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Adenylyl Cyclase Functions Downstream of the Gα Protein Gpa1 and Controls Mating and Pathogenicity of *Cryptococcus neoformans*

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The signaling molecule cyclic AMP (cAMP) is a ubiquitous second messenger that enables cells to detect and respond to extracellular signals. cAMP is generated by the enzyme adenylyl cyclase, which is activated or inhibited by the Gα subunits of heterotrimeric G proteins in response to ligand-activated G-protein-coupled receptors. Here we identified the unique gene (*CAC1*) encoding adenylyl cyclase in the opportunistic fungal pathogen *Cryptococcus neoformans*. The *CAC1* gene was disrupted by transformation and homologous recombination. In stark contrast to the situation for *Saccharomyces cerevisiae*, in which adenylyl cyclase is essential, *C. neoformans cac1* mutant strains were viable and had no vegetative growth defect. Furthermore, *cac1* mutants maintained the yeast-like morphology of wild-type cells, in contrast to the constitutively filamentous phenotype found upon the loss of adenylyl cyclase in another basidiomycete pathogen, *Ustilago maydis*. Like *C. neoformans* mutants lacking the Gα protein Gpa1, *cac1* mutants were mating defective and failed to produce two inducible virulence factors: capsule and melanin. As a consequence, *cac1* mutant strains were avirulent in animal models of cryptococcal meningitis. Reintroduction of the wild-type *CAC1* gene or the addition of exogenous cAMP suppressed *cac1* mutant phenotypes. Moreover, the overexpression of adenylyl cyclase restored mating and virulence factor production in *gpa1* mutant strains. Physiological studies revealed that the Gα protein Gpa1 and adenylyl cyclase controlled cAMP production in response to glucose, and no cAMP was detectable in extracts from *cac1* or *gpa1* mutant strains. These findings provide direct evidence that Gpa1 and adenylyl cyclase function in a conserved signal transduction pathway controlling cAMP production, hyphal differentiation, and virulence of this human fungal pathogen.

The conversion of intracellular ATP to cyclic AMP (cAMP), catalyzed by adenylyl cyclase, is a central reaction in eukaryotic signal transduction. The control of cAMP concentration is principally determined by the precise regulation of adenylyl cyclase activity (54). Adenylyl cyclases are either activated or inhibited by interactions with Gα or βγ subunits liberated from heterotrimeric G proteins in response to ligand-activated G-protein-coupled receptors. The molecular basis of G protein activation of adenylyl cyclase was recently determined by X-ray crystallographic analysis of activated Gα subunits with the catalytic domains of the enzyme (55). Here we present studies that illustrate how the regulation of adenylyl cyclase by Gα proteins has been conserved between microorganisms and mammals.

Although the basic catalytic functions of adenylyl cyclases are shared in fungi, the mechanisms of activation and the downstream targets of these enzymes differ among divergent fungal species. In the budding yeast *Saccharomyces cerevisiae*, the single gene encoding adenylyl cyclase, *CYR1*, is essential (40, 57). Two partially redundant Ras proteins, Ras1 and Ras2, and the Gα protein Gpa2 activate adenylyl cyclase in response to nutrient conditions (10, 29, 37, 58) and intracellular acidification (10). The target of cAMP, cAMP-dependent protein kinase (PKA), plays central roles in filamentation, sporulation, and stress survival (6, 42, 47).

In contrast to the situation in budding yeast, adenylyl cyclase is not essential in the fission yeast *Schizosaccharomyces pombe* (39). Mutants lacking adenylyl cyclase (*cyr1*$^-$) exhibit precocious mating that is no longer repressed by nutrients, as in wild-type cells (39). The Gα protein Gpa2, and not Ras1, plays a central role in regulating adenylyl cyclase in *S. pombe* (17).

Pathogenic fungi have coopted these conserved signal transduction pathways to regulate their virulence (4, 5, 35). In the corn smut fungus *Ustilago maydis*, the morphological transitions involved in mating and pathogenicity are dependent upon cAMP signaling. For example, disruption of the adenylyl cyclase gene *uac1* results in constitutive filamentation (19). The *U. maydis* Gα protein Gpa3 activates adenylyl cyclase in response to specific nutritional signals and is required for pathogenesis (21, 27, 46).

Cryptococcus neoformans is an opportunistic human fungal pathogen and an excellent model system for the genetic and

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TABLE 1. Strains used

Serotype	Strain	Genotype	Reference or source
A	H99	*MAT*α	43
	H99-ura5	*MAT*α *ura5*	This study
	M001	*MAT*α *ade2*	44
	AAC1	*MAT*α *ade2 gpa1*::*ADE2*	3
	AAC3	*MAT*α *ade2 gpa1*::*ADE2 GPA1-hph*	3
	AAC17	*MAT*α *ade2 gpa1*::*ADE2 CAC1-hph*	This study
	RPC3	*MAT*α *ura5 cac1*::*URA5*	This study
	RPC7	*MAT*α *ura5 cac1*::*URA5 CAC1-hph*	This study
	LCC22	*MAT*α *ade2 cac1*::*ADE2*	This study
	LCC23	*MAT*α *ade2 cac1*::*ADE2*	This study
	LCC22-1	*MAT*α *ade2 cac1*::*ADE2 CAC1-hph*	This study
D	JEC20	*MAT***a**	31

molecular dissection of microbial pathogenicity (2). Recently, it was demonstrated that the *C. neoformans* Gα protein Gpa1 regulates mating and the expression of the inducible virulence factors melanin and capsule. As a consequence, *gpa1* mutant strains are attenuated for virulence in animal models of cryptococcal meningitis (3, 15; B. M. Allen, J. G. Kimbrough, J. Heitman, and J. A. Alspaugh, unpublished data). Because exogenous cAMP restores mating and virulence factor production in *gpa1* mutant cells, it was proposed that Gpa1 might regulate adenylyl cyclase. Interestingly, Gpa1 belongs to a conserved subgroup of fungal Gα proteins that primarily respond to nutrient deprivation signals (35). We also note that the central role that this conserved Gα protein-cAMP signaling pathway plays in pathogenesis could not have been predicted from studies of model organisms such as budding and fission yeasts.

To investigate the direct role of cAMP signaling in fungal differentiation and microbial pathogenesis, we cloned and disrupted the *C. neoformans CAC1* gene, which encodes adenylyl cyclase. Strikingly, *cac1* mutants were viable, lacked any detectable cAMP, and maintained the budding growth phenotype of wild-type cells. These findings indicate that adenylyl cyclase and cAMP are dispensable for viability and do not play a major role in determining morphology in this pathogenic basidiomycete under conditions that promote growth as a budding yeast. Mutant strains lacking adenylyl cyclase were sterile, failed to induce capsule or produce melanin, and were avirulent in animal models. Exogenous cAMP or reintroduction of the wild-type gene restored mating and virulence factor production. Genetic epistasis tests support a model in which the Gα protein Gpa1 regulates adenylyl cyclase. Taken together with recent studies on the role of PKA in *C. neoformans* (15), these findings reveal that the central features of Gα protein regulation of adenylyl cyclase are conserved between unicellular and multicellular eukaryotes. The similarities and differences among cAMP signal transduction pathways in divergent fungi demonstrate how a conserved nutrient-sensing signaling pathway that controls differentiation in nonpathogenic yeasts has been coopted for the control of virulence in pathogenic fungi.

MATERIALS AND METHODS

Strains and media. The strains used are listed in Table 1. Except for strain JEC20, a serotype D *MAT***a** strain used in all of the mating experiments (31), the *C. neoformans* strains used in these experiments were derived from the serotype A wild-type strain H99 (43). H99-ura5 is a spontaneous 5-fluoroorotic acid-resistant derivative of H99 that was isolated on 5-fluoroorotic acid-containing medium by using the method of Kwon-Chung et al. (33). Strain AAC1 is a serotype A *gpa1* mutant strain, and strain AAC3 is a *gpa1 GPA1*-reconstituted strain (3). RPC3, LCC2, and LCC23 are *cac1* mutant strains described in this study. RPC7 and LCC22-1 are *cac1 CAC1*-reconstituted strains derived from strains RPC3 and LCC22, respectively. Strain AAC17 is a *gpa1* mutant strain in which the *CAC1* gene was ectopically integrated in multiple copies.

Standard yeast media were used for most experiments as described previously (51). Niger seed agar (30), Dulbecco's modified Eagle's medium (DMEM) with 22 mM $NaHCO_3$ (20), and V8 mating medium (30) were prepared as previously described. When needed, cAMP was added at 2.5 to 5 mM.

PCR. All PCRs were performed by use of a Perkin-Elmer GeneAmp 9600 thermocycler with 50 ng of template DNA, 100 ng of each oligonucleotide primer, and standard reagents from a TaKaRa kit (Takara Shuzo Co.). For initial identification of the adenylyl cyclase gene in *C. neoformans*, primers were designed based on conserved regions of fungal adenylyl cyclases: primer DF2, 5′-AGTIAAGACIGARGGIGAYATG, and primer DF5, 5′-AYTGICCICCRT CIGC (I, inosine; R, purine; Y, pyrimidine). Genomic DNA from strain H99 was used as a template for the PCRs. The PCR conditions were 35 cycles at 94°C for 30 s, 35°C for 30 s, and 72°C for 30 s. The resulting 316-bp PCR fragment was TA cloned (Invitrogen) and sequenced.

Southern hybridization and cloning of the *CAC1* gene. Genomic DNA was isolated from strain H99 as described previously (45). Restriction digestion, gel electrophoresis, DNA transfer, prehybridization, hybridization, and autoradiography were performed as described previously (49) with the initial 316-bp *CAC1* PCR fragment as the probe. The probe was labeled by using a Random Primed DNA labeling kit (Boehringer Mannheim) and ^{32}P-dCTP (Amersham).

Based on the Southern hybridization data, subgenomic libraries of *Nhe*I-digested genomic fragments were cloned into pBluescript, and the clones containing the *CAC1* gene fragments were identified by colony hybridization.

Identification of 5′ and 3′ regions of the *CAC1* gene. Strain H99 was incubated at 30°C for 18 h in yeast extract, peptone, dextrose (YPD) medium. Aliquots were subcultured for 4 h at 30 and 37°C in YPD medium and in DMEM–22 mM $NaHCO_3$. The cells were pelleted, and total RNA was isolated by using an RNeasy Mini kit (Qiagen). The RNA samples were pooled, and the polyadenylated RNA fraction was purified by using an Oligotex mRNA Midi kit (Qiagen). This sample was used as the template for cDNA production and subsequent PCR amplification of the 5′ and 3′ ends of the *CAC1* gene message by using a Marathon cDNA amplification kit (Clontech).

Disruption of the *CAC1* gene. To create a *cac1*::*URA5* disruption construct, we subcloned a 6.7-kb *Sac*I/*Xho*I fragment of the *CAC1* locus (extending from 523 bp before the start codon to 6,234 bp after the start codon) into plasmid pUC18. The *URA5* gene was inserted as a selectable marker into the *Bam*HI-digested *CAC1* fragment, resulting in the loss of 1,652 nucleotides internal to the *CAC1* open reading frame (ORF). The *cac1*Δ::*URA5* linear fragment was precipitated onto 0.6 μg of gold microcarrier beads (Bio-Rad) and biolistically transformed into strain H99-ura5 as previously described (44). Stable transformants were selected on synthetic medium lacking uracil and containing 1 M sorbitol.

To screen for *cac1* mutant strains, genomic DNA from each transformant was isolated and used as the template for PCR amplification with the *CAC1*-specific primer 3273 (5′-CCAACATCTCTCAACGTGACG) and the *URA5*-specific primer 5151 (5′-CCTCTTCTTCATCTAGTCGG). Because the recognition sequence of primer 3273 lies outside the disruption construct, only strains in which the *cac1*::*URA5* disruption allele was integrated at the endogenous *CAC1* locus amplified a 2-kb PCR band in this reaction. One strain (RPC3) of 120 transformants screened in this manner was found by PCR to have a *cac1*Δ::*URA5* mutation. Southern hybridization was performed by using genomic DNA digested with *Pst*I and the 3.7-kb *Xba*I/*Kpn*I fragment of the *CAC1* gene (corresponding to nucleotides 1153 to 4900 of the *CAC1* GenBank sequence) as the probe. We observed that the wild-type bands at 2.2 and 1.7 kb were missing and that only the expected band at 4.1 kb was present in the samples of strain RPC3, confirming that the *CAC1* locus was replaced by the *cac1*Δ::*URA5* allele, with no ectopic integrations.

Two independent *cac1* mutant strains were made by using the *ADE2* gene as the selectable marker for gene disruption. Both of these strains were made by using the serotype A *ade2* strain M001 as the recipient for transformation (44, 53). Strain LCC22 was created by using a *cac1*Δ::*ADE2* disruption construct with the *ADE2* gene cloned into a *Stu*I site at nucleotide position 5176 of the published *CAC1* sequence. The majority of the *CAC1* open reading frame was replaced by the *ADE2* gene in strain LCC23 by using a *cac1*Δ::*ADE2* construct with the *ADE2* gene inserted into the *Xba*I-digested *CAC1* gene, resulting in the loss of 6,590 nucleotides of *CAC1* sequence.

The *cac1 CAC1*- and *gpa1 CAC1*-reconstituted strains were created by biolistically transforming the wild-type *CAC1* gene, linked to the *hph* gene conferring resistance to hygromycin B, into the *cac1* mutant strains RPC3 and LCC22 and the *gpa1* mutant strain AAC1 as previously described (3, 13).

Northern analysis. Strains were incubated in YPD medium at 30°C for 18 h. Cells were pelleted and divided equally for incubation for 4 h in synthetic complete medium with 2% glucose, synthetic complete medium with 0% glucose, or synthetic low-ammonium–dextrose (SLAD) medium (18). Cells were pelleted at 4°C and frozen on dry ice, and total RNA was isolated as previously described (3). Fifteen micrograms of RNA was analyzed for each sample. Gel electrophoresis, RNA transfer, hybridization, and autoradiography were performed as described previously (49).

Mating assays. All strains to be tested for mating were initially grown in YPD medium for 48 h at 30°C. Mating reactions were performed by coincubating cells of opposite mating types on V8 mating medium in the dark at room temperature for 1 to 2 weeks. Mating mixtures were analyzed for filamentation, and photomicroscopy was performed on representative sectors of the mating mixtures.

Capsule assessment by packed-cell-volume measurement. Packed-cell volume was assessed as previously described with modifications (20). Strains were incubated in DMEM–22 mM $NaHCO_3$ at 30°C for 24 h, treated with 10% formalin, and normalized to 10^9 cells/ml. The normalized samples were added to heparinized Microhematocrit capillary tubes (Fisher 02-668-66) and spun for 5 min in a model MB Microhematocrit centrifuge (International Equipment Co.). Packed-cell volume, or cryptocrit, was measured as the length of the packed-cell phase divided by the length of the total suspension within the capillary tube.

Determination of the intracellular cAMP concentration. Cells were preincubated at 30°C for 18 h in YPD medium. The overnight culture was inoculated into fresh YPD medium to an optical density at 600 nm of 0.05 and grown under the same conditions for 20 h. Cells were collected by centrifugation and washed twice with water and once with buffer (10 mM morpholineethanesulfonic acid [MES] [pH 6.0], 0.1 mM EDTA). Cells were resuspended in buffer and incubated at 30°C with shaking so that they would be subjected to glucose starvation. After 2 h, glucose was added to a final concentration of 2%. At various time points, 0.5 ml of cell suspension was transferred to a tube containing an equal volume of ice-cold 10% trichloroacetic acid and 0.3 ml of glass beads and was immediately frozen in liquid nitrogen. Crude cell extracts were prepared by homogenization with a bead beater at 4°C and were lyophilized. cAMP assays were performed by using a cAMP enzyme immunoassay kit (Amersham) as previously described (38).

Virulence experiments. In the murine inhalation model of systemic cryptococcosis, A/Jcr mice were intranasally inoculated with 5×10^5 cells as previously described (12). Groups of 10 mice were infected with each strain, and animals were observed twice daily. Symptoms due to the experimental infection included lethargy, ruffled fur, and inability to maintain daily care. In this model, mice develop meningitis and resulting hydrocephalus due to *C. neoformans*, mimicking the natural history of infection in humans. Moribund mice were sacrificed prior to death. The Kruskal-Wallis algorithm was used to determine the statistical significance of differences in survival.

In the rabbit model, New Zealand White rabbits were sedated with ketamine (Fort Dodge) and xylazine (Vedco) and intrathecally inoculated with the *CAC1* wild-type strain (H99), the *gpa1* mutant strain (AAC1), and the *cac1* mutant strain (LCC22) as previously described (3). Three rabbits were infected with each strain and were treated daily with 1.2 mg of betamethasone sodium-betamethasone acetate (Schering). Cerebrospinal fluid (CSF) was obtained by cisternal puncture after sedation on experimental days 4, 7, and 11 after infection, and the total CFU per milliliter of CSF was determined by quantitative culturing on YPD medium. All virulence studies were performed in compliance with institutional guidelines for animal experimentation.

Nucleotide sequence accession number. The CAC1 has been assigned GenBank accession no. AF290191.

RESULTS

Identification and disruption of the *C. neoformans* adenylyl cyclase gene. In previous studies, it was found that mutants lacking the Gα protein Gpa1 were viable and exhibited cAMP-remediable phenotypes (3; Allen et al., unpublished). Here we tested the hypothesis that Gpa1 regulates cAMP production by adenylyl cyclase. Because adenylyl cyclase is essential in *S. cerevisiae*, we wished to distinguish between two alternative models. In the first model, adenylyl cyclase is essential and Gpa1 is not essential because redundant upstream factors regulate adenylyl cyclase in *gpa1* mutants. In the second model, neither Gpa1 nor adenylyl cyclase is essential for viability in *C. neoformans*.

We identified a fragment of the gene encoding *C. neoformans* adenylyl cyclase, *CAC1* (*Cryptococcus* adenylyl cyclase), from serotype A strain H99 by using low-stringency PCR and degenerate primers based on conserved regions of other fungal adenylyl cyclase genes. Southern hybridization, under high or low stringency, revealed a single copy of the *CAC1* gene. Two adjacent *Nhe*I restriction fragments, spanning the entire *CAC1* open reading frame, were isolated from the genomic DNA of strain H99 by colony hybridization of size-selected libraries and then sequenced (GenBank accession number AF290191). The 5′ and 3′ regions of the *CAC1* gene were determined by rapid amplification of cDNA ends, and the intron-exon boundaries were identified by comparing *CAC1* cDNA fragments and the genomic sequence.

The *CAC1* gene consists of 7,188 nucleotides from the start to the termination codons, contains 7 introns, and encodes a predicted protein of 2,271 amino acids. The *C. neoformans* adenylyl cyclase shares 62% amino acid sequence similarity and 45% identity from residues 838 to 2228 with the analogous region of its closest homolog, *U. maydis* adenylyl cyclase Uac1. Like other fungal adenylyl cyclase proteins (25, 63, 64), the *C. neoformans* Cac1 enzyme lacks the hydrophobic transmembrane domains characteristic of the mammalian enzymes (28). It does, however, share the tandemly repeated leucine-rich motifs found in the *S. cerevisiae* and *S. pombe* adenylyl cyclase proteins. These regions are predicted to serve regulatory or cell localization functions, since they are not required for catalytic activity (25).

To disrupt the *C. neoformans CAC1* gene, an internal portion of the gene was replaced with the *URA5* gene. The resulting *cac1*Δ::*URA5* disruption construct was introduced into the serotype A *ura5* strain H99-ura5 by biolistic transformation. In 1 isolate (RPC3) from among 120 Ura^+ transformants, the *CAC1* gene was replaced by integration of the *cac1*Δ::*URA5* mutant allele, with no ectopic integrations (see Materials and Methods). Two independent *cac1* mutant strains (LCC22 and LCC23) were also isolated by using the *ADE2* gene as a selectable marker in *ade2* mutant strain M001 (44, 53). The in vitro phenotypes of the three independent *cac1* mutants were identical. It is important that all three mutants exhibited a budding morphology like that of wild-type cells. This observation is in contrast to the situation for another basidiomycete pathogen, *U. maydis*, in which disruption of the gene for adenylyl cyclase results in constitutive filamentation and the loss of budding growth (19).

To ensure that the phenotypes observed were attributable to the *cac1* adenylyl cyclase mutation, the wild-type *CAC1* gene was reintroduced into the *cac1* mutant background. The wild-type *CAC1* gene was linked to the *hph* gene, encoding resistance to hygromycin B, and ectopically integrated into the genome of the *cac1* mutant strains (RPC3 and LCC22) by biolistic transformation to generate *cac1 CAC1*-reconstituted strains. Three reconstituted strains in the RPC3 strain background and nine reconstituted strains in the LCC22 strain background demonstrated identical phenotypes in vitro. Therefore, one *cac1 CAC1* strain from each mutant back-

ground was selected for a more detailed evaluation (RPC7 and LCC22-1). This transformation technique has been used to integrate *C. neoformans* genes and typically results in the integration of multiple tandem copies of the linked *hph* gene, which are apparently necessary for sufficient expression to confer hygromycin B resistance (3). We note that as a consequence, the reintroduced linked *CAC1* gene is also present in multiple copies, often resulting in functional overexpression, which can be used for epistasis analysis. We have used this approach to achieve overexpression, since the serotype D *GAL7*-regulatable promoter is not tightly regulated or highly expressed in serotype A strains (14). Additionally, the pCnTel1 (16) and pPM8 (41) episomal plasmids, which have been used for overexpression studies with serotype D strains, are often unstable and integrated in serotype A strains (unpublished observations). To observe the effects of *CAC1* overexpression in the *gpa1* mutant background, the *CAC1* gene was similarly introduced into a *gpa1* mutant strain (AAC1). The in vitro phenotypes of four such *gpa1 CAC1* strains were identical, and one (AAC17) was chosen for further studies. Northern analysis confirmed that the *CAC1* gene was not expressed in the *cac1* mutant strain but was markedly overexpressed in the *gpa1 CAC1* and *cac1 CAC1* strains, albeit to different extents, independent of medium conditions (Fig. 1A). In contrast to the important role of nutrient deprivation in the transcriptional regulation of the *GPA1* gene (59), expression of the *CAC1* gene was not induced by either glucose or nitrogen limitation (Fig. 1A).

The Gα protein Gpa1 and adenylyl cyclase control intracellular cAMP production. The hypothesis that mutation of the *CAC1* gene affects cAMP production in *C. neoformans* was tested by assaying intracellular cAMP levels (Fig. 1B). The *CAC1* wild-type (H99), *cac1*Δ::*ADE2* mutant (LCC22), *cac1 CAC1*-reconstituted (LCC22-1), *gpa1* mutant (AAC1), *gpa1 GPA1*-reconstituted (AAC3), and *gpa1 CAC1* (AAC17) strains were grown overnight in YPD medium and then starved in glucose-free buffer for 2 h. Following the readdition of glucose, cells were collected and frozen, and cAMP levels were determined by an enzyme immunoassay. The cAMP concentration was modestly increased in wild-type cells in response to glucose readdition, as in previous studies with *S. cerevisiae* (56) (Fig. 1B). No cAMP was detectable in total cell extracts from either the *cac1* or the *gpa1* mutant strains under any conditions (Fig. 1B). When the wild-type *GPA1* gene was reintroduced into the *gpa1* mutant strain, the basal cAMP level was modestly increased compared to that in wild-type cells, a result which may be attributable to partial activation of the pathway by the increased level of the Gpa1 protein. cAMP production in response to glucose readdition was also restored in the *gpa1 GPA1* strain (Fig. 1B). In comparison, both the basal cAMP level and the glucose-induced cAMP increase were significantly enhanced in the *cac1 CAC1* strain, in which adenylyl cyclase is overexpressed. Importantly, overexpression of adenylyl cyclase in the *gpa1* mutant strain restored basal cAMP production but not glucose-stimulated cAMP synthesis. These findings support a model in which a receptor coupled to Gpa1 detects extracellular glucose and activated Gpa1 then stimulates cAMP production by adenylyl cyclase.

C. neoformans* adenylyl cyclase is required for efficient mating.** The *cac1* mutants lacking adenylyl cyclase showed significantly reduced mating compared to isogenic wild-type strains. The *CAC1* wild-type (H99), *cac1* mutant (RPC3), *cac1 CAC1*-reconstituted (RPC7), *gpa1* mutant (AAC1), and *gpa1 CAC1* (AAC17) strains were incubated in mating reactions with the serotype D *MATa** strain JEC20 on V8 mating medium. The mating reaction mixtures containing the wild-type and *cac1 CAC1*-reconstituted strains produced extensive mating hyphae after 7 days of incubation. In contrast, the *cac1* and *gpa1* mutant strains produced no mating hyphae after 7 days (Fig. 2). As previously described, isolated foci of mating hyphae were observed with the *gpa1* mutant strain following prolonged incubation (≥14 days); however, no significant mating was observed with the *cac1* mutant strain. The addition of 2.5 mM cAMP to V8 mating medium restored the mating of both *gpa1* and *cac1* mutant strains (Fig. 2). Similarly, overexpression of adenylyl cyclase restored the mating of the *gpa1* mutant strain. These findings further support a model in which the Gα protein Gpa1 regulates cAMP production by the Cac1 fungal adenylyl cyclase.

cAMP restores melanin production in adenylyl cyclase mutant strains. We next tested whether adenylyl cyclase is required for melanin production. The *CAC1* wild-type (H99), *cac1* mutant (RPC3), *cac1 CAC1*-reconstituted (PRC7), *gpa1* mutant (AAC1), and *gpa1 CAC1* (AAC17) strains were incubated on Niger seed medium with and without cAMP. The wild-type and *cac1 CAC1*-reconstituted strains produced similar amounts of melanin after 3 to 4 days of incubation at 37°C, whereas the *cac1* and *gpa1* mutant strains made little or no visible melanin, even after 7 days of incubation (Fig. 3). Exogenous cAMP restored melanin production by both *gpa1* and *cac1* mutant strains. Interestingly, overexpression of adenylyl cyclase in the *gpa1 CAC1* strain partially restored melanin production after 72 h of incubation on Niger seed medium, and exogenous cAMP further enhanced melanin production by this strain. These observations demonstrate that adenylyl cyclase is necessary for melanin production and provide additional evidence that Gpa1 normally functions to regulate cAMP production by adenylyl cyclase.

cAMP suppresses the capsule defect of adenylyl cyclase mutant strains. The polysaccharide capsule of *C. neoformans* is induced by conditions that mimic environmental signals encountered by this pathogenic yeast in the infected host, including iron deprivation and physiological CO_2/HCO_3^- levels (20). To assess the role of adenylyl cyclase in *C. neoformans* capsule production, the *CAC1* wild-type (H99), *cac1* mutant (RPC3), *cac1 CAC1*-reconstituted (RPC7), *gpa1* mutant (AAC1), and *gpa1 CAC1* (AAC17) strains were incubated in DMEM–22 mM $NaHCO_3$ for 24 h. India ink analysis revealed a marked decrease in the size of the capsule in the *cac1* and *gpa1* mutant strains compared with the wild-type strain (Fig. 4A). When the *CAC1* gene was introduced into either the *cac1* or the *gpa1* mutant strains, capsule induction was restored (Fig. 4A). Exogenous cAMP also restored capsule production by the *cac1* and *gpa1* mutant strains and increased capsule size in all of the strains tested (Fig. 4A).

To quantify the differences in capsule production of these strains, the packed-cell volume (3, 20), which is related to capsule size, was determined for normalized suspensions of strains incubated in capsule-inducing medium in the absence or presence of cAMP. The quantitative findings indicated a

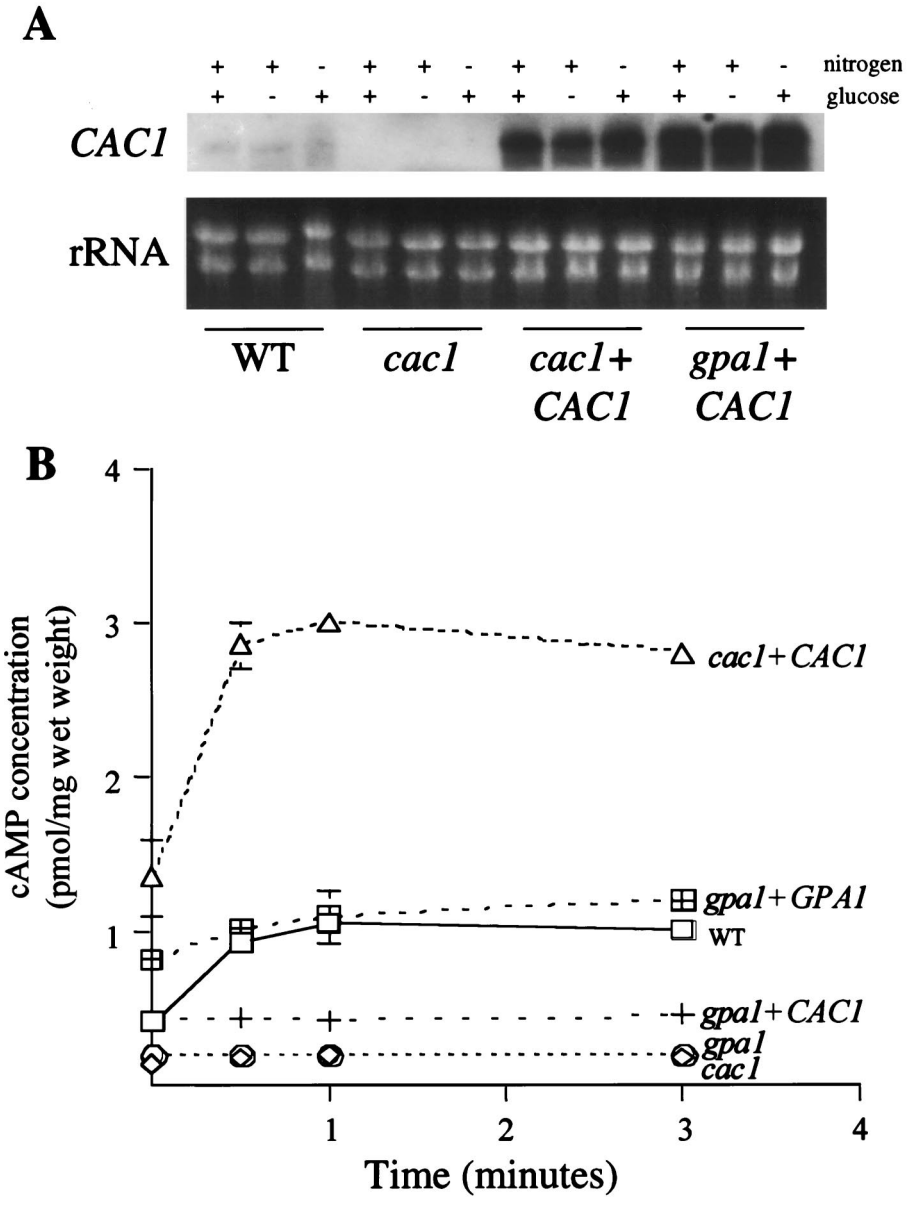

FIG. 1. Disruption of the adenylyl cyclase gene *CAC1* abolishes cAMP production. (A) *CAC1* wild-type (WT) (H99), *cac1* mutant (RPC3), *cac1 CAC1*-reconstituted (RPC7), and *gpa1 CAC1* (AAC17) strains were incubated in YPD medium for 18 h. The cells were divided for incubation for 4 h in one of three different media: synthetic complete medium with 2% glucose (nitrogen +, glucose +), synthetic complete medium with 0% glucose (nitrogen +, glucose −), or SLAD (nitrogen −, glucose +). Total RNA from each sample was assessed by Northern analysis with the *CAC1* gene as a probe. The rRNA bands of the ethidium bromide-stained gel (rRNA) are shown to demonstrate RNA loading. (B) *CAC1* wild-type (H99) (squares), *cac1* mutant (LCC22) (diamonds), *cac1 CAC1*-reconstituted (LCC22-1) (triangles), *gpa1* mutant (AAC1) (circles), *gpa1 GPA1*-reconstituted (AAC3) (crossed squares), and *gpa1 CAC1* (AAC17) (crosses) strains were starved for glucose for 2 h. At the indicated time after a glucose pulse, aliquots of the cell suspensions were frozen, and intracellular cAMP concentrations were determined. Data points represent the mean and standard deviation for duplicate samples in two identical experiments (four samples for each data point).

significant decrease in capsule size in the *cac1* and *gpa1* mutant strains as well as complete restoration of encapsulation in these mutant strains by exogenous cAMP or the *CAC1* adenylyl cyclase gene (Fig. 4B).

Adenylyl cyclase is required for *C. neoformans* virulence. The roles of melanin and the polysaccharide capsule in the pathogenesis of cryptococcal infections are well established. Strains deficient in either melanin or capsule production are dramatically attenuated for virulence in animal models of cryptococcosis (7–9, 32, 48). Moreover, *C. neoformans gpa1* mutant strains are significantly less virulent than wild-type parent strains in both rabbit and murine models of cryptococcal meningitis (3, 15). Based on models in which Gpa1 regulates adenylyl cyclase, we hypothesized that disruption of the *CAC1* gene would similarly impair virulence.

Ten A/Jcr mice were infected by inhalation with the *CAC1*

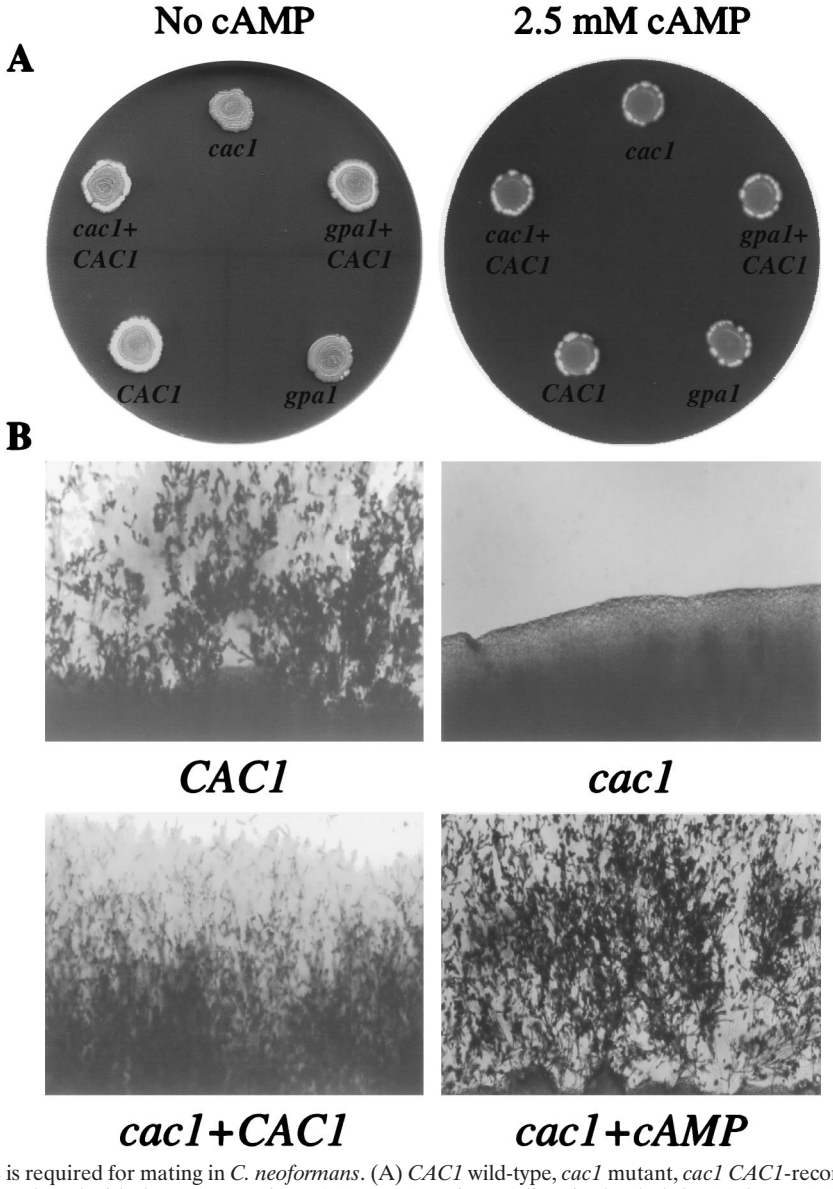

FIG. 2. Adenylyl cyclase is required for mating in *C. neoformans*. (A) *CAC1* wild-type, *cac1* mutant, *cac1 CAC1*-reconstituted, *gpa1* mutant, and *gpa1 CAC1* strains were coincubated with the *MAT***a** strain JEC20 on V8 mating medium in the dark for 14 days at 30°C with and without cAMP (2.5 mM). (B) The edges of the mating mixtures were examined microscopically each day for mating hyphae and photographed after 7 days (×61).

wild-type (H99), *cac1* mutant (RPC3), and *cac1 CAC1*-reconstituted (RPC7) strains. In this model, inhaled cells initially infect the lung and then disseminate hematogenously to infect the brain, resulting in meningoencephalitis in all infected animals. Survival was monitored over the course of a 60-day infection. Mice infected with the *CAC1* wild-type strain survived a median of 20 days (Fig. 5). In contrast, the *cac1* mutant strain was avirulent, and no lethal infection was observed in any infected animal ($P < 0.01$) (Fig. 5). Importantly, reintroduction of the wild-type *CAC1* gene restored the virulence of the *cac1* mutant strain to the wild-type level (median survival, 21 days; $P = 0.076$ in a comparison with the wild-type strain).

Two independent *cac1*Δ::*ADE2* mutant strains were also found to be avirulent. In the murine inhalation model of systemic cryptococcosis, the *cac1*Δ::*ADE2* mutant strains (LCC22 and LCC23) produced no lethal infections after 120 days of observation. Isogenic *CAC1* wild-type strains induced lethal infections in all animals by day 23 in the same experiment. Additionally, in the rabbit model of cryptococcal meningitis, *cac1* mutant strain LCC22 showed a significant reduction in the ability to survive in the host compared to the wild-type strain. The number of viable cells recovered from the CSF of animals infected with *cac1* mutant strain LCC22 was reduced approximately 10,000-fold compared to the results obtained with the wild-type strain after 10 days of infection (1.5×10^5 CFU/ml of CSF for the wild-type strain; 60 CFU/ml of CSF for the *cac1* mutant strain). In summary,

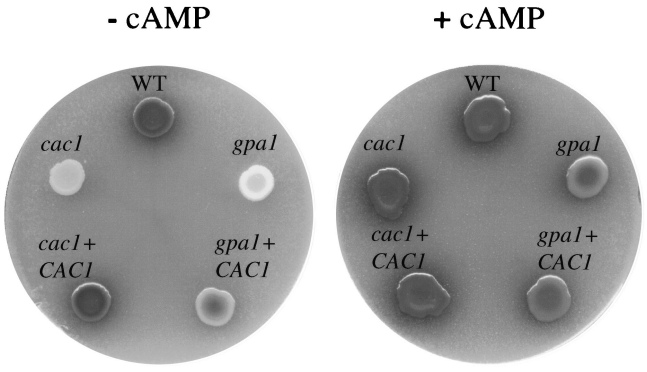

FIG. 3. Adenylyl cyclase mutants have defects in melanin production. *CAC1* wild-type (WT), *cac1* mutant, *cac1 CAC1*-reconstituted, *gpa1* mutant, and *gpa1 CAC1* strains were grown on Niger seed medium with (+) and without (−) cAMP (2.5 mM) at 37°C and photographed after 4 days. Strains that produce melanin are brown (gray on figure), whereas strains that produce less or no melanin are white.

in three separate experiments with three independent *cac1* mutant strains, adenylyl cyclase was found to be required for *C. neoformans* virulence.

DISCUSSION

All cells must sense and respond to changes in the extracellular environment. In pathogenic microorganisms, dramatic cellular adaptation occurs as these organisms infect their host. An understanding of the mechanisms by which these organisms adapt to and infect their host underlies the basis for the molecular dissection of microbial pathogenesis.

Here we demonstrate that the enzyme adenylyl cyclase and its second messenger product, cAMP, play a central role in the differentiation and virulence of the opportunistic fungal pathogen *C. neoformans*. First, we identified a single, nonessential gene encoding adenylyl cyclase in this fungus. Second, we showed that the adenylyl cyclase Cac1 plays a central role in

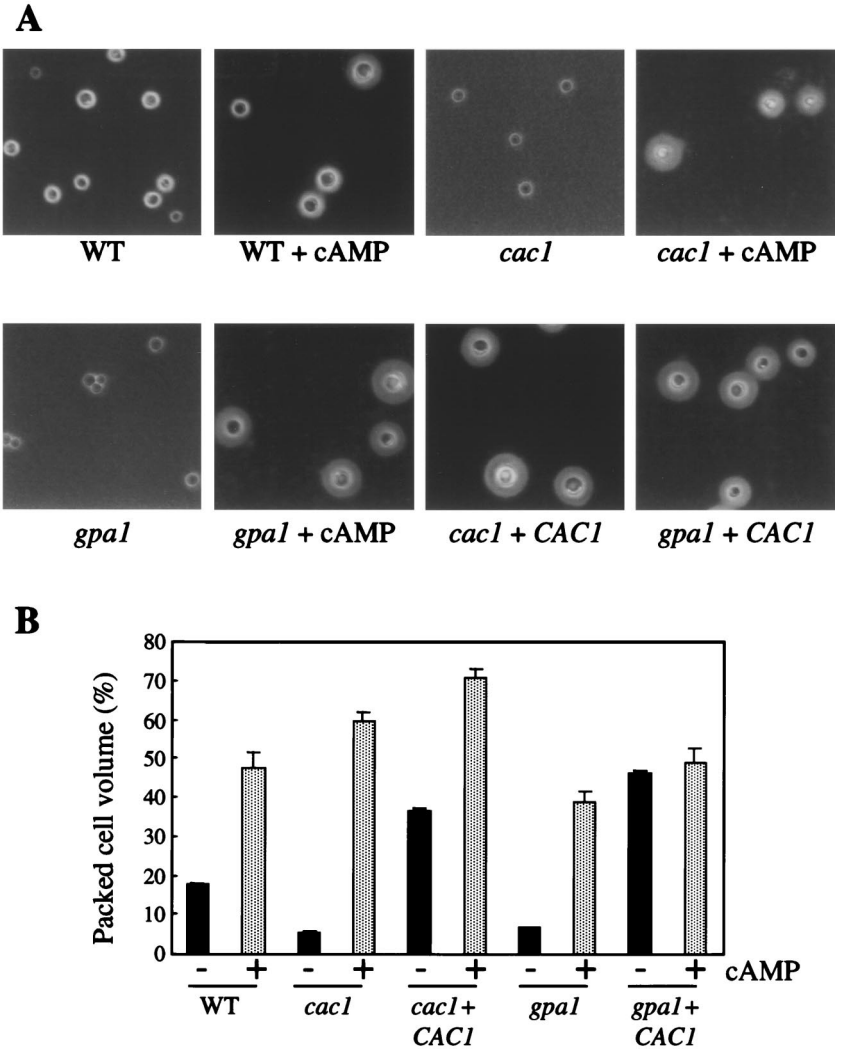

FIG. 4. Adenylyl cyclase mutants fail to induce capsule. (A) *CAC1* wild-type (WT), *cac1* mutant, *cac1 CAC1*-reconstituted, *gpa1* mutant, and *gpa1 CAC1* strains were incubated for 2 days under capsule-inducing conditions (DMEM–22 mM $NaHCO_3$) with and without cAMP (20 mM). Capsule induction was qualitatively assessed with a standard India ink preparation and photographed (×61). (B) Capsule size was quantified by determining the packed-cell volume of normalized cell suspensions (10^9 cells/ml) for each sample. Data points represent the mean and standard error for triplicate samples.

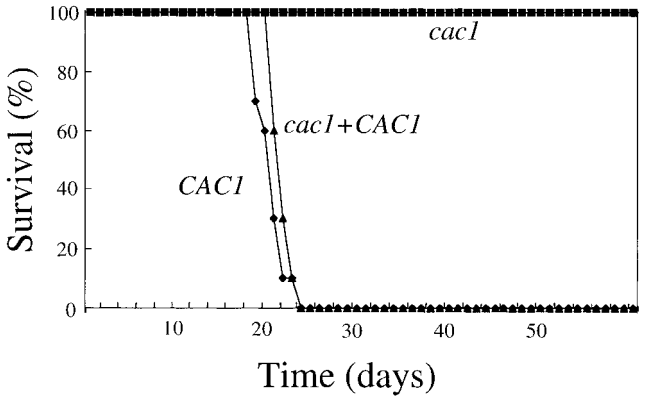

FIG. 5. Adenylyl cyclase is required for virulence of *C. neoformans*. Ten A/Jcr mice were intranasally inoculated with the *CAC1* wild-type strain (diamonds), the *cac1* mutant strain (squares), or the *cac1 CAC1*-reconstituted strain (triangles). The three groups of mice (30 total) were monitored for survival over 60 days.

the induction of two virulence factors, capsule and melanin. Third, we demonstrated that adenylyl cyclase is required for virulence. Mutant phenotypes conferred by the adenylyl cyclase mutation were completely remediated by cAMP in vitro, indicating that the enzyme is catalytic and that it is not required for scaffolding of other signaling components.

Previously it was found that the differentiation and virulence of *C. neoformans* are controlled by the Gα protein Gpa1 (3; Allen et al., unpublished). Because the *gpa1* mutant phenotypes were suppressed by exogenous cAMP, it was proposed that Gpa1 might function by regulating cAMP production. Here we isolated the *CAC1* gene encoding adenylyl cyclase and, by molecular genetic approaches, provide compelling support for this model. Strains lacking the adenylyl cyclase gene were sterile, similar to *gpa1* mutants. Additionally, both the *gpa1* and the *cac1* mutant strains failed to induce the expression of the major virulence determinants capsule and melanin. All mutant phenotypes were suppressed by exogenous cAMP or by overexpression of adenylyl cyclase. These findings support a model in which the Gpa1 and Cac1 proteins function in a linear pathway controlling cAMP production. Our studies and recent findings obtained with budding and fission yeasts underscore how this pathway is conserved between microorganisms and humans.

Determination of intracellular cAMP levels provides additional evidence that the *C. neoformans* Gα protein Gpa1 and adenylyl cyclase functionally interact to regulate cAMP production. The cAMP level increased when glucose was added to glucose-starved *C. neoformans* cells, directly implicating cAMP as a central element in *C. neoformans* nutrient-sensing pathways. Similar responses to glucose have been observed for *S. cerevisiae* (56). In contrast, no cAMP was detectable in the *cac1* mutant strain. That no cAMP was produced in the *gpa1* mutant strain further supports a central role for the Gα protein Gpa1 in cAMP signaling. Interestingly, the basal cAMP level was restored in the *gpa1* mutant strain overexpressing adenylyl cyclase, but glucose failed to stimulate cAMP production. In the same strain background, reintroduction of a wild-type *GPA1* gene complemented the mutant cAMP defect to wild-type levels. Although the interpretation of these results may be limited by the number of strains tested, together these findings suggest that the Gpa1 protein is required to link a glucose-sensing G-protein-coupled receptor to adenylyl cyclase. This receptor has not yet been identified for *C. neoformans*, but the corresponding receptors in budding yeast (Gpr1) and fission yeast (git3) are known (61, 62).

Other fungal G protein subunits in this conserved signal transduction pathway have been identified. The fission yeast Gβ subunit git5 and the Gγ subunit git11 interact with the Gα protein gpa2 and are required for adenylyl cyclase activation by glucose (34). Thus far, only one Gβ subunit (Gpb1) and no Gγ subunits have been identified for *C. neoformans* (60). The Gpb1 protein clearly functions in pheromone sensing and mating and is not coupled to the Gpa1-cAMP cascade (60). Its role is analogous to those of the βγ subunits Ste4 and Ste18 in pheromone sensing in *S. cerevisiae* but is quite distinct from the role of the βγ subunits git5 and git11 that function with gpa2 in nutrient sensing in *S. pombe*. Therefore, in budding yeast and in *C. neoformans*, the nutrient-sensing Gα subunits function in the absence of other G protein subunits or with novel subunits that remain to be identified (reviewed in reference 35).

Mating in the fission yeast *S. pombe* requires a nutrient-poor medium, and the *S. pombe* gpa2-adenylyl cyclase pathway plays a central role in signaling nutrient-rich conditions. cAMP levels are regulated in response to carbon source, although nitrogen source may also play a role (52). Mutation of the *S. pombe cyr1* adenylyl cyclase gene leads to starvation-independent mating on nutrient-rich medium (39). Similarly, *gpa2* mutant cells mate and sporulate in rich medium and fail to produce cAMP in response to glucose (23). Thus, the cAMP pathway in *S. pombe* functions to signal the presence of abundant nutrients, either carbon source or nitrogen source, and mutations in this pathway result in starvation-independent mating (52).

Like fission yeast mating, *C. neoformans* mating can occur on a nutrient-poor medium limiting for nitrogen source but containing abundant fermentable carbon source, such as SLAD medium. However, disruption of cAMP signaling in *C. neoformans* impairs mating. These observations could suggest that the *C. neoformans* Gpa1-Cac1-cAMP pathway is activated by nutrient deprivation signals rather than the presence of abundant nutrients. Alternatively, the Gpa1-Cac1-cAMP pathway might function to sense abundant fermentable carbon sources and thereby stimulate the mating of *C. neoformans*. In this model, the pathway inhibits mating in *S. pombe* and stimulates mating in *C. neoformans*. Nitrogen limitation stimulates mating and meiosis in *S. pombe*, pseudohyphal differentiation in *S. cerevisiae*, and haploid fruiting and mating in *C. neoformans*. These events are also regulated by fermentable carbon sources that are sensed by the cAMP signaling cascade. cAMP inhibits mating in *S. pombe*, possibly to restrict mating and sporulation until both nitrogen and fermentable carbon sources have been exhausted. In contrast, pseudohyphal growth in *S. cerevisiae* and filamentous differentiation and mating in *C. neoformans* are stimulated by fermentable carbon sources and activated by cAMP. Thus, mutations in the Gα protein and adenylyl cyclase lead to a loss of carbon source sensing and defects in development in budding yeast and *C. neoformans* on minimal medium and yet to precocious mating of *S. pombe* on rich medium. Although the precise roles of components of the cAMP

pathway differ between organisms, the basic functions of Gα proteins and cAMP in nutrient sensing and mating are conserved among divergent fungal species.

The mating processes of other fungi are also dependent on nutrient-sensing Gα protein-cAMP signal transduction pathways. In *U. maydis*, a basidiomycete plant pathogen, a Gα protein-cAMP pathway regulates mating. Strains with mutations in the *ubc1* gene, encoding a PKA regulatory subunit, are mating defective (19). Additionally, the Gα protein Gpa3, homologous to *C. neoformans* Gpa1, regulates cAMP signaling, mating, and virulence in this organism (27). The cAMP pathway also plays a major role in morphogenesis in *U. maydis* because mutants defective in adenylyl cyclase display a constitutively filamentous phenotype (19). As we have demonstrated here, disruption of the *CAC1* gene does not trigger filamentous growth in *C. neoformans*.

G protein activation of adenylyl cyclases has been intensively investigated in other systems, including the slime mold *Dictyostelium discoideum*. Cell surface receptors sense extracellular cAMP to monitor cell density signals (22, 26, 50). The cAMP receptors in turn control intracellular adenylyl cyclase and PKA activities through G protein activation. The molecular dissociation and reassociation of heterotrimeric G protein subunits in response to receptor activation by the cAMP ligand were recently demonstrated by fluorescence resonance energy transfer (24). At present, there is no evidence that fungi express extracellular cAMP receptors. However, the basic signaling machinery by which G proteins regulate adenylyl cyclases in response to extracellular signals to control developmental processes is remarkably conserved.

In addition to regulating mating and differentiation, the *C. neoformans* cAMP pathway controls two major virulence factors: capsule and melanin. There was no difference in the growth rates of the isogenic wild-type and *cac1* mutant strains at 30 or 37°C, arguing that the phenotypes of the mutant are not simply attributable to defects in growth. In animal experiments, the *cac1* adenylyl cyclase mutant strain had defects in capsule and melanin that conferred a severe disadvantage in the host. This strain was completely avirulent in two animal models with different modes of infection. Therefore, a functioning cAMP pathway is necessary for the expression of virulence irrespective of animal host or site of infection. Furthermore, in comparison to *C. neoformans* mutants lacking Gpa1 (15), phospholipase B (11), or urease (12), which are attenuated but not avirulent, the *cac1* mutant strain was more severely compromised in the host. This mutational block may result in an in vivo fungicidal response, which could have therapeutic implications in the design of fungicidal drugs that target this enzyme.

While the phenotypes of adenylyl cyclase mutant strains are strikingly similar to those of strains lacking the Gα protein Gpa1, the *cac1* mutation confers more severe phenotypes. Some degree of mating by *gpa1* mutants is observed after prolonged incubation, whereas *cac1* mutants are completely sterile. Higher concentrations of cAMP are required to suppress the capsule defect of *cac1* mutant strains compared to *gpa1* mutant strains (3). In the murine inhalation model of cryptococcosis, *gpa1* mutant strains are attenuated for virulence but are still capable of causing lethal infections (15). In contrast, no lethal infections are observed with *cac1* mutant strains. These observations could be explained by a basal level of adenylyl cyclase activity present in *gpa1* mutants but not in *cac1* mutants, possibly implicating other signaling elements that also act on adenylyl cyclase function. For example, the *C. neoformans* RAS1 protein may play a dual role in the regulation of both cAMP and pheromone-responsive pathways (1). We note that the cAMP measurements (Fig. 1B) showing an undetectable level of cAMP in the *gpa1* and *cac1* mutants argue that the quantitative phenotypic differences between the *gpa1* and *cac1* mutant strains may not simply be attributable to differences in cAMP levels in total extracts prepared from bulk cultures. We offer three possible explanations. First, since the cAMP levels were below the limits of detection of this assay, *gpa1* mutant cells may have a higher level of cAMP than *cac1* mutant cells that we cannot detect. Second, there may be a difference in cAMP levels in some cells of the population such that some *gpa1* mutant cells have more cAMP than *cac1* mutant cells. Third, there may be differences in localized cAMP levels that are not detected in total cell extracts. The identification of other signaling elements in this conserved pathway should further illuminate the molecular regulation of microbial pathogenicity and eukaryotic cellular differentiation.

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