imposed by reactive oxygen species (ROS) and allows C. neoformans to survive in harsh environments. Melanin formation is regulated by Gpa1-cAMP signaling, as externally supplementing cAMP suppressed the defect in melanin formation that occurred in the gpa1 and cac1 mutants (9, 29). Consistent with this observation, the ric8 mutant strain lacked the melanin pigment when induced. On Nigerseed agar at 30°C (Fig. 2B, left) and on L-DOPA medium at 37°C (Fig. 2B, right), the ric8 mutant produced an opaque colony, in contrast to the wild-type, ric8 RIC8 complemented, and ric8 GPA1 Q284L strains. However, similar to capsule induction assays, the defect shown by the ric8 mutant was not as severe as that of the gpa1 and ric8 gpa1 mutants, which showed the most drastic defects (the smallest cell sizes in capsule induction medium and nearly white colonies on melanin induction plates), reinforcing the notion that Ric8 may not be the only “activator” of Gpa1.

**Ric8 functions in the Gpa1-cAMP signaling pathway.** Since the activation of the Gpa1-cAMP pathway results in increased intracellular cAMP levels and supplementation of the growth medium with cAMP can suppress pathway defects by temporarily raising cAMP levels, we added cAMP (to 10 mM) to the Nigerseed agar medium and L-DOPA agar medium to examine its effect on melanin formation. Melanin was restored in the ric8 mutant to a level similar to that of the wild-type cells (Fig. 2B), suggesting that Ric8 functions within the Gpa1-cAMP pathway.

To test if Ric8 is directly involved in the regulation of the intracellular cAMP levels, we assayed for the transient levels of cAMP following starvation and glucose triggering (37). The ric8 mutant strain posted 2.9 × 10^3, 2.5 × 10^3, 2.6 × 10^3, and 2.7 × 10^3 fmol cAMP at 0, 0.5, 1, and 3 min post-glucose stimulation, respectively, which was similar to those posted by the gpa1 mutant strain (2.4 × 10^3, 2.4 × 10^3, 2.4 × 10^3, and 2.6 × 10^3 fmol) (Fig. 3A). The wild-type strain showed a typical transient spike in cAMP levels following glucose triggering (3.0 × 10^3, 3.9 × 10^3, 4.3 × 10^3, and 3.6 × 10^3 fmol at the respective time intervals post-glucose stimulation) (Fig. 3A). This assay indicates that Ric8 has a role similar to that of Gpa1 in the positive regulation of transient cAMP levels and the cAMP signaling pathway.

In C. neoformans, Gpr4 was identified as a GPCR that senses environmental cues, such as the amino acid methionine, and functions upstream of Gpa1 (41). Disruption of *GPR4* resulted in attenuated capsule formation to a modest degree in comparison to that of the gpa1 or ric8 mutant (41) (Fig. 3B). The ric8 gpr4 mutant displayed an acapsular phenotype similar to that of the ric8 and ric8 gpa1 mutants, suggesting that Ric8 function might be independent of Gpr4. In addition, expression of the constitutively active GPA1 Q284L allele in the gpa1 mutant resulted in enhanced capsule formation, indicative of upregulated cAMP signaling. The enhanced capsule formation is not affected by disruption of the *RIC8* gene (Fig. 3B). Collectively, these findings are consistent with the hypothesis that Ric8 likely functions in Gpa1-cAMP signaling by providing a GEF function toward Gpa1 in the absence of stimulation by Gpr4.

**Ric8 has opposing roles in pheromone response and mating.** Previous studies showed that mutants such as the gpa1 mutant were also attenuated in mating, although the mechanism is unclear (29). The ric8 mutant showed similar attenuation in mating. To examine whether Ric8 functions in the pheromone-responsive mating pathway, we carried out confrontation and mating assays. *C. neoformans* secretes diffusible pheromones, and the place-
Cryptococcus Ric8 promotes cAMP and pheromone signaling

Discoveries of G protein-coupled receptors (GPCRs) that bind peptides and regulate intracellular signaling have led to insights about receptor structure, activity, interactions, and the role of calcium in cellular function (1). GPCRs have been found in both eukaryotic and prokaryotic bacteria (2, 3). These endogenous GPCRs have the potential to act as sensors of environmental information, which can contribute to the development of pathogens. In this study, we examine the role of GPCRs in Cryptococcus neoformans, a dimorphic pathogen that can cause pneumonia and meningitis in humans.

The authors investigated the role of GPCRs in C. neoformans by assessing their impact on cell growth and mating. They found that disruptions in a GPCR gene, Gpr4, significantly reduced cell growth and mating efficiency. Additionally, they observed that the disruption of another GPCR gene, Gpa1, led to increased cell growth and mating efficiency. These findings suggest that GPCRs play a critical role in regulating cell growth and mating in C. neoformans.

The authors also investigated the role of Ric8, a putative G protein signal transducer, in the GPCR signaling pathway. They found that disruption of the ric8 gene resulted in decreased cell growth and mating efficiency, indicating that Ric8 is involved in regulating GPCR signaling.

In conclusion, the study highlights the importance of GPCRs in the regulation of cell growth and mating in C. neoformans. The findings suggest that disruptions in GPCR and Ric8 genes can significantly impact cellular function, and further investigation into their role in regulating GPCR signaling is warranted.

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Mutation of ric8 results in attenuated virulence toward animals. Gpa1 and the Gpa1-mediated cAMP pathway are known to have major regulatory roles in the expression of virulence traits, including melanin formation and capsule production (10, 29). Disruption of GPA1 or PKA1 resulted in significant reductions in melanin and capsule formation, and the gpa1 and pka1 mutant strains were severely attenuated in virulence toward animals. To examine whether Ric8 plays a role in virulence, we infected 10 AJC/r mice with the ric8 mutant, the H99 strain, and two independent ric8 RIC8 complemented strains with 10^5 cells through intranasal inhalation (35). Mouse survival was monitored twice daily, and the results were plotted against time (in days). The mice infected with the ric8 mutant survived 78 days, with a median survival of 55 days, in contrast to mice infected with H99, which survived 23 days with a median survival of 20 days (P < 0.001) (Fig. 6A). Brain smears of mice infected with the ric8 mutant showed the presence of yeast cells maintaining the acapsular form in comparison to the capsular wild-type strain (Fig. 6B). The observation that the ric8 mutant had a significant delay in causing cryptococcosis of mice demonstrates that Ric8 positively regulates fungal virulence.

DISCUSSION

In eukaryotic cells, heterotrimeric G protein-mediated signaling transduction pathways play critical roles in sensing and relaying external cues so that cells can adapt. In the pathogenic fungus C. neoformans, G protein signaling also becomes an integral part of the virulence repertoire. C. neoformans encodes three Gα proteins (Gpa1, Gpa2, and Gpa3), one Gβ protein (Gpb1), one Gβ-like protein (Gib2), and two Gγ proteins (Ggp1 and Ggp2). Functionally, these proteins exhibit remarkable conservation with homologous proteins of many other eukaryotic organisms and even higher eukaryotes, such as mammals. Gpa1, Cac1, and Pka1 constitute a major cAMP-dependent signal transduction pathway that controls growth, differentiation, and the production of virulence characteristics (reviewed in references 6, 42, and 43). Mutations occurring among the components of this pathway lead to attenuated cAMP signaling and altered capsule size and melanin formation. Meanwhile, there is growing evidence indicating that G protein signaling has evolved to be quite distinct in this fungus. A GPCR homolog, Gpr4, was found to stimulate Gpa1 upon the sensing of methionine, but a GPCR capable of sensing glucose and activating the cAMP pathway identified in other fungi, such as S. cerevisiae, was not found (41). Also, a canonical Gβ protein capable of binding to Gpa1 was not found, but the noncanonical Gβ protein Gib2 proved to form a heterotrimeric complex with Gpa1 and Gpg1 or Gpg2 (38). In addition, Gib2 functions as a scaffolding protein that positively regulates cAMP levels through novel functions involving the Ras1 and Cac1 proteins (40). The current identification of the Ric8 protein provides another mechanistic explanation of how Gpa1 functions without a classic Gβ subunit.
In contrast to the Gpa1-cAMP signaling pathway, the mechanism of pheromone response and mating regulated by Gpb1 through a conventional Gα/H9251/H9252/H9253 heterotrimeric complex appears to be conserved. However, the specific functions of Gpa2 and, in particular, Gpa3 in pheromone responses and mating are still unresolved. We found that a mutation of either Gpa2 or Gpa3 results in enhanced pheromone response and mating, while a companion study showed that Gpa2 promotes mating while Gpa3 inhibits mating (13, 14). We showed that Gpa2, but not Gpa3, interacted with Gpb1 and Crg1, a regulator of G protein signaling that negatively regulates pheromone responses and mating through Y2H screening (35). Consistent with a role in pheromone responses and mating, Gpa2 interacted with the pheromone receptor homolog Ste3. In contrast, Gpa3 failed to interact with Gpb1 but interacted with Crg1. While we were unable to detect an interaction between Gpa3 and Ste3, Hsueh et al. were able to establish an interaction using a different version of Y2H screening (13). Finally, Gpa3 is able to interact with Crg2, which is the main negative regulator of Gpa1 in cAMP signaling (31, 32).

The present study shows that Ric8 has a role in promoting mating (dikaryotic-filament and basidiospore formation), and it is conceivable that Ric8 may perform this function as a GEF toward Gpa2, dependent on or independently of Ste3a. Disruption of RIC8 delays dissociation of Gpa2 from Gpb1, resulting in attenuated formation of dikaryotic filaments and basidiospores. However, Ric8 also appears to negatively regulate the secretion of pheromones and conjugation tube formation, but an interaction cannot be found between Ric8 and Gpa3, which also lacks a Gβ
FIG 7 The Ric8 protein regulates the mating and cAMP signal transduction pathways in C. neoformans. The pheromone-responsive mating pathway consists of the upstream components, including the pheromone receptor Ste3α, the GRS protein Crg1, the Go protein Gpa2, the Gβ protein Gpb1, and either the Gγ protein Gpg1 or the Gγ protein Gpg2. The cAMP signaling pathway consists of the G protein-coupled receptor homolog Gpr4, the Go protein Gpa1, the Gβ-like protein Gib2, the Gγ proteins Gpg1/Gpg2, and the RGS protein Crg2. Ric8 performs a GEF function toward Gpa1 and Gpa2. It facilitates GDP-to-GTP exchange of Gpa1 to promote cAMP signaling that regulates growth, differentiation, and the production of virulence factors. It also promotes Gpa2 activation to subsequently prolong the positive regulatory function of Gpb1 in mating. Gpa3 likely functions as a distinctive Go protein in a pathway that senses pheromones but counters the activity of Gpa2. No physical interactions have been established between Gpa3 and Ric8.

partner. This calls for additional studies to address whether the pheromone response and mating pathways are separate events regulated by different Go proteins and to further distinguish functional differences between Gpa2 and Gpa3 (Fig. 7).

Ever since being originally identified in nematodes and fruit flies, Ric8 proteins have been found in many other eukaryotes, including animals and even certain fungi, to possess a wide variety of functions. In addition to being a GEF of Gα proteins to amplify and prolong G protein-mediated intracellular signal transduction, Ric8 proteins were also found to exhibit various other functions. Ric8A was found to prevent ubiquitination and degradation of Gα12 and Gαq (22) and to be a chaperone protein for folding nascent Gα subunits (21). Our studies of C. neoformans Ric8 revealed that it has many functions, such as affecting polysaccharide capsule and melanin production, attenuating pheromone responses, and decreasing mating efficiency. Such effects could contribute to the function of Ric8 as a GEF for Gpa1 and Gpa2. Nevertheless, additional functions are likely to be found through further research efforts.

We conclude that the C. neoformans Ric8 homolog protein functions as a GEF to facilitate the binding of GTP to Gpa1. This amplifies the cAMP-dependent signaling pathway that regulates capsule size, melanin formation, and virulence. The Ric8 protein also provides a GEF function for Gpa2, affecting mating efficiency. Our study highlights the distinct, as well as conserved, G protein signaling mechanisms and contributes to research efforts leading to the discovery of novel antifungal targets.

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