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Signaling through Adenylyl Cyclase Is Essential for Hyphal Growth and Virulence in the Pathogenic Fungus *Candida albicans*

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The human fungal pathogen *Candida albicans* switches from a budding yeast form to a polarized hyphal form in response to various external signals. This morphogenetic switching has been implicated in the development of pathogenicity. We have cloned the *CaCDC35* gene encoding *C. albicans* adenylyl cyclase by functional complementation of the conditional growth defect of *Saccharomyces cerevisiae* cells with mutations in Ras1p and Ras2p. It has previously been shown that these Ras homologues regulate adenylyl cyclase in yeast. The *C. albicans* adenylyl cyclase is highly homologous to other fungal adenylyl cyclases but has less sequence similarity with the mammalian enzymes. *C. albicans* cells deleted for both alleles of *CaCDC35* had no detectable cAMP levels, suggesting that this gene encodes the only adenylyl cyclase in *C. albicans*. The homozygous mutant cells were viable but grew more slowly than wild-type cells and were unable to switch from the yeast to the hyphal form under all environmental conditions that we analyzed in vitro. Moreover, this morphogenetic switch was completely blocked in mutant cells undergoing phagocytosis by macrophages. However, morphogenetic switching was restored by exogenous cAMP. On the basis of epistasis experiments, we propose that CaCdc35p acts downstream of the Ras homologue CaRas1p. These epistasis experiments also suggest that the putative transcription factor Efg1p and components of the hyphal-inducing MAP kinase pathway depend on the function of CaCdc35p in their ability to induce morphogenetic switching. Homozygous *cacdc35*Δ cells were unable to establish vaginal infection in a mucosal membrane mouse model and were avirulent in a mouse model for systemic infections. These findings suggest that fungal adenylyl cyclases and other regulators of the cAMP signaling pathway may be useful targets for antifungal drugs.

INTRODUCTION

Candida albicans is a major fungal pathogen of humans, and infections with this fungus are a particular problem in immune compromised patients. *C. albicans* grows in several morphological forms. Under conditions of moderate temperature and low pH and in the absence of inducers such as serum or *N*-acetylglucosamine, the cells grow as budding yeasts (Castilla *et al.*, 1998). Increases in temperature to 37°C, increases in pH, and the addition of inducers can stimulate the formation of filamentous forms. These filamentous forms include pseudohyphae (chains of elongated cells) as well as true hyphae, where growth involves parallel-sided walls with the cells separated by septa (Mitchell, 1998). The finding that mutant strains defective in hyphal growth are avirulent (Leberer *et al.*, 1997; Lo *et al.*, 1997) has implicated the yeast–hyphal transition in *C. albicans* pathogenicity.

Adenosine 3′:5′-cyclic monophosphate (cAMP) plays a role in the differentiation of many fungi, and dimorphic behavior has often been linked to intracellular levels of this cyclic nucleotide. In *Schizosaccharomyces pombe*, deletion of the adenylyl cyclase gene does not affect viability but dere-

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presses conjugation and sporulation under conditions that normally inhibit these differentiation stages in wild-type cells (Kawamukai *et al.*, 1991). In contrast, adenylyl cyclase mutants of *Magnaporthe grisea* have decreased vegetative growth rate, are sterile, and are defective in forming appresoria on an inductive surface and thus are unable to infect susceptible rice leaves (Choi and Dean, 1997). In *Cryptococcus neoformans*, the cAMP signaling pathway functions to sense nutritional signals that regulate mating and the induction of virulence factors such as melanin and capsules (Alspaugh *et al.*, 1997). In *Ustilago maydis,* mutations in the gene encoding adenylyl cyclase cause constitutive growth (Gold *et al.*, 1994), whereas strains with defects in the gene encoding the regulatory subunit of protein kinase A (PKA) are incapable of forming tumors in plants (Gold *et al.*, 1997; Durrenberger *et al.*, 1998). In addition, in *Neurospora crassa* the regulatory subunit of PKA has been demonstrated to play a role in polarized growth and the localization of septa (Bruno *et al.*, 1996), whereas adenylyl cyclase has been shown to control carbon source utilization (Terenzi *et al.*, 1979). Recently, regulation of adenylyl cyclase protein levels in *N. crassa* has been shown to be controlled by a G protein α subunit (Kays *et al.*, 2000).

In the yeast *Saccharomyces cerevisiae*, cAMP signaling has been found to be essential for growth, and together with a mitogen activated protein (MAP) kinase signaling pathway, has been found to play a role in pseudohyphal differentiation (Kronstad *et al.*, 1998; Thevelein and de Winde, 1999; Borges-Walmsley and Walmsley, 2000). Deletion of the *CDC35/CYR1* gene encoding adenylyl cyclase causes yeast cells to arrest in G1 of the cell cycle (Matsumoto *et al.*, 1982; Kataoka *et al.*, 1985). The activity of adenylyl cyclase is regulated by the yeast Ras homologues Ras1p and Ras2p (DeFeo-Jones *et al.*, 1985; Toda *et al.*, 1985; Field *et al.*, 1988). Simultaneous depletion of these homologues is lethal (Kataoka *et al.*, 1984, 1985; Tatchell *et al.*, 1985). Moreover, in addition to the Ras proteins, the G protein α subunit homologue Gpa2p appears also to modulate the activity of adenylyl cyclase (Kubler *et al.*, 1997; Lorenz and Heitman, 1997; Colombo *et al.*, 1998). Mutant cells defective in either *RAS2* or *GPA2* have normal vegetative growth, but double mutant cells exhibit very slow growth even on rich medium. This defect can be suppressed by exogenous cAMP or by mutation in the *PDE2* gene encoding cAMP phosphodiesterase (Kubler *et al.*, 1997; Xue *et al.*, 1998). Ras2p and Gpa2p are both required for filamentous growth in *S. cerevisiae*, and evidence suggests that Ras2p might be the activator of both the cAMP and the MAP kinase pathways that control filamentous growth (Kronstad *et al.*, 1998; Thevelein and de Winde, 1999; Borges-Walmsley and Walmsley, 2000).

In *C. albicans*, a role for cAMP in the yeast to hyphal switch has been controversial. Some research provides evidence for a transient rise in cAMP levels during germ tube formation (Niimi *et al.*, 1980; Chattaway *et al.*, 1981; Sabie and Gadd, 1992), but other studies support a transient decrease due to increased phosphodiesterase activity (Egidy *et al.*, 1990) or provide no evidence at all for fluctuations in cAMP levels during the yeast-to-hyphal switch (Sullivan *et al.*, 1983). Genetic evidence for a potential role of cAMP in dimorphic switching has come from the cloning of the *CaTPK2* gene encoding a *C. albicans* homologue of the catalytic subunit of PKA (Sonneborn *et al.*, 2000). Deletion of both *CaTPK2* alleles interferes with morphogenesis under some environmental conditions and partially reduces virulence in a mouse model for systemic candidiasis. Biochemical characterization of a protein with PKA activity but a molecular weight size that is different from the protein encoded by *CaTPK2* suggests that a second *C. albicans* homologue of the PKA catalytic subunit might exist (Zelada *et al.*, 1998). Consistent with this view is the observation that a second gene encoding a homologue of the catalytic subunit of PKA is present in the *C. albicans* genome (http://sequence-www.stanford.edu/group/candida/index.html).

Additional support for a role of the cAMP pathway in dimorphic switching of *C. albicans* has involved the phenotypic characterization of a gene encoding a homologue of Ras. This homologue has been shown to be required for the yeast-to-hyphal switch (Feng *et al.*, 1999) and to contribute to virulence in an animal model through regulation of the MAP kinase and cAMP signaling pathways (Leberer *et al.*, 2001). In this study, we describe the isolation and functional characterization of the *CaCDC35* gene encoding a homologue of adenylyl cyclase. We provide genetic evidence that signal transduction through adenylyl cyclase contributes to vegetative growth of *C. albicans* cells and is essential for dimorphic differentiation in response to environmental cues and for virulence in mouse models for vaginal and systemic fungal infections.

MATERIALS AND METHODS

Isolation of CaCDC35

The *CaCDC35* gene was isolated in a screen searching for *C. albicans* genes capable of suppressing the temperature-sensitive growth defect of the *ras1*Δ *ras2*^{ts} *S. cerevisiae* strain TTM3–4B (Powers *et al.*, 1989) as described (Leberer *et al.*, 2001). In addition to plasmids carrying *CaRAS1* (Leberer *et al.*, 2001), we isolated from a genomic *C. albicans* library constructed in the *S. cerevisiae* vector YEp352 (Boone *et al.*, 1991) plasmids pDH222 and pDH223 carrying inserts of 2.3 and 5.2 kb, respectively. Plasmid pDH222 carried an open reading frame of 1311 bp encoding the carboxyl terminal catalytic domain of adenylyl cyclase from amino acids 1254–1690. Plasmid pDH223 contained an open reading frame of 4917 bp encoding a carboxyl terminally truncated version of adenylyl cyclase from amino acids 1–1639. To create plasmid pCR0 containing the complete coding region of *CaCDC35*, a 5.1-kb *Sph*I-*Eco*RI fragment of plasmid pDH223 was ligated to a 1.17-kb *Eco*RI fragment from pDH222 in pTZ18R (Mead *et al.*, 1986). To identify more 5′ upstream noncoding sequences of *CaCDC35*, we screened the *C. albicans* genomic library by colony hybridization under high-stringency (Feinberg and Vogelstein, 1983) and isolated plasmid pCR21 containing 2743 bp of sequence upstream of the coding region and 4743 bp of sequence within the coding region of *CaCDC35*.

Yeast Manipulations

The C. *albicans* and *S. cerevisiae* strains used in this study are listed in Table 1. Media and culture conditions for the growth of *C. albicans* and *S. cerevisiae* cells were as described (Rose *et al.*, 1990). All media were supplemented with uridine (25 μg/ml) for the growth of *C. albicans* Ura- strains. Transformation of *S. cerevisiae* and *C. albicans* cells were performed by the lithium acetate method and spheroplast methods, respectively (Rose *et al.*, 1990). Plasmid DNA was isolated from *S. cerevisiae* cells as described (Rose *et al.*, 1990).

Plasmids

All plasmids used in this study are listed in Table 1. PCRs were performed with the use of the Expand High Fidelity or Long Tem-

Table 1. *C. albicans* and *S. cerevisiae* strains used in this study

Strains	Genotype	Source
C. albicans		
SC5314	*CDC35/CDC35 URA3/URA3*	Fonzi and Irwin, 1993
CAI4	*ura3*::λ *imm434/ura3*::*1 imm434*	Fonzi and Irwin, 1993
CR20	*ura3*::λ *imm434/ura3*::*1 imm434 CDC35/cdc35*Δ::*hisG-URA3-hisG*	This study
CR20.1	*ura3*::λ *imm434/ura3*::*1 imm434 CDC35/cdc35*Δ::*hisG*	This study
CR216	*ura3*::λ *imm434/ura3*::*1 imm434 cdc35*Δ::*hisG-URA3-hisG/cdc35*Δ::*hisG*	This study
CR276	*ura3*::λ *imm434/ura3*::*1 imm434 cdc35*Δ::*hisG/cdc35*Δ::*hisG*	This study
CR293	*ura3*::λ *imm434/ura3*::*1 imm434 cdc35*Δ::*hisG/cdc35*Δ::*hisG* [pVEC-*CaCDC35*]	This study
CR323	*ura3*::λ *imm434/ura3*::*1 imm434 cdc35*Δ::*hisG/cdc35*Δ::*hisG* [pVEC]	This study
CR340	*ura3*::λ *imm434/ura3*::*1 imm434 cdc35*Δ::*hisG/cdc35*Δ::*hisG TRP1*::*CDC35 URA3*	This study
DH108+pCR2	*ura3*::λ *imm434/ura3*::*1 imm434 ras1*Δ::*hisG/ras1*Δ::*hisG* + [yPB1-ADHp-*CaCDC35*]	Leberer *et al.*, 2001
S. cerevisiae		
TTM3-4B	*MAT***a** *ras1*Δ::*HIS3* $ras2^{ts}$ *ura3 his3 leu2 trp1 ade8*	Powers *et al.*, 1989

Plasmids	Description	Source
pVEC	*CaARS CaURA3*	Magee and Magee, 1997
pYPB1-ADHpL	*CaADH1* promoter *URA3 Ca.*ARS, *S.c.* 2 μm plasmid	Leberer *et al.*, 1996
p5921	*hisG-CaURA3-hisG*	Fonzi *et al.*, 1993
pCAO1	*CaPCK1* promoter in pUC21	Leuker *et al.*, 1997
pCR0	*CaCDC35 Sph1/Eco*RI in pTZ18R	This study
pCR1	6.2 kb *Bam*HI-*Kpn*I *CDC35* in pVEC	This study
pCR2	5.9 kb *Mlu*I *CaCDC35* in pYPB1-ADHpL	This study
pCR3	5.8 kb *Kpn*I *CaCDC35* in pVEC-CaPCK1p	This study
pCR21	*CaCDC35* (7.5 kb) in YEp352	This study
pCR4	*CaARS CaURA3 CaPCK1p*	This study
pCRF3	*CaCDC35Bam*HI-*Bgl*II*hisG-CaURA3-hisGBamHI-BamHICaCDC35*	This study
PCRQ38	*CaCDC35Bam*HI-*Kpn*I *CaTRP1Kpn*I in pVEC	This study
pDH222	*CaCDC35* (2.3 kb) in YEp352	This study
pDH223	*CaCDC35* (5.2 kb) in YEp352	This study
pDH233	pVEC-*CaRAS1 CaURA3*	Leberer *et al.*, 2001
pDH240	pYPB1-ADHpL-*CaRAS1*	This study
HLB 134	4.2 kb *Hin*dIII *EFG1* fragment in pRC2312	Lo *et al.*, 1997
pADH-HST7	pYPB1-ADHpT-*CaHST7*	Leberer *et al.*, 1996
pLJ19	pYPB1-ADHpL-*CaCPH1*	Csank *et al.*, 1998
pLJ57	pYPB1-ADHpL-$CaRAS1^{G13V}$	This study
pSU1	pVEC-$CaRAS1^{G13V}$	This study
pJA39	pVEC-*CaTRP1*	This study

plate PCR system (Boehringer Mannheim, Laval, Quebec, Canada). Fragments generated by PCR were always sequenced to ensure no unanticipated mutations were introduced during the amplification procedure. To construct pCR1, we amplified a 6.2-kb fragment containing the complete open reading frame of *CaCDC35* and 242 bp of upstream regulatory region with the use of the oligodeoxynucleotides 5′-CGGGATCCGATCAACACATTTTTAAT-3′ and 5′-CGGGTACCTCATGTATCCTTTAGGAA-3′ (the newly created *Bam*HI and *Kpn*I sites, respectively, are underlined) and pCR0 as a template. The amplified fragment was then cloned into pVEC (Magee and Magee, 1997). To construct pCR2, we amplified a 5.9-kb fragment containing the complete open reading frame of *CaCDC35* with the use of the oligonucleotides 5′-CGACGCGT*ATG*AGTTTTTTAAGGAGA-3′ and 5′-CGACGCGTTAATCCAACCAATTGATT-3′ (the newly created *Mlu*I sites are underlined; the start codon is in italics) and pCR0 as template, and cloned the fragment into pYPB1-ADHpL (Leberer *et al.*, 1996). To place *CaCDC35* under the control of the *CaPCK1* promoter, we first constructed plasmid pCR4 by cloning a *Bam*HI-*Bgl*II fragment with the *CaPCK1* promoter from plasmid pCAO1 (Leuker *et al.*, 1997) into pVEC. Next, we PCR-amplified the complete open reading frame of *CaCDC35* with the use of the oligodeoxynucleotides 5′-CGGGTACC*ATG*AGTTTTTTAAGGAGA-3′ and 5′-CGGGTACCCAAATAGTATATAAATCG-3′ (the newly created *Kpn*I sites are underlined; the start codon is in italics) and cloned the amplified 5.9-kb fragment into the *Kpn*I site of pCR4.

The integration plasmid pCRQ38 contains *CaCDC35*, *CaTRP1* for integration into the *TRP1* locus, and *CaURA3* as selectable marker. It was generated by cloning, into the *Bam*HI site of pCR1, a 1.4-kb PCR fragment of the *CaTRP1* gene amplified by PCR with the use of the divergent oligodeoxynucleotide primers OJA19 (5′-CGAGATCTTAAGCCGTGCTGGCGTGAAT-3′) and OJA17 (5′-CGAGATCTCATGAGACACTGGTCTCGCGTCTG-3′) (the newly introduced *Bgl*II sites are underlined) and with the use of plasmid pJA39 as template.

Plasmid pDH240 was constructed by cloning a *Sma*I fragment containing the complete open reading frame of *CaRAS1* (Leberer *et al.*, 2001) into the *Eco*RV site of pYPBl-ADHpL. Plasmid pSU1 was derived from pDH233, which is wild-type *RAS* cloned as a *Pst*I-*Hin*dIII fragment in pVEC, by site-directed mutagenesis with the use of the QuickChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). The oligodeoxynucleotide primer pairs were

5′-GTTGTTGTTGGAGGAGTTGGTGTTGGTAAATCCGC-3′ and 5′-GCGGATTTACCAACACCAACTCCTCCAACAACAAC-3′ for replacing the glycine residue at position 13 into a valine residue. The mutant *RAS1* allele was then cut with *Sma*I and transferred to *Eco*RV cut YPB1-ADHpL to generate pLJ57. Plasmid pJA39 consists of the *C. albicans TRP1* inserted into pVEC.

Deletion of CaCDC35

To construct a *CaCDC35* deletion cassette, a DNA fragment that contained *CaCDC35* flanking sequences from nucleotide positions −242–218 and 5649–6275, respectively, joined with *Bam*HI sites was created by PCR with the use of the divergent oligodeoxynucleotide primers OCR1 (5′-CGGGATCCTTCAAATGGTGGGTAGCTGAG3′) and OCR2 (5′CGGGATCCCACCTTCAGCTGAAGCAACAC-3′; newly introduced *Bam*HI sites are underlined) and plasmid pCR0 as template. The amplified DNA was cleaved with *Bam*HI and ligated with a *Bam*HI-*Bgl*II *hisG-CaURA3-hisG* cassette from plasmid p5921 to yield plasmid pCRF3. This plasmid was linearized with *Sph*I and *Bsp*MII and transformed into the Ura$^-$ *C. albicans* strain CAI4 (Fonzi and Irwin, 1993). Transformants in which the coding region of one of the chromosomal *CaCDC35* alleles was replaced by homologous recombination with the *hisG-CaURA3-hisG* cassette were selected on Ura$^-$ medium. Integration of the cassette into the *CaCDC35* locus was confirmed by Southern blot analysis with the use of a *Sph*I to *Spe*I fragment from nucleotide positions −1366 to −630 as a probe (Figure 1, A and B). Spontaneous Ura$^-$ derivatives were then selected on medium containing 5-fluoro-orotic acid as described (Fonzi and Irwin, 1993). These clones were screened by Southern blot hybridization to identify those that had lost the *CaURA3* gene by intrachromosomal recombination mediated by the *hisG* repeats. The remaining functional allele of *CaCDC35* was then deleted by repeating the same procedure. With the use of this two-step approach, we independently isolated the homozygous *cacdc35Δ/cacdc35Δ* strains CR153 and CR216 that showed the identical structural and phenotypic behavior.

To reintegrate the *CaCDC35* gene into the *CaTRP1* locus, strain CR276 (*cacdc35Δ::hisG/cacdc35Δ::hisG*) was transformed with plasmid pCRQ38 linearized with *Bsi*WI, and recombinants were selected on -ura medium. Correct integration of *CaCDC35* at the *CaTRP1* locus was then confirmed by Southern blot analysis.

Northern Blot Analysis

Northern blots of total RNA and poly(A)$^+$ RNA from *C. albicans* were performed as described (Leberer *et al.*, 1992). The probe for *CaCDC35* was a 3.7-kb *Nhe*I-*Sac*II fragment from nucleotide positions 432-4159, and the *CaACT1* probe was an *Eco*RI-*Hin*dIII fragment of the *CaACT1* gene (Losberger and Ernst, 1989).

Phenotypic Characterization of cacdc35Δ C. albicans *Mutant Cells*

Proliferation of *C. albicans* cells was determined in YPD medium at 30°C. An overnight culture was diluted to OD_{600} = 0.1 into fresh medium and grown at 30°C. The density at 600 nm (OD_{600}) of each culture was determined every hour over a period of 8 h. For the analysis of colony morphology, microphotographs of single colonies were taken directly from Petri plates by phase contrast microscopy. To analyze filaments, microphotographs were taken with a Leitz Aristoplan microscope with the use of Nomarski optics at 1000× magnification (Leitz, Wetzlar, Germany).

Measurements of cAMP levels were performed as described (Lorenz *et al.*, 2000). Briefly, *C. albicans* cells were grown in YPD medium at 30°C to stationary phase for 48 h, washed twice with water and once with MES buffer (10 mM, pH 6, containing 0.5 mM EDTA, pH 7.4), and then resuspended into MES buffer at OD_{600} of 2. After addition of 100 mM glucose, 500-μl aliquots were transferred at different time points to test tubes each containing 600 μl of

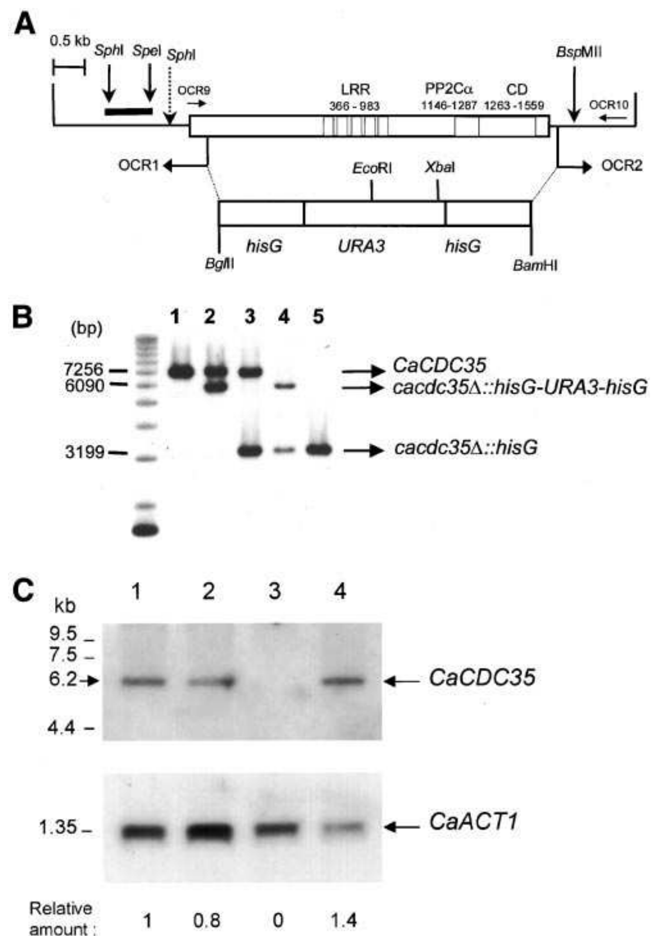


Figure 1. Deletion of *CaCDC35* in *C. albicans.* (A) Restriction endonuclease map of CaCdc35p. The white rectangle indicates the coding region of the gene. The conserved central leucine rich repeat (LRR) motif, the protein phosphatase 2Cα (PP2Cα) domain, and the catalytic domain (CD) are indicated. The PCR with the divergent oligonucleotides OCR1 and OCR2 was used to delete the coding sequence of *CaCDC35.* A *hisG-URA3-hisG* cassette was then inserted, and a two-step procedure was used to delete both alleles of *CaCDC35* by homologous recombination (see MATERIAL AND METHODS). (B) Southern blot analysis with the use of a 0.8-kb *Sph*I-*Spe*I probe of the *CaCDC35* coding region. The genomic DNA samples, digested with *Sph*I and *Bsp*MII, were prepared from strains CAI4 (*CaCDC35/CaCDC35*; lane 1), CR20 (*CaCDC35/cacdc35Δ::hisG-URA3-hisG*, lane 2), CR20.1 (*CaCDC35/cacdc35Δ::hisG*, lane 3), CR216 (*cacdc35Δ::hisG-URA3-hisG/ cacdc35Δ::hisG*, lane 4), and CR276 (*cacdc35Δ::hisG/cacdc35Δ::hisG*, lane 5). The wild-type band was 7.25 kb, whereas the KO band with the *URA3* blaster was 6.1 kb. After looping out the *URA3* fragment, the band was reduced to 3.2 kb. (C) Northern blot analysis of poly(A)$^+$ RNA isolated from strains SC5314 (*CaCDC35/CaCDC35*; lane 1), CR20 (*CaCDC35/cacdc35Δ*; lane2), CR216 (*cacdc35Δ/cacdc35Δ*; lane 3), and CR323 (*cacdc35Δ/cacdc35Δ* [CDC35]; lane 4). The blot was probed with fragments specific for *CaCDC35* or the actin gene (*CaACT1*) and quantified by phosphorimaging. The ratios at the bottom of each lane represent the amount of the *CaCDC35* transcript relative to the *CaACT1* transcript in the same lane. The relative overexpression of the reintegrant construct may be due to the consequence of having only a limited region of the promoter on the plasmid as well as to the influence of the site of integration on general expression levels.

acid-washed glass beads and 500 μl of 10% trichloroacetic acid. The tubes were mixed and immediately frozen in liquid nitrogen. After 30 min, the cells were thawed, disrupted by a bead-beater at 4°C, and centrifuged for 10 min at 20,000 $\times$ *g*. The samples were neutralized by washing the supernatant five times with water-saturated ether, lyophilized, and then resuspended in 500 μl of assay buffer (0.05 M acetate buffer, pH 5.8, 0.02% [wt/vol] bovine serum albumin). cAMP levels were determined by with the use of the EIA system–cAMP immunoassay (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

Macrophage Cytopathology Assays

The mouse macrophage cell line RAW264.7 clone D3 (kindly provided by A. Descoteaux, IAF, Laval, Canada) was cultured in an eight-chambered Permanox slide (Lab-Tek, Naperville, IL) at a cell density of 10^5 cells/well in Dulbecco's modified Eagle's medium (DMEM; Life Technologies-BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (D-10) at 37°C in a 5% CO_2 atmosphere for 24 h. Before the addition of *C. albicans* cells, the chambers were washed once with D-10 medium.

C. albicans cells were grown in YPD medium at 30°C to stationary phase and then washed twice with phosphate-buffered saline, pH 7.4 (PBS). These cells were then added to macrophages at a ratio of 2.5:1. After incubation at 37°C in 5% CO_2 atmosphere for 1–4 h, slides were washed three times with D-10 medium, incubated at 4°C for 1 h with 100 μl of rabbit anti–*C. albicans* antibodies (Accurate Chemical & Scientific Corp., Westbury, NY) diluted 1:200 in D-10 medium. Slides were washed four times with cold D-10 medium, fixed with the use of HEMA3 fixative (Biochemical Sciences Inc.) according to the manufacturer's instructions, washed three times with D-10 medium, and then incubated with FITC-conjugated $F(ab)'_2$ donkey anti-rabbit antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:100 in D-10. After 45 min of incubation at room temperature, wells were washed four times with PBS, and the slides were mounted in Prolong antifade mounting medium (Molecular Probes, Eugene, OR). Epifluorescence was monitored with the use of a Leitz Laborlux S microscope equipped with a CoolSnap CCD camera (Photometrics, Tucson, AZ) at a magnification of 1000×.

To analyze the survival rate of *C. albicans* cells exposed to macrophages, we used an end-point dilution survival assay. One milliliter of a saturated *C. albicans* culture grown in YPD was washed twice in D-PBS, sonicated, and resuspended at 10^7 cells/ml in cold D-10. Fifty microliters of the suspension was added to 150 μl of D-10 in 96-well plates containing medium only or macrophages. After serial fourfold dilutions, plates were incubated on ice for 30 min and subsequently for 48 h at 37°C in 5% CO_2 atmosphere. Colonies were visualized with the use of a Nikon (Garden City, NY) TMS inverted microscope at 20× or 100× magnification.

Virulence Studies

Eight- to 10-week-old female BALB/c mice were obtained from Charles Rivers Breeding Laboratories (Sulzfeld, Germany). Mucosal infection of the vaginal canal was initiated by inoculating mice (5–8 for each group) intravaginally with 5×10^4 stationary phase cells of the wild-type strain CR340 or 4×10^5 stationary phase cells of the homozygous *cacdc35*Δ mutant strain CR323 taken up in 20 μl of PBS (Fidel *et al.*, 1993). To induce pseudoestrus during the infection, mice were injected subcutaneously with 0.02 mg/mouse estradiol valerate (Sigma Chemical, St. Louis, MO) in 0.1 ml sesame oil 72 h before inoculation with *C. albicans* cells (Sobel *et al.*, 1985; Ryley and McGregor, 1986; Fidel *et al.*, 1993). Estradiol treatments were continued at weekly intervals thereafter. After killing, the vaginas of the animals were lavaged with 100 μl of PBS, and the vaginal fungal burden was quantified by determination of colony-forming units (CFU) as previously described (Fidel *et al.*, 1993).

For systemic infections, groups of 10 mice were inoculated with 5×10^5 wild-type cells of strain CR340 or 4×10^6 mutant cells of strain CR323 by intravenous injection and monitored for survival as described (Csank *et al.*, 1997, 1998; Timpel *et al.*, 1998). The eightfold excess of mutant cells was used to compensate for the slower growth rate of these cells. Survival curves were calculated according to the Kaplan-Meier method with the use of the PRISM program (GraphPad Software, San Diego, CA) and analyzed by with the use of the log-rank test. A p value < 0.05 was considered as significant.

Accession Number

The GenBank/EMBL Data Library accession number for *CaCDC35* is AF295379.

RESULTS

Isolation and Characterization of CaCDC35

The *CaCDC35* gene was identified in a screen searching for *C. albicans* genes capable of complementing the conditional growth defect of *S. cerevisiae ras1Δ ras2^ts^* cells. In addition to clones carrying the *CaRAS1* gene (Leberer *et al.*, 2001), we isolated two overlapping clones with the potential to encode either the carboxyl terminal catalytic domain of adenylyl cyclase from amino acid positions 1253–1690 or a carboxyl terminally truncated version of adenylyl cyclase from amino acid positions 1–1639, respectively. The alignment of both sequences generated an open reading frame that contains no introns and is predicted to encode a protein of 1690 amino acids with a calculated molecular weight of 186.9 kDa. Our sequence is identical to that recently determined independently (Mallet *et al.*, 2000).

CaCdc35p has overall sequence identities of 32, 26, and 21% with the homologues of *S. cerevisiae* (Kataoka *et al.*, 1985), *M. grisea* (Choi and Dean, 1997), and *U. maydis* (Gold *et al.*, 1994), respectively. Like the other currently known fungal homologues, CaCdc35p has a domain structure typical of peripheral membrane adenylyl cyclases lacking a transmembrane domain (Tang and Gilman, 1992). Typical for this type of adenylyl cyclases, CaCdc35p has from amino acids 366–983 a central domain composed of amphipathic leucine-rich repeats of 23 amino acids (Figure 1A). This region has 42% identity and 58% similarity to Cdc35p from *S. cerevisiae*. In *S. cerevisiae*, this region has been shown to be required for the interaction of adenylyl cyclase with the Ras homologues Ras1p and Ras2p (Suzuki *et al.*, 1990).

The carboxyl terminal half of CaCdc35p contains the ATP-binding catalytic domain from amino acid positions 1263–1559 (Figure 1A). This domain has 26% identity and 48% similarity with the homologous domain of Cdc35p from *S. cerevisiae*, but shows less homology to the catalytic domains of adenylyl cyclases from mammalian cells (Tesmer *et al.*, 1997). Like the homologue from *S. cerevisiae* (Tamura *et al.*, 1989), CaCdc35p contains a protein phosphatase 2Cα-like domain in the region between the central and catalytic domains from amino acids 1146–1287 with 31% homology to human protein phosphatase 2Cα (Figure 1A; Mann *et al.*, 1992). In comparison to the homologue from *S. cerevisiae*, CaCdc35p lacks a stretch of sequence of 381 amino acids at the amino terminus. The function of this 42-kDa amino terminal region is not known, although a 100-amino acid region just N-terminal to the central domain has been shown

Table 2. Growth at 30°C in YPD

Strain	Doubling time (h)
SC5314 (*CaCDC35/CaCDC35*)	1.46 ± 0.01
CR216 (*cacdc35Δ/cacdc35Δ*)	4.19 ± 0.01
CR340 (*cacdc35/cacdc35Δ TRP1::CDC35*)	1.54 ± 0.02
CR216 (+10 mM dibutyryl cAMP)	1.69 ± 0.03

Data represent mean values ± SD of three independent experiments.

to be required for optimal regulation of *S. cerevisiae* adenylyl cyclase by Ras (Colicelli *et al.*, 1990).

Chromosomal Deletion of CaCDC35

Both *CaCDC35* alleles were deleted in strain CAI4 by homologous recombination in a multistep procedure (Figure 1A). The deletions were confirmed by Southern blot analysis (Figure 1B) and by PCR (our unpublished results). Northern blots showed that the level of the *CaCDC35* transcript, which had a size of 6.2 kb, was reduced to ~80% in cells containing a deletion of one allele of *CaCDC35* relative to the wild-type strain, and was absent in cells deleted for both alleles (Figure 1C). This transcript was present at about a 40% increased level relative to the wild-type strain when the *CaCDC35* gene was reintegrated into the *CaTRP1* locus of homozygous mutant cells (Figure 1C, lane 4).

We found that cells deleted for both alleles were viable but grew ~2.5-fold slower than wild-type cells (Table 2). This attenuated growth was observed in media containing glucose as well as in media containing galactose, glycerol, or ethanol as carbon sources (our unpublished results). The growth defect could be complemented by either reintegration of the wild-type gene into the *CaTRP1* locus of homozygous mutant cells or addition of exogenous cAMP (Table 2),

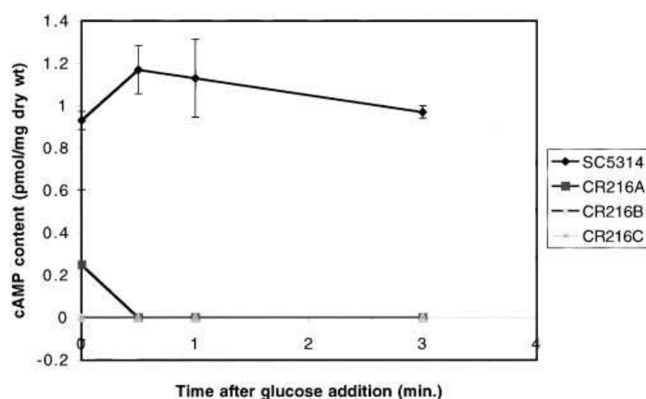


Figure 2. Intracellular cAMP concentrations in strains SC5314 (wild-type) and three isolated colonies A, B, and C of CR216 (*cacdc35Δ::hisG-URA3-hisG/cacdc35Δ*). After addition of 100 mM glucose to the medium, cells were harvested at the indicated time points, and cAMP levels were determined as described in MATERIAL AND METHODS. The data represent mean values of two independent measurements.

demonstrating that the defect in growth of mutant cells was caused by disruption of the function of adenylyl cyclase.

As shown in Figure 2, cAMP levels were undetectable in homozygous mutant cells demonstrating that deletion of both copies of *CaCDC35* resulted in a complete loss of cAMP production. In agreement with previous work (Niimi, 1984), we found that the glucose-induced cAMP burst normally observed in *S. cerevisiae* cells (Mbonyi *et al.*, 1988) is not well developed in *C. albicans* cells (Figure 2).

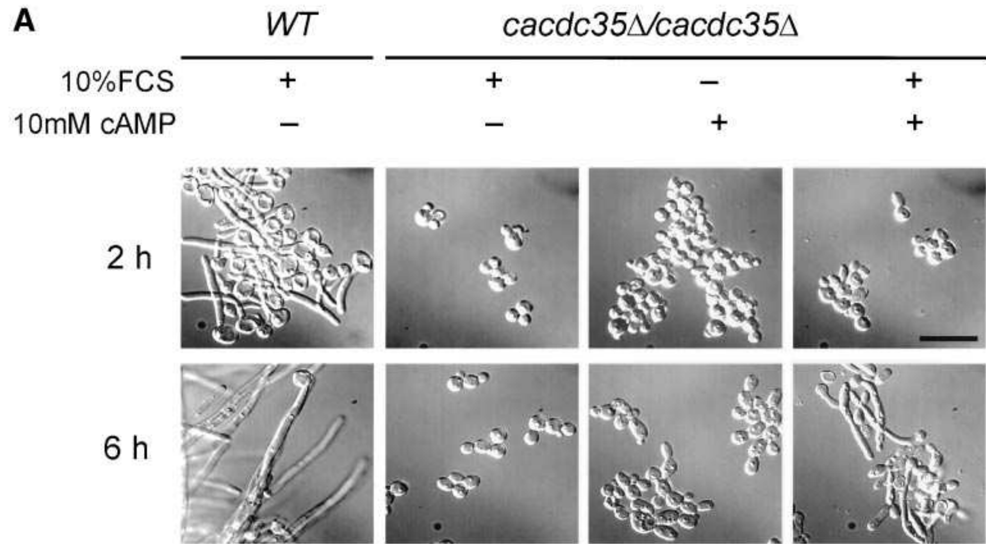
The ability of *C. albicans* cells to switch from a yeast-like to a hyphal mode of growth was not affected by deletion of only one allele of *CaCDC35* (our unpublished results). However, deletion of both alleles completely blocked the yeast-to-hyphal transition in all media and under all conditions that we investigated. When morphological switching was induced in liquid media by either serum or Lee's medium, the homozygous mutant cells were completely defective in the formation of germ tubes or filaments (Figure 3A). On solid agar plates containing serum or solid Lee's medium (as well as SLAD or Spider media), the normal formation of hyphae was completely suppressed by deletion of both alleles of *CaCDC35* (Figure 3B). These defects were reversed by either addition of exogenous cAMP (Figure 3A) or by reintegration of the wild-type *CaCDC35* gene into the *CaTRP1* locus of the homozygous mutant cells (our unpublished results).

The inability of homozygous mutant cells to switch into the hyphal mode of growth was also corrected by introduction of the *CaCDC35* gene on an autonomously replicating plasmid (Figure 4A). However, overexpression of either the *CaRAS1, HST7,* or *CPH1* genes driven by the strong ADH1 promoter or moderate overexpression of the *EFG1* gene driven by the *CaPCK1* promoter failed to suppress the hyphal switch defect (Figure 4A).

In agreement with previous observations (Feng *et al.*, 1999; Leberer *et al.*, 2001), we found that expression of the hyperactive G13V mutant version of CaRas1p in wild-type cells both induced the formation of hyphae under conditions that favor the yeast mode of growth and enhanced the formation of hyphae under inducing conditions (Figure 4B). These effects of the hyperactive CaRas1p mutant allele were completely blocked by deletion of both *CaCDC35* alleles (Figure 4B).

Role of CaCdc35p in Phagocytosis of C. albicans *Cells by Macrophages*

We monitored the uptake of *C. albicans* cells by macrophages through an immunofluorescence procedure that distinguished between *C. albicans* cells that were either free in the medium or attached to the external surface of macrophages from those cells that were undergoing phagocytosis. Cells that were not yet undergoing phagocytosis were accessible to antibodies and hence could be immunostained, whereas cells already engulfed by the macrophages were not stained by this procedure. Candida cells, either in yeast or hyphal form, could be phagocytosed (Figure 5 and our unpublished results). *C. albicans* wild-type cells developed long hyphal tubes either inside or outside of macrophages. This growth allowed them to escape the macrophage engulfment. In contrast, the homozygous *cacdc35Δ/cacdc35Δ* mutant cells engulfed by macrophages were completely blocked in the formation of hyphae, and this inability to form hyphae prevents them from escaping phagocytosis. Their slower



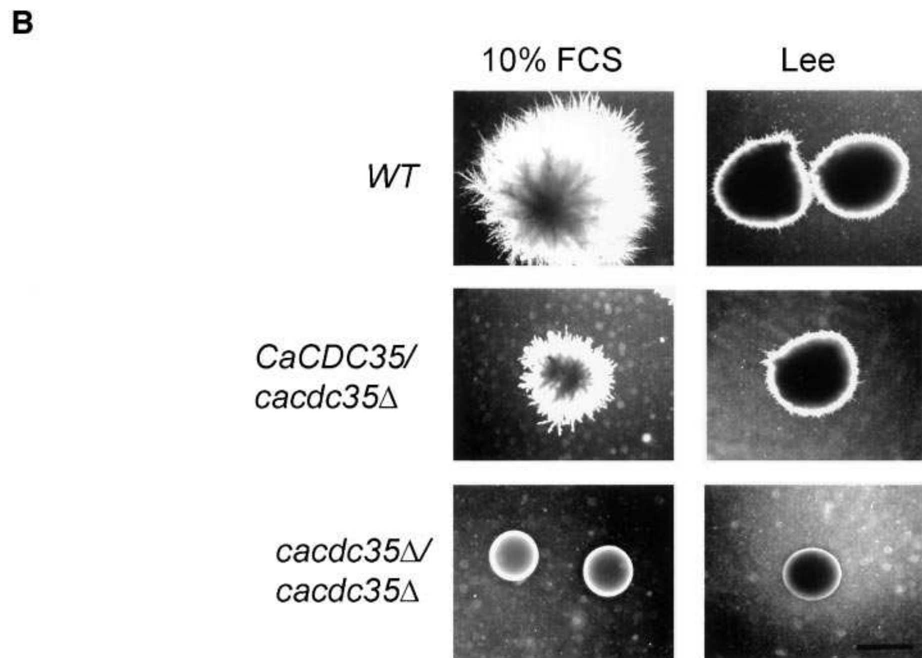


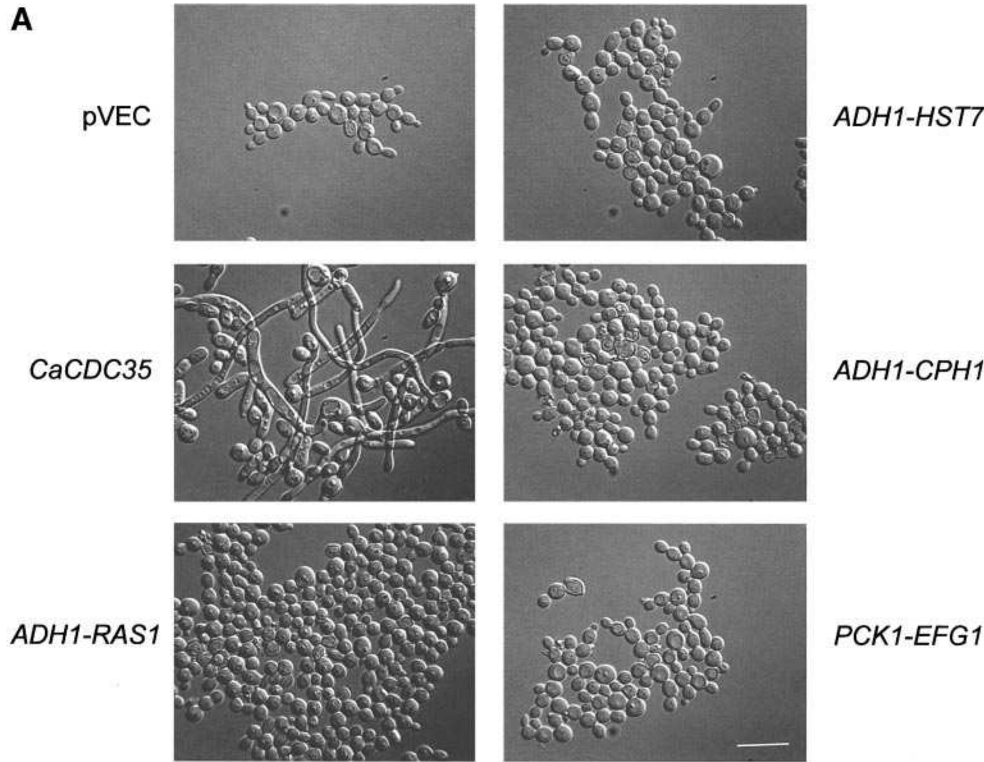
Figure 3. Defects in hyphal formation caused by deletion of both *CaCDC35* alleles. (A) The *CaCDC35* wild-type strain *SC5314* (WT) and strain CR216 deleted for both alleles of *CaCDC35* (*cacdc35Δ/cacdc35Δ*) were grown for 2 or 6 h at 37°C in liquid Lee's medium with or without (+/−) 10% fetal calf serum (FCS) and with or without (+/−) 10 mM dibutyryl-cAMP. Photomicrographs were taken by Nomarski optics at 1000× magnification. Scale bar, 10 μm. (B) The *CaCDC35* wild-type strain *SC5314* (WT), the heterozygous strain CR20 (*CaCDC35/cacdc35Δ*), and the homozygous strain CR216 (*cacdc35Δ/cacdc35Δ*) were grown for 5 d at 37°C on either solid agar medium containing 10% fetal calf serum (FCS) or on solid Lee's medium (Lee *et al.*, 1975). The same defects in hyphal growth were observed on solid low ammonia dextrose nitrogen starvation medium (SLAD; Gimeno *et al.*, 1992) and Spider medium (Liu *et al.*, 1994; our unpublished results). Scale bar, 2 mm. Photomicrographs were taken with the use of phase contrast at 20× magnification.

growth rate may also contribute to their susceptibility. Quantification of this host–pathogen interaction with the use of an end-point dilution survival assay (see MATERIALS AND METHODS) indicated that *C. albicans* adenylyl cyclase defective cells were more readily killed through interaction with macrophages than were the wild-type cells (Table 3).

Requirement of Adenylyl Cyclase for Virulence

To investigate whether CaCdc35p is required for virulence in a mucosal model of murine candidiasis, we inoculated mice intravaginally with *C. albicans* cells and monitored for fungal survival on the vaginal mucosa. In contrast to homozygous mutant cells transformed with a plasmid carrying the wild-type *CaCDC35* gene, mutant cells carrying an empty control plasmid were completely cleared from the vaginal mucosa by day 15, although the initial number of cells applied to the mucosa was eightfold higher for mutant cells than for wild-type cells to compensate for their slower growth rate (Figure 6).

To investigate whether CaCdc35p is required for virulence in systemic candidiasis, mice were inoculated intravenously with *C. albicans* cells and monitored for survival. As illustrated in Figure 7, inoculation with homozygous mutant cells transformed with a plasmid carrying the *CaCDC35* gene resulted in complete mortality after 16 d. However, all



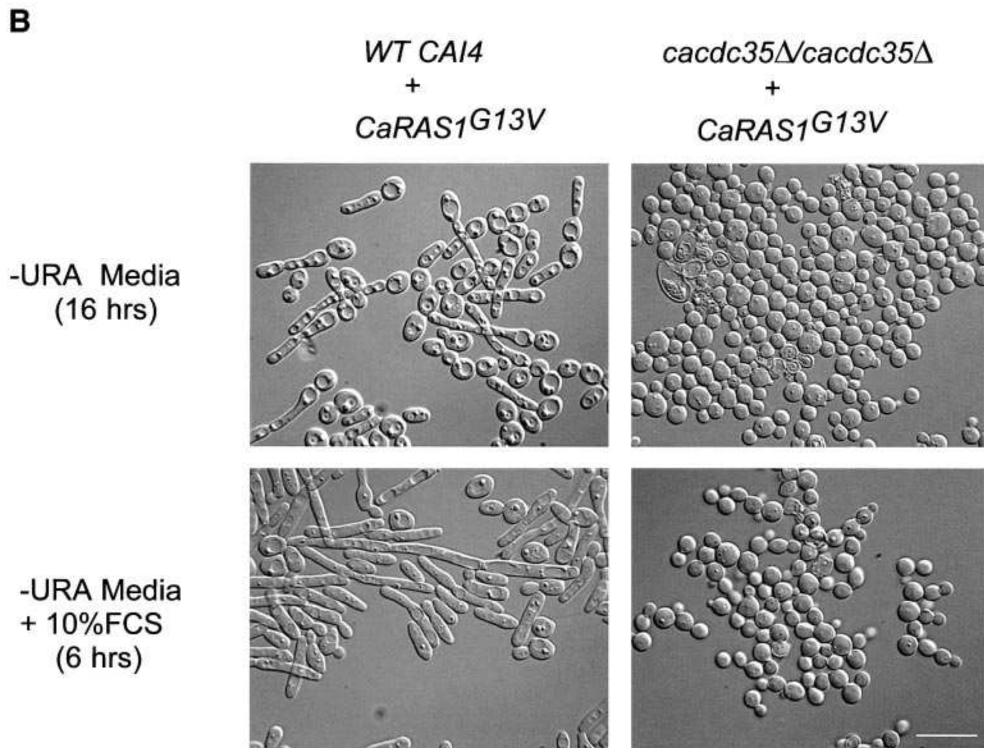


Figure 4. Epistasis analysis. (A) *C. albicans* strain CR216 deleted for both alleles of *CaCDC35* was transformed with the empty control plasmid pVEC and plasmids pVEC-*CaCDC35*, pYPB1-ADHpL-*CaRAS1*, pYPBL-ADHpT-*HST7*, pYPBl-ADHpL-*CPH1*, and pRC2312–*EFG1* carrying the indicated *C. albicans* genes. Transformants were grown for 6 h at 37°C in selective medium containing 10% fetal calf serum (FCS). To induce expression of *EFG1*, 2% casamino acids were added to the medium. (B) Strains CAI4 (WT) and CR216 deleted for both alleles of *CaCDC35* were transformed with pYPBl-ADHpL-*CaRAS1*^{G13V} carrying the hyperactive mutant version of *CaRAS1* and grown for 16 h in selective medium at 30°C (two top panels). The overnight cultures were transferred to fresh -URA medium containing 10% FCS and grown for 6 h at 37°C (two bottom panels). Photomicrographs were taken by Nomarski optics at a 1000× magnification (scale bar, 10 μm) and are representative of many cells.

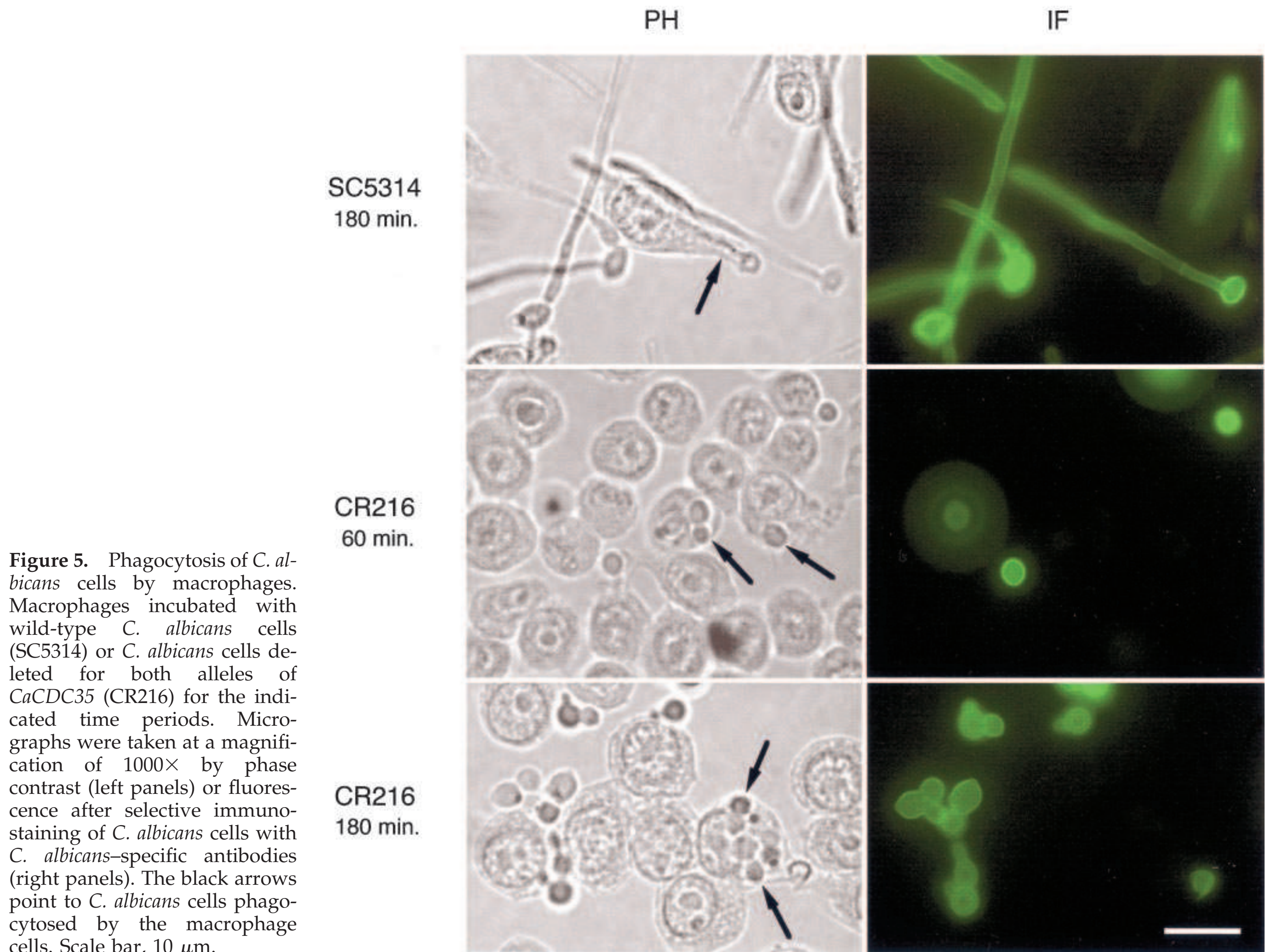


Figure 5. Phagocytosis of *C. albicans* cells by macrophages. Macrophages incubated with wild-type *C. albicans* cells (SC5314) or *C. albicans* cells deleted for both alleles of *CaCDC35* (CR216) for the indicated time periods. Micrographs were taken at a magnification of 1000× by phase contrast (left panels) or fluorescence after selective immunostaining of *C. albicans* cells with *C. albicans*–specific antibodies (right panels). The black arrows point to *C. albicans* cells phagocytosed by the macrophage cells. Scale bar, 10 μm.

mice that were infected with mutant cells transformed with an empty control plasmid survived for at least 42 d, even when the number of *C. albicans* mutant cells injected into the animals was eightfold higher than the inoculation number of wild-type cells to compensate for their slower growth rate (Figure 7). All of the animals infected with mutant cells showed absolutely no clinical signs of disease.

DISCUSSION

We have cloned and sequenced the *C. albicans CaCDC35* gene, which encodes a homologue of fungal adenylyl cyclases. In contrast to mammalian adenylyl cyclases that are characterized by two blocks of transmembrane segments, fungal adenylyl cyclases lack transmembrane domains and

Table 3. Survival of *Candida albicans* cells cultured with macrophages (cell line RAW264.7)

	No. of colonies in presence (+) or absence (−) of macrophages						
	Exp. 1		Exp. 2		Exp. 3		
Strains	−	+	−	+	−	+	% Survival[a]
SC5314 (wild type)	272	191	153	109	436	281	68.6 ± 3.6
CR216 (*cacdc35*Δ/*cacdc35*Δ)	141	4	177	9	489	27	4.5 ± 1.5

Survival of *C. albicans* cells was determined as described in MATERIALS AND METHODS and is expressed as the number of colonies in the presence of macrophages divided by the number of colonies in the absence of macrophages.
[a] Data represent mean value ± SD of three independent experiments.

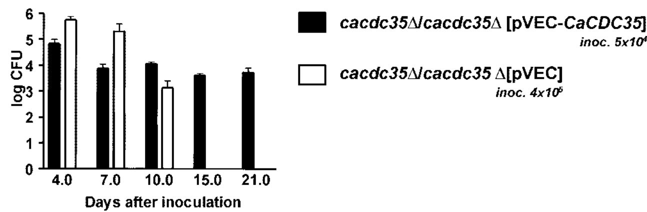


Figure 6. Vaginal infection. Mice were intravaginally inoculated with 5×10^4 cells of strain CR340 (*cacdc35Δ/cacdc35Δ* [pVEC-CaCDC35]; dark bars) and 4×10^5 cells of strain CR323 (*cacdc35Δ/cacdc35Δ* [pVEC]; open bars). *C. albicans* cells were then recovered from the vaginal canal at the indicated time points and colony forming units (CFU) were determined. The data represent log mean values ± SD of 10 independent experiments.

appear to be peripherally bound to the cell membrane (Tang and Gilman, 1992). CaCdc35p has a domain structure typical of the currently known fungal adenylyl cyclases (Figure 1A) and has some similarity to the mammalian isoforms around the catalytic core. Other similarity found with mammalian proteins is located in a region of unknown function that shows 31% homology with protein phosphatase 2Cα. In contrast to the mammalian enzymes, the fungal isoforms appear to be regulated by Ras through the interaction of this small GTP binding protein with a leucine-rich repeat domain in the central region of adenylyl cyclase (Suzuki *et al.*, 1990). This Ras binding domain is highly conserved in CaCdc35 suggesting that the *C. albicans* protein has the potential to interact with the recently identified *C. albicans* Ras homologue CaRas1p (Feng *et al.*, 1999).

Deletion of both *CaCDC35* alleles completely abolished detectable levels of intracellular cAMP (Figure 2), suggesting that CaCdc35p is the only enzyme capable of producing cAMP in *C. albicans* cells. The mutant cells were viable, indicating that cAMP is not essential for vegetative growth in *C. albicans* cells. However, mutant cells exhibited significantly reduced growth rates (Table 2). This mutant phenotype could be complemented by addition of exogenous cAMP (Table 2), suggesting that cAMP-dependent mechanisms contribute to not yet identified steps during vegetative growth of *C. albicans*. In this context, *C. albicans* resembles *M. grisea, U. maydis, S. pombe,* and *N. crassa,* where adenylyl cyclase genes are not essential (Terenzi *et al.*, 1976; Kawamukai *et al.*, 1991; Gold *et al.*, 1994; Choi and Dean, 1997) but differs from *S. cerevisiae,* where Cdc35p is essential for progression of cells through G1 of the cell cycle (Matsumoto *et al.*, 1982; Kataoka *et al.*, 1985). It is tempting to speculate that the reason for this functional diversity may be an important attribute in *C. albicans* for resisting stresses such as nutritional limitation. Although in *S. cerevisiae* the cAMP signaling pathway is primarily involved in mediating nutritional signals to the cell cycle machinery, in *C. albicans* the main function of this pathway could be to mediate stress signals to the morphogenetic machinery that controls the yeast-to-hyphal transition as part of a survival strategy. The observation that the highly similar cyclase proteins of *C. albicans* and *S. cerevisiae* provide an essential function in one organism and not the other suggests that it is the function of the downstream targets that determine whether cAMP formation is required for viability.

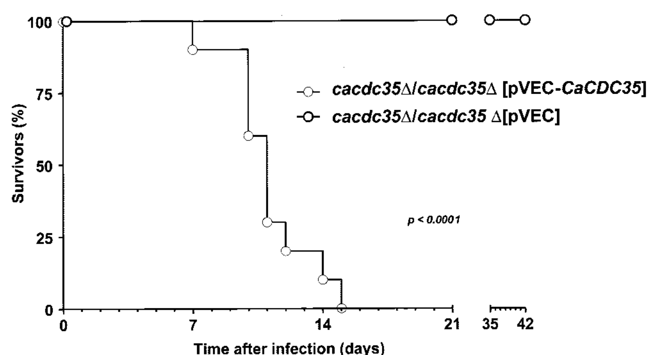


Figure 7. Survival curves of mice (10 for each group) intravenously infected with 5×10^5 cells of *C. albicans* strains CR340 (*cacdc35Δ/cacdc35Δ* [pVEC-CaCDC35]; light open circle) and 4×10^6 cells of strain CR323 (*cacdc35Δ/cacdc35Δ* [pVEC]; bold open circle).

This interpretation is consistent with our finding that *C. albicans* cells deleted for both *CaCDC35* alleles are completely deficient in the ability to switch from the yeast mode of growth into the hyphal mode under all environmental conditions that we investigated (Figure 3). This morphogenetic defect could be cured by addition of exogenous cAMP (Figure 3A), demonstrating that the catalytic function of CaCdc35p is responsible for the induction of hyphal formation in response to environmental cues. Consistent with the view that Ras is a regulator of fungal adenylyl cyclases (DeFeo-Jones *et al.*, 1985; Toda *et al.*, 1985; Broek *et al.*, 1987; Field *et al.*, 1988), the filament-inducing activity of the hyperactive G13V mutant version of CaRas1p was completely blocked in CaCdc35p-deleted mutant cells (Figure 4B). This epistatic relationship places the function of CaRas1p upstream of adenylyl cyclase.

We have previously proposed that coordinated activation of both the filament-inducing MAP kinase cascade and the cAMP signaling pathway initiates morphogenetic processes in a Ras-dependent manner (Leberer *et al.*, 2001). The requirement for dual regulation of morphogenetic switching is corroborated by our studies reported here. In contrast to the morphogenetic switching defect of homozygous *caras1Δ* mutant cells (Feng *et al.*, 1999), the switching defect of homozygous *cacdc35Δ* mutant cells could not be complemented by overexpression of Hst7p or Cph1p (a protein kinase and a transcription factor, respectively, in the filament inducing MAP kinase cascade; Liu *et al.*, 1994; Kohler and Fink, 1996; Leberer *et al.*, 1996) or Efg1p (a putative transcription factor believed to respond to the cAMP signaling pathway; Stoldt *et al.*, 1997; Whiteway, 2000; Figure 4A). A plausible interpretation for this observation is that stimulation of the filament-inducing MAP kinase cascade is only capable of inducing hyphal formation during simultaneous elevation of cAMP levels and that Efg1p is a direct target of the cAMP pathway requiring cAMP-dependent activation. By analogy with the dual regulation of the *S. cerevisiae* adenylyl cyclase by Ras and Gpa2p (Thevelein and de Winde, 1999), it is tempting to speculate that in homozygous *caras1Δ* mutant cells this requirement is fulfilled through stimulation of CaCdc35p by a G protein α subunit homologue similar to Gpa2p in *S. cerevisiae*. This explanation is

supported by the findings that overexpression of either components of the filament-inducing MAP kinase cascade or of Efg1p can complement the yeast-to-hyphal switching defect of homozygous *caras1*Δ mutant cells (Leberer *et al.*, 2001). In addition, overexpression of Efg1p complements the switching defect of mutant cells deleted for a homologue of protein kinase A (Sonneborn *et al.*, 2000).

Our finding that adenylyl cyclase mutants defective in the formation of hyphae are more vulnerable to phagocytosis by macrophages (Table 3) supports the hypothesis that the yeast-to-hyphal transition is part of a survival strategy of *C. albicans* to escape the cellular immune system (Lo *et al.*, 1997). This view is supported by our in vivo studies in mouse models for mucosal and systemic infections. In contrast to wild-type cells, mutant cells were rapidly eradicated from the mucosa after vaginal infection (Figure 6). Mice intravenously infected with mutant cells survived without any clinical signs of disease, whereas mice infected with wild-type cells were efficiently killed (Figure 7). It is unclear whether the defects in virulence are caused by reduced growth of the mutant cells or by defects in morphogenetic switching. However, because we have used eight times more mutant cells than wild-type cells for infection of the animals, it is more likely that the defects in virulence were caused by defects in morphological transitions than by the reduced growth.

In summary, we propose that CaCdc35p represents a key regulatory element in the cAMP signaling pathway and is part of a sensory system involved in detecting changes in the environment and sending signals to a morphogenetic machinery that controls the mode of growth. These interconnected signaling and morphogenetic systems are part of a strategy of *C. albicans* to resist environmental stresses and thereby contribute to the virulence of this human pathogen. Because CaCdc35p and other fungal adenylyl cyclases differ significantly in their type of regulation from their mammalian counterparts, the fungal adenylyl cyclases and their regulators could represent very attractive targets for the identification of specific inhibitors with antifungal activity.

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