G Protein-coupled Receptor Gpr4 Senses Amino Acids and Activates the cAMP–PKA Pathway in *Cryptococcus neoformans*[D]

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The Gα protein Gpa1 governs the cAMP-PKA signaling pathway and plays a central role in virulence and differentiation in the human fungal pathogen *Cryptococcus neoformans*, but the signals and receptors that trigger this pathway were unknown. We identified seven putative proteins that share identity with known G protein-coupled receptors (GPCRs). One protein, Gpr4, shares limited sequence identity with the *Dictyostelium discoideum* cAMP receptor cAR1 and the *Aspergillus nidulans* GPCR protein GprH and also shares structural similarity with the *Saccharomyces cerevisiae* receptor Gpr1. *gpr4* mutants exhibited reduced capsule production and mating defects, similar to *gpa1* mutants, and exogenous cAMP suppressed both *gpr4* mutant phenotypes. Epistasis analysis provides further evidence that Gpr4 functions upstream of the Gα subunit Gpa1. Gpr4-Gpr4 homomeric interactions were observed in the yeast two-hybrid assay, and Gpr4 was shown to physically interact with Gpa1 in the split-ubiquitin system. A Gpr4::DsRED fusion protein was localized to the plasma membrane and methionine was found to trigger receptor internalization. The analysis of intracellular cAMP levels showed that *gpr4* mutants still respond to glucose but not to certain amino acids, such as methionine. Amino acids might serve as ligands for Gpr4 and could contribute to engage the cAMP-PKA pathway. Activation of the cAMP-PKA pathway by glucose and amino acids represents a nutrient coincidence detection system shared in other pathogenic fungi.

INTRODUCTION

Cryptococcus neoformans is a basidiomycete human fungal pathogen that infects the CNS to cause meningoencephalitis (Casadevall and Perfect, 1998). The medical importance of *C. neoformans* has increased dramatically as a consequence of the acquired immune deficiency syndrome (AIDS) pandemic. Recent studies in *C. neoformans* have begun to delineate the signal transduction cascades that regulate morphological differentiation and virulence factor development. Many of these signaling cascades are conserved in other human and plant fungal pathogens. Among them, heterotrimeric G protein signaling has attracted considerable attention because of its conservation among organisms and its importance in fungal development and virulence (Lengeler *et al.,* 2000).

Three G protein α subunits (Gpa1, Gpa2, Gpa3) and one Gβ subunit (Gpb1) have been identified in *C. neoformans* (Alspaugh *et al.,* 1997; Wang and Heitman, 1999; Wang *et al.,* 2000). Gpa1 governs the cAMP-protein kinase A (PKA) signaling together with the adenylyl cyclase associated protein Aca1 (Bahn *et al.,* 2004) and plays a central role in the development of two major virulence factors, melanin and capsule, which are crucial for the pathogenicity of this or-

ganism (Alspaugh *et al.,* 1997, 2002; D'Souza *et al.,* 2001). Gpa1 also contributes to mating of *C. neoformans*. In contrast, the functions of Gpa2 and Gpa3 are less well established. The single Gβ subunit Gpb1 is required to mediate pheromone sensing during mating via the Cpk1 MAP kinase pathway that shares features conserved with the pheromone response pathway in *Saccharomyces cerevisiae* (Wang *et al.,* 2000, 2002; Davidson *et al.,* 2003).

G protein-coupled receptors (GPCRs) represent the largest family of transmembrane receptors responsible for transducing extracellular signals into intracellular responses and signal in response to stimuli as diverse as light, protons, Ca^{2+}, odorants, amino acids, nucleotides, proteins, polypeptides, steroids, and fatty acids (Maller, 2003). GPCRs mediate myriad intracellular responses and thereby regulate cellular function via activation of G protein-dependent and -independent pathways. In mammalian cells, ~720 GPCRs have been identified (Kostenis, 2004). Despite their importance in signaling regulation, only a few GPCRs other than pheromone receptors have been studied in any detail in most fungal systems (Xue *et al.,* 1998; Lorenz *et al.,* 2000; Han *et al.,* 2004; Lemaire *et al.,* 2004; Miwa *et al.,* 2004; Maidan *et al.,* 2005a, 2005b). Reported fungal GPCRs can be grouped into four groups (Han *et al.,* 2004) including GPCRs similar to the following: group i: the pheromone sensing Ste2 and Ste3 receptors of *S. cerevisiae*; group ii, the glucose sensing Gpr1 receptor of *S. cerevisiae*; group iii, the proposed nutrient sensor Stm1 of *Schizosaccharomyces pombe*; and group iv, the cAMP receptors of *Dictyostelium discoideum*.

In *S. cerevisiae*, both the Gpa2-activated cAMP pathway and the Ste4/Ste18-activated pheromone response pathway use GPCRs as ligand sensors. In the mating pathway, two

GPCRs, Ste2 and Ste3, sense pheromones produced by cells of the opposite mating type and activate the MAP kinase pathway (Versele *et al.*, 2001; Dohlman, 2002). The *S. cerevisiae* pheromone receptors are well conserved in other fungi, including *Schizosaccharomyces pombe* (Tanaka *et al.*, 1993), *Aspergillus nidulans* (Seo *et al.*, 2004), and *Neurospora crassa* (Kim and Borkovich, 2004). Ste3 homologues have also been reported in basidiomycetes, such as Pra-1 in *Ustilago maydis* (Bolker *et al.*, 1992). In *C. neoformans*, pheromone receptors expressed by α and **a** cells (Ste3α/Cprα and Ste3**a**/Cpr**a**) have been identified; both are encoded by the mating type locus and are important for mating (Chung *et al.*, 2002), and Cpr**a** also may play a role in virulence (Chang *et al.*, 2003). An additional pheromone receptor-like GPCR (Cpr2) is present in *C. neoformans* but its gene is not mating type specific and its functions are unknown.

In addition to the pheromone GPCRs, an unusual sugar sensing receptor (Gpr1) has also been identified in *S. cerevisiae*. Gpr1 senses glucose and sucrose and activates cAMP signaling through Gpa2 (Yun *et al.*, 1997, 1998; Xue *et al.*, 1998; Kraakman *et al.*, 1999; Lorenz *et al.*, 2000; Lemaire *et al.*, 2004). Glucose-sensing receptor homologues have also been identified from several other yeasts. In *Candida albicans*, a homolog of the *S. cerevisiae* Gpr1 receptor also senses low concentrations of glucose and amino acids such as methionine to activate cAMP signaling (Miwa *et al.*, 2004; Maidan *et al.*, 2005a). In *S. pombe*, a related glucose sensing receptor, Git3, has been identified (Welton and Hoffman, 2000). Several GPCRs have also been reported that are important for morphological development in filamentous fungi. In *A. nidulans*, two pheromone receptors, GprA and GprB, and seven other putative GPCRs have been identified. One of these GPCRs, GprD, was found to function as a negative regulator of sexual development (Seo *et al.*, 2004). Based on genome sequence analysis, nine potential GPCR genes have been identified in *N. crassa* (Galagan *et al.*, 2003). In the slime mold *D. discoideum*, four cAMP signal receptors and three cAMP receptor-like GPCRs have been identified (Raisley *et al.*, 2004). But it remains a mystery which proteins sense environmental signals to activate the cAMP signaling pathway in other fungal systems. We hypothesized that related GPCRs could function to activate cAMP signaling in *C. neoformans*.

In this study, we identified seven GPCR proteins and generated gene-deletion mutants for all of these GPCRs. Both phenotypic assays and biochemical analysis revealed that one GPCR, Gpr4, is important for capsule production and mating. This GPCR has a gene structure similar to *S. cerevisiae* Gpr1 and *A. nidulans* GprH. Gpr4 is physically and functionally associated with the G-protein α subunit Gpa1 and functions in sensing the amino acid methionine to activate cAMP-PKA signaling, in accord with recent studies on Gpr1 in *C. albicans* (Maidan *et al.*, 2005a, 2005b).

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

C. neoformans strains used in this study are listed in Table 1. *gpr4 gpa1*, *gpr4 pkr1*, *gpr4 pka1*, and *gpr4 crg1* double deletion mutants were all generated by screening the progeny basidiospores from mating crosses of two individual mutants. *ura5* auxotrophic mutants were generated by screening 5-FOA resistant yeast cells. Strains were grown at 30°C on yeast extract-peptone-dextrose (YPD) agar medium and synthetic (SD) medium. V8 medium (pH 5.0) was used for mating assays. Niger-seed medium was used to test for melanin production. Dulbecco's modified Eagle's (DME) medium for capsule production was prepared as previously described (Bahn *et al.*, 2004). All other media preparations were followed as described previously (Granger *et al.*, 1985; Alspaugh *et al.*, 1997; Bahn *et al.*, 2005).

Disruption of the GPR4 Gene and Construction of a gpr4 + GPR4 Complemented Strain

The *gpr4* null mutant was generated in the congenic *C. neoformans* serotype A *MAT*α (H99) and *MAT***a** (KN99**a**) strains by overlap PCR as previously described (Davidson *et al.*, 2002). The 5′ and 3′ regions of the *GPR4* gene were amplified with primers JH12678/JH12679 (see Supplementary Table 1 for primer sequences) and JH12680/JH12681 from H99 or KN99 genomic DNA, whereas the dominant selectable markers (Nat^r or Neo^r) were amplified with the M13 primers (M13F/M13R) from plasmid pNATSTM#209 or pJAF1 (Fraser *et al.*, 2003), respectively. The *GPR4* gene replacement cassette was generated by overlap PCR with primers JH12678/JH12681, precipitated onto 600-μg gold microcarrier beads (0.8 μm; Bioworld, Dublin, OH) and biolistically transformed into strains H99 or KN99**a** as described previously (Davidson *et al.*, 2000). Stable transformants were selected on YPD medium containing nourseothricin (100 mg/L) or G418 (200 mg/L). To screen for *gpr4* mutants, diagnostic PCR was performed by analyzing the 5′ junction of the disrupted *gpr4* alleles with primers JH12683/JH8994. Positive transformants identified by PCR screening were further confirmed by Southern blot.

To construct the *gpr4* complemented strains, H99 genomic DNA containing the entire *GPR4* gene was isolated from a *C. neoformans* BAC library using a *GPR4*-specific probe amplified with primers JH13137/JH13138. The 5.2-kb *Xho*I-*Kpn*I fragment containing the *GPR4* gene was cloned into plasmid pJAF7 containing the NAT selectable marker (Fraser *et al.*, 2003). The construct was linearized by *Kpn*I digestion and transformed into a *gpr4* mutant using biolistic transformation. The Gpr4 complemented strain was selected from transformants containing the full-length *GPR4* gene.

Assays for Melanin and Capsule Production

Melanin production was assayed by inoculating *C. neoformans* strains into 2 ml YPD liquid medium and incubating overnight at 30°C. Five microliters of each overnight culture was placed on Niger-seed agar medium. The agar plates were incubated at 30 or 37°C for 2 d, and pigmentation of fungal colonies was assessed.

To examine capsule production, 5 μl of overnight cultures was inoculated onto DME agar medium and incubated at 30 or 37°C for 3 d. The capsule was visualized with India ink staining and observed with a 100× Zeiss Axioskop 2 equipped with an AxioCam MRM digital camera (Carl Zeiss, Thornwood, NY). Quantitative measurement of capsule size was performed as described previously (Zaragoza *et al.*, 2003) by measuring the diameters of the capsule and the cell using Axio Vision 3.1 software (Carl Zeiss). The relative capsule diameter ($100(Dw - Dc)/Dw$, where Dw indicates the diameter of the cell plus capsule and Dc indicates the diameter of the cell only) was statistically compared between each mutant and wild-type strains by the Student's *t* test. $p < 0.05$ was considered significant.

Assays for Mating and Cell Fusion

C. neoformans cells of opposite mating type were homogenized and cocultured on V8 agar medium at 25°C in the dark for several days, and filamentation was examined under light microscopy.

Cell fusion efficiency was measured by mixing 2×10^6 cells of two strains. Culture suspension, 5 μl, was inoculated onto V8 agar medium (pH 5.0) and incubated for 24 h at room temperature in the dark. Four replicate plates were prepared for each of the three cell mixtures. Cells were then harvested and resuspended in 1 ml dH$_2$O and 200 μl of the suspension (~10^5 total cells) was plated onto YPD medium containing NAT and G418. The number of colonies on each plate was determined after incubation at 37°C for 3 d.

Generation of GPA1 Dominant Active Allele

The *GPA1* dominant active allele (*GPA1^{Q284L}*), corresponding to the *GPA2^{Q300L}* mutant in *S. cerevisiae*, was amplified by overlap PCR using primers JH12497/JH12500 and JH12499/JH12498. The overlap PCR product was amplified with primers JH12497/JH12498, cloned into the integrating vector pRCD83 (Davidson *et al.*, 2002), generating pDX10. This expression construct was transformed into wild-type and the *gpa1*, *gpr4*, *gpr4 gpa1*, and *pka1* mutants to generate dominant active strains.

Construction of GFP::Gpr4 and Gpr4::DsRED Fluorescent Strains

The *GPR4* cDNA was amplified from H99 first-strand cDNA with primers JH12961/JH12759 and cloned into plasmid pTH74 (Harashima and Heitman, 2002) between the *Sma*I and *Pst*I sites, fused to the C-terminus of the *GFP* gene in the vector, under the control of the *S. cerevisiae ADH1* promoter. The *GFP::GPR4* fusion construct was transformed into the *S. cerevisiae gpr1* mutant strain MLY232.

GPR4 genomic DNA was also amplified from H99 genomic DNA with primers JH15264/JH15307. The *DsRED* open reading frame was amplified from plasmid pDsRED2 with primers JH15305/JH15306. The *GPR4::DsRED* overlap PCR product was amplified with primers JH15264/JH15306 and cloned into plasmid pXL1 (Lin *et al.*, unpublished results) between the *Fse*I and *Pac*I sites, under the control of the *C. neoformans GPD1* promoter. The

Table 1. Strains used in this study

C. neoformans strains	Genotype	Source/reference
H99	*MATα*	Perfect *et al.* (1993)
KN99a	*MAT**a***	Nielsen *et al.* (2003)
JEC21	*MATα serotype D*	Moore and Edman (1993)
F99	*MATα ura5*	Wang *et al.* (2002)
YSB83	*MATα gpa1::NAT-STM#5*	Bahn *et al.* (2004)
YSB85	*MAT**a** gpa1::NEO*	Bahn *et al.* (2004)
YSB42	*MATα cac1::NAT-STM#159*	Bahn *et al.* (2004)
YSB119	*MATα aca1::NAT-STM#43 ura5 ACA1-URA5*	Bahn *et al.* (2004)
YSB121	*MAT**a** aca1::NEO ura5 ACA1-URA5*	Bahn *et al.* (2004)
JKH7	*MATα pka1::NAT-STM#191 ura5*	J. K. Hicks
CDX1	*MATα gpr1::NAT-STM#242*	This study
CDX3	*MATα gpr2::NEO*	This study
CDX5	*MATα gpr3::NEO*	This stiudy
CDX6	*MATα gpr4::NEO*	This study
CDX7	*MATα gpr4::NAT-STM#209*	This study
CDX9	*MAT**a** gpr4::NAT-STM#209*	This study
CDX10	*MAT**a** gpr5::NAT-STM#58*	This study
CDX12	*MAT**a** cpr2::NAT-STM#249*	This study
CDX14	*MATα gpr6::NAT-STM#218*	This study
CDX16	*MATα gpr7::NAT-STM#191*	This study
CDX18	*MATα gpr4::NEO gpr5::NAT-STM#58*	This study
CDX19	*MAT**a** gpr4::NEO gpr5::NAT-STM#58*	This study
CDX20	*MATα gpr4::NEO gpa1::NAT-STM#5*	This study
CDX21	*MAT**a** gpr4::NEO gpa1::NAT-STM#5*	This study
CDX24	*MATα gpr4::NAT-STM#209 pkr1::URA5*	This study
CDX25	*MATα gpr4::NAT-STM#209 pka1::URA5*	This study
CDX26	*MATα gpr4::NAT-STM#209 crg1::URA5*	This study
CDX27	*MAT**a** gpr4::NAT-STM#209 crg1::URA5*	This study
CDX8	*MATα gpr4::NAT-STM#209 ura5*	This study
CDX47	*MATα gpr4::NAT-STM#209 ura5 GPA1^{Q284L}-URA5*	This study
CDX45	*MAT**a** gpr4::NAT-STM#209 GPR4-NEO*	This study
CDX46	*MATα gpr4::NAT-STM#209 GPR4-NEO*	This study
CDX78	*MATα gpr4::NAT-STM#209 GPR4-NEO ura5*	This study
CDX79	*MATα gpr4::NAT-STM#209 GPR4-NEO ura5 GPA1^{Q284L}-URA5*	This study
CDX36	*MATα gpa1::NAT-STM#5 ura5*	This study
CDX37	*MAT**a** gpa1::NEO ura5*	This study
CDX38	*MAT**a** gpa1::NEO ura5 URA5*	This study
CDX40	*MAT**a** gpa1::NEO ura5 GPA1^{Q284L}::FLAG-URA5*	This study
CDX42	*MAT**a** gpa1::NEO ura5 GPA1-URA5*	This study
CDX80	*MATα gpa1::NAT ura5 GPA1^{Q284L}-URA5*	This study
CDX63	*MATα cac1::NAT-STM#159 ura5 GPA1^{Q284L}::FLAG-URA5*	This study
CDX64	*MATα pka1::NAT-STM#191 ura5 GPA1^{Q284L}::FLAG-URA5*	This study
CDX75	*MATα gpr4::NEO gpa1::NAT-STM#5 ura5*	This study
CDX76	*MATα gpr4::NEO gpa1::NAT-STM#5 ura5 GPA1^{Q284L}-URA5*	This study
CDX77	*MATα ura5 GPA1^{Q284L}-URA5*	This study
CDX70	*MATα gpr4::NAT-STM#209, serotype D*	This study
CDX73	*MATα gpr4::NAT-STM#209 GPR4-DsRED-NEO, serotype D*	This study

Each NAT-STM# indicates the Nat^r marker with a unique signature tag. Unless labeled specifically, all strains are from the serotype A H99/KN99**a** background.

GPR4::DsRED fusion construct was transformed into the *gpr4* mutant strain. Yeast cells were stained with the yeast vacuole membrane marker dye MDY-64 (Molecular Probes, Eugene, OR). Fluorescence was observed using a Zeiss Axioskop 2 fluorescent microscopy.

Assays for cAMP Production

cAMP assays were conducted as described previously (Bahn *et al.,* 2004). Briefly, a single colony of each *C. neoformans* strain was inoculated into 10 ml of YPD liquid medium and incubated for 24 h at 30°C with shaking. Cells were collected and washed twice with dH$_2$O, once with MES buffer (10 mM MES, 0.5 mM EDTA), and resuspended in 20 ml MES buffer. Diluted cells (15 ml; OD$_{600}$ = 2.0) were incubated for 2 h at 30°C for glucose starvation. One milliliter of cells was filtered through a wet Millipore filter on a vacuum manifold (pore size, 0.45 μm, HVLP02500) for the 0 time point. Twenty percent glucose, 1.5 ml, was added to the remaining 14 ml of cell suspension. One milliliter was removed and filtered at 30 s, 1 min, and 3 min. At each time point, filters were immediately removed, placed into Petri-dishes containing 1 ml of formic acid (9.2 ml of 100% formic acid, 190.8 ml dH$_2$O, and 50 ml butanol), and agitated for 1 h to lyse cells on a table-top orbital shaker. Cell suspensions were spun down and the supernatants were transferred to fresh tubes and lyophilized. Pellets were resuspended in 400 μl of assay buffer and 100 μl was used for each sample. cAMP concentrations were determined using the cAMP Biotrak Enzyme immunoassay (EIA) system (Amersham, Piscataway, NJ) and normalized to the wet weight of the cells.

Protein-Protein Interaction Assays Using the Yeast Two-hybrid and Split-ubiquitin Systems

Yeast two-hybrid interaction assays were performed as described (Osman, 2004). cDNAs of the *GPR4* 3rd cytoplasmic loop and C-terminal cytoplasmic tail were cloned into the bait vector pGAD424 and the prey vector pGBT9, respectively. The full-length *GPA1* cDNA was cloned into plasmid pGBT9. All inserted cDNA sequences were confirmed by sequencing. Both bait constructs and prey constructs were cotransformed into yeast strain PJ69-4A. Transformants growing on medium lacking histidine were considered positive interactions. Positive interactions were further confirmed and quantified by

Table 2. GPCR genes in *C. neoformans*

C. neoformans	*A. nidulans*	*S. cerevisiae*	*S. pombe*	*D. discoideum*
No homolog	GprA (AN2520.2)	Ste2	Mam2	
Cprα (163.m06349)	GprB (AN7743.2)	Ste3	Map3	
Cpr2 (186.m03862)	GprB (AN7743.2)	Ste3	Map3	
Gpr1 (164.m02000)	GprC (AN3765.2)	Gpr1	Git3	
No homolog	GprD (AN3387.2)			
No homolog	GprE (AN9199.2)			
Gpr2 (179.m00299)	GprF (not defined)		Stm1	
Gpr3 (186.m04059)	GprG (not defined)		Stm1	
Gpr4 (185.m02504)	GprH (AN8262.2)			cAR1
Gpr5 (184.m04563)	GprI (AN8348.2)			cAR1

The GPCR sequences from *A. nidulans* were used in BLASTP searches of the *C. neoformans* annotated protein database. The GPCRs in *A. nidulans* were found by BLASTN searches using GPCRs from *S. cerevisiae* or *S. pombe* and the *A. nidulans* genome database (Han *et al.*, 2004).

β-galactosidase enzyme activity assays using chlorophenolred-β-D-galacto-pyranoside (CPRG; Calbiochem, San Diego, CA) as substrate as described previously (Idnurm and Heitman, 2005).

The split-ubiquitin system was also utilized to investigate the interaction between Gpr4 and Gpa1. Vectors and yeast strains were included in the DUALmembrane Kit 2 (Dualsystem Biotech, Zürich, Switzerland). *GPR4* (full-length cDNA) and *GPR4$^{81–840}$* (first transmembrane domain deletion) were cloned into pNCW (the C-terminal half of the ubiquitin Cub protein was fused to the N-terminus of Gpr4), or pCCW (Cub was fused to the C-terminus of Gpr4), respectively. *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 G proteins C-termini). 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 or adenine and also by measuring β-galactosidase activity.

Virulence Study

Yeast strains were grown at 30°C overnight and cultures were washed twice with 1× phosphate-buffered saline and resuspended at a final concentration of 2×10^6 CFU/ml. Groups of 10 female A/Jcr mice (NCI/Charles River Laboratories, Wilmington, MA) were intranasally infected with 10^5 yeast cells of each strain as previously described (Cox *et al.*, 2000). Animals that appeared moribund or in pain were killed by CO_2 inhalation. Survival data from the murine experiments were statistically analyzed between paired groups using the long-rank test using the PRISM program 4.0 (GraphPad Software, San Diego, CA. $p < 0.05$ were considered significant.

RESULTS

GPCR Proteins in C. neoformans

Capitalizing upon the now completed *C. neoformans* genome (Loftus *et al.*, 2005), we identified over 60 putative proteins that have 7 transmembrane domains (TMs), a structural feature of GPCRs, based on a hidden Markov model (TM-HMM) program (Krogh *et al.*, 2001; Han *et al.*, 2004; see Supplementary Table 2). Among these proteins, seven proteins (Ste3α/Cprα, Cpr2, and Gpr1–5) share homology with GPCR proteins identified in other organisms (Table 2). Compared with the reported classification of fungal GPCRs (Han *et al.*, 2004), Ste3α/Cprα (Chung *et al.*, 2002), Ste3**a**/Cpr**a** (Chang *et al.*, 2003), and Cpr2 can be grouped into the pheromone receptor group i; Gpr1 shares some sequence identity to GprC in *A. nidulans*, but is grouped in the third group in the phylogenetic tree based on Clustal X (Thompson *et al.*, 1997), indicating that it is more closely related to proteins in this group; Gpr2 and Gpr3 have sequence identity to Stm1 in *S. pombe* and belong to the third group iii; and Gpr4 and Gpr5 are more similar to the cAMP receptors in group iv (Figure 1A). Gene deletion mutants for these 7 GPCRs were generated. *gpr4* and *ste3α/cprα* mutations produce phenotypes involved in virulence factor production or mating, whereas the other 5 GPCR deletion mutants have no obvious phenotype. Twenty-nine hypothetical proteins with 7 TMs have homology with proteins other than GPCRs; the other twenty-four hypothetical proteins with 7 TMs have no homology to any known protein sequence in GenBank (http://www.ncbi.nlm.nih.gov/), and these could represent additional GPCR candidates. Gene deletion mutants for two such genes (*GPR6* and *GPR7*) have also been generated; however, no obvious phenotype was apparent.

Gpr4 Is Important for Capsule Formation and Mating via the cAMP-PKA Pathway

Among the GPCRs identified, Gpr4 was found to play an important role in capsule production and mating. Gpr4 is a novel GPCR and does not share overall sequence identity with the *S. cerevisiae* Gpr1 receptor but rather has homology with the *A. nidulans* GprH GPCR protein (Han *et al.*, 2004) and the *D. dicoideum* cARl cAMP receptor (Pupillo *et al.*, 1992; Raisley *et al.*, 2004) and thus can be grouped into receptor group iv (Table 2; Figure 1B). Gpr4 contains 840 amino acids and has a large third cytoplasmic loop and C-terminal cytoplasmic tail, similar to Gpr1 of *S. cerevisiae* (Figure 1, B and C). Therefore, we hypothesized that Gpr4 might function as a receptor for cAMP signaling and proceeded to compare the phenotypes of *gpr4* and *gpa1* mutants.

When incubated on DME medium, *C. neoformans* produces a polysaccharide capsule that surrounds and protects the cell. *gpr4* mutants exhibit reduced capsule size, a phenotype similar to that of *gpa1* mutants (Figure 2A). The average relative size of the capsule produced by *gpr4* mutant cells was reduced by 30% compared with the wild-type isogenic strain H99 (Figure 2B). This reduction is statistically significant ($p < 0.01$) based on a Student's *t*-test. In contrast to *gpa1* mutants, melanin production was not altered in *gpr4* mutants (Figure 2C). Thus, Gpr4 could be one of several receptors coupled to Gpa1 that activate the cAMP signaling pathway, or the loss of Gpr4 could result in partial constitutive active of Gpa1 sufficient to drive melanin but not capsule production.

In addition to a defect in capsule production, *gpr4* mutants also exhibit a mating defect (Figure 3). During unilateral mating between *gpr4* mutants and a wild-type strain of opposite mating type, no obvious mating defect was observed. But in a bilateral *gpr4* × *gpr4* cross, a significant mating defect with reduced filamentation was apparent (Figure 3A). To further define whether the mating defect observed in *gpr4* mutants results from defects in cell fusion,
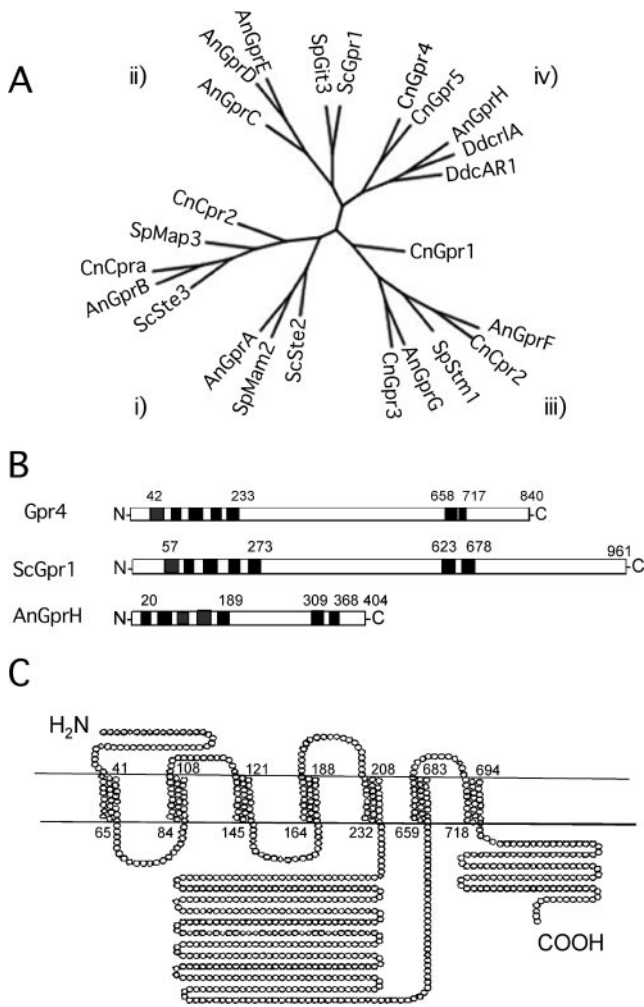
Figure 1. Overall similarity between Gpr4 and other reported GPCRs and the predicted protein structure of Gpr4. (A) Phylogenetic tree of the fungal GPCR family. Classification of fungal GPCRs was carried out by analysis of GPCRs from *C. neoformans* (Cprα, Cpr2, Gpr1–5), *A. nidulans* (GprA–H), *S. cerevisiae* (Ste2, Ste3, Gpr1), *S. pombe* (Mam2, Map3, Git3, Stm1), and *D. discoideum* (cAR1, crlA) using Clustal X. The tree diagram was created with TreeView software. (B) The primary protein structures of Gpr4 in *C. neoformans*, Gpr1 in *S. cerevisiae*, and GprH in *A. nidulans*. ■, transmembrane regions (TMs). Numbers indicate the beginning of the first TM, the end of TM-V, the beginning of TM-VI, and the end of TM-VII, as well as the total number of amino acids in each protein. (C) Proposed two-dimensional model of the transmembrane topology of Gpr4 in the plasma membrane. ○, amino acid residues. Amino acid residue numbers around the predicted seven TMs are indicated.

mating filamentation, or both, cell fusion assays were performed. *gpr4* mutants were less efficient in cell fusion (25.4%), similar to *gpa1* mutants (15.4%), indicating that the *gpr4* deletion results in a modest defect in cell fusion (Figure 3B). When the wild-type *GPR4* gene was reintroduced, both mutant phenotypes were complemented and wild-type levels of capsule production and mating were restored. The addition of 1 mM cAMP to DME medium restored capsule production in the *gpa1* and *gpr4* mutant strains, providing further evidence that both Gpr4 and Gpa1 act via the cAMP-PKA pathway (Figure 2). The mating defects of both *gpa1* and *gpr4* mutants were also suppressed by 1 mM cAMP in

V8 agar medium, further implicating Gpr4 in a functional role in the cAMP-PKA pathway (Figure 3).

Gpr4 Functions Upstream of the Gα Protein Gpa1

To further corroborate a functional relationship between Gpr4 and Gpa1, *gpr4 gpa1* double mutants were generated. A *MAT***a** *gpr4* mutant was crossed with a *MAT*α *gpa1* mutant. Single basidiospores were isolated and *gpr4 gpa1* double mutants were identified by double dominant marker (Nat[r] and Neo[r]) selection. Analysis of capsule formation, melanin production, and mating of *gpr4 gpa1* double mutants supports the hypothesis that Gpr4 functions upstream of Gpa1. *gpr4 gpa1* mutants exhibited identical phenotypes with *gpa1* mutants, consistent with models in which Gpr4 functions upstream of Gpa1 (Figure 2A). However, because *gpa1* mutant phenotypes are more severe than those of *gpr4* mutants, the possibility that Gpr4 and Gpa1 signal in different pathways could not be excluded.

To address this caveat, additional epistasis tests were performed. In *S. cerevisiae*, the $GPA2^{Q300L}$ mutation produces a dominant active form of Gpa2 (Sprang, 1997; Harashima and Heitman, 2002). The corresponding Q284L point mutation was introduced into *C. neoformans* Gpa1 using overlap PCR. Wild type, *gpa1,* and *gpr4* mutant *ura5* auxotrophic strains were transformed with this dominant active *GPA1* allele. Expression of the $GPA1^{Q284L}$ allele in the wild-type strain produced enlarged capsules in capsule induction conditions, indicating that this dominant active form of Gpa1 is functional. Expression of the $GPA1^{Q284L}$ dominant active allele suppressed both the *gpa1* and the *gpr4* mutant defects, restoring both capsule production (Figure 2A) and mating (Figure 3A) to a level similar to the wild-type strain H99 expressing $GPA1^{Q284L}$ and supporting models in which the Gpr4 receptor functions upstream of the Gα subunit Gpa1.

Gpr4 Forms Homodimers and Interacts with Gpa1

To assess the potential for physical interactions between Gpr4 and Gpa1, conventional yeast two-hybrid interaction assays were performed with Gpa1 and Gpr4. Because Gpr4 is a membrane-bound protein, we made several truncated Gpr4 constructs by using gene fragments encoding portions of the receptor located in the cytosol, including the third cytoplasmic loop ($GPR4^{233-658}$ and $GPR4^{384-658}$) and the C-terminal cytoplasmic tail ($GPR4^{696-840}$, $GPR4^{718-840}$, $GPR4^{754-840}$, and $GPR4^{781-840}$). No interaction was observed between any of these fragments and Gpa1 (or Gpa2 or Gpa3). However, we did find that the 3rd cytoplasmic loop of Gpr4 could interact with itself or with the C-terminal tail (Figure 4A), indicating that the Gpr4 receptor may form an oligomeric structure, similar to reports with other GPCRs (Overton *et al.,* 2003; Bai, 2004; Park *et al.,* 2004; Ladds *et al.,* 2005).

To further explore the potential for interactions between Gpr4 and Gpa1, the split-ubiquitin system was used. The split-ubiquitin system has been developed to assess interactions between membrane proteins (Johnsson and Varshavsky, 1994; Stagljar *et al.,* 1998; Iyer *et al.,* 2005). In this system, interactions between membrane-bound proteins can be detected by the release of an artificial transcription factor consisting of protein A, LexA, and VP16, which then activates nuclear reporter genes (*HIS3, ADE2,* and *LACZ*) resulting in expression of His3, Ade2, and β-galactosidase.

Three versions of Gpr4 were tested in the split-ubiquitin system. A *Cub::GPR4* fusion plasmid was generated by fusing the C-terminal half of ubiquitin (Cub) to the N-terminus of full-length *GPR4* cDNA, which resulted in an 8TM structural fusion protein based on topology prediction programs TMpred (Ikeda *et al.,* 2002) and HMMTOP (Tusnady and
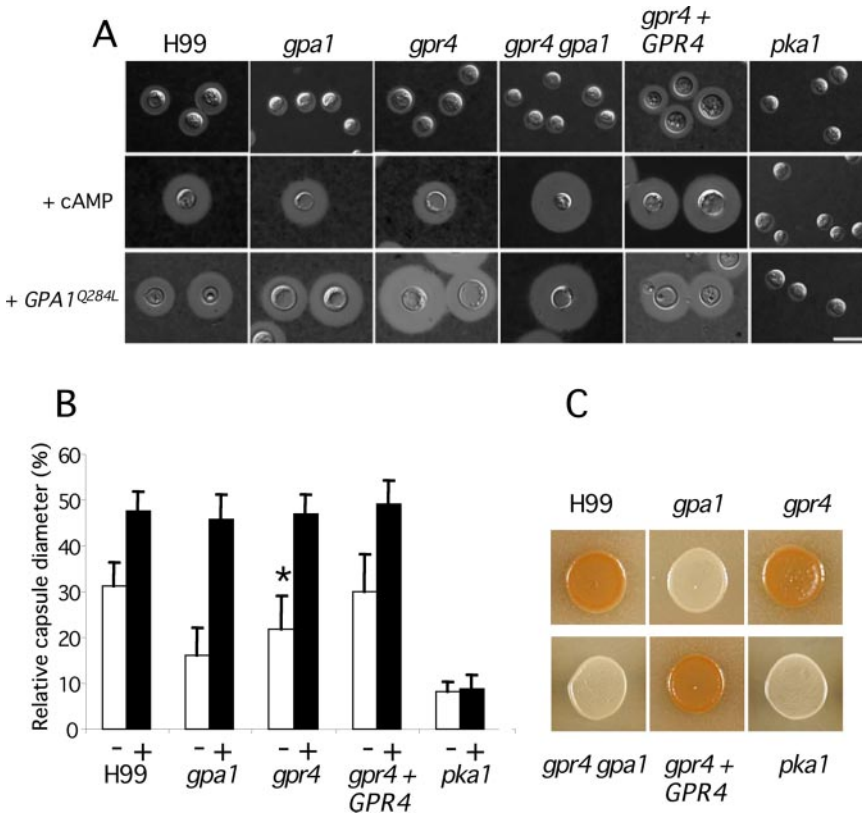
Figure 2. Gpr4 is important for capsule formation but not melanin production. (A) Capsule production was visualized by India ink staining of wild-type strain H99, *gpa1* (YSB83), *gpr4* (CDX6), *gpr4 gpa1* (CDX20), and *pka1* (JKH7) mutant strains, and the *gpr4* + *GPR4* complemented strain (CDX46) after growth on DME medium for 3 d at 37°C (top row). Capsule production by the same series of yeast strains was examined in the presence of 10 mM cAMP (middle row) or expressing the *GPA1* dominant active allele (*GPA1*^{Q284L}; bottom row) grown under the same conditions. Bar, 10 μm. (B) Quantitative measurements of the relative capsule diameter under standard conditions without cAMP (□) or with 10 mM cAMP (■). A total of 50 cells were measured for each strain, and error bars indicate the SD of the mean. *t*-test was performed for the statistical significance of the relative capsule sizes between H99 and *gpr4* mutants (* $p < 0.01$). (C) Melanin produced by the wild-type strain H99, *gpa1* (YSB83), *gpr4* (CDX6), *gpa1 gpr4* (CDX20), and *pka1* (JKH7) mutant strains, and the *gpr4* + *GPR4* complemented strain (CDX46) was photographed after cells were grown on Niger-seed agar for 36 h at 37°C.

Simon, 2001; Figure 4B). A *GPR4::Cub* plasmid was constructed by fusing Cub to the C-terminus of full-length *GPR4* cDNA. A *Cub::GPR4*$^{73–840}$ plasmid was generated by fusing Cub to the N-terminus of Gpr4 lacking the first TM domain sequence to ensure the N-terminus of the fusion protein is in the cytosol (Figure 4B). All these fusion alleles were able to interact with protein expressed from the control construct pAI-Alg5 (expresses the native N-terminal half of ubiquitin)
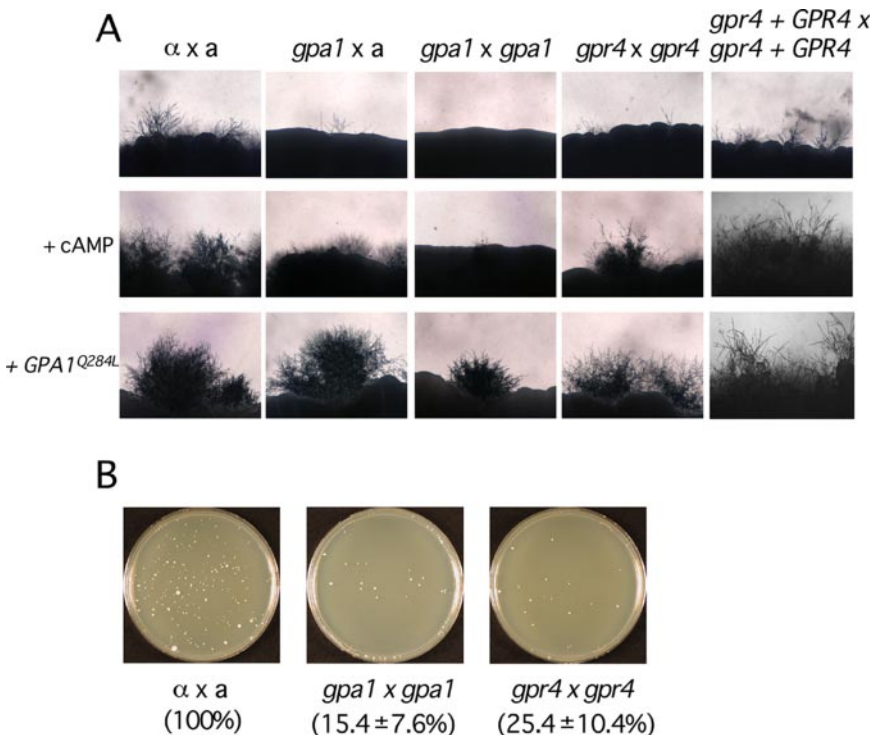

Figure 3. *gpr4* mutants exhibit a bilateral mating defect. (A) The following strains were cocultured on V8 agar medium (pH 5.0) for 6 d in the dark at room temperature: Top row: H99 × KN99**a**, α *gpa1* (YSB83) × KN99**a**, α *gpa1* (YSB83) × **a** *gpa1* (YSB85), α *gpr4* (CDX6) × **a** *gpr4* (CDX9), and α *gpr4* + *GPR4* (CDX46) × **a** *gpr4* + *GPR4* (CDX45). The same series of yeast strains was also cocultured under the same conditions in the presence of 1 mM cAMP (middle row) or expressing the *GPA1* dominant active allele (*GPA1*^{Q284L}) in α cells (bottom row). (B) Cell fusion assays were performed with wild-type strains α (YSB119) × **a** (YSB121), α *gpa1* (YSB83) × **a** *gpa1* (YSB85), and α *gpr4* (CDX6) × **a** *gpr4* (CDX9). In each experiment, the percentage of cell fusion products relative to the α × **a** mixture (100%) was calculated by averaging results from duplicate plates from three independent experiments and calculating the SD of the mean.
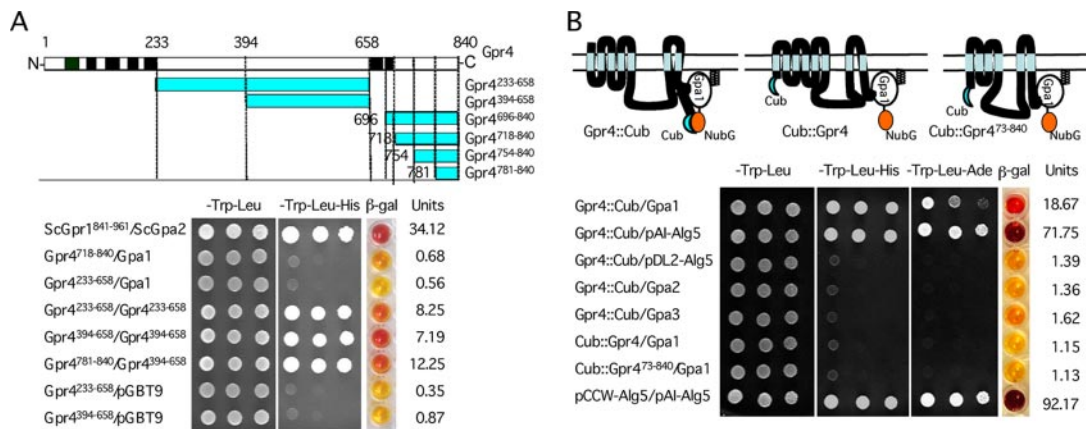
Figure 4. The GPCR Gpr4 interacts with itself and the Gα protein Gpa1. (A) Dimerization of Gpr4. Yeast two-hybrid interaction assays were performed using portions of the Gpr4 protein, including the 3rd cytoplasmic loop (Gpr4$^{233-658}$ and Gpr4$^{394-658}$), and C-terminal cytoplasmic tail (Gpr4$^{696-840}$, Gpr4$^{718-840}$, Gpr4$^{754-840}$, and Gpr4$^{781-840}$) as baits, and full-length Gpa1, Gpr4$^{233-658}$, and Gpr4$^{394-658}$ as preys. An interaction between the C-terminal 120 amino acids of the *S. cerevisiae* Gpr1 receptor (ScGpr1$^{841-961}$) with ScGpa2 served as a positive control, and Gpr4$^{233-658}$, Gpr4$^{394-658}$ with the empty vector pGBT9 served as negative controls. Yeast transformants were grown on selective medium lacking histidine as serial dilutions (1:1, 1:10, and 1:100). β-galactosidase activity assays (see *Materials and Methods*) were performed to further verify the interactions. The portions of Gpr4 analyzed are indicated schematically in blue, and the TMs of Gpr4 are marked in black. (B) Physical interactions between Gpr4 and Gpa1 in the split-ubiquitin system. The C-terminal half of ubiquitin (Cub) was fused to the N-terminus (Cub::Gpr4) or C-terminus (Gpr4::Cub) of the full-length Gpr4 cDNA or to the N-terminus of Gpr4 without TM-I (Cub::Gpr4$^{73-840}$). The N-terminal half of ubiquitin (NubG) was fused to the C-terminus of full-length Gpa1 (Gpa1::NubG), Gpa2 (Gpa2::NubG), and Gpa3 (Gpa3::NubG). Gpr4::Cub interaction with the control vector pAI-Alg5 served as a control to ensure the correct topology of the Gpr4:Cub fusion protein, Gpr4::Cub interaction with the empty vector pDL2-Alg5 served as a negative control, and the pCCW-Alg5 interaction with pAI-Alg5 served as a positive control for the assay. Yeast transformants contained both a Cub fusion and a NubG fusion construct and were grown on selective medium lacking histidine or adenine after serial dilution (1:1, 1:10, and 1:100). β-galactosidase activity assays were performed to further verify the interaction. A schematic of interactions between Gpr4::Cub, Cub::Gpr4, or Cub::Gpr4$^{73-840}$ and Gpa1 are presented at the top of B.

and grew on selective medium, indicating that the Cub fusion proteins were localized inside the cell and ubiquitin was successfully reconstituted. None of these fusion alleles showed interaction with the negative control pDL2-Alg5, indicating that they were not self-activated. G protein constructs were generated by fusing full-length Gpa1, Gpa2, and Gpa3 with the mutated N-terminal half of ubiquitin (NubG), respectively. Transformants coexpressing Gpr4::Cub and Gpa1::NubG grew on medium lacking histidine or adenine and produced robust β-galactosidase enzyme activity, indicating that Gpr4 interacts with Gpa1 directly. Gpr4 did not interact with Gpa2 or Gpa3 in this assay (Figure 4B).

Gpr4 Is Membrane Localized and Rapidly Internalized in Rich Medium

A *GFP::GPR4* fusion allele was expressed in *S. cerevisiae* and found to target GFP to the plasma membrane. The *GFP::GPR4* expression plasmid was transformed into the *S. cerevisiae gpr1* deletion strain MLY232. By direct fluorescence microscopy, cells containing the *GFP::GPR4* expression plasmid exhibited a robust GFP signal localized exclusively on the cell membrane, whereas cells containing the control GFP vector had fluorescence throughout the cell, indicating that the *GFP::GPR4* fusion construct is expressed and that Gpr4 localizes to the plasma membrane (Figure 5A).

A *GPR4::DsRED* construct was also generated and expressed in *C. neoformans*. When this expression allele was transformed into a *gpr4* mutant strain, robust red fluorescence was observed on the plasma membrane of cells grown in minimal medium without amino acids (YNB). When cells were shifted to grow in YPD or YPgly medium, within 30 min the majority of cells exhibited fluorescence in the cytosol, especially concentrated in the vacuole, which was

visualized with the vacuole membrane marker dye MDY-64 (Figure 5, B and C). These results suggest that the Gpr4::DsRed receptor fusion is properly expressed and localized to the cell membrane and that YP medium contains components that can serve as ligands for Gpr4 and trigger its internalization, which is one key step in GPCR trafficking and recycling. We also tested the possibility of the involvement of glucose in Gpr4 internalization by adding 2% glucose to cells grown in YNgly medium, and no clear fluorescence localization change was observed. Together with the result that Gpr4 is localized to the plasma membrane when grown in YNB medium containing 2% glucose, we conclude that glucose is not an important factor for Gpr4 trafficking.

Amino Acids But Not Glucose May Function as Ligands for the Gpr4 Receptor

In *S. cerevisiae*, Gpr1 senses glucose and sucrose and activates cAMP signaling (Lemaire *et al.*, 2004). To identify potential Gpr4 ligands, we first measured cAMP production in response to glucose added to starved cells. As expected, neither *gpa1* nor adenylyl cyclase (*cac1*) mutants responded to glucose re-addition (Figure 6). But surprisingly, these cAMP assays consistently showed that *gpr4* mutants still respond to glucose, although there may be a very modest reduction compared with wild type (Figure 6). This result indicates that Gpr4 is not a major glucose sensor, and other receptors may be involved in glucose sensing and Gpa1 activation. The possibility that other carbon sources may function as potential agonists for Gpr4 and activate downstream G protein signaling was also examined. cAMP assays showed that neither H99 nor *gpr4* mutants responded to sucrose, indicating sucrose cannot activate cAMP signaling in *C. neoformans* (Supplementary Figure 1), and thus differ-
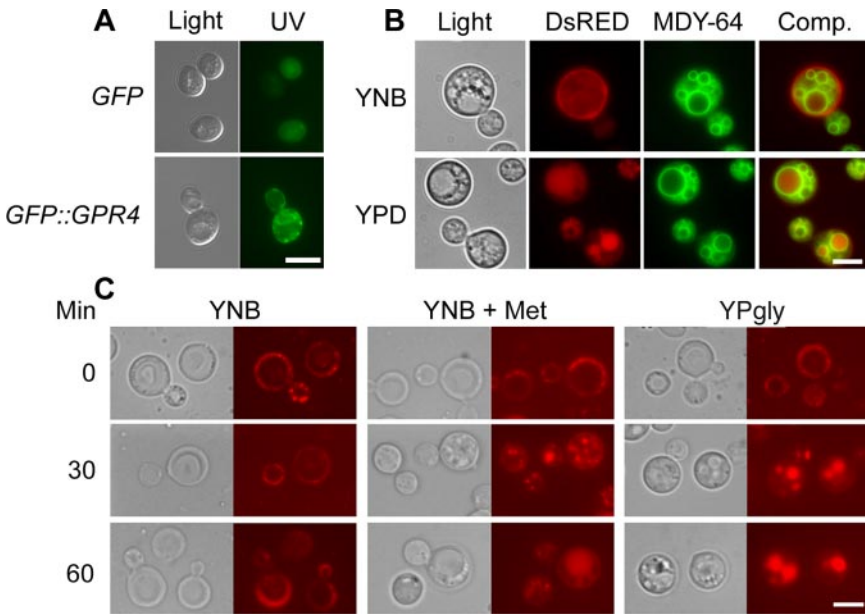
Figure 5. Gpr4 is membrane localized and rapidly internalized in response to rich medium or methionine. (A) A *GFP::GPR4* fusion construct was expressed in the *S. cerevisiae gpr1* mutant strain MLY232. Control transformants (*pADH1-GFP*) expressed GFP alone. (B) A *GPR4::DsRED* fusion construct was expressed in the *C. neoformans gpr4* mutant strain (CDX70), and cells were grown in minimal medium (YNB, 2% glucose, no amino acid) or rich medium YPD. Cells were stained with the vacuole membrane marker dye MDY-64 and the fluorescent signals were monitored by direct fluorescence microscopy. Red channel fluorescence, green channel fluorescence, and their superimposition, as well as the corresponding light microscopy images are presented. (C) Gpr4 internalization was induced by methionine or YPgly medium (glycerol as carbon source). Cells were grown in 5 ml YNB and incubated overnight at 30°C. Cells were collected and resuspended into 2 ml fresh YNB medium and incubated for 2 h. Cells were then collected and resuspended in YNB, YNB plus 10 mM methionine, or YPgly medium. Gpr4 localization was examined by direct fluorescence microscopy at 0-, 30-, and 60-min time points. Left panel, DIC images; right panel, red fluorescence images. Bars, (A–C) 5 μm.

ent mechanisms may be involved in cAMP pathway activation in *S. cerevisiae* and *C. neoformans*. Taking advantage of the interaction between Gpr4 and Gpa1 in the split-ubiquitin system, we cultured this yeast strain (Gpr4::Cub with Gpa1::NubG) on media with different carbon sources and measured β-galactosidase activity to quantify the interaction. If any of these carbon sources were to activate Gpr4, the degree of the interaction between Gpr4 and Gpa1 might be reduced. We failed to detect significant differences in the interactions between Gpr4 and Gpa1 in response to glucose, galactose, mannose, fructose, or maltose (unpublished data). We concluded that Gpr4 is not a major receptor for carbon sources.

Recently, several reports indicated that in *C. albicans*, Ras proteins but not Gpr1 are critical for glucose sensing (Leberer *et al.*, 2001; Maidan *et al.*, 2005a). However, Gpr1 signaling can b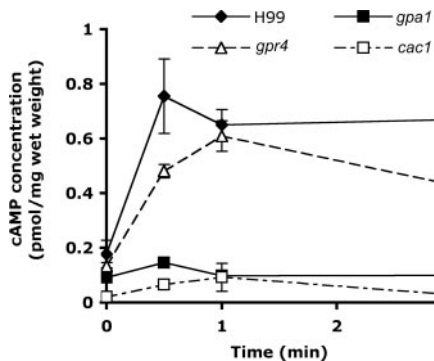e activated by amino acids such as methionine and alanine (Maidan *et al.*, 2005a, 2005b). To address the hypothesis that Gpr4 may also function as an amino acid sensor, cAMP assays were performed by adding different amino acids and measuring the production of cAMP. Fifteen amino acids were divided into three groups and amino acid mixtures for these three groups of amino acids were generated. Group A contained isoleucine, valine, histidine, and leucine; group B contained lysine, methionine, phenylalanine, tryptophan, alanine, and tyrosine; and group C contained serine, glutamic acid, arginine, threonine, and aspartate. Only group B resulted in an altered cAMP level between the isogenic *GPR4* wild-type strain H99 and a *gpr4* mutant (Figure 7A). Several amino acids in this group were tested individually for affects on cAMP production, and methionine was found to induce cAMP accumulation in H99 but not the *gpr4* mutant in our study (Figure 7B). cAMP production was induced in both strains by tryptophan (unpublished data). However, neither wild-type nor the *gpr4* mutant responded to alanine (Figure 7B), which differs from Gpr1 activation in *C. albicans* (Maidan *et al.*, 2005a). Taking advantage of the interaction between Gpr4 and Gpa1 in the split-ubiquitin system, we also cultured this yeast strain (Gpr4::Cub with Gpa1::NubG) on media with or without methionine and measured β-galactosidase activity to quantify the interaction. We observed an approximately twofold reduction of β-galactosidase activity for the samples grown on medium with methionine (average 15.5 U/ml) compared with that grown on medium without methionine (average, 30.3 U/ml), furthering support our hypothesis that Gpr4 is involved in methionine sensing.

Because the cAMP accumulation pattern showed some modest differences in responses to glucose induction between the *gpr4* mutant and wild type, we hypothesized that cAMP pathway activation might be affected by both glucose and amino acids through Gpr4. cAMP assays were performed using a glucose and methionine mixture as the inducer, and cAMP accumulation in the wild-type strain H99 attained a higher level (average 1.64-fold increase after 30 s of induction) than either glucose or methionine alone, which
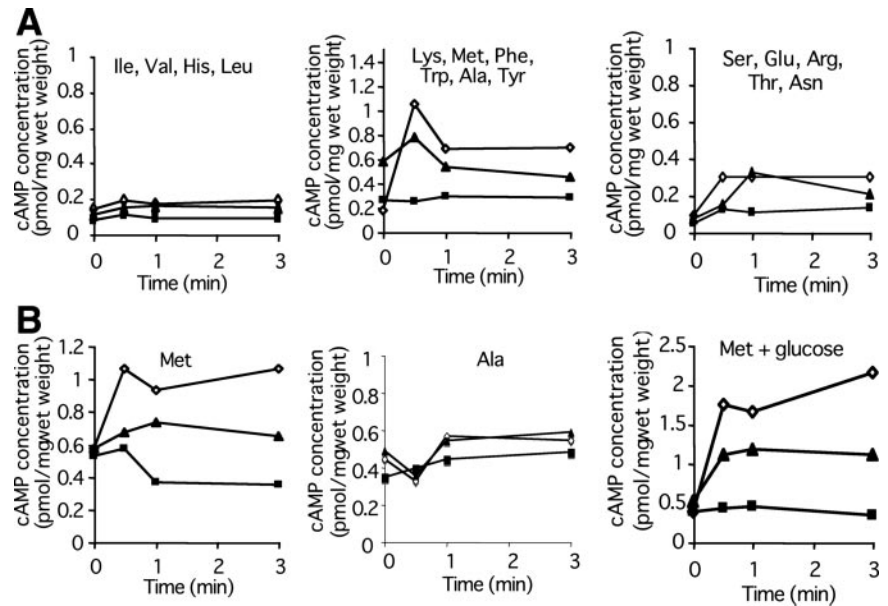

Figure 6. Gpa1 but not Gpr4 is required for cAMP production in response to glucose. The wild-type strain H99, and *gpa1* (RSB83), *gpr4* (CDX6), and *cac1* (YSB42) mutants were starved for glucose for 2 h. One milliliter of cell suspension for each strain was extracted and cAMP levels were measured at the indicated time points after glucose re-adding. Each data point and error bar indicates the SD of the mean for three independent experiments.

Figure 7. Gpr4 is required for cAMP induction in response to methionine. (A) Fifteen amino acids were divided into three groups (groups A–C) and each mixture was added to amino acid starved cells of H99 (◇), the *gpr4* mutant strain CDX6 (▲), or the *gpa1* mutant strain YSB83 (□) and cAMP levels were assayed. (B) Methionine, alanine, and methionine + glucose were tested individually by re-adding each to starved cells and conducting cAMP assays. All data presented is representative of three independent experiments.

is statistically significant in a Student's *t* test ($p < 0.05$), indicating that *C. neoformans* can sense both glucose and methionine to activate the cAMP-PKA pathway (Figure 7B).

Gpr4 Is Rapidly Internalized in Response to Amino Acids

GPCR internalization is one key step in GPCR trafficking, which is a fundamental biological process and a conserved mechanism leading to GPCR signaling desensitization. Briefly, when ligands bind to a GPCR receptor, the GPCR changes conformation and can be phosphorylated by GPCR kinases, releasing its interactions with G proteins and allowing internalization. We can visualize this internalization process by fusing a GPCR to a fluorescent tag such as GFP or DsRed and verify the ligand of a specific GPCR by testing the ability of the compound to trigger the internalization of the corresponding GPCR. To further elucidate the potential biological role of amino acids on Gpr4 activation, we tested whether Gpr4 trafficking would respond to different amino acids. Red fluorescence can be observed on the plasma membrane when a strain expressing the Gpr4::DsRED fusion protein (strain CDX75) is grown in minimal medium lacking amino acids. Within 30 min after addition of 10 mM methionine, red fluorescence was observed in endocytic vesicles or vacuoles, indicating that the Gpr4 protein has been internalized (Figure 5C). We also tested several other amino acids, including alanine, but no clear fluorescence internalization was observed. These results further confirm that methionine could function as a ligand for the Gpr4 receptor.

Methionine Stimulates Filamentation during Mating through Gpr4

We used defined filamentation agar medium (amino acid free) to further investigate the biological role of methionine on mating. Without any amino acid, mating crosses between wild-type strains produced robust filament growth after 6-d incubation. *gpr4* mutants produced less and shorter filaments, indicating a mating defect. By adding 20 μM methionine, a significant stimulation of filamentous growth could be observed from the mixture of wild-type strains, but not from the mixture of *gpr4* or *gpa1* mutants, indicating that neither *gpr4* nor *gpa1* mutants respond to methionine (Figure

8). We also test additional concentrations (50 μM, 100 μM, 1 mM, and 10 mM) and observed an inhibition effect on filamentation during mating when the methionine concentration was higher than 1 mM. This result further confirms that methionine plays a role in mating via the Gpr4, Gpa1-activated cAMP pathway in *C. neoformans* in a dose-dependent manner.

Gpr4 Is Not Essential for Virulence

The involvement of Gpr4 in capsule production and cAMP signaling prompted us to investigate its possible role in virulence using a murine inhalation model of systemic *C. neoformans* infection (Figure 9). Female A/Jcr mice were intranasally inoculated with 10^5 yeast cells, and animals were monitored twice daily. As previously demonstrated, all mice infected with the wild-type strain H99 survived between 18 and 26 d after infection, whereas mice infected with the attenuated *gpa1* mutant survived for more than 60 d (Alspaugh *et al.*, 1997). Surprisingly, mice infected with the *gpr4* mutant exhibited mortality similar to those infected with the wild-type H99 strain, indicating that Gpr4 is not critical for virulence of *C. neoformans*. Because the GPCR Gpr5 shares sequence identity with Gpr4, we generated a *gpr4 gpr5* double mutant strain and its virulence was also evaluated. No significant mortality difference was observed
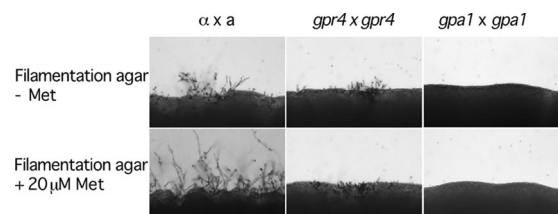

Figure 8. Gpr4 is required for methionine-induced mating stimulation. The following strains were cocultured on filamentation agar medium for 7 d in the dark at room temperature without (top row) or with 20 μM methionine (bottom row): H99 × KN99**a**, α *gpr4* (CDX6) × **a** *gpr4* (CDX9) and α *gpa1* (YSB83) × **a** *gpa1* (YSB85).
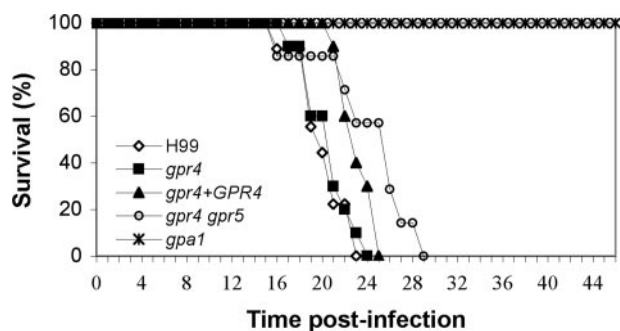
Figure 9. The GPCR Gpr4 is not essential for virulence. Female A/Jcr mice were intranasally inoculated with 10^5 cells of the following strains: H99, *gpa1* mutant (YSB83), *gpr4* mutant (CDX6), *gpr4 gpr5* mutant (CDX18), and *gpr4* + *GPR4* complemented strain (CDX46). Animals were monitored for clinical signs of cryptococcal infection and sacrificed at predetermined clinical end points that predict imminent mortality.

compared with H99 and the *gpr4* mutant, indicating that Gpr4 and Gpr5 are not functionally redundant (Figure 9).

DISCUSSION

The cAMP pathway is well conserved from mammalian cells to microbes such as fungi. In *C. neoformans*, the cAMP pathway plays a central role in pathogenicity. In this pathway, the Gα protein Gpa1 and the cyclase associated protein Aca1 activate adenylyl cyclase (Cac1) and stimulate the production of cAMP, which binds to the regulatory subunit of PKA (Pkr1), and releases the PKA catalytic subunits (Pka1/2). Although many components in this pathway have been characterized in several model fungal systems, little is known as to how most fungi sense extracellular signals to activate cAMP signaling. The Gpr1 receptor in *S. cerevisiae* is the best characterized nutrient-sensing GPCR system. Gpr1 senses glucose and sucrose and functions as the receptor for the cAMP pathway (Xue *et al.*, 1998; Yun *et al.*, 1998; Lemaire *et al.*, 2004). In this study, we investigated the GPCR Gpr4 as a potential receptor for cAMP signaling in *C. neoformans*. This protein drew our attention because it has structural similarity with Gpr1 in *S. cerevisiae*, and it also shares sequence identity with the cAMP receptor in *D. discoideum*. Gpr4 has a very long cytoplasmic loop (encompassing more than half of the total protein) between TM-V and TM-VI, but unlike the large 3rd cytoplasmic loop in the *S. cerevisiae* Gpr1 receptor, which contains a long asparagine residue (N) repeat, no conserved sequence was identified.

The *gpr4* null mutant phenotypes are related to cAMP signaling and involve both capsule production and mating. But Gpr4 does not play a role in regulating melanin production, which is another important virulence factor controlled by the Gpa1-cAMP pathway. Epistasis analysis supports a model in which Gpr4 functions upstream of Gpa1, but Gpr4 may not be the only receptor coupled to Gpa1 (see model in Figure 10). cAMP assays after glucose induction revealed Gpr4 is not a major glucose sensor, which is functionally distinct from *S. cerevisiae* Gpr1, but similar to the Gpr1 receptor of *C. albicans*. That Gpr4 is not required for glucose sensing may further explain why *gpr4* mutants produce wild-type levels of melanin because melanin induction is known to be triggered by low glucose levels. Other receptors may function to control melanization and act as glucose sensors because Gpa1 responds to glucose induction in a
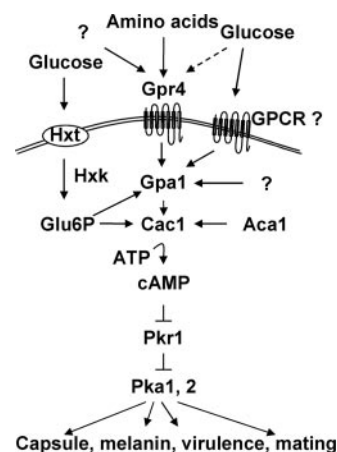

Figure 10. Proposed model for the Gpr4, Gpa1-activated cAMP signaling pathway in *C. neoformans*. Two signal inputs to the cAMP signaling pathway are proposed in this model and involve nutrient transport and sensing. Fermentable carbon sources, such as glucose, are transported into the cell via hexose transporters and phosphorylated by hexose kinases to produce glucose-6-phosphate (Glu6P), which in turn regulates Cac1 or Gpa1 activation to engage cAMP signaling. Glucose may also be sensed by an unknown receptor to activate the cAMP pathway. Gpr4 may also play a role in low glucose concentration sensing based on the cAMP assay results. In this model, Gpr4 is an amino acid sensor and interacts with Gpa1 to regulate downstream elements of the cAMP pathway. This model does not exclude that other proteins such as methionine permease may participate in ligand sensing via direct or indirect interactions with Gpr4. Hxt, hexose transporters; Hxk, hexose kinases; Glu6P, glucose-6-phosphate.

cAMP signaling-dependent manner. In addition to Gpr4, we also identified additional proteins having seven TMs, a feature of GPCRs. Gene deletion mutants were generated for seven of these putative GPCRs, but none appears to be involved in melanin or capsule production. Among them, Gpr5 has both sequence identity and structural similarity with Gpr4. However, *gpr4 gpr5* double mutants produced phenotypes similar to that of *gpr4* single mutations, indicating that Gpr4 and Gpr5 are not functionally redundant (unpublished data).

Direct interactions between Gpr4 and Gpa1 provide further evidence that Gpr4 is a receptor upstream of Gpa1 and acts through cAMP signaling. The interactions between Gpr4 fragments indicate that Gpr4 may form dimeric or oligomeric structures, a property shared with other GPCR receptors. The importance of receptor oligomerization has only recently become apparent and is now a focus of study for GPCR activation (George *et al.*, 2002; Bai, 2004; Ladds *et al.*, 2005). One reason we did not detect an interaction between Gpr4 and Gpa1 using the conventional yeast two-hybrid assay could be that we did not use the proper fragment for interaction or those portions isolated from Gpr4 are not sufficient to mediate physical interactions with Gpa1. In contrast, the split-ubiquitin system is designed to monitor interactions between membrane proteins. With the split-ubiquitin system, we observed a physical interaction between Gpa1 and the Gpr4::Cub fusion construct, in which the Cub domain was fused to the C-terminus of Gpr4. This result supports our model that Gpr4 is a receptor of Gpa1-activated cAMP signaling. It also suggests that this interaction does not require the free carboxyl terminal tail, which is different to GCR1-Gpa1 interaction studies in *Arabidopsis*, which required the free C-tail of GCR1 (Pandey and Ass-

mann, 2004). The failure to observe any interaction between Cub::Gpr4 or Cub::Gpr4[73–840] (N-terminal Cub fusions) and Gpa1 could indicate that the N-terminus is important for Gpr4 activation and other functions. Alternatively, the two halves of ubiquitin (Cub and NubG) might not be suitably oriented to activate the ubiquitin protease, which would result in a failure to release the artificial transcription factor (LexA-VP16).

Our study demonstrates that Gpr4 and Gpa1 play different roles in glucose sensing. Gpr4 is not essential for glucose sensing because a cAMP increase in response to glucose induction is still observed in the *gpr4* mutant, even though its cAMP induction pattern is modestly different from that of H99. But Gpa1 is essential for glucose-mediated cAMP induction. There are several possibilities for the differing roles of Gpr4 and Gpa1 in glucose sensing. One is that other receptor(s) may exist to sense glucose and trigger cAMP signaling. There is no ScGpr1 homolog in *C. neoformans,* based on genomic sequence comparison. Although Gpr4 is structurally related to the *S. cerevisiae* Gpr1 receptor, no significant sequence identity was found between the two. Therefore, if there is a glucose receptor in *C. neoformans,* it may be very divergent from *S. cerevisiae* Gpr1, or multiple proteins might share glucose sensing functions. A second possibility is that *C. neoformans* and some other fungi may use a different mechanism to sense glucose, such as using glucose-6-phosphate to trigger Gpa1 activity instead of sensing extracellular glucose via receptors (see model in Figure 10). In *S. cerevisiae,* glucose-6-phosphate is produced in the cell by phosphorylation of glucose after its import by hexose transporters and is involved in promoting the cAMP pathway through adenylyl cyclase although the precise mechanism is as yet unclear (Rolland *et al.,* 2002). Similar machinery may exist in *C. neoformans* because hexose transporters and hexose kinases homologues can also be found in the genome. To test this possibility in *C. neoformans,* generation of hexose transporter or hexose kinase mutants would be one approach. Caution will be required as functional redundancy may exist because both gene families have multiple members. A third model is that loss of Gpr4 may lead to partial constitutive activation of Gpa1. This may result in sufficient cAMP signaling to support melanin production but not mating or capsule production. In this model, the amplitude of pathway signaling (off, low, high) could enable at least three distinct biological readouts from a single GPCR-Gα module rather than a simple binary switch with only off and on states.

The nature of the ligands for Gpr4 is a central question. In *C. albicans,* recent reports reveal that Cdc25 and Ras but not Gpr1 are responsible for glucose sensing, and instead Gpr1 may sense amino acids (Maidan *et al.,* 2005a). In *C. neoformans,* Ras proteins are unlikely to play a role in glucose sensing because Ras1 has been reported to act independently from the cAMP signaling pathway (Alspaugh *et al.,* 2000; Bahn *et al.,* 2004). Amino acid rich media are known to induce morphological transitions in *C. albicans.* Van Dijck and colleagues further discovered that methionine is required for hyphal induction and both Gpr1 and Mup1 (a high-affinity methionine permease) are responsible for methionine induced filamentation in *C. albicans.* Deletion of *GPR1* causes marked defects in true hypha formation and invasive growth, whereas the defect of the *mup1* mutant strain in morphological transitions is in a methionine concentration-dependent manner. Their study revealed Gpr1 may sense methionine (Maidan *et al.,* 2005a, 2005b). Methionine is also important for sexual development in the fission yeast *S. pombe* via a cAMP-dependent Ste11-signaling path-

way (Schweingruber *et al.,* 1998). In *C. neoformans,* we also observed that methionine can stimulate filamentation during mating filaments in a Gpr4, Gpa1-dependent manner, indicating that methionine is also important for sexual development in *C. neoformans* and could be one extracellular signal involved in Gpr4, Gpa1-activated cAMP signaling.

To test the possibility that Gpr4 may also function as a nutrient sensor, we tested cAMP production in response to amino acid induction and found that Gpr4 is important for sensing methionine. This conclusion is further supported by Gpr4 internalization assays, wherein Gpr4 is also rapidly internalized in response to high concentrations of methionine, indicating that amino acids can activate the Gpr4, Gpa1-activated cAMP pathway. Interestingly, we observed an additive increase in cAMP production in response to both glucose and methionine, indicating that cAMP signaling may be activated by multiple extracellular signals.

We also measured the expression of two glucose regulated genes, *CAS8* (glucose inducible gene) and *LAC1* (glucose reducible gene), using wild-type, *gpr4,* and *gpa1* mutant strains in the presence and absence of either glucose and methionine. Expression of *CAS8* was modestly induced by glucose both in the wild-type strain and the *gpr4* mutant strain, consistent with a previous report (Pukkila-Worley *et al.,* 2005). The wild type and the *gpr4* mutant shared similar expression patterns indicating that Gpr4 is not involved in glucose sensing, and methionine is not important for *CAS8* gene expression. No significant difference in expression was observed in the *gpa1* mutant strain under these conditions. The expression of the *LAC1* gene was significantly repressed by the glucose in wild-type and the *gpr4* mutant strain, similar to the previous report (Pukkila-Worley *et al.,* 2005). Surprisingly, we also observed a significant difference in *LAC1* gene expression in the *gpa1* mutant strain with or without glucose, indicating glucose signaling may in part bypass the Gpa1 protein to repress downstream target genes. These results also suggest that Gpr4 is not important for glucose sensing or glucose regulated gene expression, consistent with our findings in this study (see Supplementary Figure 2).

Gpr4 is not essential for virulence and H99 and the *gpr4* mutant strain were indistinguishable in our virulence study. Virulence is controlled by several attributes, including melanin production, capsule formation, and growth at body temperature. The *gpr4* mutant strain produces reduced capsule size but has normal melanin production compared with H99, and no growth defect at 37°C. The virulence test results indicate that reduction of capsule size may not be sufficient to affect virulence if enough capsule is present to provide protection against the host immune response and secrete normal amounts of glucuronoxylomannan (GXM). As an important virulence factor, the presence of a capsule is required for optimal pathogenicity in *C. neoformans,* but its size is not necessarily essential to produce clinical disease. In nonisogenic strains there is no direct correlation between the size of the capsule and the virulence of the strain (Dykstra *et al.,* 1977), so the relative size of the capsule does not ensure pathogenicity.

Overall, our study identified a novel GPCR, which is a receptor for the cAMP pathway that can sense amino acids such as methionine. Our study also indicates that *C. neoformans* may have a more complicated signal sensing system than *S. cerevisiae* and could involve multiple sensors for the cAMP signal pathway. To fully understand the extracellular signals and their sensors that activate the cAMP pathway, additional receptor proteins may need to be identified, es-

pecially those involved in glucose sensing and that may potentially affect the production of melanin.

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