G protein signaling governing cell fate decisions involves opposing $G\alpha$ subunits in *Cryptococcus neoformans*^D

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Communication between cells and their environments is often mediated by G protein-coupled receptors and cognate G proteins. In fungi, one such signaling cascade is the mating pathway triggered by pheromone/pheromone receptor recognition. Unlike *Saccharomyces cerevisiae*, which expresses two $G\alpha$ subunits, most filamentous ascomycetes and basidiomycetes have three $G\alpha$ subunits. Previous studies have defined the $G\alpha$ subunit acting upstream of the cAMP-protein kinase A pathway, but it has been unclear which $G\alpha$ subunit is coupled to the pheromone receptor and response pathway. Here we report that in the pathogenic basidiomycetous yeast *Cryptococcus neoformans*, two $G\alpha$ subunits (Gpa2, Gpa3) sense pheromone and govern mating. *gpa2 gpa3* double mutants, but neither *gpa2* nor *gpa3* single mutants, are sterile in bilateral crosses. By contrast, deletion of *GPA3* (but not *GPA2*) constitutively activates pheromone response and filamentation. Expression of *GPA2* and *GPA3* is differentially regulated: *GPA3* expression is induced by nutrient-limitation, whereas *GPA2* is induced during mating. Based on the phenotype of dominant active alleles, Gpa2 and Gpa3 signal in opposition: Gpa2 promotes mating, whereas Gpa3 inhibits. The incorporation of an additional $G\alpha$ into the regulatory circuit enabled increased signaling complexity and facilitated cell fate decisions involving choice between yeast growth and filamentous asexual/sexual development.

INTRODUCTION

The ability to sense environmental stimuli and appropriately respond is vital for all organisms. A large proportion of this communication between cells and the environment is mediated via G protein-coupled receptors (GPCRs) and their cognate heterotrimeric G proteins, which consist of α , β , and γ subunits. GPCRs comprise the largest superfamily of cell surface receptors, responding to a panoply of extracellular stimuli as diverse as hormones, neurochemicals, odorants, nutrients, and light (Bockaert and Pin, 1999). G proteins can bind to and hydrolyze GTP and alternate between the GTP-bound “on” and the GDP-bound “off” states. They function as molecular switches in signal transduction pathways to elicit diverse physiological processes. Ligand activation of GPCRs triggers the coupled $G\alpha$ subunit to release GDP and bind to GTP, transforming them into the $G\alpha$ -GTP active state. This results in a conformational change of the $G\alpha$ subunit, which promotes dissociation from the $G\beta\gamma$ complex. The liberated $G\alpha$ and $G\beta\gamma$ subunits may then interact with downstream effectors to switch on or off signaling cascades.

Two major signal transduction pathways, one responding to pheromone and the other to nutrients, are both governed by G proteins in *Saccharomyces cerevisiae* (Lengeler *et al.*, 2000; Harashima and Heitman, 2004). These two pathways, the pheromone response pathway and the cAMP pathway, are among the most well understood signaling pathways in eukaryotes. Studies of this model have enormously contrib-

uted to the understanding of the mechanisms of G protein signaling and regulation. The G protein α subunit Gpa1 is activated when pheromone binds to the pheromone receptors Ste2 or Ste3; the GTP-bound Gpa1 releases the $G\beta\gamma$ complex (Ste4/Ste18) to allow activation of their downstream effectors, including Ste20, Far1/Cdc24, and Ste5 to initiate mating responses (Dohlman and Thorner, 2001). In the cAMP-protein kinase A (PKA) pathway, the sugar receptor Gpr1 senses glucose and activates Gpa2 to stimulate adenylyl cyclase and increase intracellular cAMP levels, leading to PKA activation (Kubler *et al.*, 1997; Lorenz and Heitman, 1997; Kraakman *et al.*, 1999; Lorenz *et al.*, 2000).

The GPCR and G protein (GPCR-G) repertoire in *S. cerevisiae* is extremely simple; only three GPCRs (Ste2/3 and Gpr1) and two G protein α subunits (Gpa1 and Gpa2) are expressed. Many higher eukaryotes have an enormous complexity of GPCRs signaling. For example, 57 GPCRs and 14 $G\alpha$ subunits are encoded in the slime mold *Dictyostelium discoideum* genome, and these numbers are further expanded to hundreds and even thousands for GPCRs and more than 20 $G\alpha$ subunits in the nematode and human, respectively (Simon *et al.*, 1991; Jansen *et al.*, 1999; McCudden *et al.*, 2005). As a consequence, it is a considerable challenge to study GPCR-G protein coupling specificity in these higher organisms, even though the number of expressed GPCRs and G proteins in each cell type may be more limited.

Several fungal genomes that have been annotated thus far reveal that the GPCR-G protein signaling networks in the filamentous ascomycetes and basidiomycetes are more complex than the *S. cerevisiae* paradigm, but not yet as extreme as that found in the multicellular eukaryotes. For example, in *Neurospora crassa* 10 potential GPCRs and 3 $G\alpha$ subunits are present in the genome, and in *A. nidulans* there are 16 GPCRs and 3 $G\alpha$ subunits (Borkovich *et al.*, 2004; Yu, 2006). In the basidiomycete human pathogen *Cryptococcus neoformans*, ~31 genes are predicted to be potential GPCRs and 3 $G\alpha$

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subunits were identified (Loftus *et al.*, 2005; Xue *et al.*, 2006). Furthermore, the fungal G proteins do not fall into discrete G protein categories; they all are most closely related to G_i mammalian proteins, based on sequence homology. Thus, these fungal species, with more complicated G protein signaling circuits than *S. cerevisiae*, yet still genetically tractable, can serve as models with which to dissect GPCR- and G protein-coupling specificity.

Among the three G protein α subunits expressed in *C. neoformans* (Gpa1, Gpa2, and Gpa3), only Gpa1 has a defined function. Genetic and biochemical analyses established that Gpa1 functions upstream of adenylyl cyclase and PKA and is required for pathogenesis and development of *C. neoformans* (Alspaugh *et al.*, 1997, 2002; D'Souza *et al.*, 2001). In a more recent study, the GPCR Gpr4 was shown to interact with Gpa1 and in response to methionine binding, Gpa1 initiates cAMP-PKA signaling (Xue *et al.*, 2006). Conversely, it has been uncertain which G α protein is coupled to the pheromone receptor, because neither of the other two G protein α subunit null mutants (*gpa2* or *gpa3*) exhibits a sterile phenotype. In fact, except in the budding and fission yeast, which only have two G α subunits, no studies have conclusively shown the coupling/interactions between the pheromone receptors and the G α subunits in any other fungus. In contrast, several G α subunits have been identified that signal through the cAMP-PKA pathway in both ascomycetes and basidiomycetes (Regenfelder *et al.*, 1997; Kruger *et al.*, 1998; Kays *et al.*, 2000; Shimizu and Keller, 2001; Han *et al.*, 2004; Maidan *et al.*, 2005).

Basic principles of G protein signaling and regulation in *S. cerevisiae* were elucidated over a decade ago, yet recently several unexpected facets were revealed. First, Gpa1 was for a long time considered to solely negatively regulate pheromone signaling by sequestering the G $\beta\gamma$ complex, but Slessareva *et al.* (2006) demonstrated that GTP-bound Gpa1 transduces a positive signal leading to mating events via the phosphatidylinositol 3-kinase Vps34 located on endosomal membranes. Second, a recent study showed that the RGS (regulator of G protein signaling) protein Sst2, which functions as a GAP for the G α subunit Gpa1 to desensitize cells from pheromone signaling, binds to the activated pheromone receptor via its DEP domain (Ballon *et al.*, 2006). This interaction localizes Sst2 in close proximity to its target Gpa1, presumably enhancing target specificity (Ballon *et al.*, 2006). Similar to the expansion of G proteins found in the filamentous fungi, the RGS protein family has also expanded. For example, five distinct RGS proteins were identified in the *A. nidulans* genome (Yu, 2006). In *C. neoformans*, Crg1 was identified as an Sst2 homolog that functions to desensitize pheromone responses, but its target substrates are not yet well understood (Wang *et al.*, 2004).

In this study, we dissected G protein signaling regulation of the pheromone response pathway in *C. neoformans*. Two G protein α subunits, Gpa2 and Gpa3, were found to be critical to enable pheromone sensing and mating in haploid yeast cells. *gpa2 gpa3* double mutants exhibit a cell-cell fusion defect and a bilateral sterile phenotype, indicating that both G protein α subunits share an overlapping role in mating. However, Gpa2 promoted filamentous growth in its activated form, whereas dominant active Gpa3 inhibited mating. In addition to the G α proteins, a G γ subunit Gpg2 was identified to be essential for mating. Furthermore, the novel RGS protein Crg2 was found to negatively regulate pheromone response during the early stages of mating and is required to complete sexual development in the terminal stage of mating. The pheromone receptor Ste3 α interacts with both G α subunits and RGS proteins in vivo.

Our study demonstrates that *C. neoformans*, and likely also in other fungal species that express more than two G α subunits, additional G proteins are involved in the mating pathway regulatory circuits. We propose that this may be a consequence of the requirement for cells to choose their fate between budding yeast growth and asexual or sexual filamentous growth. Our studies also have implications for an understanding of the redundancy and specialized signaling roles of G protein-mediated GPCR signaling networks in multicellular eukaryotes.

MATERIALS AND METHODS

Strains and Media

Strains used in this study are listed in Table 1. Yeast extract-peptone-dextrose (YPD) and synthetic (SD) media were used for strain growth and maintenance. Mating assays were conducted on 5% V8 juice agar medium (pH 5 for serotype A and pH 7 for serotype D strains) or Murashige and Skoog (MS) medium minus sucrose (Sigma-Aldrich, St. Louis, MO).

Gene Disruption of GPA2, GPA3, CRG2, GPB1, and GPG2

All mutations in the serotype A background were generated with dominant selectable markers *NAT* or *NEO* using an overlap PCR approach as previously described (Davidson *et al.*, 2002; Fraser *et al.*, 2003). Briefly, the 5' and 3' flanking regions of the target gene were amplified with gene-specific primers and the dominant *NAT* or *NEO* selectable markers were amplified with the universal M13F and M13R primers. These amplified 5' and 3' flanking regions, together with one of the dominant markers, were used as the DNA template for the second overlap PCR. The products of overlap PCR were purified and directly transformed into the cells by biolistic transformation (Davidson *et al.*, 2000). Primers used for amplification of the 5' and 3' flanking regions of each gene disruption cassette are described in Supplementary Table 1.

Complementation of *gpb1* and *gpg2* Mutations

The wild-type *GPB1* and *GPG2* genes, including 1-kb upstream promoter and 0.5-kb downstream terminator regions were amplified with primer pairs JOHE17978/JOHE17979 and JOHE17947/JOHE17948 using wild-type genomic DNA as template. A second overlap PCR was conducted using the amplified wild-type *GPB1* and *GPG2* DNA fragment and the M13F/M13R amplified *NEO* marker as template with primers JOHE17978/M13R and JOHE17947/M13R. The overlap PCR products were purified and transformed into strains YPH312 and YPH272 to complement the *gpb1* and *gpg2* mutations. Southern analysis was performed to determine the copy number of the reintroduced wild-type alleles. Transformants with one copy of the wild-type *GPB1* or *GPG2* gene were analyzed phenotypically.

Double or Triple Deletion Mutant Generation

The serotype D *MATa* and *MATa gpa2 gpa3* double mutants (YPH88 and YPH97) were isolated from the progeny of the cross *MATa gpa2* $\times$ *MATa gpa3* (WSC26 $\times$ YPH86) and the serotype A *MATa gpa2 gpa3* double mutant (YPH106) was also a progeny derived from the cross *MATa gpa2* $\times$ *MATa gpa3* (YSB25 $\times$ YSB136). Basidiospores were micromanipulated according to methods described previously (Hsueh *et al.*, 2006), and the genotype of each progeny was determined by PCR analyses. Serotype A *MATa gpa2 gpa3* double mutant (YPH308) was created by deleting the *GPA2* gene in the *gpa3* mutant (YPH105) background using an overlap PCR and biolistic transformation approach. The *crg1 gpa2* double mutants (YPH113 and YPH114) were progeny derived from the cross PPW196 (*MATa crg1*) $\times$ YSB25 (*MATa gpa2*) and *crg1 gpa3* double mutants (YPH116 and YPH117) were progeny from the cross PPW111 (*MATa crg1*) $\times$ YSB136 (*MATa gpa3*). The *crg1 gpa2 gpa3* triple mutants (YPH118 and YPH380) were progeny derived from the cross YPH114 (*MATa crg1 gpa2*) $\times$ YSB136 (*MATa gpa3*). The *crg2 gpa2*, *crg2 gpa3*, and *crg2 gpa2 gpa3* mutants were isolated from the cross CDX50 (*MATa crg2*) $\times$ YPH106 (*MATa gpa2 gpa3*). Primer pairs JOHE6013/JOHE6014, JOHE8994/JOHE14774, JOHE8994/JOHE10923, and JOHE8994/JOHE12228 were used to identify the *crg1*, *crg2*, *gpa2*, and *gpa3* mutant alleles, respectively. Sequences of these primers are listed in Supplementary Table 1.

Cell Fusion and Confrontation Assays

Cell fusion assays for *gpa2*, *gpa3*, and *gpa2 gpa3* mutants were conducted with serotype D auxotrophic strains. To introduce a *ura5* allele into the *MATa gpa2*, *MATa gpa3*, and *MATa gpa2 gpa3* mutants, cells were grown on 5-FOA medium for 3–5 d, and spontaneous resistant colonies were selected and tested for their ability to grow on the SD-uracil medium. On the other hand, the *MATa gpa2 lys1*, *MATa gpa3 lys1*, and *MATa gpa2 gpa3 lys1* strains were selected progeny from crosses YPH94 (*MATa gpa2*) $\times$ JEC31 (*MATa lys1*),

Table 1. Strains used in this study

Strain	Genotype	Sources/References
<i>C. neoformans</i> var. <i>neoformans</i> (serotype D)		
JEC20	MATa	Kwon-Chung <i>et al.</i> (1992)
JEC21	MATα	Kwon-Chung <i>et al.</i> (1992)
JEC30	MATa <i>lys1</i>	J. Edman
JEC31	MATα <i>lys1</i>	J. Edman
JEC34	MATa <i>ura5</i>	Moore and Edman (1993)
JEC43	MATα <i>ura5</i>	Moore and Edman (1993)
JEC50	MATα <i>ade2</i>	Moore and Edman (1993)
RCD10	MATα <i>P_{MFα1}::lacZ</i>	Shen <i>et al.</i> (2002)
WSC26	MATα <i>gpa2::ADE2 ade2</i>	Shen <i>et al.</i> , unpublished data
YPH128	MATa <i>gpa2::ADE2 ade2?</i>	This study
WSC34	MATα <i>gpa3::ADE2 ade2</i>	Shen <i>et al.</i> , unpublished data
YPH86	MATa <i>gpa3::ADE2 ade2?</i>	This study
YPH97	MATα <i>gpa2::ADE2 gpa3::ADE2 ade2?</i>	This study
YPH88	MATa <i>gpa2::ADE2 gpa3::ADE2 ade2?</i>	This study
YPH312	MATα <i>gpa3::ADE2 gpb1::NAT</i>	This study
YPH381	MATα <i>gpa3::ADE2 gpb1::NAT GPB1-NEO</i>	This study
YPH130	MATα <i>gpa2::ADE2 ura5 ade2?</i>	This study
WSC75	MATα <i>gpa3::ADE2 ura5 ade2</i>	Shen <i>et al.</i> , unpublished data
YPH132	MATα <i>gpa2::ADE2 gpa3::ADE2 ura5 ade2?</i>	This study
YPH131	MATa <i>gpa2::ADE2 gpa3::ADE2 ura5 ade2?</i>	This study
YPH95	MATa <i>gpa2::ADE2 lys1 ade2?</i>	This study
YPH96	MATa <i>gpa3::ADE2 lys1 ade2?</i>	This study
YPH99	MATα <i>gpa2::ADE2 gpa3::ADE2 lys1 ade2?</i>	This study
YPH100	MATa <i>gpa2::ADE2 gpa3::ADE2 lys1 ade2?</i>	This study
YPH328	MATα <i>P_{GPD1}::GPA2^{Q203L} P_{MFα1}::lacZ</i>	This study
YPH329	MATα <i>P_{GPD1}::GPA3^{Q206L} P_{MFα1}::lacZ</i>	This study
WSC129	MATα <i>gpb1::URA5 ura5</i>	Shen <i>et al.</i> (2002)
YPH330	MATα <i>P_{GPD1}::GPA2^{Q203L} gpb1::URA5 ura5</i>	This study
RCD25-1	MATα <i>ste7::ADE2 ade2</i>	Davidson <i>et al.</i> (2003)
YPH332	MATα <i>P_{GPD1}::GPA2 Q203L ste7::ADE2 ade2</i>	This study
YPH471	MATα <i>P_{GPD1}::GPA2</i>	This study
YPH472	MATα <i>P_{GPD1}::GPA3</i>	This study
<i>C. neoformans</i> var. <i>grubii</i> (serotype A)		
H99	MATα WT	Perfect <i>et al.</i> (1993)
KN99a	MATa WT	Nielsen <i>et al.</i> (2003)
F99	MATα <i>ura5</i>	Wang <i>et al.</i> (2004)
YSB25	MATα <i>gpa2::NEO</i>	This study
YSB110	MATa <i>gpa2::NEO</i>	This study
YPH105	MATα <i>gpa3::NEO</i>	This study
YSB136	MATa <i>gpa3::NEO</i>	This study
YPH308	MATα <i>gpa2::NAT gpa3::NEO</i>	This study
YPH106	MATa <i>gpa2::NEO gpa3::NEO</i>	This study
PPW111	MATα <i>crg1::URA5 ura5</i>	Wang <i>et al.</i> (2004)
PPW196	MATa <i>crg1::URA5 ura5</i>	Wang <i>et al.</i> (2004)
YPH114	MATα <i>crg1::URA5 gpa2::NEO ura5?</i>	This study
YPH113	MATa <i>crg1::URA5 gpa2::NEO ura5?</i>	This study
YPH116	MATα <i>crg1::URA5 gpa3::NEO ura5?</i>	This study
YPH117	MATa <i>crg1::URA5 gpa3::NEO ura5?</i>	This study
YPH380	MATα <i>crg1::URA5 gpa2::NEO gpa3::NEO ura5?</i>	This study
YPH118	MATa <i>crg1::URA5 gpa2::NEO gpa3::NEO ura5?</i>	This study
CDX50	MATα <i>crg2::NAT</i>	This study
CDX51	MATa <i>crg2::NEO</i>	This study
YPH299	MATα <i>crg2::NAT gpa2::NEO</i>	This study
YPH303	MATα <i>crg2::NAT gpa3::NEO</i>	This study
YPH305	MATα <i>crg2::NAT gpa2::NEO gpa3::NEO</i>	This study
YPH272	MATα <i>gpg1::NAT</i>	This study
YPH314	MATα <i>gpg1::NAT GPG1::NEO</i>	This study
<i>S. cerevisiae</i>		
NMY32	MATa <i>his3Δ200 trp1-901 leu2-3,112 LYS2::(lexAop)</i> <i>4-HIS3URA3::(lexAop)8-lacZ (lexAop)8-ADE2 GAL4</i>	DUALmembrane Kit 2 (Dualsystem Biotech, Zürich, Switzerland)

YPH86 (MATa *gpa3*) × JEC31 (MATα *lys1*), and YPH88 (MATa *gpa2 gpa3*) × JEC31 (MATα *lys1*). Spores were isolated by micromanipulation, germinated, and analyzed by PCR and growth assays on SD-lysine medium.

To perform the cell fusion assays, MATα *ura5* wild-type or mutant strains (JEC43, YPH130, WSC75, and YPH132) and MATa *lys1* wild-type or mutant strains (JEC30, YPH95, YPH96, and YPH100) were grown in YPD liquid medium overnight. The next day, cells were collected, washed, and adjusted to 1×10^7 cells/ml. Equal numbers of cells of opposite mating type were

mixed on V8 mating medium for 24 h in the dark. Cells were removed from the surface of medium and spread onto SD-uracil-lysine medium to select dikaryotic fusion products. The number of colonies from each cross was counted, and fusion efficiency calculated in proportion to wild type. To measure the fusion ability of the *gpg2* mutant, equal numbers of strains YPH272 (MATα *gpg2::NAT*) and YSB121 (MATa *NEO*) were mixed and incubated on V8 mating medium for 24 h, and the mating colonies were harvested and spread on YPD medium containing nourseothricin and G418. The num-

ber of the colonies was determined and compared with that from the fusion between wild-type strains YSB119 (*MATa* NAT) and YSB121 (*MATa* NEO).

For confrontation assays, cells of opposite mating type were streaked in parallel, ~3–4 mm apart, on V8 medium (pH 5) and incubated at 25°C in the dark for 3–4 d. Images were captured with a Nikon Eclipse E400 microscope equipped with a Nikon DXM1200F digital camera.

RNA Extraction and Northern Blot Analysis

Cells for RNA extraction were grown in YPD liquid culture overnight and the next day were harvested, washed, and adjusted to a density of 1×10^7 cells/ml. Drops of 5 μ l of cells were spotted on V8 mating medium alone or with a mating partner for the nonmating and mating conditions, respectively. For both conditions, cells were incubated at 25°C for 24 h and harvested. RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Total RNA, 10 μ g, was separated by denaturing agarose gel electrophoresis and blotted to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and probed with [³²P]dCTP-radiolabeled DNA fragments. Primers used for probe amplification are listed in Supplementary Table 2. Quantification of the expression signals was conducted with a Typhoon 9200 imager and Image Quantifier 5.2 software (Molecular Dynamics, Sunnyvale, CA).

Overexpression of Wild-Type and Dominant Active *Gpa2*^{Q203L} and *Gpa3*^{Q206L} Alleles

The dominant active *GPA2*^{Q203L} and *GPA3*^{Q206L} alleles were obtained by overlap PCR. Primers JOHE17654/JOHE17655 (for *GPA2*) and JOHE17656/JOHE17657 (for *GPA3*) were designed to introduce the Q-to-L substitution at the conserved position. The N-terminal half of the *GPA2*^{Q203L} allele was amplified with primer pair JOHE17646 and JOHE17655, using H99 genomic DNA as template. The C-terminal half was amplified with primer pair JOHE17647 and JOHE17654. Primers JOHE17646 and JOHE17647 were then used for the second overlap PCR. By the same method, *GPA3*^{Q206L} allele was amplified with primer pairs JOHE17648/JOHE17657 and JOHE17649/JOHE17656 (Supplementary Table 1). These amplified *GPA2*^{Q203L} and *GPA3*^{Q206L} alleles were digested with the restriction enzymes FseI and PacI and cloned into pXL1 driven by the constitutively expressed *GPD1* promoter to generate the *GPA2*^{Q203L} and *GPA3*^{Q206L} overexpression plasmid YPP34 and YPP35. These plasmids were sequenced to confirm that the Q-to-L mutation (and no extraneous mutations) was present and then transformed into different backgrounds including wild-type, *gpb1*, and *ste7* mutants. The transformants were analyzed with Northern analysis to determine the abundance of the *GPA2*^{Q203L} and *GPA3*^{Q206L} transcripts. Strains that expressed similar levels of *GPA2*^{Q203L} and *GPA3*^{Q206L} message were subjected to further β -galactosidase activity assays and morphological analyses. Wild-type *GPA2* and *GPA3* alleles were amplified with primers JOHE17646/JOHE17647 and JOHE17648/JOHE17649, respectively. The DNA was digested with FseI and PacI and cloned into plasmid pXL1 driven by the constitutively expressed *GPD1* promoter, resulting in the *GPA2* and *GPA3* overexpression constructs YPP44 and YPP47. These two constructs were transformed into strain JEC21 and the transformants that expressed a high abundance of *GPA2* or *GPA3* transcripts as detected by Northern analysis were further analyzed for their mating ability.

Split-Ubiquitin Protein-Protein Interaction Assays

The split-ubiquitin system was utilized to investigate the potential interactions between pheromone receptor Ste3 and G protein α subunits Gpa1, Gpa2, and Gpa3, as well as interactions between Ste3 and RGS proteins Crg1 and Crg2. Vectors and yeast strains were included in the DUALmembrane Kit 2 (Dualsystem Biotech, Zurich, Switzerland). *STE3* full-length cDNA was cloned into pCCW (the C-terminal half of the ubiquitin Cub protein was fused to the C-terminus of *STE3*). *CRG1*, *GPA1*, *GPA2*, and *GPA3* full-length cDNAs were cloned into the pDL2XN vector (the mutated N-terminal half of ubiquitin NubG protein was fused to the test proteins' C-termini). *CRG2* full-length cDNA was cloned into the pDSL-NX vector (the NubG protein was fused to the N-terminus of *CRG2*). All cDNA sequences were confirmed by DNA sequencing. Cub and NubG fusion constructs were cotransformed into host yeast strain NMY32. Interaction was determined by the growth of yeast transformants on medium lacking histidine and also by measuring β -galactosidase activity. Primers used for the generation of the fusion alleles are listed in Supplementary Table 1.

RESULTS

gpa2 gpa3 Double Mutants Are Bilaterally Sterile

Three G protein α subunits are expressed in *C. neoformans*. Gpa2 and Gpa3 were identified in the genome sequence and share homology with *S. cerevisiae* Gpa1 (GenBank Accession numbers AAQ62550 and AAQ74379). Gpa2 and Gpa3 are 45% identical to each other, and both have the conserved GTPase motif and N-terminal Met-Gly sequence and an

adjacent Cys residue that are required for myristoylation and palmitoylation. To identify which G protein α subunit governs the pheromone response pathway, *gpa2* and *gpa3* null mutants were generated in both **a** and α cells in the *C. neoformans* var. *grubii* (serotype A) and var. *neoformans* (serotype D) backgrounds. Surprisingly, neither the *gpa2* or *gpa3* single mutants exhibited mating defects, even in bilateral crosses, suggesting that cells can sense pheromone without either G α subunit (Figure 1, A and B). We also considered and excluded the hypothesis that Gpa2 and Gpa3 might play cell type-specific roles in mating (i.e., α *gpa2* $\times$ **a** *gpa3* and α *gpa3* $\times$ **a** *gpa2* crosses were fertile). These results led us to hypothesize that the two G α subunit might be functionally redundant in pheromone sensing or that no G α subunits regulate mating.

To test the functional redundancy model, double mutants lacking both G α subunits were isolated and examined for their mating properties. In unilateral crosses (mutant $\times$ wild-type), the *gpa2 gpa3* mutants were still fertile in both mating types and serotypes. Sexual structures, including dikaryotic filaments, fused clamp cells, basidia, and basidiospores were all observed, but were less abundant when compared with a wild-type cross (Figure 1, A and B). Similarly, *gpa2 gpa3* double mutants are still fertile when crossed to *gpa2* or *gpa3* single mutants (Supplementary Figure 1). Nonetheless, in the bilateral cross (mutant $\times$ mutant), *gpa2 gpa3* double mutants exhibited severe mating defects. No mating structures were observed in serotype A mutants, whereas in serotype D mutants, because of their self-filamentous phenotype (Figure 2A), filaments and basidia were still observed but no basidiospores (meiotic progeny) were produced (Figure 1B). DAPI staining showed that filaments produced in the serotype D *gpa2 gpa3* double mutant bilateral cross were monokaryotic instead of dikaryotic, demonstrating that these hyphae are not mating structures (Figure 1B).

To establish that the bilateral sterile phenotype and the *gpa2 gpa3* double deletion are linked, a genetic analysis was conducted. The serotype A *MATa gpa2 Δ ::NAT gpa3 Δ ::NEO* double mutant was crossed to the wild-type *MATa* strain KN99a, and 42 basidiospores were isolated. Among these progeny, 12 wild-type, 10 *gpa2 Δ ::NAT* single mutant, 16 *gpa3 Δ ::NEO* single mutant, and 4 *gpa2 Δ ::NAT gpa3 Δ ::NEO* double mutants were found. The wild-type progeny of different mating types were crossed to each other, and all crosses were fertile. Thus, the sterile phenotype displayed by *gpa2 gpa3* double mutants is not attributable to a background mutation. On the other hand, when the four *gpa2 Δ ::NAT gpa3 Δ ::NEO* double mutants of opposite mating types were crossed to each other or to the parental *gpa2 gpa3* mutants, all exhibited a bilateral sterile phenotype. Thus, the bilateral sterile phenotype is linked to the *gpa2* and *gpa3* mutations.

The bilateral sterile phenotype of the *gpa2 gpa3* mutant strongly suggests that both G α subunits play a role in the mating pathway, and the double mutants are likely to be insensitive to the pheromone secreted by the mating partner. One of the first steps during sexual development in *Cryptococcus* involves the fusion of **a** and α cells to form a heterokaryon, and defects in pheromone sensing compromise this process. To determine whether the sterile phenotype of a *gpa2 gpa3* mutant is caused by an inability to undergo cell-cell fusion, quantitative cell-cell fusion assays were performed using different auxotrophic strains (see *Materials and Methods*). In a *gpa2 gpa3* double mutant bilateral cross, the fusion efficiency is dramatically reduced by at least 10^5 fold (0.0008% compared with wild-type; 100%). In unilateral mutant crosses, the *MATa gpa2 gpa3* mutant fused to wild-type α cells at a level of 90% efficiency and the *MATa gpa2 gpa3*

Figure 1. *gpa2 gpa3* double mutants exhibit a bilateral mating defect. (A) Serotype A strains of the indicated genotypes were crossed on V8 mating medium. The photos of the mating colonies (in the top panels) were taken after 2-wk incubation in the dark at 25°C. Chains of basidiospores were photographed at 200× magnification. (B) Serotype D strains of the indicated genotypes were crossed on V8 mating medium and photographed at 40× (the first row of panels) or 200× (the second row of panels) magnification after 72 h. The number of nuclei in the hyphal cells was determined by DAPI staining and differential interference contrast imaging at 1000× magnification. ▲ fused clamps; △, unfused clamps (third and fourth rows of panels). The fusion efficiency in isogenic strain backgrounds was determined by quantitative cell–cell fusion assays (bottom row of panels).

double mutant fused 51% as efficiently as wild type (Figure 1 B, bottom panel). This analysis demonstrates that if both *a* and α cells are insensitive to pheromone, they are unable to fuse with each other; however, if pheromone sensing is only perturbed in one of the two mating partners, a substantial proportion of the cells are still capable of completing the sexual cycle. This genetic evidence supports the hypothesis that both Gpa2 and Gpa3 are involved in pheromone sensing and are at least in part functionally redundant.

gpa3 Deletion Leads to a Constitutively Active Pheromone Response

In *S. cerevisiae*, one major function of the $G\alpha$ subunit Gpa1 is to sequester the $G\beta\gamma$ complex to prevent pheromone signaling under nonmating conditions (Nomoto *et al.*, 1990). As a consequence, the pheromone response pathway is constitu-

tively activated in *gpa1* mutant cells in which the $G\beta\gamma$ complex is liberated. In *C. neoformans*, an analogous positively acting $G\beta$ subunit, Gpb1, has been characterized previously (Wang *et al.*, 2000). Thus, we hypothesized that a constitutively active pheromone response would be observed when the Gpb1-interacting $G\alpha$ subunit was removed.

One prominent phenotype of the *gpa3* mutant in the serotype D background (but not the serotype A background) was dramatically enhanced self-filamentation. After 48-h incubation on V8 medium, prolific filamentation was observed along the peripheries of *gpa3* mutant colonies, whereas in the wild-type strain, filamentation was only observed after 2 wk. This hyperfilamentation phenotype is dependent on the $G\beta$ subunit Gpb1 because *gpa3 gpb1* double mutants were nonfilamentous and reintroduction of the *GPB1* gene (*gpa3 gpb1 GPB1*, Figure 2A, right panel) complemented this defect and restored fila-

Figure 2. *gpa3* and *gpa2 gpa3* double mutants are self-hyperfilamentous and *MATα* cells produce longer hyphae than *MATa* cells. (A) *MATα* Serotype D strains of the indicated genotypes were grown on V8 mating medium and photographed at 40× magnification after 4-d incubation at 25°C. (B) DAPI staining showed that the filaments are monokaryotic. Filaments were removed from V8 mating medium after 4-d incubation, fixed, stained with DAPI, and photographed at 400× magnification. Δ , unfused clamps. (C) *MATα* and *MATa* *gpa3* and *gpa2 gpa3* double mutants were cultured on V8 mating medium for 48 h at 25°C and photographed at 100× magnification.

mentous growth. The hyperfilamentous phenotype of *gpa3* cells does not require *Gpa2* as the *gpa2 gpa3* double mutant showed a similar phenotype as the *gpa3* single mutant (Figure 2A). DAPI staining showed that the hyphal cells produced by *gpa3* single or *gpa2 gpa3* double mutants contained a single nucleus, unfused clamp cells, and short basidiospore chains, all of which are hallmarks of monokaryotic fruiting (Figure 2B). Fruiting is a same-sex mating process that involves cells of only one mating type, and so far has only been routinely observed in serotype D strains (Lin *et al.*, 2005). Several components that function in the pheromone signaling have been shown to contribute to this process (Shen *et al.*, 2002; Davidson *et al.*, 2003). Interestingly, the monokaryotic hyphae produced by *MATα gpa3* and *MATα gpa2 gpa3* double mutants were more prolific than *MATa gpa3* or *gpa2 gpa3* strains, indicating that the *MAT* locus may play a role in filamentous growth (Figure 2C). Indeed, it was recently shown that the *MATα* allele serves as one of several quantitative trait loci that promote filamentation (Lin *et al.*, 2006).

We suspected that the hyperfilamentation phenotype of the *gpa3* mutant was a result of a constitutively active pheromone response because it has been shown that overexpression of the pheromone genes enhances monokaryotic fruit-

ing (Shen *et al.*, 2002). Thus, the abundance of pheromone transcripts in the wild-type, *gpa2*, *gpa3*, or *gpa2 gpa3* double mutant cultured alone or with a mating partner on V8 medium was determined. As shown in Figure 3, in wild-type cells, the pheromone genes were expressed at a basal level in monoculture and were induced during mating coculture conditions (Figure 3). In contrast, *gpa3* or *gpa2 gpa3* double mutant cells exhibited a constitutively active pheromone response; in both α and α cell types, cultured alone, the expression of the pheromone genes was dramatically induced. *Gpb1*, the G protein β subunit required for mitogen-activated protein kinase (MAPK) cascade activation of the pheromone response pathway, was also induced under mating conditions. Quantification using a phosphorimager documented that the *GPB1* transcripts were 1.6-fold more abundant during mating, whereas the expression of *GPB1* in the *gpa3* or *gpa2 gpa3* double mutant was 1.3–1.8-fold higher than in wild-type cells, even in the absence of a mating partner (Figure 3). The expression pattern of the *MFα1* and *MFa1* pheromone genes and *GPB1* demonstrates that in the *gpa3* and *gpa2 gpa3* double mutants, the pheromone signaling pathway is engaged without the prerequisite of pheromone activation. These results suggest that although *Gpa2*

Figure 3. Expression of G protein α subunits and RGS proteins is differentially regulated in response to pheromone activation. RNA from serotype D strains with the indicated genotypes was extracted and analyzed by Northern blot hybridization. RNA was extracted from cells cultured alone on V8 medium (*MATα*, left; *MATa*, middle) or from *MATα* and *MATa* cocultures (right). Probes used are indicated to the left; an *MFα1* gene probe was used in the *MATα* strains and a probe for *MFa1* in the *MATa* and coculture cells.

and Gpa3 play redundant roles in pheromone sensing, Gpa3 has a prominent function in repressing pheromone signaling via sequestration of Gpb1 in nonmating cells.

GPA2 and GPA3 Are Differentially Regulated during Mating

Several lines of evidence support the hypothesis that the two G protein α subunits play overlapping roles in pheromone sensing. However, deletion of *GPA3* leads to a constitutively active pheromone response, whereas deletion of *GPA2* does not, suggesting they serve different roles during *C. neoformans* sexual development. To further investigate how these two G protein α subunits are regulated, the same RNA blot that was probed for pheromone gene transcripts was used to analyze *GPA2* and *GPA3* expression. In wild-type cells, growth alone on V8 mating medium induced *GPA3* expression, and no further induction was apparent following exposure to an opposite mating type partner. In contrast, the *GPA2* transcripts were undetectable in monoculture and were strongly induced by the opposite mating partner (Figure 3). The *GPA2* expression pattern is quite similar to that of the *MF α* /*MFa* pheromone genes. In addition, in the *gpa3* mutants, in which the pheromone response pathway is constitutively activated, even without pheromone stimulation

from opposite mating-type cells, *GPA2* was also induced (Figure 3). This demonstrates that *GPA2* expression is specifically triggered by pheromone signaling pathway activation. Therefore, the expression of *GPA2* and *GPA3* is temporally regulated during mating in response to different cues. Cells initially express *GPA3* in response to signals present in the mating medium and *GPA2* is subsequently induced when the mating partner is encountered.

Two RGS Proteins, Crg1 and Crg2, Negatively Regulate Pheromone Responses

The RGS protein Crg1 has been previously characterized as a negative regulator of the pheromone response pathway in *C. neoformans*; cells that lack Crg1 are hypersensitive to pheromone, resulting in the formation of conjugation tubes observed in a confrontation assay (Nielsen *et al.*, 2003; Wang *et al.*, 2004). To determine which G α subunit Crg1 acts on, we generated *crg1 gpa2*, *crg1 gpa3* double mutants and *crg1 gpa2 gpa3* triple mutants in both mating type backgrounds to examine whether the *crg1* phenotype is dependent on either or both G α subunits. These strains were isolated in the serotype A background in order to circumvent the self-filamentous phenotype of the serotype D *gpa3* or *gpa2 gpa3* double mutants.

Figure 4. The RGS proteins Crg1 and Crg2 negatively regulate pheromone signaling via Gpa2 and Gpa3. (A) Confrontation assays were performed with serotype A strains of the indicated genotypes. Cells were grown on V8 mating medium and photographed at 40 $\times$ magnification after 4-d incubation in the dark at 25 $^{\circ}$ C. (B) *MAT α* *crg2* mutants in the serotype A background were crossed to wild-type *MATa* strains on V8 mating medium. Colonies were photographed after 1-wk incubation in the dark at 25 $^{\circ}$ C, and the basidia structures were photographed at 200 $\times$ magnification. (C) Northern blot analysis of *MF α* expression in the serotype A wild-type, G α , and RGS protein deletion mutants in α strains grown on V8 medium (left) or in $a \times \alpha$ cocultures (right).

As shown in Figure 4A, in confrontation assays both *crg1 gpa2* and *crg1 gpa3* double mutants could still produce conjugation tubes in response to cells of the opposite mating-type, indicating that both are capable of generating and responding to pheromones. In confrontation assays the *crg1 gpa2 gpa3* triple mutant still secreted pheromones (based on the formation of conjugation tubes by the *crg1* partner), but they failed to sense pheromone and did not produce conjugation tubes (Figure 4A). These findings indicate that the pheromone hypersensitive phenotype of *crg1* mutants requires Gpa2 or Gpa3 in cells of both mating types.

A second RGS protein, Crg2, was identified in the genome based on its homology to the RGS domain. To investigate the functions of Crg2 and to determine the interacting G α subunit, *CRG2* was deleted in the wild-type serotype A background, and the resulting *crg2* mutants were phenotypically analyzed. The most prominent phenotype for a *crg2* mutant was enhanced filamentation in mating that was readily observed macroscopically (Figure 4B). After coculturing wild-type α and α cells on V8 mating medium, filaments at the edge of the colony were apparent after 5 d, whereas in a *crg2* unilateral cross, filaments were much more prolific and could be observed as early as 48 h of coculture. This phenotype argues that Crg2, like Crg1, negatively regulates the pheromone response pathway by desensitizing the G α subunits. Although filamentation was accelerated and enhanced in the *crg2* mutant, sporulation was impaired and most basidia bore only a few spores, in contrast to the four long spore chains observed in a wild-type cross. This result suggests that Crg2 may play additional positive roles at the later stages of mating. To dissect the genetic relationship between Crg2 and Gpa2 and Gpa3, double and triple mutants were generated and analyzed for mating. As shown in Figure 4B, the hyperfilamentation phenotype of *crg2* is dependent on the presence of either one of the two G α subunits and a *crg2 gpa2 gpa3* triple mutant exhibiting mating defects, resembling the phenotype of a *gpa2 gpa3* double mutant.

The expression of both *CRG1* and *CRG2* was examined in wild-type, *gpa2*, *gpa3*, and *gpa2 gpa3* double mutant cells in the presence or absence of a mating partner. *CRG1* transcripts were detected in the *gpa3* and *gpa2 gpa3* double mutant monocultures and also in wild-type cells during mating (Figure 3). This pattern indicates that the expression of *CRG1* is pheromone responsive, similar to the regulation of the pheromone genes and *GPA2*. *CRG2* transcripts, on the other hand, were present in cells before mating, and their abundance did not increase when cells were exposed to a mating partner (Figure 3). Therefore, like *GPA3*, *CRG2* was expressed at a constant basal level irrespective of pheromone signaling. These analyses revealed that the expression of the G α subunits and RGS proteins are regulated coordinately (*CRG2* like *GPA3*, and *CRG1* like *GPA2*). Interestingly, genetic analysis revealed that *CRG1* is linked to *GPA2*. In an α *gpa2* $\times$ α *crg1* cross, only 2 of 84 progeny analyzed were wild-type or *gpa2 crg1* mutant. This suggested that the two genes are 2.4 cm apart, and analysis of the completed serotype A and D genomes revealed that they are separated by a 40-kb interval.

To provide further evidence to support the idea that both Crg1 and Crg2 are involved in the pheromone response pathway, we analyzed pheromone gene expression in the wild-type and mutant strains. Similar to findings shown in Figure 3, the expression of the pheromone gene was elevated under mating conditions and in the *gpa3* mutant cultured alone (Figure 4C). The level of induction was lower in the serotype A strains (Figure 4C) than that observed in serotype D strains (Figure 3), in accord with the more proficient

mating of serotype D strains compared with serotype A strains in the laboratory. On the other hand, the pheromone transcripts were 4.6- and 7.3-fold, respectively, more abundant than wild-type in the *crg1* and *crg2* mutant crosses (Figure 4C). This indicates that cells lacking either of the RGS proteins fail to desensitize the pheromone response. However, it remains to be determined if the elevated pheromone expression in *crg2* mutants is an indirect consequence of Gpa1 activation.

Dominant Active *GPA2*^{Q203L} and *GPA3*^{Q206L} Alleles Display Opposing Signaling Capacities

Studies in *S. cerevisiae* showed that overexpression of a dominant active (GTPase-deficient) Gpa1 mutant led to a subset of the mating responses induced by Ste4-Ste18, suggesting that in addition to sequestering the G $\beta\gamma$ complex, Gpa1 positively signals to the pheromone response pathway (Metodieff *et al.*, 2002; Guo *et al.*, 2003). Recent studies further demonstrated that the GTP-bound Gpa1 translocates onto endosomes, activates phosphatidylinositol 3-kinase, and enhances pheromone responsiveness of the cell (Slessareva *et al.*, 2006).

To investigate whether *C. neoformans* Gpa2 and Gpa3 have any intrinsic signaling roles, dominant active *GPA2*^{Q203L} and *GPA3*^{Q206L} alleles were overexpressed under the control of a constitutive *GPD1* promoter in a strain that also bears an *Mfa1* pheromone promoter-driven β -galactosidase reporter gene. Cells that overexpressed *GPA2*^{Q203L} were self-filamentous on V8 mating medium and had 4.2-fold higher β -galactosidase activity compared with wild-type cells (Figure 5A). The β -galactosidase activity in the *GPA2*^{Q203L} strain in the absence of a mating partner was similar to the level of wild-type cells under mating conditions, indicating that a mating response was induced in *GPA2*^{Q203L} cells alone. In the presence of a mating partner, overexpression of *GPA2*^{Q203L} elicited a higher level of β -galactosidase activity than wild type, and these cells mated more robustly and prolific filamentation was observed (Figure 5A). In contrast, the *GPA3*^{Q206L} allele exhibited an opposing signaling activity. In the absence of a mating partner on V8 medium, overexpression of *GPA3*^{Q206L} conferred no difference in β -galactosidase activity compared with wild-type cells. However, during mating, when pheromone expression was highly elevated in wild-type cells, it was inhibited in cells expressing the *GPA3*^{Q206L} allele; as a consequence, mating ability was diminished and less filamentation was observed (Figure 5A).

On the basis of the findings presented above, we conclude that Gpa2 and Gpa3, although redundant for pheromone sensing, display opposing signaling activities in their active forms. Thus, upon pheromone activation, although the signaling is dominantly transmitted via the G $\beta\gamma$ complex, fine tuning can be achieved via the G α subunits. Gpa2^{Q203L} positively regulates mating responses, whereas Gpa3^{Q206L} inhibits mating. This result prompted us to examine in more detail the pheromone expression pattern in the *gpa2* mutant, in which the negative signaling role of Gpa3 would be unopposed. As seen in Figure 4C, pheromone expression was moderately decreased in the *gpa2* $\times$ *gpa2* bilateral mutant cross when compared with wild-type in the serotype A strains. The Northern blot analysis was repeated to quantify the pheromone and actin signals. The result revealed that pheromone gene expression was 2.34-fold lower in the *gpa2* $\times$ *gpa2* cross, indicating that pheromone induction during the early stages of mating was modestly diminished, although this difference was not observed in the serotype D strains in which mating is more robust (Figure 3). In accord with the decreased level of pheromone expression, serotype A *gpa2* $\times$ *gpa2* crosses exhibited less robust filamentation when compared with a wild-type cross, which was in con-

Figure 5. Dominant active $Gpa2^{Q203L}$ activates pheromone expression and mating, whereas $Gpa3^{Q206L}$ inhibits. (A) β -galactosidase activity of the pheromone reporter gene in the wild-type, dominant active $GPA2^{Q203L}$, and dominant active $GPA3^{Q206L}$ overexpression serotype D strains in the absence (left) or presence (right) of the opposite mating partner grown on V8 mating medium. Error bars, SD of the mean for triplicate assays. Photos demonstrate the colony morphology of strains of the indicated genotype. (B) The hyperfilamentous phenotype caused by $GPA2^{Q203L}$ overexpression is dependent on the G protein β subunit Gpb1 and the MAPKK Ste7. Cells were grown on V8 mating medium and photographed at 40 $\times$ magnification after 48-h incubation in the dark at 25°C.

trast to the enhanced mating observed in the $gpa3 \times gpa3$ cross (Figure 1 A, first panel).

To further determine whether the self-filamentous phenotype caused by $GPA2^{Q203L}$ overexpression required the G β subunit or MAPK module, we overexpressed the $GPA2^{Q203L}$ allele in the $gpb1$ and $ste7$ mutant strains and monitored colony morphology on V8 mating medium. As shown in Figure 5B, in contrast to wild-type cells, $gpb1$ and $ste7$ mutant overexpressing $GPA2^{Q203L}$ were not self-filamentous, indicating that Gpb1 and Ste7 are needed to sustain $GPA2^{Q203L}$ signaling or that Gpa2-GTP signaling must occur in parallel with the MAPK cascade. Furthermore, it shows that both Gpa2-GTP and Gpb1 function coordinately to activate MAPK signaling. This requirement was also seen for Gpa1 signaling in *S. cerevisiae* (Guo *et al.*, 2003) and suggests that the regulation of a positive signal evoked by the dominant active G α mutant is evolutionarily conserved.

Pheromone Receptor $Ste3\alpha$ Interacts with Both $Gpa2$ and $Gpa3$

Genetic analysis demonstrates that both $Gpa2$ and $Gpa3$ participate in the *Cryptococcus* mating pathway and a double mutant loses the ability to sense pheromone from the mating partner. We therefore hypothesized that the pheromone receptor $Ste3\alpha/a$ is likely coupled to both G protein α subunits, which then transmit signals to the downstream MAPK cascade to control genes involved in mating.

To corroborate this hypothesis, we used the split-ubiquitin two-hybrid system that was developed to assess physical interactions for membrane-associated proteins in a physiological setting (Stagljari *et al.*, 1998). The C-terminal fragment of ubiquitin carrying the artificial transcription factor LexA-VP16 (Cub) was fused to a bait protein and the mutated N-terminal fragment (NubG) was fused to a prey. Protein-protein interactions enable reassociation between the two halves of split ubiquitin, resulting in cleavage and release of LexA-VP16, which then activates the *HIS3*, *ADE2*, and *lacZ* nuclear reporter genes. In our assays, the Cub domain was fused to the C-terminus of

the pheromone receptor $Ste3\alpha$, and the NubG domain was fused to $Gpa1$, $Gpa2$, or $Gpa3$. The $Ste3\alpha::Cub$ plasmid was cotransformed with one of the G α -NubG plasmids, and as shown in Figure 6, cells that coexpressed $Ste3\alpha::Cub$ and $Gpa2::NubG$, or $Ste3\alpha::Cub$ and $Gpa3::NubG$, grew on medium lacking histidine, whereas cells coexpressing $Ste3\alpha::Cub$ and $Gpa1::NubG$ failed to grow. This finding indicates that the pheromone receptor $Ste3\alpha$ interacts with both $Gpa2$ and $Gpa3$, but not $Gpa1$ in vivo. The magnitude of the interactions was further quantified by β -galactosidase activity assays (Figure 6). Compared with the positive control, the interactions between the pheromone receptor and both G α subunits were more moderate. In accord with this result, cells coexpressing $Ste3\alpha$ and $Gpa2$ or $Gpa3$ could not grow on medium lacking adenine, consistent with a less stable or transient interaction.

Crg1 and *Crg2* Interact with $Ste3\alpha$

The RGS protein Sst2 has been shown to bind to the pheromone receptor through its DEP domain, which places it in close proximity with its substrate $Gpa1$ (Ballon *et al.*, 2006). *Crg1*, like Sst2, also possesses a DEP domain; *Crg2*, although it does not carry a DEP domain, has three predicted C-terminal transmembrane helices. Therefore, we hypothesized that both RGS proteins have the potential to bind to pheromone receptors. To examine this hypothesis, the split-ubiquitin assay was used to determine if $Ste3\alpha$ and *Crg1*/*Crg2* interact. As shown in Figure 6, *Crg1* displayed a stronger interaction in the split ubiquitin assay with the pheromone receptor $Ste3\alpha$ compared with *Crg2*. Cells that coexpressed $Ste3\alpha::Cub$ and *Crg1*::NubG or *Crg2*::NubG both grew on medium lacking histidine. β -galactosidase activity assays, however, revealed that *Crg1* yielded a stronger signal with the pheromone receptor $Ste3\alpha$. We therefore conclude that similar to what was seen in *S. cerevisiae*, *Crg1* is in a complex that also contains $Ste3\alpha$, likely through the DEP domain and *Crg2*, which is membrane-bound, also interacts with the pheromone receptor, possibly with a lower affinity.

Figure 6. $G\alpha$ subunits Gpa2 and Gpa3 and the RGS proteins Crg1 and Crg2 physically interact with the pheromone receptor Ste3 α in *S. cerevisiae*. (A) A schematic presentation of the split-ubiquitin assay with the $G\alpha$::NubG or RGS::NubG and the Ste3 α ::Cub fusion proteins. (B) Serial dilution assays of cells expressing both a Cub fusion and a NubG fusion construct. Plasmids Ste3 α ::Cub and pAI-Alg5, which expresses a fusion of endogenous ER protein Alg5 to the wild-type Nub portion of ubiquitin, were used as a positive control to confirm the correct expression and topology of Ste3 α ::Cub. The wild-type Nub has strong affinity to Cub so the ubiquitin can reform even Ste3 α does not interact with Alg5. pDL2xN (NubG empty vector) were used as a negative control. Interactions were quantified by β -galactosidase activity assays.

Figure 7. The $G\gamma$ subunit Gpg2 is essential for mating. Serotype A wild type, *gpg2* mutant, and *gpg2* + GPG2 complemented MAT α strains were crossed to the congenic wild-type MAT α strain KN99a on V8 mating medium. Mating colonies (top) were photographed after 2-wk incubation in the dark at 25 $^{\circ}$ C and basidiospores (middle) were photographed at 200 $\times$ magnification. Bottom, cell-cell fusion products grown on double selection medium (G418 + nourseothricin).

G γ Subunit Gpg2 Is Essential for Sexual Development

The G protein β subunit Gpb1 activates the MAPK cascade to trigger mating responses in *C. neoformans* similar to the signaling cascade in *S. cerevisiae*, but the Gpb1-interacting $G\gamma$ subunit has been elusive (Wang *et al.*, 2000). A putative open reading frame (ORF) annotated in the genome of the sequenced serotype D strain B3501 (CNBL0880) was found to share similarities with known fungal $G\gamma$ subunits such as Ste18 in *S. cerevisiae*, including the hallmark C-terminal CAAX motif (Supplementary Figure 2). This gene, GPG2, was also independently identified by Palmer *et al.* (2006),

and it was shown that Gpg2 physically interacts with Gpb1 in a yeast two-hybrid assay.

To investigate whether this gene indeed encodes a $G\gamma$ subunit that activates mating responses via pheromone stimulation, the GPG2 gene was deleted in the wild-type serotype A MAT α background, and the mutant was crossed to wild-type *a* cells. The *gpg2* mutation completely abolished mating, even in a unilateral cross, a phenotype highly reminiscent of the *gpb1* mutant (Figure 7). Based on a quantitative cell-cell fusion assay, it was determined that the α *gpg2* mutant was unable to fuse with wild-type *a* cells and hence was sterile (Figure 7). Complementation of the *gpg2* mutation with the wild-type GPG2 allele restored the mating ability of the *gpg2* mutant, demonstrating that this mating defect is attributable to the loss of GPG2 gene function (Figure 7). Thus, we conclude that Gpg2 is a γ subunit that is essential for pheromone signaling in *C. neoformans*.

DISCUSSION

The goal of this study was to answer the simple question: which G protein α subunit is coupled to the pheromone receptor and governs the pheromone signaling cascade in *C. neoformans*? Surprisingly, all lines of evidences indicate that both Gpa2 and Gpa3 are recruited into the signaling circuit, with both overlapping and specialized signaling activities.

These findings allow us to propose a model of the pheromone signaling network in *C. neoformans* (Figure 8). Compared with *S. cerevisiae*, in which only one G protein α subunit (Gpa1) and an RGS protein (Sst2) are involved in pheromone signaling regulation, two G protein α subunits Gpa2 and Gpa3 and two RGS proteins Crg1 and Crg2 were identified to regulate the mating pathway in *C. neoformans*. Under nutrient limiting conditions, cells in the serotype D lineage express Gpa3 to inhibit basal signaling and filamentous growth via sequestration of the $G\beta\gamma$ complex. Meanwhile, the RGS protein Crg2 functions to terminate signaling input from spontaneously activated Gpa3 and therefore, loss of either component leads to an activation of the pheromone response. When a mating partner is encountered, pheromone binding to the Ste3 α / α receptor on the cell membrane triggers Gpa3 to dissociate from the Gpb1/Gpg2 $\beta\gamma$ complex, activating the MAPK signaling cascade and inducing the expression of pheromone-responsive genes, including GPA2, MF α /a, and CRG1. After this first wave of

Figure 8. Models on the dualism of G protein signaling in the pheromone response pathway in *C. neoformans*. Under nutrient limiting conditions, Gpa3 mainly serves to repress $G\beta\gamma$ signaling and prevent induction of pheromone response. On pheromone activation, the expression of Gpa2 is induced and in addition to functioning passively through releasing the $G\beta\gamma$ subunit, the active form of Gpa2 also plays a positive signaling role, contributing to the pheromone response that leads to mating, whereas the activated form of Gpa3 inhibits mating. The RGS proteins Crg1 (in the pheromone activated state) and Crg2 (in the basal and activated states) both function to constrain Gpa2 and Gpa3 signaling by stimulating GTPase activity.

pheromone induced-signaling, Gpa2, now in much higher abundance, may generate a second wave of signaling contributing to mating in its GTP-bound state. On the other hand, the RGS protein Crg1, a pheromone-inducible gene and a built-in feedback regulator, facilitates signal termination by shortening the lifetime of GTP-bound Gpa2 and desensitizes cells to pheromone secreted by the mating partner.

The bilateral mating defect observed in the *gpa2 gpa3* double mutants is in contrast with the unilateral sterility observed in mutants that are defective in pheromone signaling (*ste11*, *ste7*, and *cpk1*; Wang *et al.*, 2002; Davidson *et al.*, 2003). One major factor that may contribute to this difference is the constitutive pheromone signaling in the *gpa2 gpa3* mutants due to the release of $G\beta\gamma$. In the *ste11*, *ste7*, and *cpk1* mutants, pheromone signaling is blocked and cells exhibit a unilateral fusion defect; conversely, because the pheromone signaling is constitutively active in the *gpa2 gpa3* mutants, they do not have the fusion defect in a unilateral cross.

The molecular mechanisms regarding how Gpa2 positively signals mating responses in *C. neoformans*, although unclear, are of considerable interest. Three models are proposed here. In the first model, Gpa2 binds to the Gpb1/Gpg2 $\beta\gamma$ complex and pheromone activation of Gpa2 triggers its dissociation from $\beta\gamma$ and both Gpa2-GTP and free $\beta\gamma$ activate downstream effectors to induce mating responses; this model is most analogous to pheromone signaling in *S. cerevisiae*. In accord with this hypothesis, we found that when *GPA2* or *GPA3* is overexpressed in the wild-type, mating is reduced (Supplementary Figure 3). This suggests

that both $G\alpha$ subunits may have the capacity to sequester the $G\beta\gamma$ complex. On the other hand, the higher expression levels of both of the pheromone genes and *GPA2* in the *gpa3* mutant background suggest alternative models. If Gpa2 indeed forms a complex with the Gpb1/Gpg2 $\beta\gamma$ complex, the highly expressed *GPA2* is likely to restrain the availability of free $\beta\gamma$ in the *gpa3* mutant and reduce activation of pheromone signaling. However, the pheromone genes are highly expressed in the *gpa3* mutant. Thus, in the second and third model, we propose that Gpa2 could form a complex with other $G\beta$ like proteins or it could act as a solo $G\alpha$.

A central question is the identity of the downstream effectors of Gpa2. Studies in *S. cerevisiae* revealed that potential $G\alpha$ effectors include the MAPK Fus3, an RNA-binding protein Scp160, and phosphatidylinositol 3-kinase Vps34 (Metodiev *et al.*, 2002; Guo *et al.*, 2003; Slessareva *et al.*, 2006). These are candidate effectors of *C. neoformans* Gpa2 and, in addition, our results show that Gpa2 activity requires functional Gpb1 and Ste7. A recent report showed that a mammalian G_o subunit can directly target PKA (Ghil *et al.*, 2006). Thus, we do not exclude the possibility that Gpa2 may act via other signaling cascades, such as the cAMP-PKA pathway that also contributes to govern mating. Future investigations will be required to discover the downstream effectors of Gpa2.

Mating in *C. neoformans* is regulated by both pheromones and nutrients, and a principle role of Gpa3 is to inhibit filamentous growth in environments in which cells are prepared for mating but a mating partner is not present. Thus, Gpa3 is a negative regulator for the yeast to hyphal dimor-

phic transition. We propose that an analogy may exist with those human fungal pathogens that are dimorphic and able to switch between yeast and hyphal growth. Examples include *Penicillium marneffei*, *Histoplasma capsulatum*, and *Coccidioides* spp. One critical signal for this transition is temperature; cells grow as mycelia at room temperature and switch to yeast growth when temperature is elevated. Similarly, *C. neoformans* can only grow filamentously below 25°C. The *gpa3* mutant is hyperfilamentous at permissive temperature (18–25°C), but it only grows as a yeast at 37°C. These dimorphic fungi all have three G protein α subunits and are thought to have cryptic sexual cycles, and studies from *P. marneffei* show that one of the G α subunits (GsaA) functions as a negative regulator for asexual development (Borneman *et al.*, 2001; Zuber *et al.*, 2002). We hypothesize that in the dimorphic fungi, one of the G protein α subunits may function analogously to Gpa3 in *C. neoformans*.

Functional parallels for the Gpa2/Gpa3 signaling paradigm may be found in other organisms. In chemotactic cells such as neutrophils and the slime mold *D. discoideum*, cell polarity and movement signaling is positively transmitted by G $\beta\gamma$, and the G α subunits may not be required (Wu *et al.*, 1995; Neptune and Bourne, 1997; Neptune *et al.*, 1999). However, more recent studies in both systems have suggested that G α subunits are more than just passive components in the signaling networks (Brzostowski *et al.*, 2002; Xu *et al.*, 2003). In *Dictyostelium*, two G α subunits (G α 2 and G α 9) were shown to couple to the cAMP receptor CAR1 and mediate cAMP chemotaxis. G α 2 is mainly responsible for the release of G $\beta\gamma$, whereas G α 9 negatively regulates multiple downstream pathways activated by G $\beta\gamma$ (Brzostowski *et al.*, 2004). Another analogy is evident in mammalian β 2 adrenergic receptor (β 2-AR)-mediated signaling events. On agonist binding, β 2-AR activates Gs to activate the cAMP-PKA pathway. Activated PKA then phosphorylates β 2-AR and switches its coupling specificity to another class of G α (Gi), which in turn activates MAPK via the released G $\beta\gamma$ (Daaka *et al.*, 1997). In addition to β 2-AR, PKA-mediated G protein-coupling switching is also observed with the prostacyclin receptor (Lawler *et al.*, 2001). Through switches in G protein-coupling specificity, receptors can govern distinct signaling pathways and generate temporal cellular signaling waves.

Functional redundancy between G proteins has been previously described in some organisms. In the filamentous ascomycete *N. crassa* the G α subunits Gna-1 and Gna-2 share overlapping functions in hyphal extension, growth under stress conditions, and female fertility (Baasiri *et al.*, 1997; Kim and Borkovich, 2004). In *C. elegans*, two G α subunits (GPA-16 and GOA-1) were shown to coregulate spindle position and orientation in embryos, and inactivation of both genes causes embryonic lethality (Gotta and Ahringer, 2001). Furthermore, like Gpa2 and Gpa3 in *C. neoformans* and in both *N. crassa* and the nematode, the above-mentioned G proteins share the highest level of similarity to each other and may have resulted from gene duplication events.

It is well understood that the budding yeast only requires one G α (Gpa1) for mating; on the other hand, in *C. neoformans*, two G α subunits with both overlapping and divergent functions are involved in sexual development. One of the subunits promotes mating, whereas the other inhibits filamentous growth, yet either one is sufficient to enable mating. Analyzing the number of G α subunits present in most annotated fungal genomes suggests that one G α subunit has been lost in the hemiascomycete lineage. This also suggests that the pattern of three G α subunits is more ancient. We propose that the involvement of two G α subunits in sexual development may be evolutionarily conserved among other

fungi that express three different G α proteins. The cell fate decision in *S. cerevisiae* is a simple binary choice; cells grow either as asexual budding yeasts, or they undergo mating when an appropriate partner is present. Conversely, *C. neoformans* and other dimorphic fungi have a ternary choice regarding their cell fate; they can grow as budding yeasts or switch to asexual or sexual filamentous development. The addition of an extra G α subunit enables increased signaling complexity, which allows cells to make an appropriate ternary choice during the various stages of the life cycle. Finally, our study provides insights into GPCR and G protein-coupling specificity relevant to an understanding of similar signaling circuits operating in higher multicellular organisms, including slime molds, nematodes, flies, and even mammals.

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