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The G-Protein β Subunit GPB1 Is Required for Mating and Haploid Fruiting in *Cryptococcus neoformans*

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***Cryptococcus neoformans* is an opportunistic fungal pathogen with a defined sexual cycle. The gene encoding a heterotrimeric G-protein β subunit, GPB1, was cloned and disrupted. *gpb1* mutant strains are sterile, indicating a role for this gene in mating. GPB1 plays an active role in mediating responses to pheromones in early mating steps (conjugation tube formation and cell fusion) and signals via a mitogen-activated protein (MAP) kinase cascade in both *MAT*α and *MAT*a cells. The functions of GPB1 are distinct from those of the Gα protein GPA1, which functions in a nutrient-sensing cyclic AMP (cAMP) pathway required for mating, virulence factor induction, and virulence. *gpb1* mutant strains are also defective in monokaryotic fruiting in response to nitrogen starvation. We show that *MAT*a cells stimulate monokaryotic fruiting of *MAT*α cells, possibly in response to mating pheromone, which may serve to disperse cells and spores to locate mating partners. In summary, the Gβ subunit GPB1 and the Gα subunit GPA1 function in distinct signaling pathways: one (GPB1) senses pheromones and regulates mating and haploid fruiting via a MAP kinase cascade, and the other (GPA1) senses nutrients and regulates mating, virulence factors, and pathogenicity via a cAMP cascade.**

Cryptococcus neoformans is an opportunistic fungal pathogen that infects the central nervous system to cause meningoencephalitis in individuals with compromised immune function (26, 39). Virulence is associated with mating type (28), production of melanin (29, 30, 48, 56) and a polysaccharide capsule (4, 16, 30), and growth at 37°C (30, 45).

The life cycle of this organism has been defined (25). Mating occurs between *MAT***a** and *MAT*α cells and involves cell fusion, filamentation, nuclear migration and fusion, meiosis, and sporulation. Mating type is linked to physiology and virulence. *MAT*α strains are more prevalent in the environment, and most clinical isolates are *MAT*α (27); *MAT*α strains are more virulent in mice than are congenic *MAT***a** strains (28). In response to nitrogen starvation, *MAT*α cells differentiate to form filaments, basidia, and spores (haploid fruiting) (62). Thus, genes linked to the *MAT*α locus regulate the physiology and virulence of *C. neoformans*. A homolog of the *Saccharomyces cerevisiae* and *Candida albicans* STE12 transcription factor is encoded by the *C. neoformans MAT*α locus (61), and *ste12* mutant strains have defects in haploid fruiting (67). Recent studies of a GTP-binding protein, GPA1, underscore the importance of signaling cascades in *C. neoformans* virulence (2, 54).

Heterotrimeric guanine nucleotide binding proteins interact with G-protein-coupled receptors to sense external signals and regulate cell growth and development (14). G-protein-mediated signals include responses to hormones and neurotransmitters, vision and olfaction, and pheromone-induced mating in *S. cerevisiae* and *Schizosaccharomyces pombe*. Heterotrimeric G proteins are comprised of alpha (α), beta (β), and gamma (γ) subunits. In response to binding of ligand to receptors, the Gαβγ complex is recruited, leading to GDP-GTP exchange on Gα and release of Gβγ.

In most examples, the Gα-GTP subunit actively transduces signals. However, Gβγ subunits can also signal (5, 21–23, 32, 33, 36, 52, 64). For example, the *S. cerevisiae* Gβγ complex Ste4-Ste18 is released from the Gα subunit Gpa1 by pheromone (6, 32, 33, 59). The Gβγ complex recruits the Ste5 scaffold, allowing activation of the Ste5-bound kinase Ste11 by membrane-localized Ste20 kinase (32–34, 47). The Gα subunit Gpa1 plays a negative role in *S. cerevisiae* mating (9, 40). In contrast, in *Schizosaccharomyces pombe*, the Gα subunit Gpa1 positively signals mating and, together with Ras1, activates a mitogen-activated protein (MAP) kinase cascade (8, 42, 44, 58, 65). In the chestnut blight fungus *Cryphonectria parasitica*, the Gβ subunit CPGB-1 regulates sporulation and virulence, likely with the Gα subunit CPG-1 (13, 21).

In *C. neoformans*, the Gα subunit GPA1 is required for mating and virulence (2). GPA1 regulates responses to nutritional starvation signals required for mating and induction of the virulence factors capsule and melanin. Cyclic AMP (cAMP) suppresses the mating and virulence defects of *gpa1* mutant cells, suggesting that GPA1 activates adenylyl cyclase similarly to Gαs in mammals and Gpa2 during *S. cerevisiae* pseudohyphal growth (24, 37). In the present study, we investigated the roles of a G-protein β subunit, GPB1, in *C. neoformans*.

MATERIALS AND METHODS

Strains and media. *C. neoformans* strains used in this study included H99 (serotype A, *MAT*α) and the isogenic *ade2* mutant M049. Strains JEC34 and JEC43 are isogenic *ura5* mutant serotype D *MAT***a** and *MAT*α strains, respectively (41). Strain BAC20 is a *gpa1*::*ADE2* mutant of the *MAT***a** strain JEC20 (provided by B. Allen and A. Alspaugh). Yeast extract-peptone-dextrose (YPD) and yeast nitrogen base (YNB) media, synthetic medium, V8 agar, filament agar, niger seed agar for melanin production, and low-iron medium plus 56 μM ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) for induction of capsule formation were as described in references 2 and 55.

Isolation of the *C. neoformans GPB1* gene. Primers 5′-AT(ATC)TA(TC)GC (GATC)ATGCA(TCT)TGG and 5′-AA(AG)TC(AG)TA(GATC)CC(GATC)

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GC encompassed conserved residues IYAMHW and AGYDDF, respectively, of Gβ subunits. PCR parameters were as follows: 94°C for 40 s, 40°C for 1 min, and 72°C for 2 min (40 cycles). *C. neoformans* cDNA (strain B3501; 200 ng) served as the template. PCR products were excised, cloned, and used to clone the *GPB1* gene. For size-selected libraries, DNA was cleaved with *Hin*dIII and electrophoresed, and 4.9-kb fragments were excised. DNA was recovered by using a QIAEX DNA extraction kit (Qiagen), ligated in *Hin*dIII-cleaved plasmid pUC18, and transformed into *Escherichia coli*, and bacterial colonies were screened with the *GPB1* PCR product as a probe (49).

Nucleic acid manipulations. DNA and RNA were extracted from cells that were lyophilized overnight and broken with glass beads (4 mm diameter) by the use of a Vortex mixer. Total DNA for Southern blot analysis was isolated as described in reference 46. Total DNA for PCRs was obtained as described in reference 18. Total RNA was extracted with a buffer containing 150 mM sodium acetate, 100 mM LiCl, 4% sodium dodecyl sulfate, 10 mM EDTA, 10 mM EGTA, and 20 mM β-mercaptoethanol, extracted with phenol (pH 4.0), and precipitated with LiCl.

The *GPB1* cDNA clone was obtained in two steps. First, cDNA was synthesized from total RNA of strain H99 by using a reverse transcription-PCR kit (Stratagene) with random primers to generate cDNA from the 5′ region and oligo(dT) primers to obtain 3′ cDNA. Second, the two cDNA pools were used as templates for PCR with primers corresponding to the *GPB1* gene based on the genomic sequence to amplify 5′- and 3′-proximal fragments of the gene which span an internal *Eco*RI site. The full-length *GPB1* cDNA was obtained by ligating these two *Eco*RI fragments. Southern and Northern blot analyses and hybridizations were performed by standard procedures (49).

Two-hybrid assays. For two-hybrid interactions, a *GPB1* cDNA was cloned in plasmids pGBT9 and pGAD424 (Clontech) to yield plasmids pGBT9::GPB1 and pGAD424:GPB1, expressing GAL4(DB)-GPB1 and GAL4(AD)-GPB1 fusion proteins, respectively. DNA of plasmids pGAD424::GPB1 and pGBT::GPA1 (2) or of plasmids pGBT9::GPB1 and pGAD424::GPA1 (2) was used to transform the yeast strain PJ69-4A (20).

***GPB1* gene disruption.** pCnGPB1 is a pUC18-derived clone containing a 4.9-kb *Hin*dIII fragment spanning the *GPB1* gene from strain H99. For the *gpb1*::*ADE2* gene disruption, pCnGPB1 was digested with *Apa*I (for which there is a unique cleavage site in the *GPB1* gene), blunt ended with T4 DNA polymerase, and dephosphorylated with calf intestinal alkaline phosphatase. Two plasmids were constructed for the *GPB1* gene disruption. Either a 2.4-kb *Xho*I or 2.9-kb *Kpn*I-*Bam*HI DNA fragment containing the *ADE2* gene from *C. neoformans* serotype D strain B3501 (51, 53) was blunt ended and inserted at the blunted *Apa*I site in plasmid pCnGPB1 to yield the *gpb1*::*ADE2* disruption alleles. The *ade2* serotype A strain M049 was grown for 40 h in liquid YPD and transformed with the *gpb1*::*ADE2* disruption allele by the use of a biolistic DNA delivery apparatus (Bio-Rad) as described elsewhere (53). Transformants were selected on synthetic medium lacking adenine but containing 1 M sorbitol. Primers used for PCRs to verify the presence of *gpb1*::*ADE2* alleles were 5′-AGAGAGCTCAGCGCACAC-3′ and 5′-GTAGTCATCGTAGCCGGC-3′.

Mating assays. Mating assays were conducted by coculturing *MAT*α strains with the tester *MAT***a** strain JEC20 on V8 or filament agar medium containing 0.5% galactose (inducing) or 0.5% glucose (repressing) (62). Plates were incubated at 22°C, and resultant colonies were examined by using a Nikon Eclipse E400 microscope.

Expression of *GPB1*, *GAL7-GPB1*, *GAL7-CPK1*, *GAL7-STE12*α, *MF*α*1*, and *Ras1-Q67L*. A *ura5* derivative of the *gpb1* mutant strain was obtained by plating cells on 5-fluoroorotic acid medium, and the *ura5* mutation was then complemented by introducing plasmid pCnTel1 (10, 31). A 2.5-kb *Xba*I fragment containing the wild-type *GPB1* gene was inserted into plasmid pCnTel1 (pCnTel1::GPB1) for complementation tests. The genomic clone of the *C. neoformans* Fus3/Kss1 MAP kinase homolog *CPK1* (pCnTel1::CPK1) and the *CPK1* cDNA clone (R. Davidson and J. Heitman, unpublished data) were cloned under the control of the *C. neoformans GAL7* promoter (60), and the resulting plasmids (pCnTel1::GAL7-CPK1) were used for epistasis. The cDNA clone of the *C. neoformans STE12*α gene expressed from the *GAL7* promoter in plasmid pCGS-1 (61) was also used. Plasmids were transformed in circular form by biolistic transformation into the *gpb1 ura5* mutant. The *GPB1* cDNA clone was placed under the control of the *GAL7* promoter (pCnTel1Δ::GAL7-GPB1) and used to transform a *ura5* strain of H99, the *gpb1 ura5* mutant, and the *ura5* serotype D *MAT***a** and *MAT*α strains JEC34 and JEC43. pCnTel1Δ differs from pCnTel1 in that it lacks the *Not*I fragment containing telomeric sequences. The dominant active Ras1 Q67L mutant was expressed with the *C. neoformans* actin gene promoter and was introduced with the hygromycin B resistance or *URA5* gene as a marker. The cloned *MF*α*1* gene (plasmid pCnTel1::MFα1) (41) was expressed in the serotype D strain JEC34 (*MAT***a** *ura5*) and the isogenic *gpa1* mutant strain BAC20 (*MAT***a** *gpa1*::*ADE2 ura5*).

Haploid fruiting assays. For haploid fruiting assays, the Ras1 Q67L mutant protein was expressed by introducing linear DNA fragments containing the mutant *RAS1* gene, linked to the hygromycin resistance or *URA5* gene as a marker, by biolistic transformation. Isolates containing the mutant allele for Ras1 Q67L were identified by PCR of genomic DNA with primers flanking the *RAS1* gene and by *Xba*I cleavage to detect the Q67L mutation. Haploid fruiting was assayed by incubating spotted suspensions of cells on filament agar at 24°C for up to 4 weeks.

For confrontation assays, isolated colonies were streaked on the surface of filament agar as lines with sterile toothpicks. In some cases, a sterile dialysis membrane was interposed between the cell types by inserting it with sterile forceps into an agar cut that was sealed with molten agar.

Virulence test. Virulence was evaluated using a rabbit model of cryptococcal meningitis (2, 45). Cells of the isogenic wild-type strain (H99) and the *gpb1* mutant strain were grown for 48 h in liquid YPD medium and resuspended in 15 mM phosphate-buffered saline. New Zealand White male rabbits (four in a group for each strain) weighing 2 to 3 kg were administered cortisone acetate (2.5 mg/kg of body weight) intramuscularly 1 day prior to inoculation of *C. neoformans* and then daily for 14 days. Twenty-four hours following initial steroid treatment, rabbits were anesthetized with xylazine and ketamine intramuscularly and inoculated intracisternally with 0.3 ml of cell suspension (3×10^8 cells/ml). Rabbits were sedated on days 4, 7, 10, and 14 postinoculation, and cerebrospinal fluid (CSF) was withdrawn. Cell cultures were performed by plating dilutions of CSF (in phosphate-buffered saline) on YPD medium, incubating the plates at 30°C for 3 days, and counting viable colonies.

Nucleotide sequence accession number. The *GPB1* gene sequence has been submitted to GenBank under accession no. AF091120.

RESULTS

Identification of the *C. neoformans* G-protein β subunit GPB1. Previous studies revealed that the Gα protein GPA1 (54) regulates mating and virulence in *C. neoformans* (2). To further address the role of G proteins in mating and physiology, we identified a heterotrimeric G-protein β subunit from *C. neoformans*.

Oligonucleotides were designed against conserved regions of Gβ subunits and used as primers in low-stringency PCRs with a *C. neoformans* cDNA library or *C. neoformans* genomic DNA as a template. Primers encompassing two conserved peptides, IYALHW and AGYDDY, amplified a partial Gβ cDNA homolog from the serotype D strain B-3501. This cDNA clone was sequenced and then used to probe a Southern blot of genomic DNA isolated from the serotype A *MAT*α strain H99 and from the congenic serotype D strains JEC20 (*MAT***a**) and JEC21 (*MAT*α). The *GPB1* gene was present in a single copy in both mating types and serotypes (data not shown).

The complete *GPB1* genomic locus was cloned from a size-selected genomic library. Sequence analysis revealed an open reading frame of 1,059 nucleotides encoding a 352-amino-acid protein (GenBank accession no. AF091120). Four introns were identified by sequence comparison with a cDNA clone from strain H99. The predicted GPB1 protein shares marked identity with G-protein β subunits from other organisms, including Gβ subunits from humans (68%), *Drosophila melanogaster* (67%), *C. parasitica* (70%), *Schizosaccharomyces pombe* (40%), and *S. cerevisiae* (38%) (Fig. 1).

Disruption of the *C. neoformans GPB1* gene. The *GPB1* gene was disrupted by inserting the *ADE2* gene into the *GPB1* open reading frame, and the resulting *gpb1*::*ADE2* disruption allele was introduced into the *ade2* strain M049 by biolistic DNA transformation and homologous recombination. Genomic DNA was extracted from candidate *gpb1*::*ADE2* strains (18). PCRs with primers flanking the *ADE2* gene insertion were used to identify *gpb1* mutations and generate a 550-bp product from the *GPB1* allele and a 3,450-bp product from the *gpb1*::*ADE2* allele.

In total, six *gpb1*::*ADE2* mutant strains were identified from 306 adenine-prototrophic transformants by PCR analysis. In subsequent analyses, independent *gpb1* mutations conferred the same phenotypes. Southern blot analysis confirmed that the *GPB1* gene had been replaced by the *gpb1*::*ADE2* disruption allele by homologous recombination at the *GPB1* locus in all six mutant strains (Fig. 2). The wild-type *GPB1* gene is located on a 4.9-kb *Hin*dIII fragment and a 1.6-kb *Not*I-*Xba*I fragment. In the *gpb1*::*ADE2* mutant, the wild-type 4.9-kb *Hin*dIII fragment is replaced by 2.9- and 5.0-kb *Hin*dIII fragments (Fig. 2). In addition, the 1.6-kb *Not*I-*Xba*I wild-type

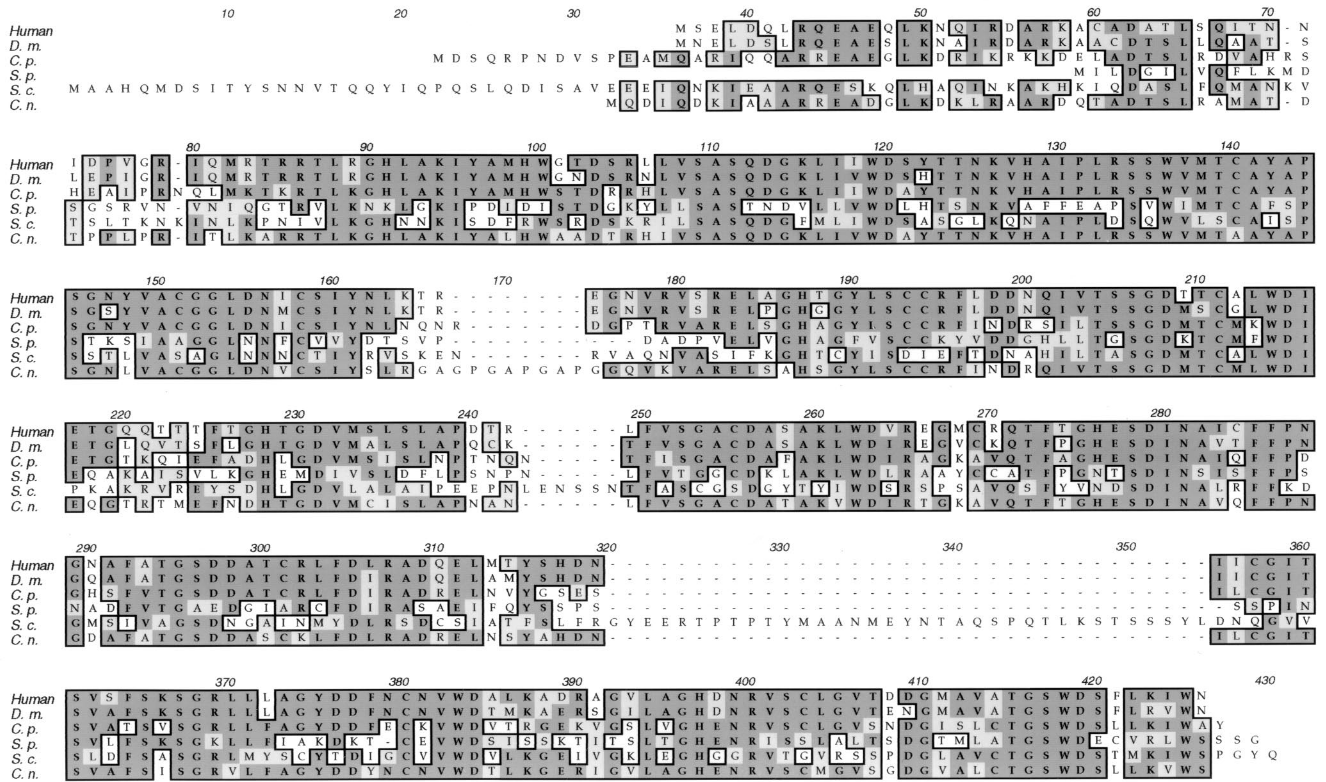

FIG. 1. *C. neoformans* GPB1 exhibits identity to G-protein β subunits. The sequences of Gβ subunits from humans (12), *D. melanogaster* (*D.m.*) (66), *Cryphonectria parasitica* (*C.p.*) (21), *Schizosaccharomyces pombe* (*S.p.*) (23), and *S. cerevisiae* (*S.c.*) (59) were aligned with that of the *C. neoformans* (*C.n.*) GPB1 protein. Identical amino acids are boxed and darkly shaded; conservative amino acid substitutions are boxed and lightly shaded.

GPB1 locus is missing from the *gpb1*::*ADE2* mutant, having been replaced by 4.5- and 5.1-kb *Not*I-*Xba*I fragments (Fig. 2).

GPB1 is required for mating in *C. neoformans*. We tested whether GPB1 regulates mating in *C. neoformans*. *MAT*α and *MAT***a** strains of *C. neoformans* mate when cocultured on nutrient-limiting medium (25). Mating consists of conjugation tube formation, cell fusion, and filamentation (1). Subsequent nuclear migration results in the formation of dikaryotic filaments that differentiate to form terminal basidia, in which nuclear fusion, meiosis, and sporulation occur. When one or both parents are sterile, few or no filaments or spores are produced.

The wild-type *GPB1 MAT*α serotype A strain (H99) yielded abundant filaments and basidiospores when crossed with the *MAT***a** serotype D strain JEC20 (Fig. 3). In contrast, no filaments or spores were ever observed when any of the independent *gpb1* mutant *MAT*α strains were mated with their *MAT***a** mating partners (Fig. 3). Reintroduction of the wild-type *GPB1* gene into the *gpb1* mutant strain restored filamentation and spore production to the wild-type level (Fig. 3).

GPB1 and the Gα subunit GPA1 play different roles in mating. Several findings suggest that the Gα protein GPA1 and the Gβ protein GPB1 function in distinct pathways to regulate mating. First, the *gpb1* mutation confers an absolute mating defect, whereas, following prolonged incubation, *gpa1* mutants eventually mate to a limited extent with a wild-type mating partner, forming filaments, basidia, and recombinant basidiospores (Fig. 3) (2). Second, cAMP suppresses the mating defect of *gpa1* mutants, but not that of *gpb1* mutants (Fig. 3) (2). Third, no interaction between GPA1 and GPB1 was detected in the two-hybrid system (data not shown) (see Materials and Methods).

Several additional findings indicate the Gα subunit GPA1 is not required for pheromone sensing. First, in confrontation assays, the congenic *MAT*α strain JEC21 and the *MAT***a** strain JEC20 both produced conjugation tubes in response to pheromone secreted by their mating partners (Fig. 4A). Most importantly, when the wild-type *MAT*α strain JEC21 was grown in confrontation with a *gpa1 MAT***a** mutant strain (BAC20), both the *gpa1* mutant and the wild-type strain produced conjugation tubes (Fig. 4A). Second, when a plasmid expressing the MFα1 pheromone was introduced into wild-type and *gpa1* mutant *MAT***a** strains, both produced conjugation tubes (Fig. 4B). The response of *gpa1* mutants to pheromones was somewhat reduced from that of the wild type, but taken together these findings indicate that GPA1 is not required for pheromone sensing. In an assay that detects cell fusion during mating (*MAT*α *ura5* strains were coincubated with *MAT***a** *lys1* strain JEC30 on V8 agar, and prototrophic self-filamenting heterokaryons were detected by replica plating to YNB medium), the *gpb1* mutation prevented cell fusion whereas the *gpa1* mutation reduced but did not block fusion (data not shown). In a mating assay in which recombinant basidiospores were quantified (*MAT*α prototrophic strains were mated with *MAT***a** *ura5 lys1* strain JEC53 on V8 agar, and *LYS1 ura5* recombinants were selected on 5-fluoroorotic acid–lysine medium), no recombinant basidiospores were produced by the *gpb1* mutant whereas the *gpa1* mutant produced a reduced number of basidiospores.

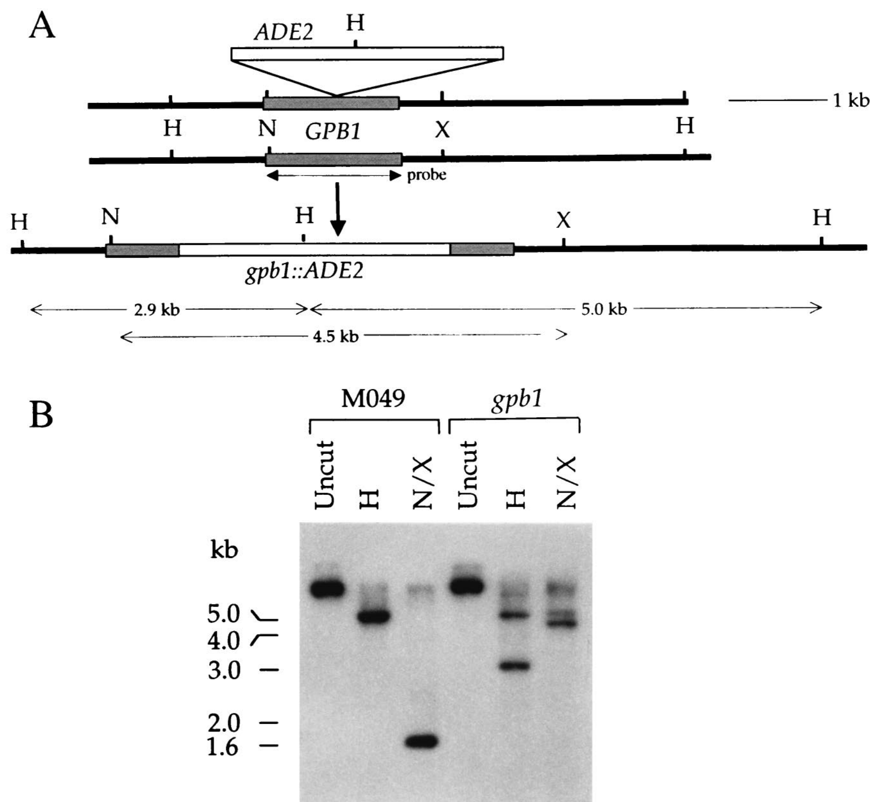

FIG. 2. Disruption of the *C. neoformans GPB1* gene. (A) A schematic illustration of the *GPB1* gene replacement; (B) Southern analysis of the wild type and the *gpb1* mutant. The *ADE2* gene was inserted at an *Apa*I site in the *GPB1* coding domain, and the *gpb1*::*ADE2* disruption allele was used to biolistically transform the Δ*ade2* strain M049 to adenine prototrophy. Genomic DNAs from the isogenic *GPB1* wild-type strain H99 and the *gpb1*::*ADE2* disruption mutant were isolated, cleaved with *Hin*dIII (H) or with *Not*I (N) and *Xba*I (X), separated by 1% agarose gel electrophoresis, transferred to a nylon membrane, and probed with the ^{32}P-labeled *GPB1* open reading frame (indicated by an arrow labeled "probe"). Sizes of DNA fragments resulting from gene disruption are indicated by horizontal arrows. The positions of DNA molecular size standards are indicated on the left.

GPB1 is not required for melanin or capsule production or virulence. The Gα protein GPA1 regulates the production of the virulence factors melanin and capsule in response to nutrient limitation (2). To determine whether the functions of GPB1 and GPA1 are distinct, we tested whether the *gpb1* mutation alters virulence factors or virulence.

C. neoformans produces melanin when grown in the presence of diphenolic precursors under carbohydrate-limiting conditions. Melanin is required for virulence and may protect cells from nitrogen- and oxygen-derived radicals produced by host immune cells (56, 57). When cultured on a medium containing niger seed extract as a source of diphenolic compounds, *gpa1* mutants did not produce melanin (Fig. 5A) (2). In contrast, *gpb1* mutant strains produced melanin to the same extent as the *GPB1* wild-type strain (Fig. 5A). By a quantitative spectrophotometric assay, it was determined that *gpb1* mutant and *GPB1* wild-type cells produced similar levels of laccase activity (data not shown) (63).

C. neoformans is distinguished from many pathogenic yeast by its polysaccharide capsule, which inhibits phagocytosis by host cells and is required for virulence (3). Formation of the capsule is induced during infection or in response to low-iron or elevated-CO_2 conditions in vitro (16, 55). To assess capsule production, the wild-type strain H99 and the *gpa1* and *gpb1* mutant strains were grown in liquid iron-limiting medium. Capsule production in wild-type cells was readily observed by staining with India ink, and the capsule size was decreased in *gpa1* mutant cells (Fig. 5B) (2). In contrast, *gpb1* mutant cells produced capsules similar to those of wild-type cells (Fig. 5B).

We next tested whether the *gpb1* mutation alters virulence. An animal model of cryptococcal meningitis was employed in which glucocorticoid-immunosuppressed rabbits were inoculated intrathecally with *C. neoformans* strains and survival in the central nervous system was determined by removing CSF and quantifying yeast cells by serial dilution and culture (2, 45). As shown in Fig. 5C, virulence of the *gpb1* mutant was similar to that of the *GPB1* wild-type strain H99. Both wild-type and *gpb1* mutant cells persisted for up to 14 days in the CSF, and they were recovered in similar quantities, although cell counts for the *gpb1* mutant were slightly reduced on days 4 and 7. Similar results were obtained with a second *gpb1* mutant, as well as when the inoculum size was reduced 10-fold. *gpb1* mutant cells recovered from infected animals still exhibited a mating defect in vitro. In summary, in contrast to GPA1, GPB1 is not required for melanin or capsule production and is not a major virulence determinant.

GPB1 regulates mating upstream of a MAP kinase cascade. Our findings suggested that the Gβ subunit GPB1 activates a signaling pathway that regulates mating in parallel with the GPA1-cAMP-regulated nutrient-sensing pathway. We tested whether the Gβ protein GPB1 regulates a MAP kinase cascade during mating in *C. neoformans*.

In addition to the G-protein β subunit, two other MAP kinase cascade components have been identified in *C. neofor-*

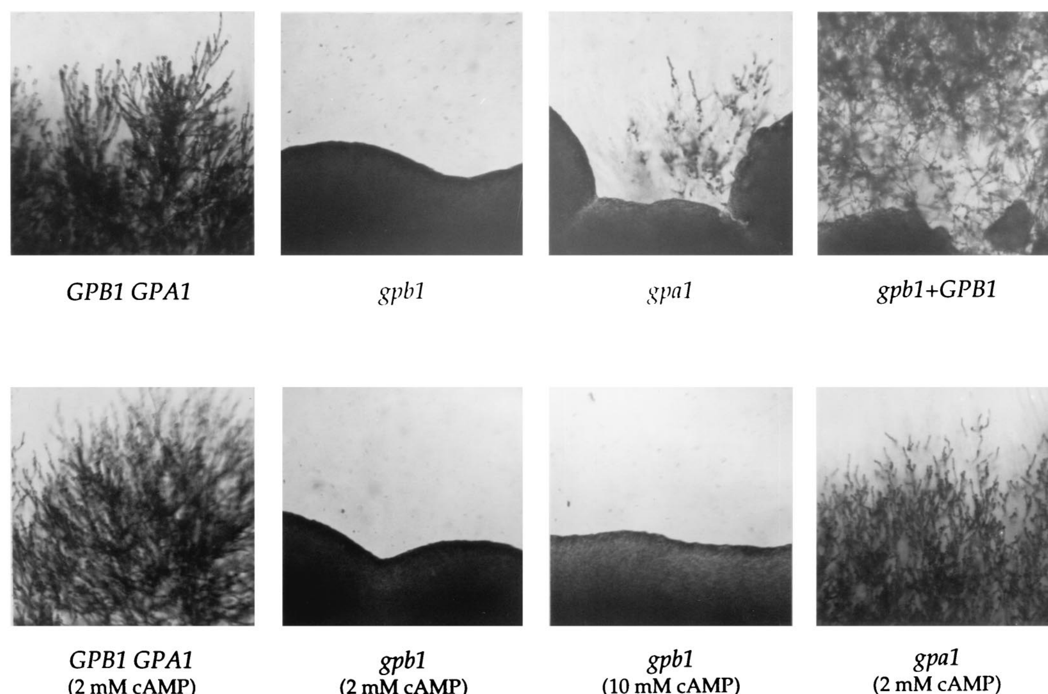

FIG. 3. The *C. neoformans* G-protein β subunit GPB1 is required for mating. The isogenic *C. neoformans* wild-type *MAT*α strain H99 (*GPB1 GPA1*) and the *gpb1*::*ADE2* (*gpb1*) and *gpa1*::*ADE2* (*gpa1*) *MAT*α mutant strains were mated with the *MAT***a** strain JEC20 on V8 agar medium (upper panels) and V8 agar medium supplemented with 2 or 10 mM cAMP as indicated (lower panels). The wild-type *GPB1* gene was reintroduced into the *gpb1* mutant strain as described in Materials and Methods (*gpb1*+*GPB1*). Mating was at 22°C for 7 days. Magnification, ×25.

mans: a MAP kinase homolog, CPK1 (R. Davidson and J. Heitman, unpublished data), and a homolog of the STE12 transcription factor (61, 67). We tested whether CPK1 or STE12α functions downstream of GPB1 by epistasis, using cloned genes under the control of the *C. neoformans GAL7* promoter, which is induced by galactose and repressed by glucose (62).

When the *gpb1* mutant strain was transformed with the *GAL7-CPK1* gene fusion, mating with a *MAT***a** strain was restored on galactose filament agar but not on glucose (Fig. 6A). Thus, expression of the CPK1 MAP kinase suppresses the *gpb1* mating defect, providing evidence that GPB1 functions upstream of this MAP kinase. The *GAL7-CPK1* gene fusion did not restore mating in *gpa1* mutants (data not shown), indicating that CPK1 functions downstream of GPB1 but not of GPA1.

In contrast to the effects of CPK1, the *GAL7-STE12*α gene fusion did not restore mating of the *gpb1* mutant strain on glucose or galactose filament agar (data not shown). The functions of STE12α likely involve haploid fruiting and not mating, because STE12α overexpression stimulates haploid fruiting (61) whereas *ste12*α mutations block haploid fruiting but not mating (67).

GPB1 stimulates conjugation tube formation in *MAT*α and *MAT*a cells. We next tested whether GPB1 plays an active signaling role upstream of the MAP kinase cascade, analogous to that of the Gβγ complex in *S. cerevisiae* (50). During mating in *C. neoformans*, the mating partners secrete pheromones that trigger the formation of conjugation tubes in the opposite cell type (1, 41; R. Davidson and J. Heitman, unpublished data). We tested whether GPB1 overexpression stimulates conjugation tube formation in cells not exposed to pheromones.

The *GAL7* promoter was fused upstream of the *GPB1* gene, and the *GAL7-GPB1* gene fusion was introduced into congenic *MAT*α and *MAT***a** serotype D strains. Growth on galactose filament agar induced the formation of conjugation tubes in both *MAT***a** and *MAT*α strains (Fig. 6B). Conjugation tubes produced in response to *GPB1* overexpression were similar to those observed in confrontation assays or in *MAT***a** cells in response to expressed or synthetic MFα1 pheromone (1, 41) (Fig. 4). *MAT***a** cells produced more conjugation tubes than did *MAT*α cells, suggesting that the mating responses of the two cell types differ (Fig. 6B).

GPB1 and *MAT*a cells regulate monokaryotic fruiting. Mating of *MAT***a** and *MAT*α cells of *C. neoformans* is regulated by both pheromones and nitrogen starvation. In contrast, in response to nitrogen starvation alone, *MAT*α haploid strains differentiate, forming monokaryotic filaments, basidia, and spores by haploid fruiting (62). This filamentous differentiation shares some features with pseudohyphal growth in *S. cerevisiae* (15). Components of the mating pheromone response pathway are required for pseudohyphal growth, whereas mating pheromones, pheromone receptors, and the coupled heterotrimeric G protein are not (35). We therefore hypothesized that the Gβ protein GPB1 would not be required for haploid fruiting in *C. neoformans*.

To our surprise, we found that GPB1 is required for haploid fruiting in *C. neoformans*. Similar to the many lab strains of *S. cerevisiae* which do not undergo pseudohyphal growth, *C. neoformans* strains also differ in their ability to form filaments in response to nitrogen starvation. The serotype A strain H99 does not exhibit haploid fruiting under a variety of conditions. Introduction of a dominant active RAS1 mutant (Ras1 Q67L) does stimulate haploid fruiting of strain H99 (Fig. 7A) (J. A. Alspaugh and J. Heitman, unpublished data). However, the dominant active Ras1 Q67L mutant protein did not stimulate haploid fruiting in the *gpb1* mutant strain (Fig. 7A). Reintroduction of the wild-type *GPB1* gene restored haploid fruiting

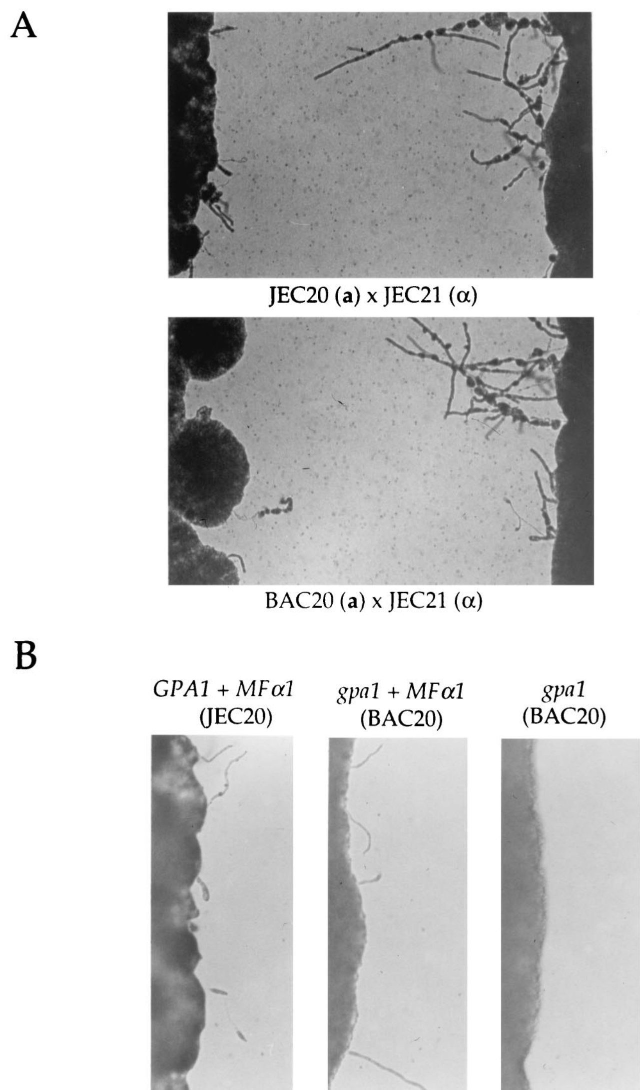

FIG. 4. The Gα subunit GPA1 is not required for responses to pheromones. (A) Cells of the wild-type *MAT*α serotype D strain JEC21 were grown in confrontation with the isogenic *MAT***a** *GPA1* wild-type strain JEC20 (upper panel) or the *gpa1* mutant strain BAC20 (lower panel), with incubation for 3 days at 24°C on filament agar, and conjugation tubes were photographed. Magnification, ×25. (B) A *ura5* derivative of the *GPA1* wild-type strain JEC20 (*MAT***a** *ura5*) and the isogenic *gpa1* mutant strain BAC20 (*MAT***a** *gpa1*::*ADE2 ura5*) were transformed with plasmid pCnTel1 lacking or expressing the *MF*α*1* pheromone gene, grown on filament agar for 2 days at 24°C, and photographed. Magnification, ×50.

of the *gpb1* mutant (Fig. 7A). The *GAL7-STE12*α gene fusion (Fig. 7A) and the *GAL7-CPK1* gene fusion (data not shown) suppressed the haploid fruiting defect of *gpb1* mutants on galactose filament agar. Thus, GPB1 is required for monokaryotic fruiting and functions upstream of CPK1 and STE12α.

We next addressed why the pheromone-sensing Gβ protein is required for haploid fruiting if this process normally occurs in response to nitrogen limitation. We found that when *MAT*α cells are grown in confrontation with *MAT***a** cells, monokaryotic fruiting of the *MAT*α cells is dramatically stimulated and abundant filaments, basidia, and basidiospores are produced (Fig. 7B). In contrast, a much lower level of monokaryotic fruiting is observed when *MAT*α cells are grown in isolation or when *MAT*α cells are grown in confrontation with *MAT*α cells (Fig. 7B). The response of *MAT*α cells to confronting *MAT***a** cells does not require cell-cell or cell-filament contact, and it occurs before any of the projecting filaments touch the confronting cells. Moreover, monokaryotic fruiting was still observed when a dialysis membrane with a molecular mass cutoff of 3,800 Da was interposed between *MAT*α and *MAT***a** cells (data not shown). The *C. neoformans* mating pheromones are predicted to diffuse through this membrane.

By microscopic observation and nuclear staining with the DNA-specific dye DAPI (4′,6′-diamidino-2-phenylindole), it was determined that the filament cells are linked by unfused clamp connections and are monokaryotic, hallmarks of monokaryotic fruiting. In addition, micromanipulation and mating type tests confirmed that basidiospores produced by *MAT*α cells in response to confronting *MAT***a** cells are all *MAT*α and are thus products of asexual monokaryotic fruiting (data not shown). Our findings indicate that monokaryotic fruiting of *MAT*α cells is stimulated by *MAT***a** cells, possibly in response to *MAT***a** pheromones sensed by a receptor coupled to GPB1.

DISCUSSION

We have identified the gene encoding a heterotrimeric G-protein β subunit, GPB1, from *C. neoformans*. GPB1 is required for mating and plays a role in the pheromone response in both *MAT*α and *MAT***a** cells by activating a MAP kinase cascade leading to conjugation tube formation and cell fusion. Two distinct signal transduction pathways regulate mating: one involves pheromone sensing and requires GPB1, and the second senses nutrients via the Gα protein GPA1-cAMP pathway and is also required for virulence factor production and pathogenicity. These signal transduction cascades coordinately regulate mating in *C. neoformans*, analogous to the role of the MAP kinase and Gα-cAMP signal transduction cascades in development in other organisms, including pseudohyphal growth in *S. cerevisiae* and mating in *Schizosaccharomyces pombe*. We found a novel role for the pheromone-sensing Gβ subunit GPB1 in haploid fruiting in *C. neoformans*. We have also discovered that monokaryotic fruiting of *MAT*α cells is dramatically stimulated by *MAT***a** cells, suggesting that this differentiation cascade may function in mating. Finally, we have shown that *gpb1* mutant strain virulence is similar to that of wild-type strains, indicating that this component of the mating pathway does not play a prominent role in virulence.

***C. neoformans* GPB1 Gβ and GPA1 Gα subunits have distinct functions.** Our studies support a model in the which the GPB1 Gβ subunit and the GPA1 Gα subunit function in two different signal transduction cascades that regulate different steps in mating. Several observations indicate that GPB1 functions in the pheromone response pathway and regulates early steps in mating involving conjugation tube formation and cell fusion. First, *gpb1* mutants are completely sterile and exhibit a profound defect in a cell fusion assay. Second, GPB1 overexpression stimulates conjugation tube formation. Third, overexpression of the MAP kinase CPK1 suppresses the mating defect of *gpb1* mutant strains, whereas cAMP does not. Later in mating, the pheromone response pathway likely also plays a second role involving the fusion of the clamp cells during filament formation.

Our findings also contribute to the understanding of the role of GPA1-cAMP signaling in mating. The mating defect of *gpa1* mutant strains is suppressed by cAMP but not by the MAP kinase CPK1. In addition, *gpa1* mutants can respond to pheromones in confrontation assays and in response to expression of the MFα1 pheromone gene (Fig. 4). In quantitative mating assays, the *gpa1* mutation reduces but does not block cell

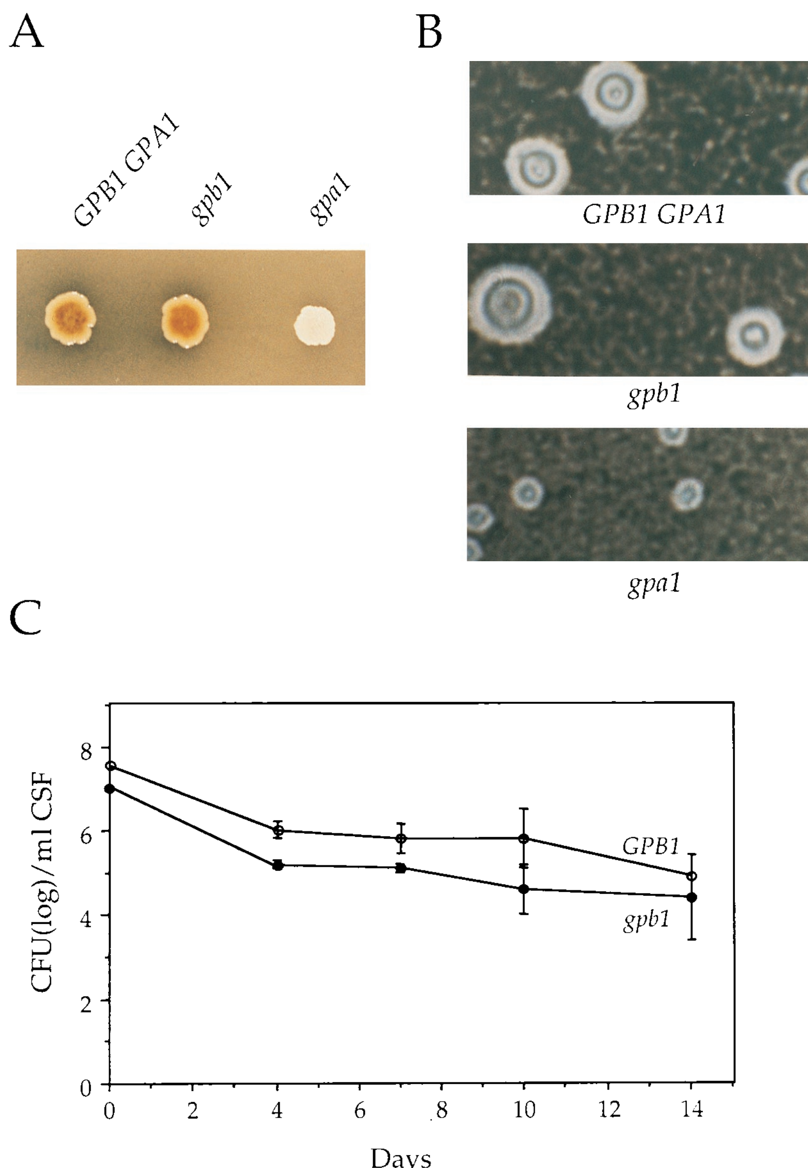

FIG. 5. GPB1 is not required for virulence factors or virulence in *C. neoformans*. (A) The isogenic *GPB1 GPA1* wild-type strain H99 and the *gpb1*::*ADE2* (*gpb1*) and *gpa1*::*ADE2* (*gpa1*) mutant strains were grown on niger seed agar for 72 h at 37°C. Strains that produce melanin (*GPB1 GPA1*, *gpb1*) form brown colonies on this medium, whereas strains that do not produce melanin (*gpa1*) are white. (B) Cells of the wild-type strain H99 (*GPB1 GPA1*) and the *gpb1*::*ADE2* (*gpb1*) and *gpa1*::*ADE2* (*gpa1*) mutant strains were grown in low-iron medium plus EDDHA at 30°C for 48 h to induce capsule synthesis. The polysaccharide capsule was identified by India ink staining and photographed. Magnification, ×200. (C) The *GPB1* wild-type (H99) and *gpb1* mutant strains were inoculated intracisternally into immunosuppressed rabbits. CSF was withdrawn on days 4, 7, 10, and 14 postinfection, and the numbers of surviving yeast cells were determined by plating serial dilutions of CSF on YPD medium. The mean cell count for each strain was plotted with the standard error of the mean.

fusion and also reduces filamentation and the production of recombinant basidiospores. The nutrient-sensing GPA1-cAMP cascade is required for melanin and capsule production and virulence, whereas GPB1 is not.

These findings support a model in which the GPA1 Gα and GPB1 Gβ subunits are components of two different signaling cascades. We propose that GPA1 and GPB1 are components of two different G proteins and function in distinct signaling cascades, one that senses nutrients via a cAMP pathway (GPA1) and another that senses mating pheromones and signals via a MAP kinase cascade (GPB1).

MAP kinase signaling in *MAT*α and *MAT*a cells. Our findings reveal that the Gβ subunit GPB1 regulates conjugation tube formation in both *MAT***a** and *MAT*α cells. The *GPB1* gene is present in both *MAT*α and *MAT***a** strains and is expressed in cells of both mating types. This is in contrast to other MAP kinase cascade components recently identified in *C. neoformans*, including STE11α, STE12α, and STE20α homologs, which are encoded by the *MAT*α locus and are specific to *MAT*α cells (61; B. Wickes and J. Edman, personal communication; P. Wang and J. Heitman, unpublished results). These findings raise the conundrum of how signaling occurs in *MAT***a** cells during mating if several components are present only in *MAT*α cells. Our findings suggest that the MAP kinase cascade functions during mating in both *MAT*α and *MAT***a** cells. We propose that mating in both cell types is regulated by GPB1 signaling via two divergent versions of a conserved MAP kinase cascade: one, containing components encoded by the *MAT*α

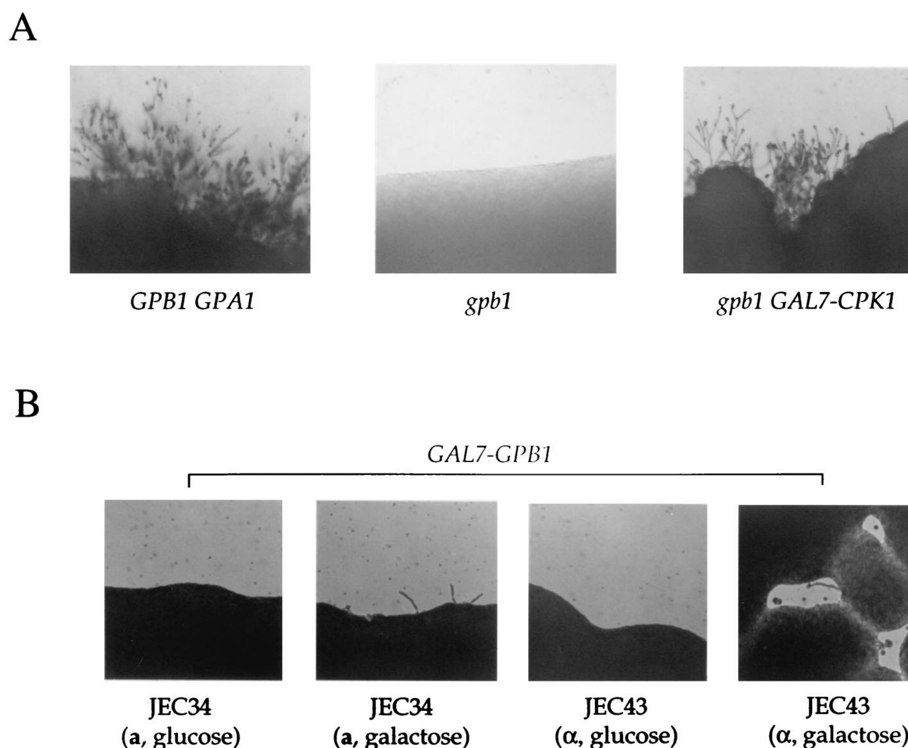

FIG. 6. GPB1 activates a MAP kinase cascade involving the CPK1 kinase. (A) The *CPK1* gene expressed from the *C. neoformans GAL7* promoter in the *URA5* plasmid pCnTel1 was introduced into a *gpb1 ura5* mutant strain (see Materials and Methods) by biolistic transformation. The isogenic *MAT*α wild-type strain H99 (*GPB1 GPA1*), the *gpb1* mutant strain, and the *gpb1* mutant strain transformed with the *GAL7-CPK1* gene fusion (*gpb1 GAL7-CPK1*) were cocultured with a *MAT***a** mating partner (JEC20). Mating was for 21 days at 22°C on filament agar containing 0.5% galactose (shown here) or 0.5% glucose (data not shown). Magnification, ×25. (B) The congenic serotype D *MAT***a** *ura5* strain JEC34 and the *MAT*α *ura5* strain JEC43 were transformed with the *GAL7-GPB1* gene fusion linked to the *URA5* gene and grown for 72 h at 24°C on filament agar with glucose or galactose. Conjugation tubes emanating from cell patches were photographed. Magnification, ×25.

locus, that supports mating and can also function in haploid fruiting and virulence, and another, with components encoded by the *MAT***a** locus, that plays a more restricted role in mating, does not support haploid fruiting, and remains to be identified. Such a model may be related to the situation in the yeast *S. cerevisiae*, which expresses two related MAP kinases with divergent functions: Fus3, which is specialized for mating of haploid cells, and Kss1, which regulates pseudohyphal differentiation of diploid cells (7, 38).

G-protein signaling roles in other yeasts. Our studies on G-protein function are relevant to previous studies of G proteins in other yeasts. In *S. cerevisiae*, two G proteins regulate responses to pheromone and nutrients (50). During pseudohyphal differentiation, nutrients regulate the Gα protein Gpa2, which signals via a cAMP cascade (24, 37). During mating, pheromone binding to the Ste2 or Ste3 receptors recruits the Gαβγ complex (Gpa1-Ste4-Ste18), and the released Ste4-Ste18 βγ complex activates the MAP kinase cascade by recruiting signaling components to the membrane (32–34, 47). The Gα subunit Gpa1 inhibits signaling by βγ. *C. neoformans* Gβ subunit GPB1 functions analogously to the Ste4 Gβ subunit in *S. cerevisiae* mating, whereas the functions of the *C. neoformans* Gα subunit GPA1 are analogous to nutrient sensing by *S. cerevisiae* GPA2.

In the fission yeast *Schizosaccharomyces pombe*, mating is also regulated by two G proteins, composed of the Gα subunit Gpa1 and the Gα and Gβ subunits Gpa2 and Gpb1 (65). Gpa1 is required for the pheromone response during mating and, in contrast to the situation for *S. cerevisiae*, plays a positive role in activating the MAP kinase cascade. Gpa2 plays a role analogous to that of the *S. cerevisiae* Gpa2 and *C. neoformans* GPA1 subunits, and it functions in a nutrient-sensing cAMP pathway regulating mating (19, 43). Mutants lacking the Gβ subunit Gpb1 exhibit a phenotype similar to that of *gpa2* mutants and mate and sporulate under nutrient-rich conditions (23). The sensing of pheromones by the Gβ subunit GPB1 in *C. neoformans* appears to be distinct from the role for the *Schizosaccharomyces pombe* Gβ subunit in nutrient sensing.

GPB1 and *MAT*a cells regulate haploid fruiting in *C. neoformans*. We found that the Gβ subunit GPB1 is also required for haploid fruiting in *C. neoformans*. Haploid fruiting is a differentiation pathway whereby *MAT*α cells can form filaments and sporulate in response to nitrogen starvation in the absence of a mating partner (62). Haploid fruiting shares features with pseudohyphal differentiation in the yeast *S. cerevisiae*, which is regulated by a MAP kinase cascade and induced by nitrogen limitation (15). However, the mating pheromones, receptors, and the coupled G protein are not required for filamentation in *S. cerevisiae* (35).

Although haploid fruiting occurs to a limited extent in some *MAT*α strains in response to nitrogen starvation alone (61), we found that monokaryotic fruiting of *C. neoformans MAT*α cells is markedly stimulated by confrontation with *MAT***a** cells. This stimulation does not require cell-cell or filament-filament contact. We propose that *MAT***a** cells stimulate haploid fruiting of adjacent *MAT*α cells by secreting a peptide mating pheromone. Stimulation of monokaryotic fruiting by this pheromone may function to disperse *MAT*α spores to locate uncommon *MAT***a** mating type cells at a distance. Thus, haploid fruiting

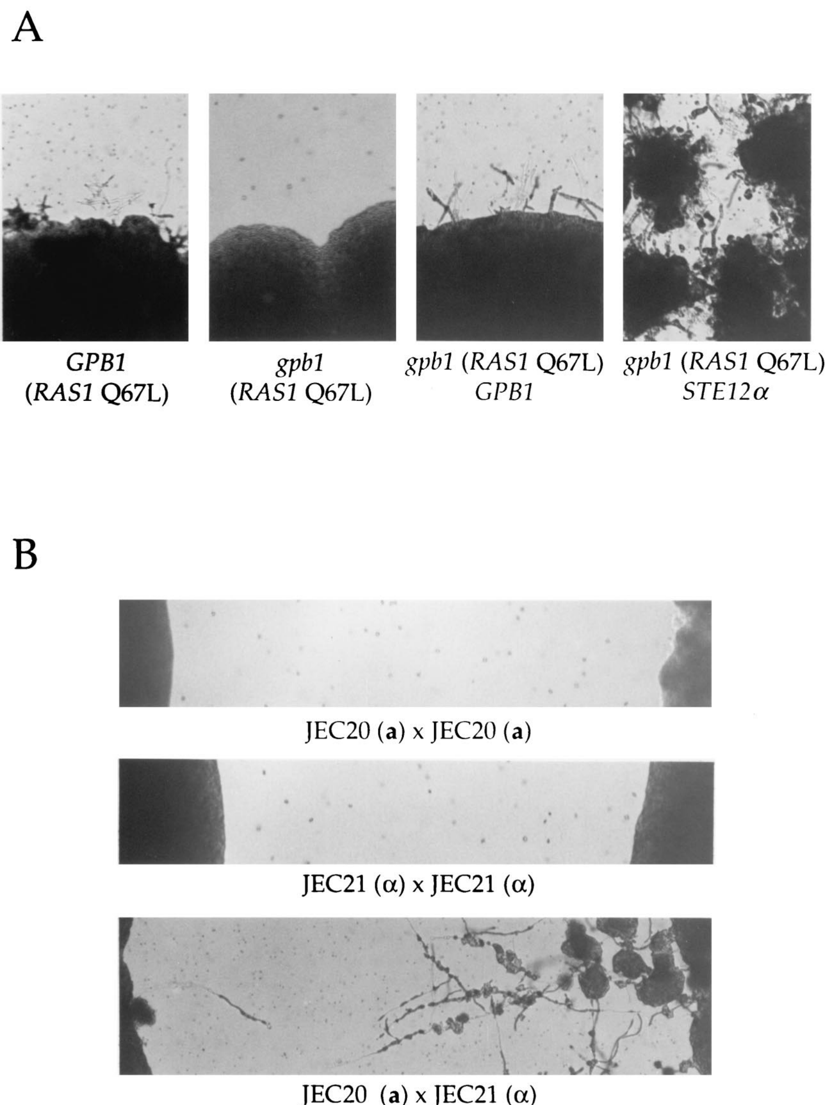

FIG. 7. GPB1 and *MAT***a** cells regulate haploid fruiting. (A) The isogenic *GPB1* wild-type strain H99 (far-left panel), the *gpb1*::*ADE2* mutant strain (second panel from left), and the *gpb1*::*ADE2* mutant strain reconstituted with the *GPB1* wild-type gene (third panel from left) were transformed with the dominant active *Ras1* Q67L mutant gene, grown on glucose filament agar medium for 7 days at 24°C, and photographed. The *gpb1* mutant strain was also transformed with plasmid pCGS-1 expressing the *GAL7-STE12* fusion gene and grown on galactose filament agar (far-right panel) for 7 days at 24°C. Magnification, ×25. (B) Cells of the serotype D *MAT*α strain JEC21 were grown in confrontation with themselves (middle panel) or with congenic cells of the opposite (*MAT***a**) mating type (strain JEC20) (lower panel). As a control, the *MAT***a** strain JEC20 was grown in confrontation with itself (upper panel). Cells were incubated for 10 days at 24°C on filament agar and photographed. Magnification, ×25.

could function as a prelude to mating in an organism in which *MAT*α cells are more abundant than *MAT***a** cells.

We propose that haploid fruiting occurs in isolated *C. neoformans MAT*α cells at a low level due to basal signaling of the pheromone-responsive MAP kinase pathway in the absence of pheromone and is then markedly stimulated by a pheromone produced by adjacent *MAT***a** cells. *MAT***a** cells do not undergo haploid fruiting, in part because they lack the *MAT*α locus-encodes transcription factor STE12α required for haploid fruiting (67). The model in which haploid fruiting is regulated by pheromones makes the testable prediction that the MFa1 pheromone and its receptor are also required.

Although haploid invasive growth and diploid filamentous growth in *S. cerevisiae* are not normally regulated by the Gβ subunit Ste4, our findings may be related to studies of altered differentiation in mutant *S. cerevisiae* strains and the basal signaling state of the yeast pheromone response pathway. First, in *fus3* mutant yeast strains, haploid invasive growth and expression of filamentous reporter genes are increased, and this requires the Gβ subunit Ste4 and results from inappropriate cross-talk between the pheromone-responsive MAP kinase cascade and the filamentous growth pathway (38). Second, even in the absence of pheromones, the *S. cerevisiae* pheromone response pathway is active at a basal level. The Gβ subunit Ste4, the kinases Ste11 and Ste7, the scaffold Ste5, and the Ste12 transcription factor are required for basal signaling (11, 17, 50). By analogy, we propose that signaling by the Gβ subunit GPB1 in the absence of pheromones supports a basal level of monokaryotic fruiting in *C. neoformans* that is then stimulated by pheromones.

Perspective. The *MAT*α locus has been linked to virulence in *C. neoformans*. Our finding that *gpb1* mutant strains are defective in mating but not impaired in virulence indicates that mating is not required for virulence, and further studies are needed to establish the link between the *MAT*α locus and virulence.

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