Canonical Heterotrimeric G Proteins Regulating Mating and Virulence of *Cryptococcus neoformans*[□]

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Perturbation of pheromone signaling modulates not only mating but also virulence in *Cryptococcus neoformans*, an opportunistic human pathogen known to encode three G α , one G β , and two G γ subunit proteins. We have found that G α s Gpa2 and Gpa3 exhibit shared and distinct roles in regulating pheromone responses and mating. Gpa2 interacted with the pheromone receptor homolog Ste3 α , G β subunit Gpb1, and RGS protein Crg1. Crg1 also exhibited in vitro GAP activity toward Gpa2. These findings suggest that Gpa2 regulates mating through a conserved signaling mechanism. Moreover, we found that G γ s Gpg1 and Gpg2 both regulate pheromone responses and mating. *gpg1* mutants were attenuated in mating, and *gpg2* mutants were sterile. Finally, although *gpa2*, *gpa3*, *gpg1*, *gpg2*, and *gpg1 gpg2* mutants were fully virulent, *gpa2 gpa3* mutants were attenuated for virulence in a murine model. Our study reveals a conserved but distinct signaling mechanism by two G α , one G β , and two G γ proteins for pheromone responses, mating, and virulence in *Cryptococcus neoformans*, and it also reiterates that the link between mating and virulence is not due to mating per se but rather to certain mating-pathway components that encode additional functions promoting virulence.

INTRODUCTION

Cryptococcus neoformans is an encapsulated yeast-like fungus capable of infecting both immunocompromised and healthy individuals to cause life-threatening meningoencephalitis (Mitchell and Perfect, 1995; Chayakulkeeree and Perfect, 2006). The organism belongs to basidiomycota taxonomically and has a defined life cycle and a bipolar mating system with MAT α being the predominant mating type in both environmental and clinical settings (Hull and Heitman, 2002; Wang and Fox, 2005; Nielsen and Heitman, 2007). Like other fungi and higher eukaryotes, heterotrimeric G protein-mediated signaling pathways are central for *C. neoformans* to sense environmental- and host-imposed cues and to respond through regulation of developmental processes such as mating and haploid differentiation, as well as the production of several virulence factors including melanin and capsule (Alspaugh *et al.*, 1998; Wang and Fox, 2005).

The lower eukaryotic organisms such as fungi also encode multiple G α subunits, similar to higher eukaryotic organisms such as animals and plants (Lengeler *et al.*, 2000). For example, the budding yeast *Saccharomyces cerevisiae* and the

fission yeast *Schizosaccharomyces pombe* encode two G α subunits, the plant pathogen *Ustilago maydis* encodes four G α subunits, and many other fungal species contain three G α subunits. The *S. cerevisiae* G α Gpa1 is a negative regulator of the pheromone-responsive mating pathway (Whiteway *et al.*, 1989; Whiteway *et al.*, 1990; Clark *et al.*, 1993; Kübler *et al.*, 1997; Colombo *et al.*, 1998; Thevelein and Winder, 1999), whereas G α Gpa2 positively controls a glucose-sensing cAMP pathway for pseudohyphal differentiation and invasive growth (Kübler *et al.*, 1997; Lorenz and Heitman, 1997). Distinct, but largely similar, signaling pathways mediated by two G α subunits were also found in *S. pombe* (Stiefel *et al.*, 2004; Hoffman, 2005). Unlike the two yeast models, only the G α subunit that functions in the conserved cAMP-dependent signaling pathway was better understood in fungi that contain three or more G α s (Gao and Nuss, 1996; Choi and Dean, 1997; Liu and Dean, 1997; Regenfelder *et al.*, 1997; Ivey *et al.*, 2002; Kays and Borkovich, 2004).

Despite encoding multiple G α s, only a single G β was found in fungi. Extensive genetic and biochemical studies have shown that *S. cerevisiae* G β Ste4 couples with G γ Ste18 as a protein heterodimer to regulate mating in response to pheromone stimulation. In comparison, the *S. pombe* G β Git5 and G γ Git11 heterodimer functions in a cAMP-signaling pathway mediated by Gpa2, instead of the mating pathway mediated by Gpa1 (Stiefel *et al.*, 2004). In *Neurospora crassa*, G β Gnb1 and G γ Gng1 were found to maintain the stability of all three G α s during multiple signaling, although the molecular basis for such signaling mechanisms is not clear (Yang *et al.*, 2002; Krystofova and Borkovich, 2005).

Previous studies have demonstrated that there exist two distinct G protein-mediated signaling pathways in *C. neoformans*, similar to those described for *S. cerevisiae* and *S. pombe* (Alspaugh *et al.*, 1998; Wang and Heitman, 1999). Gpa1 mediates a conserved cAMP-dependent signaling pathway

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Abbreviations used: GAP, GTPase-activating protein; RGS, regulator of G protein signaling.

to regulate morphogenesis and virulence traits (Alspaugh *et al.*, 1997, 2002; D'Souza *et al.*, 2001; Bahn *et al.*, 2004; Xue *et al.*, 2006), and Gpb1 plays a positive role in governing the mating response, such as conjugation tube formation, and mating (Wang *et al.*, 2000). The G γ subunits Gpg1 and Gpg2 were found to associate with Gpb1 and the G β -like/RACK1 (receptor for activated C kinase 1) Gib2, but their functions remain uncharacterized (Palmer *et al.*, 2006).

With two functionally distinct G protein-signaling pathways (cAMP signaling and mating) and a single G β subunit, why does *C. neoformans* encode three G α and two G γ subunits? Here we show that Gpa2, Gpa3, Gpg1, and Gpg2 all have a role in pheromone response and mating. We provide evidence suggesting that Gpa2, Gpb1, and Gpg1 or Gpg2 could form a heterotrimeric G protein complex and that Gpa2 regulates mating through a conserved mechanism. Despite interactions detected between Gpa3 and Ste3 α , and Gpa3 and Crg1, a functional mechanism for Gpa3 remains to be delineated. Finally, we provide direct evidence demonstrating that Gpa2 and Gpa3, but not Gpg1 and Gpg2, collectively regulate virulence of *C. neoformans* in a murine virulence model.

MATERIALS AND METHODS

Strains, Media, and Plasmids

C. neoformans consists of two highly related, but distinct varieties (serotypes): var. *grubii* (serotype A) and var. *neoformans* (serotype D), with var. *grubii* being the predominant type. The wild-type MAT α (hereafter expressed as α) H99 and MAT α (hereafter α) KN99a, as well as their ura- derivatives F99 and F99a, were described previously (Perfect *et al.*, 1993; Nielsen *et al.*, 2003; Wang *et al.*, 2004b). The *ura5 ade2* strain was created by transformation of an F99a strain with the *ade2* knockout allele linked to a nourseothricin-resistant marker (NAT1; McDade and Cox, 2001). All of the strains in this study were of the var. *grubii*, and their genotypes and sources are listed in Supplementary Table 1 (Supplementary Data). Oligonucleotide primers for PCR amplification are listed in Supplementary Table 2 (Supplementary Data).

Yeast extract-peptone-dextrose (YPD), synthetic medium (SD), 10% V8 agar (pH 5.0) for mating, Niger seed agar for melanin production and filament agar for conjugation tube formation were prepared following standard protocols or as described previously (Wang *et al.*, 2000, 2004b).

cDNA for GPA1, GPA2, GPA3, GPG1, and GPG2 was synthesized by RT-PCR and inserted into the pGBKT7 plasmid as described previously (Palmer *et al.*, 2006). GPB1 and CRG1 cDNA was inserted into pGADT7 (BD Biosciences, San Jose, CA). Partial cDNA encoding the C-terminal domains of Ste3 α (GenBank AAN75177) and Cpr2 (H99 sequence, homologous to GenBank XM_569248) was synthesized with primers PW347 and PW348, and PW434 and PW435, respectively, and inserted in pGADT7.

Nucleic acid manipulation procedures were performed according to standard protocols (Sambrook and Russell, 2001).

Identification and Disruption of GPA2, GPA3, GPG1, and GPG2 Genes

GPA2, GPA3, GPG1, and GPG2 genomic DNA was identified from the database at <http://cneo.genetics.duke.edu> (var. *grubii*), and the sequences were obtained by PCR amplification using primers PW366 and PW117 (GPA2), PW436 and PW141 (GPA3), PW237 and PW249 (GPG1), and PW239 and PW248 (GPG2), respectively. The *gpa2::URA5* gene disruption-specific mutation allele was constructed as follows. First, a 1.4-kb fragment containing the GPA2 coding domain was amplified into two partially overlapping fragments that were each ~700 base pairs in length. The first fragment was amplified using primers PW114 and PW223, and the second fragment was amplified using PW224 and PW117. A SmaI restriction site was incorporated into primers PW223 and PW224, so that the URA5 gene could be inserted into the site creating the *gpa2::URA5* disruption allele. The *gpa3::URA5* knockout allele was created similarly with the following primers: PW47, PW141, PW51, and PW52. The *gpg1::URA5* gene knockout allele was constructed with primers PW237, PW362, PW363, and PW249. The *gpg2::URA5* allele was created using primers PW239, PW 364, PW365, and PW248. Mutant alleles were introduced into F99 and F99a strains by biolistic transformation to obtain *gpa2*, *gpa3*, *gpg1*, and *gpg2* strains of both α and α types. The *gpa2 crg1* and *gpa3 crg1* mutant strains were constructed by transformation of the *gpa2* (PWC279) and *gpa3* (PWC201) mutant strains with the *crg1::NAT1* allele. The *gpa2 gpa3* double mutant strains were obtained by crossing α *gpa3* (PWC201) to a *gpa2* (PWC569) followed by microdissection of the basidiospore progenies as described previously (Sia *et al.*, 2000; Palmer *et al.*, 2006).

The α *gpa2* and α *gpa3* mutant strains were complemented by reintroducing a 3.0-kb fragment containing the wild-type GPA2 gene and a 2.8-kb fragment containing the GPA3 gene linked to the NAT1 marker. The α and α *gpg1* and *gpg2* mutant strains were complemented similarly.

The *gpg1::NAT1* and *gpg2::NAT1* alleles were also created to obtain an additional set of *gpg1* and *gpg2* mutants in α strains. α *gpg1 gpg2* mutant strains were obtained by transformation of a *gpg2::URA5* strain, PWC424, with the *gpg1::NAT1* allele and screened for *gpg1::NAT1 gpg2::URA5* transformants. All transformant and segregant strains were verified by PCR amplification and Southern blot hybridization analysis.

Phenotypic Characterization

Pheromone responses were monitored through formation of conjugation tubes that were induced by streaking cells adjacent to those of the opposite mating type on standard filament agar containing 0.5% glucose (Wang *et al.*, 2000). Mating was performed by mixing and patching α and α cells on standard V8 agar. For quantitative assessment of mating, 5 μ l of α cells (10^6 cell/ml) was spotted in triplicate mixing with an equal number of α cells (*ura5 ade2*) on synthetic low ammonium dextrose agar (SLAD). After incubation at 22°C for 7 d, colonies were extracted and suspended in sterile water. Cells were then serially diluted and spread on SD-Ade medium containing 5-FOA (5-fluoroorotic acid), and surviving meiotic progenies (*ADE2 ura5*) were counted as a quantitative measurement for mating efficiency.

Production of melanin was observed by growing cells on standard Niger seed agar at 30°C for 48 h (Wang *et al.*, 2004a,b). The wild-type strains exhibited dark brown pigments, whereas white opaque colonies indicated a defect in melanin production. The polysaccharide capsule was induced by incubating cells in Dulbecco's modified Eagle's medium (DMEM) at 30°C for 48 h, negatively stained with India ink, and visualized using an Olympus microscope (BX51; Melville, NY) equipped with a digital camera (Wang *et al.*, 2004a).

Virulence tests were carried out in female A/JCR and male severe combined immunodeficiency disease (SCID) mice using a murine inhalation model, and mouse survival was analyzed by the Kaplan-Meier method using Prism 4.0 software (GraphPad Software, San Diego, CA), as described previously (Wang *et al.*, 2004b). Animal testing was carried out in compliance with the Institutional Animal Care and Use Committee (IACUC).

Yeast Two-Hybrid, Protein Expression, and Binding Assays

The two-hybrid assay was carried out in a PJ69-4a yeast host as described previously (James *et al.*, 1996; Palmer *et al.*, 2006).

To express Gpa2, Gpa3, and Gpb1 proteins in yeast, cDNA was inserted into the expression vector pYES2/NT, and the corresponding plasmids were transformed into a yeast strain (INVSc1, Invitrogen, Carlsbad, CA). Yeast transformants were grown in liquid SD-Ura medium containing 2% glucose for 24 h at 30°C, and cells were precipitated, washed, and grown in SD-Ura medium containing 2% galactose and 1% raffinose overnight in a 30°C shaker (225 rpm). To extract proteins, cells were collected, suspended in lysis buffer (phosphate-buffered saline [PBS], pH 7.4, 1% Nonidet P-40, and a cocktail of protease inhibitors [Roche, Indianapolis, IN]), mixed with an equal volume of acid-washed glass beads (~400 μ M, Sigma, St. Louis, MO), and homogenized in a Fast-Prep cell disrupter (Bio101, Carlsbad, CA) four times for 40 s at 4°C. Homogenized samples were centrifuged at 13,000 rpm for 30 min at 4°C, and supernatants were recovered. For protein purification, cell extracts were incubated with Co $^{2+}$ affinity resin (Clontech, Palo Alto, CA) for 1.5 h at 4°C and washed, and proteins were eluted with buffer containing 200 mM imidazole. Proteins were then concentrated through a centrifugal filter (Millipore, Bedford, MA), measured using a BCA protein assay kit (Pierce, Rockford, IL), and verified by SDS-PAGE analysis and Western blotting using the anti-Xpress mAb (Invitrogen).

For protein production in *Escherichia coli*, cDNA for CRG1, RGS1 (partial CRG1 sequence encoding the RGS domain), GPB1, GPG1, and GPG2 was inserted into the pET41b vector, and plasmid DNA was transformed into Rosetta2(DE3) cells (Novagen, Madison, WI). Protein expression was induced for 4 h at room temperature after the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. Cells were collected by centrifugation, washed, and stored at -70°C. To extract proteins, cells were suspended in PBS buffer (pH 7.3) also containing 1 mM EDTA, 0.5% Nonidet P-40, 0.5 mg/ml lysozyme, and protease inhibitors (Roche) and incubated for 30 min at 4°C with gentle shaking. Samples were centrifuged at 13,000 rpm for 20 min at 4°C and supernatants were recovered. For protein purification, supernatants were mixed with glutathione-Sepharose resin (Amersham Pharmacia, Piscataway, NJ) for 1.5 h, washed, and proteins eluted with 15 mM glutathione. Proteins were also verified by SDS-PAGE analysis and Western blotting with the anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CASC-138). The GST-Gpg1 and GST-Gpg2 fusion proteins were produced using a similar approach.

For binding, 400 μ l of the GST-Gpb1 protein extract was added to glutathione-Sepharose resin and washed with buffer C (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl $_2$, 1 mM EDTA, 1 mM PMSF, and proteinase inhibitors). Gpa2 and Gpa3 proteins were first preconditioned in buffer A (50 mM

Figure 1. Gpa2 and Gpa3 regulate pheromone responses in *C. neoformans*. The α and **a** *gpa2* and *gpa3* mutant strains were streaked, respectively, on filament agar next to **a** and α *crg1* strains. The α *gpa2 crg1* and α *gpa3 crg1* mutants were also streaked next to the **a** *crg1* strain. The wild-type α and **a** strains are H99 and KN99a, respectively. Plates were incubated in the dark for 2 d (48 h) at room temperature, and representative sections were photographed using an Olympus microscope (BX51) equipped with a digital camera. Insets are partial images enlarged by threefold. The shade of conjugation tubes (transparent or dark) resulted from variations in light reflection. The test was repeated at least three times with similar results.

NaHepes, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 25 mM β -glycophosphate, 100 μ M Na₂VO₄, 10 μ M GDP, and the Roche protease inhibitor cocktail) for 30 min at room temperature before adding to resin containing Gpb1. The protein mixture was incubated overnight with gentle rotation at 4°C, precipitated, and washed twice with PBS buffer containing 0.2% Nonidet P-40 and 1 mM PMSF. SDS-containing gel loading buffer, 50 μ L, was added to the resin, and, after a brief boiling, 20 μ L was analyzed by SDS-PAGE electrophoresis and Western blotting using the anti-Xpress and -GST antibodies. Buffer B (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 10 μ M GDP, 30 μ M AlCl₃, 10 mM NaF, and proteinase inhibitors) was used to promote the transition state of G α s in the assay for binding between G α proteins and GST-Rgs1. Binding assays for His₆-Gpb1 and GST-G γ proteins were performed similarly.

GAP Activity Assay

To measure the GAP activity, the steady-state GTP hydrolysis assay was performed following the method described previously (Apanovitch *et al.*, 1998; Versele *et al.*, 1999; Lan *et al.*, 2000). His₆-Gpa2 and GST-Crg1 proteins were prepared as described above. Gpa2 (200 nM) and [γ -³²P]GTP (7500 cpm/pmol, 2 μ M final concentration) were mixed at room temperature in 2 $\times$ GTP buffer (50 mM Na-HEPES, pH 8.0, 1 mM dithiothreitol [DTT], 1 mM EDTA, 2 μ M GTP- γ -S, and 10 mM MgCl₂) for 2 min. Crg1 was then added, and samples were withdrawn at 2, 4, 6, and 8 min. Samples (in triplicate) were mixed with 5% charcoal (Fisher Scientific, Pittsburgh, PA) in 50 mM NaH₂PO₄, pH 2, on ice and precipitated for 15 min at 13,000 rpm. Supernatant (400 μ L) was transferred to a scintillation vial, and free phosphates were measured using a liquid scintillation counter (Cobra II, Packard Instrument, Meriden, CT). The mean value was plotted with error bars representing the SEM. Statistical significance was calculated using Student's *t* test through Prism 4.0 software (GraphPad Software). Assays were repeated twice that yielded comparable results.

RESULTS

Gpa2 and Gpa3 Exhibit Distinct Roles in Pheromone Responses

C. neoformans is divided into two distinct, but highly related, varieties: var. *grubii* (serotype A) and var. *neoformans* (serotype D). *C. neoformans* respond to the presence of the opposite mating partner by producing conjugation tubes that lead to cell fusion and formation of dikaryotic filaments under conditions such as nitrogen deprivation and desiccation (Wang *et al.*, 2000). In var. *grubii*, conjugation tubes are induced in α strains that carry a mutation in the *CRG1* gene (*crg1*) (Figure 1, bottom row), in contrast to var. *neoformans*, whose conjugation tubes are readily inducible in the wild-type α strain. No changes were found in α *gpa2*, α *gpa3*, or α

gpa2 gpa3 mutants when facing **a** *crg1* cells, similar to the wild-type strain (Figure 1, top row). However, conjugation tube formation was seen in the **a** cells facing α *gpa3* or α *gpa2 gpa3* mutants, suggesting an increased pheromone production by the α mutants. The large number of conjugation tubes stimulated by the α *gpa2 gpa3* mutant suggested that the level of pheromones it produced is equal to that of the α *crg1* mutant (Figure 1, top and bottom rows). Inclusion of the *GPA3* mutation, but not the *GPA2* mutation, in the *crg1* mutant blocked conjugation tube formation, indicating that Gpa3 is required for this pheromone response (Figure 1, middle row).

In comparison to α cells, phenotypic changes occurring in **a** cells as a result of *GPA2* and *GPA3* mutations were less apparent (Figure 1, middle row). However, our test did reveal that Gpa2 has a positive role in pheromone production, as a reduction of conjugation tube formation was seen in the α *crg1* cells facing the **a** *gpa2* mutant, in contrast to the same cells facing the **a** *gpa3*, **a** *gpa2 gpa3*, and wild-type **a** strains (Figure 1, middle and bottom rows).

On the basis of these observations, we concluded that Gpa2 and Gpa3 play distinct roles in regulating pheromone response in *C. neoformans*. Gpa2 plays a positive role and Gpa3 a negative one in pheromone production, and Gpa3 is also required for conjugation tube formation.

Gpa2 and Gpa3 Share a Conserved Regulatory Role and Are Both Required for Normal Mating

Mating in *C. neoformans* is traditionally assessed by coculturing α and **a** cells on solid V8 agar and observing formation of mating-specific dikaryotic filaments, basidia, and basidiospores. The α and **a** *gpa2* and *gpa3* mutant strains each exhibited a moderate increase in mating when compared with wild-type strains with no visible variation in either the structure or the amount of dikaryotic filaments between the cross (Figure 2, top and bottom rows). To confirm that the cross involving *gpa2* and *gpa3* strains resulted in increased mating efficiency, a quantitative mating assay was carried out by crossing the mutant strains to an **a** *ade2 ura-* (FOA⁺) strain and detecting Ade2 FOA^R progenies as a representation of mating efficiency. After coculture on

Figure 2. Gpa2 and Gpa3 regulate mating in *C. neoformans*. The α *gpa2*, α *gpa3*, α *gpa2 gpa3*, α *gpa2 crg1*, and α *gpa3 crg1* mutants were crossed with the wild-type *a* strain, and *a* *gpa2*, *a* *gpa3*, and *a* *gpa2 gpa3* strains were crossed with the wild-type α strain. The α *gpa2 crg1* and α *gpa3 crg1* strains were also crossed with the wild-type *a* strain. Plates were also incubated in the dark for 7 d at room temperature, and representative data are shown. Dikaryotic filaments, basidia, and basidiospores were visible in all crosses at day 7, with the exception that no filaments or spores were seen in crosses involving *gpb1* or between two compatible *gpa2 gpa3* mutants. The test was repeated three times yielding similar results.

SLAD medium for 7 d, crosses involving *gpa2* and *gpa3* strains, respectively, produced 112 and 107 Ade2 FOA^R progenies, whereas crosses involving the wild-type α and *gpa1*, a control strain, generated 38 and 5 Ade2 FOA^R strains, respectively.

Intriguingly, both the α and *a* *gpa2 gpa3* strains exhibited attenuated mating when crossed to a wild-type mating part-

ner, and they were unable to produce any dikaryotic filaments when crossed with each other (Figure 2, top and bottom rows), suggesting Gpa2 and Gpa3 are collectively required for normal mating.

Association between G α Subunits and G α -interacting Proteins

To help reveal the mechanism by which Gpa2 and Gpa3 regulate pheromone responses and mating, we utilized the yeast two-hybrid and protein pulldown assays to examine whether Gpa2 and Gpa3 physically interact with proteins known to also play a regulatory role in mating, such as the pheromone receptor homolog Ste3 α , Gpb1, and the RGS protein Crg1.

Ste3 α /*a* (Cpr α /*a*) and Cpr2 are the two pheromone receptor homologues in *C. neoformans*. Mutation of the *STE3 α /*a** genes attenuated mating, whereas Cpr2 remains uncharacterized (Chung *et al.*, 2002, 2003; Loftus *et al.*, 2005). We found that Gpa2 interacted with the C-terminal domain of Ste3 α (Ste3 α -C) in a yeast two-hybrid assay, suggesting that Ste3 α could activate Gpa2 in response to pheromone stimulation (Figure 3A). We also found that Gpa3 interacted with Ste3 α (Figure 3A), suggesting a similar function, although the interaction was less robust in comparison to that between Ste3 α and Gpa2.

A previous study has shown that Gpb1 is a positive regulator of pheromone responses and mating in *C. neoformans*, analogous to Ste4 of *S. cerevisiae* (Wang *et al.*, 2002). Accordingly, Gpa2 was found to interact with Gpb1 in both in vivo and in vitro assays. As shown in Figure 3A, the growth of the yeast host was robust in the presence of 1 mM 3-amino-1,2,4-triazole (3-AT), suggesting a tight binding. This binding was further demonstrated by an in vitro binding assay in which His₆-Gpa2 bound with the GST-Gpb1 fusion protein (Figure 3B, top panel, lane 4), in contrast to that involving the His₆-Gpa2 and GST proteins (Figure 3B, lane 3). Surprisingly, an interaction was not found for His₆-Gpa3 and GST-Gpb1 in both in vivo and in vitro assays, suggesting that Gpa3 may not couple to Gpb1 for function.

Crg1 Accelerates the GTPase Activity of Gpa2 In Vitro

We previously characterized the RGS protein homolog Crg1 as a negative regulator for mating, because the *crg1* mutant

Figure 3. Interactions between G α subunits and G α -interacting proteins. (A) Gpa2 interacts with Ste3 α , Gpb1, and Crg1, whereas Gpa3 only interacts with Ste3 α and Crg1 in a yeast two-hybrid assay. *GPA1*, *GPA2*, and *GPA3* cDNA was inserted into pGBKT7 (BD; Palmer *et al.*, 2006) and cDNA for the C-terminus of Ste3 α (Ste3 α -C), *GPB1*, and *CRG1* were inserted into pGADT7 (AD). The AD and BD plasmids were cotransformed into yeast PJ69-4a, and transformants were plated on SD-Leu-Trp for 3 d and on selective SD-Leu-Trp-His-Ade, with or without 3-AT, for 5 d. (B) Gpa2, but not Gpa3, interacts with Gpb1 in vitro. His₆-Gpa2, -Gpa3, and GST-Gpb1 were expressed and purified by affinity chromatography, and binding was carried out (see text). Bound proteins were separated by SDS-PAGE in duplicate and analyzed by Western blotting. Membrane blots were incubated with the anti-Xpress antibody (top panel) and the anti-GST mAb (bottom panel). The latter blot was scanned instead of autoradiographed for better visibility. Gpa2 and Gpa3 share approximately the same molecular mass of 40 kDa. Lanes 1 and 5 are direct input controls for His₆-Gpa2 and His₆-Gpa3 (20 μ l), respectively.

Figure 4. Both Gpa2 and Gpa3 interact with Crg1 in vitro and Crg1 also accelerates the GTPase activity of Gpa2. (A) His₆-Gpa2 and -Gpa3 were expressed in yeast and purified using Co²⁺ affinity resin chromatography. The Crg1 RGS domain, Rgs1, was expressed in *E. coli* and purified by glutathione affinity chromatography. After Western blotting, membranes were incubated with either the anti-Xpress antibody (top panel) or the anti-GST antibody (bottom panel). Lanes 1 and 5 are direct input controls for His₆-Gpa2 and His₆-Gpa3. (B) The steady-state GTPase assay measures the accumulation of free phosphates (Pi) over an extended time period of 8 min. His₆-Gpa2 and GST-Crg1 were expressed and purified by affinity chromatography as described in text. Sampling was carried out at 2, 4, 6, and 8 min in triplicate, and mean values were plotted against time, with SEM as error bars. Student's *t* test was performed for statistical analysis (see text). Controls contain boiled Gpa2 and Crg1. The assay was repeated multiple times with reproducible results.

strains were enhanced in pheromone response and mating (Nielsen *et al.*, 2003; Wang *et al.*, 2004b). Consistent with the observation that Gpa2 and Gpa3 are mating-specific, we found that they both associate with Crg1 in a yeast two-hybrid assay (Figure 3A). Although the interaction between Gpa3 and Crg1 was less prominent than that between Gpa2 and Crg1, Gpa3, in its transition state (GDP-AlF₄ bound), was found to bind to the RGS domain of Crg1 (Rgs1) in a pulldown assay (Figure 4A, top panel, lanes 4 and 8), similar to Gpa2. Thus, these assays indicate that Crg1 could desensitize or inactivate pheromone signaling through direct interaction with Gpa2 and Gpa3.

To determine whether Crg1 also functions as a GTPase-activating-protein (GAP) by accelerating the hydrolysis of GTP-bound Gα to GDP-bound Gα, we measured the GTPase activity of Gpa2, with and without the presence of Crg1. There are two comparable methods to evaluate the RGS

proteins GAP function: a single GTP turnover method detecting the transient release of free phosphates upon addition of the RGS protein to a mixture of preconditioned Gα and [³²P]GTP, and a steady state method measuring the accumulation of free phosphates over an extended time period. We performed the second method as the extended time allowed easier handling for multiple sampling. The recombinant Crg1 (GST-Crg1) and Gpa2 (His₆-Gpa2) proteins were purified by affinity chromatography as described in *Materials and Methods*. As seen in Figure 4B, the intrinsic GTPase activity of Gpa2 appeared to be minimal, whereas addition of the recombinant Crg1 protein prompted an increase in the GTPase activity of Gpa2 by nearly fivefold (*p* < 0.0005). The GST-Crg1 exhibited a higher than background, but less than Gpa2, GTPase activity (Figure 4B), and the *p* value was <0.0002 between GST-Crg1 and GST-Crg1 plus His₆-Gpa2, suggesting that the activity by Crg1 alone was minimal and not significant (Figure 4B).

Gpg1 and Gpg2 Exhibit Redundant and Distinct Roles in Mating

C. neoformans encodes two Gγs, in comparison to other fungal species in which a single Gγ subunit has been reported (Lengeler *et al.*, 2000). Gpg1 and Gpg2 were shown to interact with Gpb1 in a yeast two-hybrid assay (Palmer *et al.*, 2006), but the interaction was not confirmed by in vitro protein pulldown experiments. Additionally, functions for both Gγs were unknown.

The GST-Gpg1 and GST-Gpg2 fusion proteins were therefore produced and used for binding with His₆-Gpb1 through the protein pulldown assay. As shown in Figure 5A, GST-Gpg1 and GST-Gpg2 fusion proteins were able to bind to His₆-Gpb1 in vitro (lanes 2 and 3), whereas no binding was found between GST and His₆-Gpb1 (lane 6), suggesting that Gpg1 and Gpg2 can both couple to Gpb1.

Moreover, the *gpg1* and *gpg2* mutants of both α and a mating types, and the α *gpg1 gpg2* mutants were created. In confrontation testing, mutation of the *GPG1*, *GPG2*, and *GPG1 GPG2* genes in α cells did not reveal any detectable phenotypic changes associated with pheromone responses (Figure 5B, top row). However, confrontation of the α *gpg1* and α *gpg2* mutants to α *crg1* showed, respectively, less and short (α *gpg1*) or barely visible (α *gpg2*) conjugation tubes in α *crg1*, indicating that both Gpg1 and Gpg2 play a positive

Figure 5. Gpg1 and Gpg2 share a role in mating of *C. neoformans*. (A) Gpg1 and Gpg2 interact with Gpb1 in vitro. His₆-Gpb1 was expressed in yeast using the pYES2/NT vector, and Gpg1 and Gpg2 were expressed as the GST-fusion proteins in *E. coli*. Binding and detection were as the same as those described in Figures 3B and 4A. Lane 1 is the His₆-Gpb1 direct input control (20 μl). Lanes 2 and 3 are the same. (B) α *gpg1* and α *gpg2* mutants were streaked next to α *crg1* cells, and α *gpg1* and α *gpg2* mutant strains to α *crg1* cells on filament agar. Plates were incubated in the dark at room temperature for 48 h. The confrontation test between α *crg1* and α wild type is from Figure 1. Insets show the images enlarged by 2.5-fold. (C) The α *gpg1*, α *gpg2*, and corresponding complemented strains, as well as the α *gpg1 gpg2* mutant, were crossed to the wild-type α strain. The α *gpg1*, α *gpg2*, and corresponding complemented strains were crossed to the wild-type α strain. Plates were incubated in the dark for 7 d at room temperature. Dikaryotic filaments, basidia, and basidiospores were visible in crosses involving *gpg1*, but not *gpg2* or *gpg1 gpg2* mutants.

Figure 6. Mating-specific G protein subunits do not regulate virulence factors melanin and capsule formation in vitro. (A) The α *gpa2*, *gpa3*, *gpa2 gpa3* (PWC 573), *gpg1* (PWC564), *gpg2* (PWC509), and *gpg1 gpg2* mutants were spotted on Niger seed agar, and plates were incubated at 30°C for 3 d before being photographed. The *gpa1* mutant is defective in melanin production as its colony appeared pigment free. (B) The same set of mutant strains plus *gpa1* were grown in liquid DMEM medium for 2 d at 30°C (225 rpm), and cells were stained with India ink. The *gpa1* mutant is also defective in capsule formation and serves as a negative control. The capsule appears as a “white” halo surrounding the cell and the image was documented using an Olympus microscope (BX51) and a digital camera. The scale for magnification is indicated.

role in pheromone production, with the role for Gpg2 being more prominent (Figure 5B, bottom row).

Consistently, the *gpg1* mutant strains showed attenuated formation of mating-specific dikaryotic filaments and basidia, whereas the *gpg2* and *gpg1 gpg2* (α) mutant strains were sterile when crossed to strains of the opposite mating type (Figure 5C). A small amount of mating-specific structures was still seen when α *gpg2* was crossed to *a crg1*, or *a gpg2* crossed to α *crg1*, after an incubation period of 2 wk or more (data not shown), suggesting that the *gpg2* mutant is conditionally sterile. However, no mating structures were seen in the cross involving the *gpg1 gpg2* strain, indicating that the *gpg1 gpg2* mutant is sterile, similar to that of Gpb1. Results from quantitative mating by crossing the G γ mutants to the *a* tester strain (*ade2 ura-*) yielded 13 (WT α), 9 (*gpg1*), 2 (*gpg2*), and 0 (*gpg1 gpg2*) progenies after a 3-wk incubation on SLAD medium, which were consistent with those from the plate assay.

Individual Mating-specific G Proteins Are Not Required for Virulence

The previous study of *C. neoformans* Gpb1 demonstrated uncoupling between mating and virulence (Wang *et al.*, 2000). To investigate whether Gpa2, Gpa3, Gpg1, and Gpg2 proteins play any role in virulence, the corresponding mutant and complemented strains were examined for the production of virulence factors: melanin and capsule, and also virulence using a murine model. All *gpa2*, *gpa3*, *gpa2 gpa3*, *gpg1*, *gpg2*, and *gpg1 gpg2* mutants exhibited normal melanin formation on Niger seed agar (Figure 6A, α strains shown), and none of the mutants showed any abnormal formation of capsule after standard incubation of 48 h in liquid DMEM medium (Figure 6B, also α strains).

For direct virulence assessment, the α *gpa2*, *gpa3*, *gpg1*, and *gpg2* mutant strains were each introduced into five female A/JCR mice by the nasal route of inoculation, and mouse survival was monitored twice daily after infection. In the first test, mice infected with the wild-type strain had a survival of 23 d, whereas mice infected with *gpa2* and *gpa3* survived 20 and 22 d, respectively (Figure 7A). A second test revealed that mice infected with *gpg1*, *gpg2*, and wild type had survived 27, 25, and 24 d, respectively (Figure 7B). The survival was analyzed using the Kaplan-Meier method and

p values were estimated to be 0.5 (*gpa2* vs. wild type), 0.5 (*gpa3* vs. wild type), 0.25 (*gpg1* vs. wild type), and 0.6 (*gpg2* vs. wild type), indicating similar survivals (Figure 7B). In the third test that involved male mice with SCID, animals infected with *gpa2*, *gpa3*, *gpg1*, *gpg2*, *gpg1 gpg2*, and the wild-type control all had similar survival times (21, 22, 20, 21, 21, and 21, respectively). Mice infected with two independent *gpg1 gpg2* knockout mutants (nos. 53 and 55) had an identical survival (Figure 7C, no. 55, not shown). However, mice infected with two independent *gpa2 gpa3* mutant segregants (nos. 22 and 23) showed longer survival (25 and 27 d, respectively, Figure 7C), in comparison to the wild-type control (21 d, *gpa2 gpa3* vs. WT, $p < 0.05$, Figure 7C), indicating that Gpa2 and Gpa3 collectively regulate fungal virulence.

Figure 7. Gpa2 and Gpa3 collectively regulate fungal virulence. Mice were inoculated via nasal cavities, and mouse survival was monitored twice daily and plotted against time (Wang *et al.*, 2004b). (A and B) α *gpa2*, *gpa3* (PWC 573), *gpg1* (PWC564), *gpg2* (PWC509), and wild-type control were each inoculated into five 5-wk-old female A/JCR mice via nasal cavities. (C) α *gpa2*, *gpa3*, *gpa2 gpa3* (PWC572 and PWC573), *gpg1* (PWC564), *gpg2* (PWC509), *gpg1 gpg2* (PWC574, PWC575 not shown), and wild-type strains were inoculated into five 6-wk-old male SCID mice via nasal cavities. Survival was analyzed using the Kaplan-Meier method (see text).

DISCUSSION

Two $G\alpha$ Subunits Regulate Pheromone Responses and Mating

In *C. neoformans*, $G\alpha$ Gpa1 and $G\beta$ Gpb1 govern a conserved cAMP-dependent signaling and a pheromone responsive mating pathway, respectively (Alspaugh *et al.*, 1998; Wang and Heitman, 1999). Gpa1 did not interact with Gpb1, but it interacted with a $G\beta$ -like/RACK1 protein homolog, Gib2, that regulates cAMP production in strains defective for Gpa1 signaling (Palmer *et al.*, 2006). Although it is apparent that Gpa1- and Gpb1-mediated signaling pathways are analogous to those of Gpa2 and Gpa1 in *S. cerevisiae* and *S. pombe*, it is not known whether or which of the remaining two $G\alpha$ s, Gpa2 or/and Gpa3, functions in mating by coupling to Gpb1. Our study revealed that both Gpa2 and Gpa3 appear to have a role in regulating pheromone responses and mating.

The mechanism by which Gpa2 and Gpa3 regulates the pheromone response and mating is apparently complicated, and this complexity is further compounded by the inherent difference between α and *a* cells in sensing and responding to pheromones (Figure 1, bottom row). Nevertheless, studies of α cells allowed us to show that Gpa3 has a negative role in pheromone production, and Gpa3 is required for conjugation tube formation. Accordingly, studies of *a* cells revealed that Gpa2 plays a positive role in pheromone production. The abundance of conjugation tubes in a *crg1* stimulated by α *gpa2 gpa3*, suggesting an enhanced pheromone production and the opposite effect on mating by single and double $G\alpha$ mutation are all indicative of cross-talk between Gpa2- and Gpa3-mediated pathways. A simplified view for how Gpa2 and Gpa3 might function to regulate pheromone response and mating is given in Figure 8.

Gpa2 Regulates Mating through a Conserved Functional Mechanism

Mating assays of both α and *a* *gpa2* and *gpa3* mutant strains revealed an increase in mating efficiency, suggesting that Gpa2 and Gpa3 couple to Gpb1 and that mutation of one of the $G\alpha$ subunits could activate Gpb1 and the conserved downstream MAP kinase pathway. Also, previous studies

have shown that other $G\alpha$ -interacting proteins, such as the pheromone receptor Ste3 α and the RGS protein Crg1, also regulate the pheromone responses and mating in *C. neoformans* (Fraser *et al.*, 2003; Nielsen *et al.*, 2003; Wang *et al.*, 2004b). Protein-protein binding assays were therefore carried out to test possible interactions between Gpa2 and Gpa3 and the above-mentioned proteins. Consistent with a role for Gpa2 in regulating mating through a conserved signaling mechanism, it interacted with Ste3 α , Gpb1, and Crg1. Moreover, Crg1 exhibited in vitro GAP activity toward Gpa2.

Intriguingly, despite interacting with Ste3 α and Crg1, Gpa3 was not found to interact with Gpb1, suggesting that Gpa3 may function independent of Gpb1, or alternately, the in vivo and in vitro conditions do not foster an environment in which Gpa3 and Gpb1 can interact. We have recently obtained evidence indicating that the GDP-ALF₄ bound Gpa3, but not Gpa2, interact with a RGS protein homolog involved in the Gpa1-cAMP pathway of *C. neoformans* (Shen and Wang, unpublished observations). These observations indicate that Gpa3 may be involved in multiple signaling processes through either conserved or unique functional mechanisms.

Redundant and Unique Functions in Mating by Gpg1 and Gpg2

We previously identified Gpg1 and Gpg2 and showed that both proteins can associate with Gpb1 in a yeast two hybrid assay (Palmer *et al.*, 2006). Here, we show that both Gpg1 and Gpg2 can interact with Gpb1 in vitro and they share a positive regulatory function in pheromone production and mating.

Why does *C. neoformans* encode two $G\gamma$ subunits, whereas other fungi studied so far contain only a single $G\gamma$ subunit? One can hypothesize that two $G\gamma$ subunits are the product of a gene duplication event and that both $G\gamma$ s have evolved to assume distinct as well as overlapping functions. A similar proposition was made for the two cyclophilin proteins, Cpa1 and Cpa2, in this fungus (Wang *et al.*, 2001). However, unlike Cpa1 and Cpa2 that are inversely located within a distance of 21 kb on chromosome 2, Gpg1 and Gpg2 are found separately on chromosomes 4 and 7. Regardless, our study has shown that both Gpg1 and Gpg2 couple to Gpb1 and that the Gpb1-Gpg2 heterodimer plays a more prominent role in the activation of the pheromone-responsive mating pathway.

Coregulation between Mating and Virulence

One primary reason to study the mating regulatory mechanism in *C. neoformans* is that there is a close link between mating type and virulence. Kwon-Chung *et al.* (1992) reported that a var. *neoformans* α strain was approximately three times more virulent than a congenic *a* strain in an experimental murine virulence model. Such an association was further corroborated by studies of mating type locus components, such as Ste20 α , Ste11 α , and Ste12 α /Ste12 α , whose mutation attenuated fungal virulence (Chang *et al.*, 2000; Clarke *et al.*, 2001; Wang *et al.*, 2002). Mutation of nonmating type specific components that regulate mating, such as Gpb1 and the MAP kinase homolog Cpk1, resulted in no alteration in virulence (Wang *et al.*, 2000; Davidson *et al.*, 2003). Interestingly, on the other end of the spectrum, mutation of Crg1 resulted in an enhancement in both mating and virulence in an archetypical var. *grubii* strain (Wang *et al.*, 2004b). However, a similar increase in virulence was not found in the *a* *crg1* strains or the var. *neoformans* α and *a* *crg1* mutant strains (Thompson and Wang, unpublished observation).

Figure 8. Canonical heterotrimeric G protein(s) regulating pheromone responses, mating, and virulence in *C. neoformans*. The pheromone receptor Ste3 α /a is likely to be activated by pheromones that in turn activate the heterotrimeric G proteins Gpa2 and Gpa3. Gpa2 couples to Gpb1 and Gpg1/Gpg2 as a heterotrimeric G protein complex, whereas Gpa3 may or may not interact with Gpb1-Gpg1/Gpg2 for mating. Crg1 provides a mechanism for pheromone desensitization and inactivation of mating. The mechanisms by which Gpa2 inhibits, Gpa3 promotes, pheromone production, Gpa3 is required for conjugation tube formation, and Gpa2 and Gpa3 are collectively required for normal mating and virulence remain to be defined.

Neither the $G\alpha$ nor $G\gamma$ subunits under study are mating type specific. The *in vitro* production of melanin and capsule of mutants also did not reveal any significant changes when compared with wild-type strains, suggesting that virulence traits were not affected by gene mutation. *In vitro* virulence traits often provide a good indication for virulence, but they do not always correspond fully with results from animal models. For example, mutation of the Akt/PKB type protein kinase Sch9 resulted in enlarged capsules *in vitro*, but the *sch9* mutant was severely attenuated in virulence (Wang *et al.*, 2004a). Therefore, the $G\alpha$ and $G\gamma$ mutant strains were subjected to direct virulence testing in murine models. We have shown that individual $G\alpha$ and individual and collective $G\gamma$ subunits do not play any role in virulence. However, Gpa2 and Gpa3 are collectively required for full virulence expression, because two independent *gpa2 gpa3* mutants were attenuated in virulence involving male SCID mice (Figure 7C). Thus, the association between mating and virulence appears not due to mating per se but rather to certain components of the mating pathway(s) that encode functions in addition to their conserved role for mating. As a result of mating pathway alteration, decreased/increased activities of mating-associated protein kinases and transcription factors resulted in reduced/enhanced production of the factors that promote virulence.

In summary, *C. neoformans* is a unique organism in which pheromone responses and mating are regulated by two $G\alpha$ and two $G\gamma$ subunits, as well as a single $G\beta$ subunit. We found that Gpa2 functions through a conserved signaling mechanism and that there may exist an additional regulatory mechanism for Gpa3. Finally, Gpa2 and Gpa3 collectively regulate virulence even though individual Gpa2 and Gpa3 do not. Studies of these G proteins could therefore lead to further revelations of the complexity by which G protein signaling pathways govern the physiology and virulence of this pathogenic fungal organism.

Note added in proof. We acknowledge online publication of an independent study by Hsueh *et al.* (2007) on a similar subject before the final acceptance of this study.

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