Adenylyl Cyclase-Associated Protein Aca1 Regulates Virulence and Differentiation of Cryptococcus neoformans via the Cyclic AMP-Protein Kinase A Cascade†

Yong-Sun Bahn,1 Julie K. Hicks,1 Steven S. Giles,2 Gary M. Cox,2 and Joseph Heitman1,2,3,4*

Departments of Molecular Genetics and Microbiology,1 Medicine,2 and Pharmacology and Cancer Biology,3 and Howard Hughes Medical Institute,4 Duke University Medical Center, Durham, North Carolina

Received 16 July 2004/Accepted 21 August 2004

Cryptococcus neoformans is a heterothallic, basidiomycetous, pathogenic fungus that causes serious infections of the central nervous system in individuals immunocompromised by AIDS or undergoing organ transplantation, cytotoxic chemotherapy, or corticosteroid therapy (6, 31). The virulence of C. neoformans is influenced by several factors, including the production of an antiphagocytic polysaccharide capsule (7, 20, 27), the use of melanin as an antioxidant (26, 51), growth at host physiological temperature (37 to 39°C) (27, 29), and prototrophy (41). Although not directly involved in the virulence of C. neoformans, mating and filamentous growth may play a survival role in the environment and also promote dissemination of the pathogen into the host. Signaling cascades regulating virulence and differentiation of C. neoformans have been extensively studied, including a mitogen-activated protein kinase (MAPK) pathway, a G-protein-regulated cyclic AMP (cAMP) pathway, a Ras-specific pathway, and the calcineurin pathway (for reviews, see references 29 and 48).

The MAPK cascade regulates mating processes involving morphological differentiation, such as the dikaryotic mycelia, basidia, and basidiospores, which are produced in response to peptide pheromones secreted by opposite mating-type cells (24, 32, 43). The MAPK pathway is composed of mating-type-specific (Ste3α/a, Ste20α/a, Ste11α/a, and Ste12α/a) and non-specific (Gpb1, Ste7, and Cpk1) elements (12, 49). Gene disruption experiments revealed that the MAPK pathway is required for mating and cell type-specific differentiation but not for virulence (12). However, mating type has been associated with the virulence of serotype D (variety neoformans) strains by Kwon-Chung et al., who showed that the α-mating type is more virulent than the α-mating type (25). Furthermore, Del Poeta et al. demonstrated that the MFα1 pheromone gene is induced during the late stages of central nervous system infection (13). In contrast, mating type has not yet been associated with virulence in congenic serotype A (variety grubii) strains (35). In response to nitrogen limitation, desiccation, and MFA phosphomine, haploid α strains can also filament and sporulate by a process known as haploid fruiting (50, 54).

The ability to grow at high temperature is mediated by at least two signaling pathways: a Ras (Ras1/Ras2) signaling pathway and a calcineurin-dependent pathway (1, 37, 52). The Ras pathway also promotes mating via the MAPK pathway (1). Although Ras plays a prominent role in regulating adenylyl cyclase and cAMP signaling in Saccharomyces cerevisiae (22, 33, 39), in both C. neoformans and Ustilago maydis Ras lacks the domain involved in cyclase binding and no longer functions in cAMP signaling (1, 28, 29).

The cAMP-protein kinase A (PKA)-dependent signaling pathway is of particular interest based on its ability to control both virulence factors (melanin and capsule production) and morphological differentiation (mating and filamentous growth) of C. neoformans. Elements of this pathway include the α subunit Gpa1, adenylyl cyclase Cac1, and PKA consisting of the catalytic subunits Pka1/Pka2 and the regulatory subunit Pkr1 (2, 3, 14, 21). Disruption of the GPa1 or CAC1 genes confers defects in melanin and capsule formation and mating that are suppressed by exogenous cAMP (2, 3). As a result, gpa1Δ and cac1Δ mutant strains are attenuated for virulence or avirulent in rabbit and murine models of cryptococcal meningitis (2, 3). The functions of Pka1 and Pka2 have diverged.

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, 322 CARL Bldg., Box 3546, Research Dr., Duke University Medical Center, Durham, NC 27710. Phone: (919) 684-2824. Fax: (919) 684-5458. E-mail: heitm001@duke.edu.
† Supplemental material for this article may be found at http://ec.asm.org/.
between serotype A variety *C. grubii* and D variety *C. neoformans* strains. Whereas Pka1 plays an essential role in capsule and melanin production in serotype A, Pka2 has assumed this role in serotype D (21). Pka1 and Pka2 are regulated by the upstream regulatory subunit Pkr1 (14). Deletion of the *PKR1* gene results in overproduction of capsule and hypervirulence (14).

In the model yeast *S. cerevisiae*, there is a dual input to adenyl cyclase activation and cAMP production. One input is via Ras1/2 and the other via Gpa2 (for a review, see reference 29). The elements downstream of adenyl cyclase are also divergent between the model yeast and pathogenic fungus. Three PKA catalytic subunit isoforms, Tpk1, Tpk2, and Tpk3, are functionally redundant for yeast vegetative growth but specialized for pseudohyphal development. Tpk2 promotes pseudohyphal growth, whereas Tpk1 and Tpk3 inhibit filamentation (38, 42, 45). In another ascomycetous, pathogenic fungus, *Candida albicans*, only two isoforms, Tpk1 and Tpk2, have been identified and both promote hyphal differentiation, although each responds to different inducing signals (5, 44). In contrast, in *C. neoformans* the cAMP pathway appears to function as a linear Gpa1-Cac1-PKA cascade (Pka1 for serotype A or Pka2 for serotype D) (2, 3, 14, 21).

Here, we identify and characterize an upstream regulatory element of adenyl cyclase, Aca1 (for adenyl cyclase-associated protein 1). Yeast two-hybrid studies demonstrate that Aca1 binds to the C terminus of Cac1 and also forms homodimers similar to *S. cerevisiae* CAP/Srv2. Aca1 constitutes an upstream element of the Cac1-cAMP-signaling pathway independent of Gpa1 and Ras1, and regulates mating, capsule and melanin production, and virulence of *C. neoformans*. *aca1Δ gpa1Δ* double mutants exhibited phenotypes equivalent to *cac1Δ* mutants, indicating that Aca1 and Gpa1 are together necessary and sufficient for activation of the Cac1-cAMP pathway. Finally, by analyzing *pka1Δ pka2Δ* mutants we discovered that Pka2 can play a limited redundant role with Pka1 during mating and melanin production, demonstrating that under some physiological conditions the Cac1-cAMP signaling pathway is also bifurcated to Pka1 and Pka2 in *C. neoformans*.

**MATERIALS AND METHODS**

**Strains and media.** The strains used in the present study are listed in Table 1. Yeast extract-peptone-dextrose (YPD) and synthetic (SD) media, V8 mating medium (pH 5.0), Niger seed and L-DOPA media for melanin production and Dulbecco modified Eagle (DME) medium for capsule production were as described previously (2, 20, 21, 46). Agar-based DME medium for capsule production was prepared by combining filter-sterilized 2% agar solution.

| Strain | Genotype | Parental strain | Source or reference |
|--------|----------|----------------|---------------------|
| H99    | MATα     | H99            | 40                  |
| KN99   | MATα     | KN99           | 35                  |
| F99    | MATα ura5 (5-FOAr) | H99 | 49 |
| YSB6   | MATα aca1Δ::NAT-STM#43 | H99 | This study |
| YSB58  | MATα aca1Δ::NEO | KN99 | This study |
| YSB108 | MATα aca1Δ::NAT-STM#43 ura5 (5-FOAr) | YSB6 | This study |
| YSB109 | MATα aca1Δ::NEO ura5 (5-FOAr) | YSB8 | This study |
| YSB117 | MATα aca1Δ::NAT-STM#43 ACA1-NEO | YSB6 | This study |
| YSB118 | MATα aca1Δ::NEO ACA1-NAT | YSB8 | This study |
| YSB119 | MATα aca1Δ::NAT-STM#43 ura5 ACA1-URA5 | YSB108 | This study |
| YSB121 | MATα aca1Δ::NEO ura5 ACA1-URA5 | YSB109 | This study |
| YSB42  | MATα cac1Δ::NAT-STM#159 | H99 | This study |
| YSB79  | MATα cac1Δ::NEO | KN99 | This study |
| YSB83  | MATα gpa1Δ::NAT-STM#5 | H99 | This study |
| YSB85  | MATα gpa1Δ::NEO | KN99 | This study |
| YSB51  | MATα ras1Δ::NAT-STM#150 | H99 | This study |
| YSB73  | MATα ras1Δ::NEO | KN99 | This study |
| YSB49  | MATα gpb1Δ::NAT-STM#146 | H99 | This study |
| YSB76  | MATα gpb1Δ::NEO | KN99 | This study |
| YSB188 | MATα pka1Δ::NAT-STM#191 | H99 | This study |
| YSB191 | MATα pka1Δ::NEO | KN99 | This study |
| YSB194 | MATα pka2Δ::NAT-STM#205 | H99 | This study |
| YSB198 | MATα pka2Δ::NEO | KN99 | This study |
| YSB166 | MATα aca1Δ::NAT-STM#43 gpa1Δ::NEO | YSB6 | This study |
| YSB168 | MATα aca1Δ::NAT-STM#43 gpa1Δ::NAT-STM#5 | YSB8 | This study |
| YSB174 | MATα aca1Δ::NAT-STM#43 ras1Δ::NEO | YSB6 | This study |
| YSB175 | MATα aca1Δ::NAT-STM#43 ras1Δ::NAT-STM#150 | YSB8 | This study |
| YSB170 | MATα aca1Δ::NAT-STM#43 cac1Δ::NEO | YSB6 | This study |
| YSB172 | MATα aca1Δ::NEO cac1Δ::NAT-STM#159 | YSB8 | This study |
| YSB182 | MATα cac1Δ::NAT-STM#159 ras1Δ::NEO | YSB42 | This study |
| YSB185 | MATα cac1Δ::NEO ras1Δ::NAT-STM#150 | YSB79 | This study |
| YSB200 | MATα pka1Δ::NAT-STM#191 pka2Δ::NEO | YSB188 | This study |
| H99 erg1 | MATα ura5 erg1::URA5 | F99 | 35 |
| PPW196 | MATα ura5 erg1::URA5 aca1::NEO | F99a | P. Wang |
| YSB96 | MATα ura5 erg1::URA5 aca1::NEO | YSB58 × JKH43 | This study |
| CHM3   | MATα lac1Δ::NAT | H99 | 21 |
| CAP59  | MATα cap59Δ::HYG | H99 | 34 |

* Each NAT-STM# indicates the Nat r marker with a unique signature tag.
served as the host strain for transformation and propagation of all plasmids used in the present study.

**Identification of the 5’ and 3’ regions of the ACA1 gene.** Strain H99 was incubated overnight at 30°C in YPD medium, pelleted, lyophilized, and total RNA was isolated with TRIzol (Gibco-BRL) according to the manufacturer’s instructions. 5’ and 3’ rapid amplification of cDNA ends (RACE) was performed with the GeneRacer kit (Invitrogen) according to the manufacturer’s instructions. Each RACE product was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced.

**Yeast two-hybrid assay.** To construct plasmids expressing the Gal4 DNA binding domain (BD) or activation domain (AD) fused to the full-length ACA1 open reading frame (ORF), the ACA1 gene was amplified by reverse transcription-PCR (RT-PCR) with the primers 12873/12874 and cloned into pGBT9 and pGAD-ACA1. Similarly, the C-terminal region (2126 to 2260 amino acids [aa]) of the ACA1 gene was PCR amplified (1,223 bp) from the same plasmid and cloned into pGAD-ACA1. The reporter yeast strain pJAF7 containing a Natr or Neor marker was used as the host strain for transformation and propagation of all plasmids used in the present study.

**Construction of serotype A caclΔ gnp1Δ, cacr1Δ cac1Δ, rasi1Δ rasi2Δ cac1Δ, pka1Δ pka2Δ, and crglΔ caclΔ double-mutant strains.** For epistasis analysis, the GPA1, CAC1, RAS1, and GPA1 genes were further disrupted in MATa caclΔ (YS86) and MATa caclΔ (YS88) mutant strains by using the same disruption cassette described above, generating MATa caclΔ (YS92) and MATa caclΔ (YS93) double-mutant strains (Table 1). The RAS1 gene was also disrupted in MATa caclΔ (YS94) and MATa caclΔ (YS95) mutant strains, creating rasi1Δ caclΔ double mutants (Table 1). The PKA2 gene was further disrupted in the MATa pka1Δ mutant (YS888), generating pka1Δ pka2Δ double mutants (Table 1). Stable double-mutant strains (Nat’Neo’) were selected on YPD medium containing both nourseothricin and G418. Positive transformants were further screened by diagnostic PCR and confirmed by South- ern blot analysis with a PstI-digested genomic DNA fragment as probes. Cell fusion efficiency was measured as previously described (21) with minor modifications. A total of 104 cells of each MATa strain bearing Nat’ or Neo’ markers, respectively, per ml was mixed in an equal volume, and 5 μl of this cell mixture was spotted on V8 medium, followed by incubation for 24 h at room temperature in the dark. The cells were scraped and resuspended in 1 ml of distilled H2O, and 20 μl of cell suspension was plated onto YPD medium containing nourseothricin and G418. The number of colonies on each plate was determined after 4 days of incubation at room temperature.

**Disruption of the ACA1 gene.** The caclΔ-null mutant (caclΔ) was generated in the congenic C. neoformans serotype A MATa (H99) and MATa (KN99) strain backgrounds by PCR overlap as previously described (20). Amplification of regions of the ACA1 gene were generated by PCR (Ex Taq; Takara Co.) by using H99 or KN99 genomic DNA and primers (Fig. 2): 10341/10342 for the 3’ end and 10343/10344 for the 5’ end. Nat’ or Neo’ dominant selectable markers were also generated by PCR with M13 forward (M13F) and reverse (M13R) primers by using plasmid pNATSTM#45 with a unique signature tag (kindly provided by Jennifer K. Lodge, St. Louis University School of Medicine; tag available upon request) or pAf1 (17) as templates, respectively. ACA1 disruption fragments were generated by PCR overlap with the primers 10341/10344. A 4.3-kb caclΔ disruption construct was gel extracted and precipitated onto 600-μg gold microcarrier beads (0.8 μm; Bioworld, Inc.) and biologically transformed into strain H99 or KN99 as described previously (11). Stable transformants were selected on YPD medium containing nourseothricin (100 μg/ml) or G418 (200 μg/ml).

To screen caclΔ mutant strains, diagnostic PCR was performed by analyzing the 5’ end of disrupted caclΔ alleles with primers 10345 and 10949. Screened transformants were further confirmed by Southern blot analysis with a PstI-digested genomic DNA and an ACA1-specific probe made by PCR with primers 10409 and 10410. Uracl auxotrophic caclΔ mutants (YS108 and YS109) (Table 1) were generated by inducing spontaneous ura3 mutations in strains YS86 and YS858, respectively, on SD medium containing 5-fluoroorotic acid (5-FOA).

To construct the caclΔ::ACAC1 reconstituted strains, H99 genomic DNA containing the entire ACA1 gene was isolated from a C. neoformans H99 bacte- rial artificial chromosome (BAC) library using the ACA1 specific probe described above. The 3-kb Sall-HindIII fragment containing the ACA1 gene (Fig. 1) was cloned into plasmid pFA121 containing the Neo’ marker, generating pNEOSVR2. Plasmids pNATSVR2 and pURASVR2 were further constructed by inserting the 3-kb Xhol-HindIII fragment of pNEOSVR2 into pFA13 or pFA7 containing a Nat’ or URA3 selectable marker, respectively. Stbl-deflated plasmids pNEOSVR2, pNATSVR2, and pURASVR2 were biologically transformed into strains YSB6, YSB58, or ura3 YSB108/YSB109 (Table 1), respectively.

**RT-PCR confirmation of ACA1 disruption and reconstitution.** First-strand cDNA was generated by using 5 μg of DNAse I-treated total RNA according to the manufacturer’s instructions (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen). To detect the presence or absence of ACA1 messages, a portion of the ACA1 gene (733 to 1,150 bp of a 1,521-bp coding sequence) was PCR amplified (418 bp) from the first-strand cDNA by using ACA1-specific primers 11614/11615. As a control, a portion of the CAC1 gene (53 to 1,275 bp of a 1,283-bp coding sequence) was PCR amplified (1,223 bp) from the same first-strand cDNA by using CAC1-specific primers 11641/11615.

**Disruption of the GPA1, CAC1, RAS1, GPA1, PKA1, and PKA2 genes in the H99 and KN99 strain backgrounds.** Although the GPA1, CAC1, RAS1, GPA1, PKA1, and PKA2 genes have been previously disrupted and characterized in serotype A C. neoformans (1–3, 50), these mutants were isolated in mutated

auxothrophic H99 derivatives that may contain other unintended mutations. Therefore, these genes were disrupted in the congenic prototrophic H99 and KN99 genetic background by using methods equivalent to those described above. The 5’ regions of each gene were generated with the following primers: 10913/11736 for GPA1, 11608/11609 for CAC1, 11600/11601 for RAS1, 11592/11593 for GPB1, 12911/12912 for PKA1, and 12916/12917 for PKA2. The 3’ regions for each gene were generated with the following primer pairs: 11737/10587 for GPA1, 11610/11611 for CAC1, 11602/11603 for RAS1, 11594/11595 for GPB1, 12913/12956 for PKA1, and 12918/12919 for PKA2. The Neo’ marker was gen- erated as described above. Nat’ markers were similarly generated with the following plasmids as templates: pNATSTM#146 for GPB1, pNATSTM#150 for GPA1, pNATSTM#159 for CAC1, pNATSTM#191 for PKA1, and pNATSTM#205 for PKA2. Overlap PCR and biotic transfor- mation were performed as described above and the genotype of each mutant was confirmed by diagnostic PCR and Southern blot (not shown).

**Construction of reconstituted strains.** To construct the reconstituted strains, diagnostic PCR was performed by analyzing the 5’ end of disrupted caclΔ alleles with primers 10345 and 10949. Screened transformants were further confirmed by Southern blot analysis with a PstI-digested genomic DNA and an ACA1-specific probe made by PCR with primers 10409 and 10410. Uracl auxotrophic caclΔ mutants (YS108 and YS109) (Table 1) were generated by inducing spontaneous ura3 mutations in strains YS86 and YS858, respectively, on SD medium containing 5-fluoroorotic acid (5-FOA).

To construct the caclΔ::ACAC1 reconstituted strains, H99 genomic DNA containing the entire ACA1 gene was isolated from a C. neoformans H99 bacte- rial artificial chromosome (BAC) library using the ACA1 specific probe described above. The 3-kb Sall-HindIII fragment containing the ACA1 gene (Fig. 1) was cloned into plasmid pFA121 containing the Neo’ marker, generating pNEOSVR2. Plasmids pNATSVR2 and pURASVR2 were further constructed by inserting the 3-kb Xhol-HindIII fragment of pNEOSVR2 into pFA13 or pFA7 containing a Nat’ or URA3 selectable marker, respectively. Stbl-deflated plasmids pNEOSVR2, pNATSVR2, and pURASVR2 were biologically transformed into strains YSB6, YSB58, or ura3 YSB108/YSB109 (Table 1), respectively.

**RT-PCR confirmation of ACA1 disruption and reconstitution.** First-strand cDNA was generated by using 5 μg of DNAse I-treated total RNA according to the manufacturer’s instructions (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen). To detect the presence or absence of ACA1 messages, a portion of the ACA1 gene (733 to 1,150 bp of a 1,521-bp coding sequence) was PCR amplified (418 bp) from the first-strand cDNA by using ACA1-specific primers 11619/11974. As a control, a portion of the CAC1 gene (53 to 1,275 bp of a 1,283-bp coding sequence) was PCR amplified (1,223 bp) from the same first-strand cDNA by using CAC1-specific primers 11641/11615.

**Disruption of the GPA1, CAC1, RAS1, GPA1, PKA1, and PKA2 genes in the H99 and KN99 strain backgrounds.** Although the GPA1, CAC1, RAS1, GPA1, PKA1, and PKA2 genes have been previously disrupted and characterized in serotype A C. neoformans (1–3, 50), these mutants were isolated in mutated
tion and washed twice with water and once with MES buffer (10 mM morpholineethanesulfonic acid [pH 6.0], 0.1 mM EDTA). Cells were resuspended in MES buffer at an OD600 of 2.0 and further incubated at 30°C with shaking for glucose starvation. After 2 h of incubation, glucose was added to a final concentration of 2%. At various time points (0, 0.5, 1, and 3 min), 1 ml of cell suspension was sampled, filtered through 0.45-pm-pore-size membrane filters (Millipore), immediately extracted with butanol-saturated formic acid (4.1% [vol/vol]), and lyophilized. The intracellular cAMP concentration was determined by using a cAMP [125I] Direct Biotrak Scintillation Proximity Assay System (Amersham Biosciences) and normalized to the wet weight of cells.

**Virulence assays.** Yeast strains were grown at 30°C in YPD medium overnight with shaking (250 rpm), collected by centrifugation, and washed twice with sterile phosphate-buffered saline (PBS), and the final concentration was adjusted to 2 × 10^6 CFU/ml with sterile PBS. Female A/Jcr mice (NCI/Charles River Laboratories) mice (20 to 24 g each) were infected with wild-type (H99, six mice), aca1 (YSB6, nine mice), aca1 ACA1 reconstituted (YSB117, ten mice), and cac1 (YSB117, twelve mice). Mice were infected with 2 × 10^5 CFUs intranasally, and mortality was monitored daily for 10 days. The Kaplan-Meier product limit method was used to calculate the median survival time, with a Log-rank test to compare survival in different groups. All statistics were performed by using the software GraphPad Prism. Data are presented as group means ± SD. Differences were considered statistically significant when *p < 0.05.

**FIG. 1.** Aca1 associates with adenylyl cyclase and itself. (A) The amino acid sequences of the N-terminal region of adenylyl cyclase-associated proteins (upper) or the C-terminal region of adenylyl cyclases (lower) from *S. cerevisiae* (ScCap and ScCyr1), *S. pombe* (SpCap and SpCyr1), *C. albicans* (CaCap1 and CaCdc35), and *C. neoformans* (CnAca1 and CnCac1) were aligned. Hydrophobic residues in the heptad motif are in boldface. Asterisks indicate leucine residues essential for interactions between Cap and Cyr1 in *S. cerevisiae*. (B) Two-hybrid assay showing interactions of Aca1 with Cap or itself. Plasmids expressing full-length Aca1, Gpa1, and Ras1, and the C terminus (2126 to 2260 aa) of Cac1 with either the Gal4 DNA-binding domain (BD) or activation domain (AD) were: AD (pGAD424), BD (pGBT9), AD-Aca1 (pGAD-Aca1), BD-Aca1 (pGBT-Aca1), BD-Cac1 (pGBT-Cac12126-2260), BD-Gpa1 (pGBT-Gpa1), and BD-Ras1 (pGBT-Ras1). Combinations of the indicated plasmids were cotransformed into reporter strain PJ69-4A and three independent transformants isolated from SD Leu Trp medium were further grown on SD -Leu -Trp -His or SD -Leu -Trp -His -Ade to test for protein-protein interactions that allow reporter-dependent cell growth and photographed after 48 h of incubation at 30°C.

VOL. 3, 2004 ROLE OF Aca1 IN VIRULENCE OF *C. NEOFORMANS* 1479
C. neoformans Aca1 is a member of the CAP/Srv2 protein family and associates physically with adenylyl cyclase Cac1. Previous studies implied the existence of another upstream activating element for adenylyl cyclase Cac1 in addition to the Go subunit Gpa1. The mating defect of cac1Δ mutants is more severe than that of gpa1Δ mutants and cac1Δ mutants are avirulent, whereas gpa1Δ mutants are attenuated but can result in lethal infection (3, 14). Ras, which is one of the upstream activators for adenylyl cyclase in S. cerevisiae, is not an up-stream signaling component in the Cac1-cAMP-PKA pathway modulating capsule and melanin production in C. neoformans (1, 52). We focused our search for another potential upstream signaling component in the cAMP-signaling pathway on CAP/Srv2, which is known to be an effector protein between Ras and adenylyl cyclase in S. cerevisiae (15, 16). Furthermore, in C. albicans, the CAP/Srv2 homolog Cap1 mediates cAMP-dependent signaling to govern morphogenetic transitions and virulence (4). Therefore, we hypothesized that a CAP/Srv2 homolog may mediate Ras or CAMP signaling in C. neoformans. By BLAST searches, a single CAP/Srv2 homolog was identified in both the serotype A and D C. neoformans genomes. This gene was designated ACA1 for adenylyl cyclase-associated protein 1. The serotype A Aca1 protein shares 73% identity with serotype D Aca1 and 32 to 35% identity with homologs in S. cerevisiae, C. albicans, and humans.

Based on cDNA sequence analysis of 5′ and 3′ RACE products, the serotype A C. neoformans ACA1 spans a 1,908-bp ORF (from start to stop codon) and is interrupted by eight introns (GenBank accession number AY629599). The ACA1 gene encodes a 507-amino-acid protein. The C. neoformans Aca1 protein shows a domain structure typical of CAP/Srv2 homologs in other organisms. First, Aca1 contains the RLExAT/ VxRLE motif (16RLEAVTSRLE25) that mediates Ras/cAMP signaling and proper CAP/Srv2 localization (55). Second, Aca1 contains a central proline rich region (20PPP PPPPPP274), which may be involved in protein folding to enable interactions between the N- and C-terminal regions of CAP/Srv2 (55). Third, Aca1 has a single SH3 binding motif (PxxP; x, any amino acid, 335PLKP 338), which plays an important role in proper cellular localization (19). This motif is also implicated in binding to Abp1 (actin-binding protein 1), which modulates actin cytoskeleton regulation in S. cerevisiae (19). Finally, Aca1 has a highly conserved C-terminal CAP-signature sequence involved in actin-monomer binding in the model yeast (18).

To further confirm that Aca1 is an adenylyl cyclase-associate protein, we demonstrated that Aca1 and Cac1 physically interact. In S. cerevisiae, the N-terminal domain (1 to 36 aa) of CAP/Srv2 binds to the C-terminal domain (1,822 to 2,026 aa) of the adenylyl cyclase Ccr1 protein through coiled-coil interactions mediated by tandem repeats of a heptad motif (ωxωxxxxxx or ωxωxxxxxx, where ω is a hydrophobic amino acid and x is any amino acid) present on both proteins (36). We found that the heptad repeats are also highly conserved in the C. neoformans Aca1 and Cac1 proteins (Fig. 1A). Notably, two leucine residues in both ScCcr (L20 and L27) and ScCyr1 (L1916 and L1923) that are known to be essential for their interaction are highly conserved in Aca1 and Cac1 (Fig. 1A), suggesting the potential for coiled-coil interactions between the two proteins. This hypothesis was confirmed by a yeast two-hybrid assay showing an interaction between Aca1 and the C-terminal region (2,126 to 2,260 aa) of Cac1 (Fig. 1B).

Furthermore, we also found that Aca1 can associate with itself (Fig. 1B), indicative of homodimerization or multimerization of Aca1, although the Aca1-Aca1 interaction appears to be weaker than the Aca1-Cac1 interaction (Fig. 1B). In S. cerevisiae, dimer or multimer formation by CAP/Srv2 is important for localization but is not essential for cAMP signaling (55, 58). In contrast to the association of Aca1 with Cac1, no interaction was observed between Aca1 and Gpa1 or Ras1 (Fig. 1B), implying that Aca1 might activate Cac1 independently of Gpa1 and Ras1. Taken together, the high degree of structural conservation between Aca1 and other CAP/Srv2 homologs and the physical interaction observed between Aca1 and Cac1 demonstrate that C. neoformans Aca1 is a bona fide member of the CAP/Srv2 protein family.

Disruption of the ACA1 gene and recapitulation of gpb1Δ, ras1 Δ, gpa1Δ, cac1Δ, pka1Δ, and pka2Δ mutations in the congeneric serotype A MATα and MATa strains. To characterize the function of Aca1 in known signaling pathways of C. neoformans, the ACA1 gene was disrupted. aca1ΔΔ:NAT and aca1ΔΔ::NEO disruption alleles were introduced into strains H99 and KN99, respectively, by biolistic transformation, deleting an internal 1,880-bp (bp 1 to 1880 from the start) of the 1,908-bp ORF (Fig. 2A). The genotypes of two independent MATα Δaca1Δ mutants (YSB6 and YSB7) and three independent MATa Δaca1Δ mutants (YSB58, YSB59, and YSB60) were verified by diagnostic PCR (not shown) and Southern blot analysis (Fig. 2B). The wild-type ACA1 gene was re-introduced into the Δaca1Δ mutants by targeting to the ACA1 locus with linearized ACA1::NEO or ACA1::NAT constructs (Fig. 2B). Successful disruption and reintroduction of ACA1 were further confirmed by RT-PCR analysis, demonstrating the loss and recovery of ACA1 expression in the Δaca1Δ mutant and Δaca1Δ+ACA1 reconstituted strains, respectively (Fig. 2C). All of the independent MATα or MATa Δaca1Δ mutant strains showed identical in vitro phenotypes, and representative results from strains YSB6 (MATα Δaca1Δ) and YSB58 (MATa Δaca1Δ) (Table 1) are shown here.

For comparative phenotypic analysis of aca1Δ mutants with other cAMP-dependent or -independent mutants, we then re-disrupted the Gpb1, Ras1, Gpa1, Cac1, Pka1, and Pka2 genes in the congeneric serotype A MATα H99 and MATa KN99 strains, which were recently generated by Nielsen et al. (35). We regenerated these mutant strains for the following reasons. First, the previous mutants had been isolated in different mutagenized auxotropic parental strain backgrounds (ade2 or ura3), which complicates comparative phenotypic analysis between those mutants and mutants generated directly in H99 or KN99 by using dominant selectable markers. Second, utilization of auxotropic strains as parental strains for gene disruption
tion might cause misinterpretation of phenotypes through direct or indirect effects of auxotrophic mutations or unexpected background mutations introduced during their generation by UV or gamma irradiation. Finally, the GPB1, RAS1, GPA1, CAC1, PKA1, and PKA2 genes had not been disrupted previously in the KN99 MATa genetic background. Disruption of these genes in the congenic MATa background makes it possible to investigate serotype A α versus a bilateral mating efficiency, instead of by using heterologous serotype D mating-type tester strains.

We regenerated all mutants by using the Nat' and Neo' dominant selectable markers in the congenic H99 and KN99 backgrounds as described in Materials and Methods. In general, newly constructed MATa and MATa gpb1Δ (YSB49 and YSB76), ras1Δ (YSB51 and YSB73), gpa1Δ (YSB83 and YSB85), cac1Δ (YSB42 and YSB79), pka1Δ (YSB188 and
YSB191), and pka2Δ (YSB194 and YSB198) mutants (Table 1) displayed phenotypes comparable to mutants generated previously (1–3, 50), in terms of defective mating (gpb1Δ, ras1Δ, gpa1Δ, cac1Δ, and pka1Δ mutants), reduced capsule and melanin production (gpa1Δ, cac1Δ, and pka1Δ mutants), or inability to grow at high temperature (ras1Δ mutants) (data not shown). The genotypes for each mutant strain were confirmed by Southern blot analyses and at least two independent mutants for each gene exhibited identical phenotypes.

**Aca1 is not essential for growth at high temperature.** *C. neoformans ras1Δ* mutants exhibit a temperature-sensitive growth defect (1). Based on the functional connection between Ras1 and CAP/Srv2 in *S. cerevisiae*, we tested growth of *aca1Δ* mutants at high temperature to address the question of whether Aca1 is involved in Ras1 signaling. *aca1Δ* mutants newly constructed in the congenic H99 and KN99 background (YSB51 and YSB73) showed severe growth defects at 37 to 39°C (not shown), in accord with previous studies (1, 52). In contrast, *aca1Δ* mutants had no growth defect at 30, 37, or 39°C (not shown). *MATα* and *MATα* wild-type (H99 and KN99), *aca1Δ* mutants (YSB6 and YSB58), and *aca1Δ + ACA1* reconstituted strains (YSB117 and YSB118) showed similar growth rates in YPD medium at either 30 or 37°C. When the *RAS1* gene was disrupted in the *aca1Δ (aca1Δ ras1Δ* mutants YSB174 and YSB175) or *cac1Δ (ras1Δ cac1Δ* mutants YSB182 and YSB185) mutant background, the temperature-sensitive growth defect conferred by the *ras1Δ* mutant was readily apparent at 37° and 39°C. We conclude that Aca1 is not involved in the Ras1-specific signaling pathway regulating growth at high temperature.

**Aca1 promotes mating via the cAMP-signaling pathway in parallel with Gpa1.** In previous studies, Cac1 and Gpa1 were found to be involved in mating (2, 3). We hypothesized that Aca1 should play a similar role in mating if it is also a component of the cAMP signaling pathway. In unilateral mating crosses with the wild-type strains (H99 or KN99) of opposite mating type, both *MATα* and *MATα* *aca1Δ* mutants mated less efficiently on V8 medium than did the wild type (Fig. 3A). The defect in unilateral mating was more pronounced at earlier time points, and basidiospores were observed at later time points (Fig. 3A). *aca1Δ* mutants displayed more dramatic defects in bilateral mating crosses (*aca1Δ × aca1Δ*) in which only very short filaments were produced within the mating patches but no basidiospores were observed, even after 4 weeks of incubation (Fig. 3A).

To test whether the mating defects observed with *aca1Δ* mutants result from defects in cell fusion or mating filamentation, cell fusion efficiency was determined with Nat' or Neo' marked *aca1Δ* mutants (YSB6 and YSB58) and normalized to the fusion of control strains (YSB119 and YSB121, *aca1Δ + ACA1* reconstituted strains). *aca1Δ* mutants were less efficient in cell fusion compared than the wild-type strain (Fig. 3B) but still yielded fusion products (11.9% ± 1.4%), indicating that the *aca1Δ* disruption results in both defective mating filamentation and reduced cell fusion frequency. In support of this conclusion, all *aca1/aca1* diploid strains isolated from cell fusion assays exhibited less prolific filamentous growth compared to wild-type diploid control strains (Fig. 3C).

Defects in filamentous growth conferred by the *aca1Δ* mutation were further assayed by using confrontation assays. For the present study, we used pheromone hypersensitive *crg1Δ* mutants, which lack an RGS protein that normally desensitizes cells to pheromone exposure (35, 47). When *MATα crg1Δ* mutants were confronted with *MATα crg1Δ* mutants, both cell types formed filaments toward the mating partner in response to pheromone (Fig. 3D). Disruption of *ACA1* in the *MATα crg1Δ* mutant (*aca1Δ crg1Δ* double mutants; Table 1) still resulted in normal conjugation tube formation from confronting α *crg1Δ* cells but defective filamentous growth from the α *aca1Δ crg1Δ* mutant (Fig. 3D), indicating that Aca1 is not required for pheromone production but is involved in filamentation during response to pheromone.

To further determine whether Aca1 regulates mating in a cAMP-dependent manner, the mating defect of *aca1Δ* mutants was compared to those of *gpb1Δ* and *ras1Δ* (cAMP-independent) versus *gpa1Δ* and *cac1Δ* (cAMP-dependent) mutants. We found that *gpb1Δ*, *ras1Δ*, *gpa1Δ*, and *cac1Δ* mutants all showed more severe unilateral mating defects than *aca1Δ* mutants (Fig. 4A). The addition of 1 or 10 mM exogenous cAMP, however, greatly enhanced unilateral mating and bilateral mating, respectively, of *gpa1Δ*, *cac1Δ*, and *aca1Δ* mutant cells to wild type (Fig. 4A). In contrast, unilateral or bilateral mating defects of *ras1Δ* and *gpb1Δ* mutants were not rescued by 1 or 10 mM cAMP (Fig. 4B), further confirming that Ras1 and Gpb1 transmit mating signals through cAMP-independent signaling pathways.

Disruption of the *GPA1* gene resulted in reduced cell fusion, although this was less severe compared to *aca1Δ* mutants (Fig. 3B). *cac1Δ* mutants showed even more severe cell fusion defects than either *gpa1Δ* or *aca1Δ* mutants (Fig. 3B). In contrast to the less severe cell fusion defect observed in *gpa1Δ* mutants compared to *aca1Δ* mutants, *gpa1/gpa1* diploid strains were completely blocked in filamentous growth, as were *cac1/cac1* diploid strains (Fig. 3C). These results suggest that Gpa1 and Aca1 independently contribute to activate the Cac1-cAMP pathway during the mating process. Compared to these cAMP pathway mutants, disruption of the *RAS1* or *GPB1* genes conferred a complete lack of cell fusion even in unilateral mating, which contrasts with the reduced but apparent cell fusion events in cells lacking components of the cAMP pathway (Fig. 3B). Therefore, the data indicate that the Cac1-cAMP pathway controls mating, is independently activated by either (or both) Aca1 or Gpa1, and functions in a pathway distinct from Ras1 and Gpb1.

Previous studies identified the two PKA catalytic subunits Pka1 and Pka2, and Pka1 plays the major role in mating and filamentous growth in serotype A (14, 21). In accord with these results, *pka1Δ* mutant strains (YSB188 and YSB191) displayed a severe mating defect (particularly in bilateral matings), whereas *pka2Δ* mutant strains (YSB194 and YSB198) had no mating defect (Fig. 4B). However, the finding that the *pka1Δ* mutant showed less severe unilateral mating defects than the *cac1Δ* mutant suggested that Pka2 might play a limited role in mating in *pka1Δ* mutant cells (Fig. 4). In fact, *pka1Δ pka2Δ* double-mutant strains showed a more severe unilateral mating defect than *pka1Δ* mutant cells, similar to the *cac1Δ* mutant, suggesting that the mating signal from Cac1 is bifurcated into Pka1 and Pka2, with Pka1 serving as the principal signaling element of the cAMP pathway.

**Aca1 is required for capsule production via the Cac1-cAMP-Pka1-dependent signaling pathway.** Previous studies showed
**FIG. 3. Aca1 promotes cell fusion and filament formation during mating but is not required for pheromone production.** (A) Serotype A MATα and MATα strains were cocultured on V8 media (pH 5.0) for up to 4 weeks at room temperature in the dark, including H99 and KN99 (α × a), YSB6 and YSB59 (aca1 × a), YSB6 and YSB58 (aca1 × aca1), and YSB117 and YSB118 (aca1+ACA1 × aca1+ACA1). Edges of the mating patches were photographed at a magnification of ×100 (inserts in the second row at ×200). (B) Cell fusion assays were performed (see Materials and Methods) with the following strains: YSB119 and YSB121 for α × a, YSB119 and YSB58 for α × aca1, YSB6 × YSB58 for aca1 × aca1, YSB119 × YSB58 for α × gpal, YSB3 × YSB85 for gpal × gpal, YSB119 × YSB79 for α × cac1, YSB42 × YSB79 for cac1 × cac1, YSB119 × YSB73 for α × ras1, YSB51 × YSB73 for ras1 × ras1, YSB119 × YSB76 for α × gpbl, and YSB49 × YSB76 for gpbl × gpbl. In each experiment, the percentage of cell fusion relative to the α × a mating (100%) was calculated by averaging results from duplicate plates for three independent experiments with the standard deviations, as indicated. (C) Each diploid strain (WT from YSB119 × YSB121, aca1/aca1 from YSB6 × YSB58, gpal/gpal from YSB3 × YSB85, and cac1/cac1 from YSB42 × YSB79) recovered from the cell fusion assays was grown on YPD medium containing nourseothricin and G418 for 5 days at room temperature and photographed (×100 magnification). (D) MATα crg1Δ mutants were confronted with the MATα crg1Δ mutant as a control or the crg1Δ aca1Δ double-mutant strains on filamentation agar for 1 week at room temperature in the dark and photographed (×100 magnification).
that the Gpa1-Cac1-cAMP-Pka1 signaling pathway is the major cascade for capsule regulation (2, 3), whereas Ras1 is not required (1). Consistent with our assignment of Aca1 as a component of the cAMP-signaling pathway, Aca1 was required for capsule production (Fig. 5). aca1Δ mutants were highly defective in capsule production in response to a variety of capsule-inducing signals, including agar-based DME (Fig. 5), low iron, and 10% serum (not shown). These mutants were
hypocapsular and not acapsular, and a residual level of capsule was still observed, similar to other cAMP pathway mutants (i.e., gpa1Δ, cac1Δ, or pka1Δ) (Fig. 5). Based on quantitative measurements of relative capsule sizes, the defect in capsule production of aca1Δ mutants was comparable to those of gpa1Δ, cac1Δ, and pka1Δ (Fig. 5B). Addition of 10 mM cAMP to agar-based DME media completely restored capsule production in aca1Δ, gpa1Δ, and cac1Δ mutants but not of pka1Δ.
mutants, demonstrating that Aca1 functions upstream of Pka1 in the cAMP pathway.

To test by epistasis analysis whether Aca1 and Cac1 function in a linear fashion in the cAMP-PKA pathway, aca1Δ cac1Δ double-mutant strains (YSB42 and YSB79) were generated (Table 1). aca1Δ cac1Δ double mutants did not display any additive defects in capsule production relative to aca1Δ or cac1Δ single-mutant strains and still produced minimal capsules compared to a complete lack of capsule in the acapsular strain cap59Δ (Fig. 5). The data suggest then that Aca1 signals in a linear fashion with Cac1 and Pka1. As reported (1), Ras1 is not involved in capsule production, and aca1Δ ras1Δ double mutants exhibited the same level of capsule defect observed with aca1Δ single mutants (Fig. 5). Also in accord with a previous study (21), Pka2 was found to be completely dispensable for capsule production. pka2Δ mutants produced wild-type levels of capsule, and pka1Δ pka2Δ double mutants (YSB200) did not exhibit any additive defects in capsule production compared to pka1Δ single-mutant strains (data not shown). In conclusion, Aca1 transmits capsule inducing signals through the Cac1-cAMP-PKA-dependent signaling pathway.

Aca1 and Gpa1 individually contribute to melanin production via the Cac1-cAMP-PKA-dependent pathway. Melanin plays a role as an antioxidant during host infection and serves as a virulence factor regulated by the Gpa1-Cac1-cAMP-PKA signaling pathway. Therefore, we investigated the role of Aca1 in melanin production, both by visual inspection of melanin accumulation in cells on Niger seed medium and by quantitative measurements of laccase activity. Disruption of the ACA1 gene attenuated melanin production, and this defect was more apparent at 37°C than at 30°C (Fig. 6A). The defect of the aca1Δ mutant was comparable to gpa1Δ and cac1Δ mutants at 37°C. Melanin synthesis in the aca1Δ, gpa1Δ, and cac1Δ mutants was restored by exogenous cAMP (10 mM), further confirming that Aca1, Gpa1, and Cac1 function in a cAMP-dependent manner. Interestingly, we found that gpa1Δ and aca1Δ mutants exhibited only minor defects in melanin biosynthesis at 30°C compared to a more pronounced defect of cac1Δ mutants (Fig. 6A). Quantitative measurement of laccase activity in 1-DOPA medium further confirmed that aca1Δ and gpa1Δ mutants are deficient in melanin production compared to wild-type, but neither is as defective as cac1Δ mutants (Fig. 6B). These data suggest that Cac1 requires multiple inputs to activate the synthesis of melanin.

We hypothesized that Gpa1 and Aca1 provide parallel inputs to Cac1 for melanin production. gpa1Δ aca1Δ double-mutant strains showed a more severe melanin defect than either gpa1Δ or aca1Δ single mutant, and this synergistic defect was comparable to cac1Δ mutants on both Niger seed and 1-DOPA media (Fig. 6). The melanin defect of gpa1Δ aca1Δ mutants was completely rescued by addition of exogenous cAMP (10 mM) (Fig. 6A). In contrast, introducing an aca1Δ mutation into the cac1Δ mutant background did not exacerbate the melanin defect of cac1Δ mutant cells (Fig. 6). These findings support a model in which Gpa1 and Aca1 are two parallel upstream components activating the Cac1-cAMP-PKA signaling pathway for production of melanin.

As previously reported (21), pka1Δ mutants displayed defects in melanin synthesis similar to other cAMP cascade mutants at 37°C on Niger seed medium, which could not be rescued by exogenous cAMP (Fig. 6A). We note however that pka1Δ mutants showed a less severe defect in melanin accumulation at 30°C on Niger seed medium than cac1Δ mutants, implicating the potential role of the other catalytic subunit Pka2 in this pathway (Fig. 6A). Supporting this hypothesis, pka1Δ pka2Δ double mutants were found to be more melanin defective in both 30 and 37°C compared to pka1Δ mutants, similar to cac1Δ or aca1Δ gpa1Δ double mutants. Similarly, pka1Δ pka2 double mutants largely failed to produce melanin in 1-DOPA medium similar to cac1Δ mutants (Fig. 6B). The pka1Δ single-mutant strain exhibited wild-type melanin production (Fig. 6), as reported previously (21). These data demonstrate that Pka1 plays the predominant role in cAMP signaling to control melanin synthesis but, in its absence, Pka2 can fulfill a limited signaling capacity.

Aca1 is required for glucose-induced but not basal cAMP levels. The role of Aca1 in controlling cAMP signaling in conjunction with Gpa1 and Cac1 was confirmed by measuring cAMP levels during glucose sensing. In accord with a previous report (3), cAMP levels in the wild type rapidly increased after glucose readdition to glucose-starved cells (Fig. 7). In contrast, the gpa1Δ and cac1Δ mutant exhibited significantly lower cAMP concentrations at all time points and lacked any detectable cAMP pulse (Fig. 7), and thus both Gpa1 and Cac1 are required for both glucose-induced cAMP signaling and maintenance of basal cAMP levels. The aca1Δ mutant was defective in induced cAMP production but maintained basal cAMP levels equivalent to the wild-type at the zero time point (Fig. 7). cAMP levels in the aca1Δ mutant were significantly higher than those in the gpa1Δ and cac1Δ mutants, indicating that Aca1 plays a role in glucose-induced but not basal cAMP levels. Reintroduction of the ACA1 gene restored a wild-type cAMP signaling pattern in the aca1Δ mutant (Fig. 7). The basal cAMP levels in the aca1Δ mutant were further reduced to the levels of the cac1Δ mutant after disruption of the GPA1 gene (aca1Δ gpa1Δ) (Fig. 7), a finding consistent with the identical in vitro phenotypes observed in aca1Δ gpa1Δ and cac1Δ mutants. Taken together, these findings confirm that Aca1 controls cAMP signaling through Cac1 in a manner distinct from Gpa1.

Aca1 is required for virulence of C. neoformans. Because capsule and melanin are important virulence factors, we tested whether Aca1 is required for virulence of C. neoformans. To test this hypothesis, we used the murine inhalation model of systemic cryptococcosis. A/Jer mice were infected with wild-type (H99), aca1Δ (YSB6), aca1Δ + ACA1 reconstituted (YSB117), or cac1Δ (YSB42) mutant strains by intranasal inhalation, which mimics the natural route of human infection by C. neoformans. Inhaled cells first infect the lung and then disseminate to the brain, causing meningoencephalitis. Survival of infected mice was monitored for 40 days. All mice infected with the wild-type strain became moribund between days 19 and 24 postinfection (median survival, 23 days) (Fig. 8). In contrast, all mice infected with the aca1Δ mutant strains survived throughout the course of the experiment without showing any signs of illness (P < 0.0001 compared to the wild-type or reconstituted strains) (Fig. 8). The aca1Δ mutants were as avirulent as cac1Δ mutants and reintroduction of the ACA1 gene into the aca1Δ mutants completely restored virulence (Fig. 8). Animals infected with the aca1Δ + ACA1 reconstituted strain
became moribund between days 23 and 25 postinfection (median survival of 23 days; \( P = 0.2027 \) compared to wild-type strains). These findings provide evidence that Aca1 regulates virulence of \( C. \) neoformans via the Cac1-cAMP pathway.

**DISCUSSION**

**Aca1 controls virulence via cAMP signaling in \( C. \) neoformans.** We identified and characterized here the gene encoding the adenyl cyclase-associated protein (CAP/Srv2) in the basidiomycetous fungus \( C. \) neoformans. Several lines of evidence indicate that Aca1 is a homolog of the CAP/Srv2 protein found in mammalian cells and ascomycetous fungi. First, Aca1 shares structural features conserved with CAP/Srv2 proteins in other organisms. Second, Aca1 interacted with the C-terminal heptad repeat-containing domain (bp 2126 to 2260) of Cac1 in the yeast two-hybrid system. Aca1-Aca1 interactions were ob-

![FIG. 6. Aca1 regulates melanin production in a cAMP-dependent manner but in parallel with Gpa1. (A) The wild-type H99 (WT), YSB6 (aca1\( \Delta \)), YSB117 (aca1\( \Delta \)+ACA1), YSB83 (gpa1\( \Delta \)), YSB42 (cac1\( \Delta \)), YSB166 (aca1\( \Delta \) gpa1\( \Delta \)), YSB170 (aca1\( \Delta \) cac1\( \Delta \)), YSB188 (pka1\( \Delta \)), YSB194 (pka2\( \Delta \)), YSB200 (pka1\( \Delta \) pka2\( \Delta \)), and CHM3 (lac1\( \Delta \)) (Table 1) strains were grown for 16 h at 30\( ^\circ \)C in YPD medium and then spotted onto Niger seed medium with or without 10 mM cAMP at 30 or 37\( ^\circ \)C for 3 days and photographed. (B) For quantitative measurement of laccase activity, \( 10^8 \) cells of the same isogenic strain series in panel A were grown at 30\( ^\circ \)C for 16 h in L-DOPA medium, transferred to 25\( ^\circ \)C, and then further incubated for 6 h. The OD\( _{475} \) of the culture supernatant was determined. One unit of laccase was defined as an OD\( _{475} \) of 0.001. Solid bars present the average from three independent experiments, and error bars show the standard deviations from the mean.
served, as also seen with the *S. cerevisiae* CAP/Srv2 protein (55, 58). Although multimerization of CAP appears to be essential for its localization but not cAMP signaling in *S. cerevisiae*, the role of Aca1 multimerization in *C. neoformans* should be addressed in future studies. Third, as with CAP/Srv2 found in other organisms, Aca1 also modulates cAMP signaling. *aca1* mutations caused defects in mating, capsule and melanin production, and virulence of *C. neoformans*, similar to mutations in other cAMP signaling components, including Gpa1, Cac1, and Pka1. Furthermore, all of the in vitro phenotypes of the *aca1Δ* mutant were restored to wild-type by exogenous cAMP. Finally, Aca1 controls intracellular cAMP levels in response to glucose readadition. The *aca1Δ* mutant maintained a basal level of cAMP but failed to induce cAMP production after the readaddition of glucose. Similar to the *aca1Δ* mutant, the *C. albicans cap1Δ* mutant was found to be defective in induced cAMP levels, but maintained a normal basal level during the bud-hypha transition (4). Therefore, Aca1 encodes a member of CAP/Srv2 protein family regulating cAMP signaling in *C. neoformans*.

Aca1 was found to be an important virulence regulator for *C. neoformans*. The complete absence of virulence of *aca1Δ* mutant strains is likely attributable to lack of capsule and melanin production rather than to defects in mating and filamentation because other mutants showing only defects in mating and filamentous growth (e.g., *ste11Δ, ste7Δ, and cpk1Δ* mutants) are virulent (12). Previously, the adenylyl cyclase-associated protein (Cap1) was found to be a virulence regulator for the ascomycetous pathogenic fungus *C. albicans* (4). Interestingly, avirulence of the *C. albicans cap1Δ* mutant appears to result from its inability to switch between yeast and hyphal growth (4), an essential virulence factor for *C. albicans* (30). Therefore, the adenylyl cyclase-associated proteins Aca1 and Cap1 serve as important virulence regulators for *C. neoformans* and *C. albicans* via conserved cAMP-signaling pathways that drive unique developmental cascades required for the acquisition of virulence.

**Construction of ras1, gpb1, and cAMP cascade mutants by using the congeneric serotype A *C. neoformans* with dominant selectable markers.** Another major contribution of the present study is the recreation of cAMP-independent (*gpb1Δ* and *ras1Δ*)
and cAMP-dependent (gpa1Δ, cac1Δ, pka1Δ, and pka2Δ) mutants by using dominant selectable markers and the congenic serotype A C. neoformans MATα and MATα strains, H99 and KN99, as parental strains. In previous studies, the functions of Gpb1, Ras1, Gpa1, Cac1, and Pka1/2 were analyzed by using mutants created in uracil or adenine auxotrophic parental strain backgrounds (1–3, 50). Here, however, we suggest that use of auxotrophic strains as parents for mutant construction should be avoided, if possible, because earlier auxotrophic strains generated by UV or gamma irradiation may contain undesired background mutations and ura5 mutants have recently been found to be temperature sensitive (23). We found few, if any, examples of problems based on these concerns, but it seems prudent to advocate advancing the state of the art in the field at this time.

New insights into cAMP-dependent and -independent signaling pathways regulating virulence and differentiation of C. neoformans. Our studies further expand existing knowledge about signal transduction systems regulating virulence factors (melanin and capsule) and morphological differentiation (mating and filamentation), which are summarized in Fig. 9. In this model, Aca1 functions as an upstream component of the Cac1-cAMP signaling pathway in parallel with Gpa1. First, aca1Δ mutant strains showed subtle phenotypic differences compared to gpa1Δ mutant strains in cell fusion efficiency and filamentation during mating and in melanin synthesis. Second, disruption of both GPA1 and ACA1 conferred additive defects in melanin production, resulting in defects similar to those observed in cac1Δ mutants, providing evidence that Aca1 and Gpa1 are two necessary and sufficient upstream regulators for Cac1. aca1Δ cac1Δ double-mutant strains were comparable to cac1Δ single mutants, showing that Aca1 signals in a linear fashion with Cac1. We speculate that Aca1 and Gpa1 constitute two upstream components of adenyl cyclase in C. neoformans and serve roles similar to Ras1/2 and Gpa2 in S. cerevisiae.

A key downstream cAMP-signaling element in C. neoformans is PKA. PKA has two catalytic subunits, Pka1 and Pka2, and a single regulatory subunit, Pkr1. Recently, it has been demonstrated that Pka1 plays a major role in regulation of capsule and melanin production in serotype A C. neoformans, whereas Pka2 performs this role in serotype D strain (21). pka1Δ and pka2Δ mutants newly constructed in the present study showed phenotypes consistent with those of the previous study; however, we did uncover a minor role for Pka2 in mating and melanin production through generation of pka1Δ pka2Δ double-mutant strains. pka1Δ pka2Δ double-mutant strains displayed a more severe defect in unilateral mating crosses and melanin biosynthesis than pka1Δ mutants, similar to the cac1Δ mutant or aca1Δ gpa1Δ mutant strains. Thus, Pka1 plays the major and Pka2 a minor role, and the two are necessary and sufficient downstream elements of the Cac1-cAMP signaling pathway for complete mating and melanin production.

We found that Ras1 also has a minor role in melanin production, independent of cAMP signaling. Although ras1Δ single-mutant strains did not show significant defects in melanin production in accord with a previous report (1), aca1Δ ras1Δ and cac1Δ ras1Δ double-mutant strains exhibited a more severe melanin defect than aca1Δ and cac1Δ single mutants, respectively (data not shown). Therefore, Ras1 mediates melanin-inducing signals independent of the cAMP pathway. Previously, it has been shown that overexpression or disruption of STE12 results in induction or reduction of the LAC1 gene, respectively, for melanin production (8, 53) and Ste12 is in the MAPK pathway. Both the MAPK and the cAMP-signaling pathways control mating. The MAPK pathway mediates pheromone-responsive mating signals through the seven transmembrane pheromone receptors Ste3α/a, the Gs subunit Gpb1, the p20-activated protein kinase Ste20α/a, the MAPK kinase kinase Ste11α/a, the MAPK kinase Ste7, and the MAPK Cpk1. Ras1 appears to transmit mating signals through Gpb1 and downstream MAPK components. In contrast, nutritional starvation signals are transduced via the Gα subunit Gpa1, the adenyl cyclase Cac1, cAMP, and the catalytic subunits Pka1/2 and regulatory subunit Pkr1 of PKA. Aca1 appears to activate the Cac1-cAMP pathway independent of Gpa1. Solid lines or arrows indicate the major paths of signal flow and dashed arrows represent less significant events.

FIG. 9. Model of the signaling pathways regulating the virulence and differentiation of C. neoformans. Both the MAPK and the cAMP-signaling pathways control mating. The MAPK pathway mediates pheromone-responsive mating signals through the seven transmembrane pheromone receptors Ste3α/a, the Gs subunit Gpb1, the p20-activated protein kinase Ste20α/a, the MAPK kinase kinase Ste11α/a, the MAPK kinase Ste7, and the MAPK Cpk1. Ras1 appears to transmit mating signals through Gpb1 and downstream MAPK components. In contrast, nutritional starvation signals are transduced via the Gα subunit Gpa1, the adenyl cyclase Cac1, cAMP, and the catalytic subunits Pka1/2 and regulatory subunit Pkr1 of PKA. Aca1 appears to activate the Cac1-cAMP pathway independent of Gpa1. Solid lines or arrows indicate the major paths of signal flow and dashed arrows represent less significant events.
REFERENCES

1. Alspaugh, J. A., L. M. Cavallo, J. R. Perfect, and J. Heitman. 2000. RAS1 regulates filamentation, mating and growth at high temperature of Cryptococcus neoformans. Mol. Microbiol. 36:352–365.

2. Alspaugh, J. A., J. R. Perfect, and J. Heitman. 1997. Cryptococcus neoformans mating and virulence are regulated by the G-protein alpha subunit Gpa1 and cAMP. Genes Dev. 11:3206–3217.

3. Alspaugh, J. A., R. Pukkila-Worley, T. Harashima, L. M. Cavallo, D. Funelli, G. M. Cox, J. R. Perfect, J. W. Kronstad, and J. Heitman. 2002. Adenylyl cyclase functions downstream of the G protein Gpa1 and controls mating and pathogenicity of Cryptococcus neoformans. Eukaryot. Cell 1:75–84.

4. Bahn, Y. S., and P. Sondrheim. 2001. CAP1, an adenylate cyclase-associated protein gene, regulates bud-hypha transitions, filamentous growth, and cAMP levels and is required for virulence of Candida albicans. J. Bacteriol. 183:3211–3223.

5. Bockmühl, D. P., S. Krishnamurthy, M. Gerads, A. Sonneborn, and J. F. Ernst. 1998. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of Candida albicans. Mol. Microbiol. 42:1243–1257.

6. Casadevall, A., and J. R. Perfect. 1998. Cryptococcus neoformans. ASM Press, Washington, D.C.

7. Chang, Y. C., L. A. Penoyer, and K. J. Kwon-Chung. 1996. The second capsule gene of Cryptococcus neoformans, CAP64, is essential for virulence. Infect. Immun. 64:1977–1983.

8. Chang, Y. C., L. A. Penoyer, and K. J. Kwon-Chung. 2001. The second STE12 homologue of Cryptococcus neoformans is MATa-specific and plays an important role in virulence. Proc. Natl. Acad. Sci. USA 98:3258–3263.

9. Cox, G. M., J. Mukherjee, G. T. Cole, A. Casadevall, and J. R. Perfect. 2000. Urease as a virulence factor in experimental cryptococcosis. Infect. Immun. 68:443–448.

10. Davidson, R. C., J. R. Blankenship, P. R. Kraus, M. de Jesus Berrios, C. M. Hull, C. D’Souza, P. Wang, and J. Heitman. 2002. A PCR-based strategy to generate integrative targeting alleles with large regions of homology. Microbiol. 148:2607–2615.

11. Davidson, R. C., M. C. Cruz, R. A. Sia, B. Allen, J. A. Alspaugh, and J. Heitman. 2000. Gene disruption by biolistic transformation in surrogate D strains of Cryptococcus neoformans. Fungal Genet. Biol. 29:58–48.

12. Davidson, R. C., R. B. Nichols, G. M. Cox, J. R. Perfect, and J. Heitman. 2003. A MAP kinase cascade composed of cell type specific and nonspecific elements controls mating and differentiation of the fungal pathogen Cryptococcus neoformans. Mol. Microbiol. 49:469–485.

13. Del Poeta, M., D. L. Toffaletti, T. H. Rude, S. D. Sparks, J. Heitman, and J. R. Perfect. 1999. Cryptococcus neoformans differential gene expression detected in vitro and in vivo with green fluorescent protein. Infect. Immun. 67:1812–1820.

14. D’Souza, C. A., J. A. Alspaugh, C. Vu, T. Harashima, G. M. Cox, J. R. Perfect, and J. Heitman. 2001. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen Cryptococcus neoformans. Mol. Cell. Biol. 21:3179–3191.

15. Fedor-Chaiken, M. R., J. Deschenes, and J. R. Brauch. 1990. SW2, a gene required for RAS activation of adenylate cyclase in yeast. Cell 61:329–340.

16. Field, J., A. Vojtek, R. Ballester, G. Bolger, J. Colicelli, K. Ferguson, J. Gerst, T. Kataoka, T. Michaeili, S. Powers, et al. 1990. Cloning and characterization of CAP, the Saccharomyces cerevisiae gene encoding the 70 kd adenyl cyclase-associated protein. Cell 61:319–327.

17. Fraser, J. A., R. L. Subaran, C. B. Nichols, and J. Heitman. 2003. Recapitulation of the sexual cycle of the primary fungal pathogen Cryptococcus neoformans var. gattii: implications for an outbreak on Vancouver Island, Canada. Eukaryot. Cell. 21036–1045.

18. Freeman, N. L., Z. Chen, J. Horenstein, A. Weber, and J. Field. 1995. An activator monomer binding activity localizes to the carboxyl-terminal half of the Saccharomyces cerevisiae cycle-associated protein. J. Biol. Chem. 270:5680–5685.

19. Freeman, N. L., T. Lila, K. A. Mintzer, Z. Chen, A. J. Pahk, R. Ren, D. G. Drubin, and J. Field. 1996. A conserved proline-rich region of the Saccha-
J. F. Ernst. 2000. Protein kinase A encoded by TPK2 regulates dimorphism of Candida albicans. Mol. Microbiol. 35:386–396.

45. Toda, T., S. Cameron, P. Sass, M. Zoller, and M. Wigler. 1987. Three different genes in Saccharomyces cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase. Cell 50:277–287.

46. Vartivarian, S. E., E. J. Anaissie, R. E. Cowart, H. A. Sprigg, M. J. Tingler, and E. S. Jacobson. 1993. Regulation of cryptococcal capsular polysaccharide by iron. J. Infect. Dis. 167:186–190.

47. Wang, P., J. E. Cutler, J. A. King, and D. A. Palmer. 2004. Mutation of the regulator of G protein signaling Crg1 increases virulence in Cryptococcus neoformans. Eukaryot. Cell 3:1028–1035.

48. Wang, P., and J. Heitman. 1999. Signal transduction cascades regulating mating, filamentation, and virulence in Cryptococcus neoformans. Curr. Opin. Microbiol. 2:358–362.

49. Wang, P., C. B. Nichols, K. B. Lengeler, M. E. Cardenas, G. M. Cox, J. R. Perfect, and J. Heitman. 2002. Mating-type-specific and nonspecific PAK kinases play shared and divergent roles in Cryptococcus neoformans. Eukaryot. Cell 1:257–272.

50. Wang, P., J. R. Perfect, and J. Heitman. 2000. The G-protein beta subunit GPB1 is required for mating and haploid fruiting in Cryptococcus neoformans. Mol. Cell. Biol. 20:352–362.

51. Wang, Y., P. Aisen, and A. Casadevall. 1995. Cryptococcus neoformans melanin and virulence: mechanism of action. Infect. Immun. 63:3131–3136.

52. Waugh, M. S., C. B. Nichols, C. M. DeCesare, G. M. Cox, J. Heitman, and J. A. Alspaugh. 2002. Ras1 and Ras2 contribute shared and unique roles in physiology and virulence of Cryptococcus neoformans. Microbiology 148:191–201.

53. Wickes, B. L., U. Edman, and J. C. Edman. 1997. The Cryptococcus neoformans STE12a gene: a putative Saccharomyces cerevisiae STE12 homologue that is mating type specific. Mol. Microbiol. 26:951–960.

54. Wickes, B. L., M. E. Mayorga, U. Edman, and J. C. Edman. 1996. Dimorphism and haploid fruiting in Cryptococcus neoformans: association with the α-mating type. Proc. Natl. Acad. Sci. USA 93:7327–7331.

55. Yu, J., C. Wang, S. J. Palmieri, B. K. Haarer, and J. Field. 1999. A cytoskeletal-localizing domain in the cyclase-associated protein, CAP/Srv2p, regulates access to a distant SH3-binding site. J. Biol. Chem. 274:19985–19991.

56. Yue, C., L. M. Cavallo, J. A. Alspaugh, P. Wang, G. M. Cox, J. R. Perfect, and J. Heitman. 1999. The STE12a homolog is required for haploid filamentation but largely dispensable for mating and virulence in Cryptococcus neoformans. Genetics 153:1601–1615.

57. Zaragoza, O., B. C. Fries, and A. Casadevall. 2003. Induction of capsule growth in Cryptococcus neoformans by mammalian serum and CO2. Infect. Immun. 71:6155–6164.

58. Zelicof, A., V. Protopopov, D. David, X. Y. Lin, V. Lustgarten, and J. E. Gerst. 1996. Two separate functions are encoded by the carboxyl-terminal domains of the yeast cyclase-associated protein and its mammalian homologs. Dimerization and actin binding. J. Biol. Chem. 271:18243–18252.