

# Cdc24, the GDP-GTP Exchange Factor for Cdc42, Is Required for Invasive Hyphal Growth of *Candida albicans*

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***Candida albicans*, the most common human fungal pathogen, is particularly problematic for immunocompromised individuals. The reversible transition of this fungal pathogen to a filamentous form that invades host tissue is important for its virulence. Although different signaling pathways such as a mitogen-activated protein kinase and a protein kinase A cascade are critical for this morphological transition, the function of polarity establishment proteins in this process has not been determined. We examined the role of four different polarity establishment proteins in *C. albicans* invasive growth and virulence by using strains in which one copy of each gene was deleted and the other copy expressed behind the regulatable promoter *MET3*. Strikingly, mutants with ectopic expression of either the Rho G-protein Cdc42 or its exchange factor Cdc24 are unable to form invasive hyphal filaments and germ tubes in response to serum or elevated temperature and yet grow normally as a budding yeast. Furthermore, these mutants are avirulent in a mouse model for systemic infection. This function of the Cdc42 GTPase module is not simply a general feature of polarity establishment proteins. Mutants with ectopic expression of the SH3 domain containing protein Bem1 or the Ras-like G-protein Bud1 can grow in an invasive fashion and are virulent in mice, albeit with reduced efficiency. These results indicate that a specific regulation of Cdc24/Cdc42 activity is required for invasive hyphal growth and suggest that these proteins are required for pathogenicity of *C. albicans*.**

In response to intra- and extracellular signals, the Rho family GTPase Cdc42 and its GDP-GTP exchange factors are crucial for cytoskeletal reorganization and transcriptional activation in eukaryotes. In *Saccharomyces cerevisiae*, Cdc42 and its exchange factor Cdc24 are necessary for polarized growth during budding and mating (14). In addition, Cdc42 has been shown to be required for pseudohyphal growth in *S. cerevisiae* (27, 28), a developmental state similar in many respects to hyphal growth of fungal pathogens (20). At least two signaling pathways are important for pseudohyphal growth: a cyclic AMP (cAMP)-dependent protein kinase pathway and a mitogen-activated protein (MAP) kinase-dependent pathway (28, 32, 33, 36). Ras signaling, via Ras2, is important in both of these pathways (16, 23, 28). Three of the four kinases in the MAP kinase cascade are also required for signaling in response to pheromone (8, 24, 35).

Dominant *cdc42* mutants have been used to demonstrate that this G protein is involved in the MAP kinase signaling required for pseudohyphal growth (28). More recently, *cdc42* mutants specifically defective in pseudohyphal growth have been isolated (27). Characterization of these mutants suggests that interactions between this G protein and PAK family kinases (Ste20 and Skm1) and Cdc42 and the CRIB domain containing proteins Gic1 and Gic2 are important for pseudohyphal growth. An interaction between GTP bound Cdc42 and Ste20, via its CRIB domain, is necessary for pseudohyphal

growth (17). This interaction antagonizes the auto-inhibitory effect of the CRIB domain on Ste20 protein kinase activity, resulting in the activation of the kinase.

Although Cdc24 is necessary for polarized growth in *S. cerevisiae*, its role in pseudohyphal growth is less clear. In contrast to *S. cerevisiae*, *CDC24* is not essential for viability in *Schizosaccharomyces pombe* (7). However, *CDC24* appears to be essential for viability in the filamentous fungi *Ashbya gossypii* (42). Homokaryotic *A. gossypii cdc24* mutant spores grow in an isotropic fashion, are unable to polarize, and do not induce hyphal growth, suggesting that this exchange factor may be necessary for hyphal filament formation.

In contrast to *S. cerevisiae*, which can only form pseudohyphae characterized by chains of elongated buds with constrictions at cell-cell junctions, the most common human fungal pathogen (34) *Candida albicans* can form true hyphae that have parallel cell walls lacking constrictions at the point of germ tube emergence. Depending on its environment, this pathogen can exist either as an ellipsoidal yeast-form or different filamentous (hyphal and pseudohyphal) forms (5, 26). A range of host signals, including temperature, pH, and serum, trigger these morphological changes (31). Similar to *S. cerevisiae*, both a MAP kinase cascade and a cAMP signaling pathway are necessary for the *C. albicans* yeast-to-hyphal transition (2, 5, 9, 19, 21, 26, 38, 39). Ras1 has been implicated in both *C. albicans* hyphal growth pathways (10, 18). Disruption of genes encoding proteins of either pathway results in strains with reduced virulence (19, 21), suggesting that the yeast-to-hyphal transition is important for pathogenicity.

Very recently, Cdc42 function in the cellular proliferation and polarized growth of *C. albicans* has been presented (41). In this pathogen, the G protein Cdc42 is necessary for polarized

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growth, as is the case in *S. cerevisiae*. However, the function of its exchange factor, Cdc24, in the *C. albicans* transition to the hyphal state is unknown. To study the function of Cdc24 in *C. albicans*, we made strains in which the only copy of *CDC24* is under the control of a regulatable promoter. We compared the vegetative growth, the yeast-to-hyphal transition, and virulence of this strain and similar strains in which *CDC42*, *BUD1*, and *BEM1* are under the control of the same regulatable promoter. Our results show that Cdc24, like Cdc42, is necessary for *C. albicans* viability. Furthermore, strains ectopically expressing *CDC24* or *CDC42*, but not *BUD1* or *BEM1*, are unable to form invasive hyphal filaments and are avirulent. Together, our results indicate that specific regulation of Cdc24/Cdc42 activity is required for invasive hyphal growth and suggest a role for this GTPase module in pathogenicity.

#### MATERIALS AND METHODS

**Culture media.** Yeast extract-peptone-dextrose (YEPD) medium or synthetic complete (SC) medium supplemented with 80 mg of uridine/liter was used (37), and strains were grown at 30°C unless otherwise indicated. Where indicated, 2.5 mM of methionine and cysteine was included in SC media. For methionine- and cysteine-free fetal calf serum (FCS; BRL Life Technologies), serum was dialyzed twice for 6 h by using MWCO 12-kDa tubing against 2 liters of phosphate-buffered saline (PBS). YEPD FCS and SC-M/C DFCS (dialyzed FCS) contained 0.5× medium and 50% serum. Amino acid analysis of YEPD, FCS, and DFCS revealed 1.0 mM, 0.2 mM, and undetectable levels of methionine, respectively.

**Isolation of *C. albicans CDC24*, *CDC42*, *BUD1*, and *BEM1*.** Initially, we attempted to isolate *CaCDC24* by complementation of the temperature-sensitive growth of an *S. cerevisiae cdc24-4* strain by using a *C. albicans* genomic pRS202 library (gift from G. Fink). Since this approach was unsuccessful, degenerate oligonucleotides based on conserved amino acid sequences in *S. pombe Kluyveromyces lactis* (A. Nern and R. A. Arkowitz, unpublished data), and *S. cerevisiae* Cdc24s were used to PCR amplify two different regions of *CaCDC24* from a genomic library in pRS202. Exact-match oligonucleotides were then used to screen this library for a clone containing a 1-kb fragment between these two regions. This clone contained a part of the *CaCDC24* sequence, encoding the first 444 amino acids. The remaining portion of *CaCDC24* was isolated by PCR amplification of the library, and these two *CaCDC24* fragments were ligated and cloned into pBluescript, resulting in pBSCaCdc24 that has a 5.1-kb *C. albicans* genomic DNA fragment containing the 2.5-kb *CDC24* open reading frame (ORF), together with a 1.9-kb 5' sequence and a 0.7-kb 3' sequence. Genomic DNA fragments containing *CaCDC42*, *CaBUD1*, and *CaBEM1* were isolated by suppression of the temperature-sensitive growth of a *S. cerevisiae cdc24-4* strain by using the *C. albicans* genomic pRS202 library. All isolated DNA fragments were sequenced, and 1.7-kb (576-bp ORF), 2-kb (820-bp ORF), and 3-kb (1911-bp ORF) fragments were subcloned into pBluescript, yielding pBSCaCDC42, pBSCaBUD1, and pBSCaBEM1, respectively.

**Mutant strains construction.** Gene disruption cassettes were created by replacing each ORF with a 2.0-kb *CaHIS1* fragment from pGEMHIS1 (43). pBSCaCDC24::HIS1 was created by replacing a 2.7-kb *CaCDC24 HpaI* fragment (which includes 0.3 kb 5' of the *CDC24* ATG) with *HIS1*. This cassette replaces all but the last 21 amino acids of Cdc24. In order to construct *CaCDC42*, *CaBUD1*, and *CaBEM1* gene disruption cassettes, a unique *BamHI* restriction site was introduced 5' of the ATG of each ORF. Two internal *XbaI* sites in *CaCDC42* were removed by blunting and religation and subsequently a similar approach was used to introduce unique *XbaI* and *NheI* restriction sites 3' of each *CaCDC42* and *CaBUD1* stop codon, respectively. Each ORF, *BamHI/XbaI* for *CaCDC42*, *BamHI/NheI* for *CaBUD1*, and *BamHI/PacI* (a restriction site present 200 bp 5' of the stop codon) for *CaBEM1* was then replaced with the *HIS1* fragment. Deletion cassettes were released by digestion, transformed into BWP17 (a *ura<sup>-</sup> his<sup>-</sup> arg<sup>-</sup>* derivative of SC5314) (43), and selected for histidine prototrophy. Heterologous disruption strains (Wild-type/ $\Delta$ ) of *CaCDC24*, *CaCDC42*, *CaBUD1*, and *CaBEM1* grew normally. For methionine regulated expression, a fragment of each gene containing the ATG start codon was cloned into either pCaDIS or a modified version of this plasmid in which an *MfeI* site had been removed. We used 464-, 339-, 197-, and 418-bp fragments for *CaCDC24*, *CaCDC42*, *CaBUD1*, and *CaBEM1*, respectively. Each pCaDIS plasmid was linearized within the *CaCDC24*, *CaCDC42*, *CaBUD1*, and *CaBEM1* gene fragments by using a *ClaI*, *StuI*, *MfeI*, and *SacI* sites, respectively, and transformed into the appropriate heterozygote strain. Transformants were se-

lected for uridine prototrophy, resulting in  $\Delta$ /pMet gene strains. Correct integrants were identified by PCR, and two independent isolates of each strain were analyzed for growth. The *CaCDC24* ORF expressed from its native promoter was reintroduced at the *RP10* locus (29) on linearized pCaEXPA plasmid. pCaEXPA plasmid was constructed from pCaEXP (6), which contains *URA3* and a *MET3* promoter. First, a 1.4-kb *HindIII* fragment of pCaEXP, containing *URA3*, was replaced with a 2.0-kb *ARG4* fragment from pRSARG $\Delta$ *SpeI* (43). Subsequently, the *MET3* promoter was removed by digestion and a 4.7-kb *CaCDC24* fragment (including the 2.5-kb ORF and 1.5 kb of 5' sequence) from pBSCaCDC24 was inserted by ligation. pCaEXPA or pCaEXPACaCDC24 were targeted to the *RP10* locus after *StuI* digestion, transformation, and selection for arginine prototrophy. Correct integration of pCaEXPACaCDC24 was identified by PCR.

For mouse virulence assays, a *URA<sup>+</sup>* wild-type strain was made by transforming *StuI*-digested pCaEXP GFP (pRSC4b [6]) into BWP17. The resulting strain contains *URA3*, followed by the *MET3* promoter and green fluorescent protein (GFP) integrated at the *RP10* locus and was also used for immunoblot analyses. A completely prototrophic wild-type strain (*URA<sup>+</sup>*, *HIS<sup>+</sup>*, and *ARG<sup>+</sup>*) was made by sequential transformation of BWP17. First, this strain was transformed by *NruI*-digested pGEMHIS1 (43). *HIS<sup>+</sup>* prototrophs were subsequently transformed with *NotI*-digested pRS-ARG-URA-BN (pRS314 containing *CaURA3* and *CaARG4* [a gift from A. Mitchell]), and histidine, uridine, and arginine prototrophs were selected. A prototrophic  $\Delta$ *cdc24/CDC24* strain was constructed, as described above, by using pRS-ARG-URA-BN, and a prototrophic  $\Delta$ *cdc24/pMetCDC24* strain was made by using *StuI*-linearized pCaEXPA.

For Western analyses, a plasmid derived from pCaDISCaCDC24 was used in which the *MET3* promoter was replaced with the *CDC24* promoter to determine the levels of 3xHACaCdc24 from its native promoter. This was accomplished by first introducing a unique *BamHI* site 5' of the *CDC24* ATG in pCaEXPACaCDC24 by site-directed mutagenesis and subsequently replacing a 1.8-kb *AatII/BamHI* fragment from pCaDISCaCDC24 with a 0.9-kb *SwaI/BamHI* *CDC24* promoter fragment from the modified pCaEXPACaCDC24, yielding pCaCDC24. An amino-terminal triple-hemagglutinin (HA) epitope tag, followed by a Gly-Ala-Gly-Ala-Gly-Ala linker, was fused to CaCdc24 by PCR and cloned into pCaCDC24, resulting in pCa-3xHACDC24. The sequence of 3xHACDC24 was verified. This plasmid was linearized within *CDC24* with *ClaI*, transformed into BWP17, and selected for uridine prototrophy, and the resulting strain, pCDC24-3xHACdc24, was used for examination of Cdc24 levels. In order to determine the levels of 3xHACdc24 from the *MET3* promoter, a plasmid was constructed in which the 0.6-kb *BamHI/PstI* 3xHACDC24 fragment was cloned into pCaDis, resulting in pCaDis-3xHACDC24. This plasmid was then linearized within *CDC24* ORF with *ClaI* and transformed into BWP17, and uridine prototrophs were selected, yielding the strain pMet3-3xHACdc24. Epitope-tagged strains were confirmed by PCR.

**Southern and Western blot analyses.** For Southern analysis, genomic DNA was extracted from cells grown in YEPD. Five micrograms of DNA was digested with *MfeI*, and the resulting fragments were separated on a 1.2% agarose gel. DNA was transferred to Immobilon-nylon membrane (Millipore) and hybridized with a 1.1-kb *MfeI* fragment, which contained 0.4-kb 3' of the *CDC24* ATG, that was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP. Southern blots were visualized by using a Fuji phosphorimager. For immunoblot analyses, exponentially growing cells were broken with glass beads by agitation in a Ribolyser (Hybaid). Proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel and transferred onto a Protran nitrocellulose membrane (Schleicher & Schuell). The membranes were probed with either anti-HA (HA.11) monoclonal antibodies (1:1,000; Covance) or anti-GFP polyclonal antibodies (30), followed by enhanced chemiluminescence visualization (Amersham).

**Microscopy.** For colony morphology, cells grown on agar containing media for 3 to 5 days at 30°C were imaged with a Leica MZ6 dissection scope at  $\times 20$  magnification. For cell morphology, cells were grown to exponential phase in the indicated media and then shifted at 37°C for 3 h in the presence of 1:1 (vol/vol) FCS or DFCS. Cells were then fixed for 1 h with formaldehyde (7.5%), resuspended in PBS, and imaged with a Leica DMR microscope by using a  $\times 63$  N/A 1.32 objective lens, by using differential interference contrast (DIC) optics. All images were obtained with a Princeton Instruments Micromax charge-coupled device camera.

**Murine virulence assay.** Overnight cultures of *C. albicans* strains were diluted into YEPD and grown for an additional 4 h at 30°C. Cultures were collected by centrifugation and washed with PBS. Cells ( $10^6$ ) were injected into the tail vein of 21- to 25-g male CD1 mice. Cell numbers were determined by using a hemacytometer, and viability was confirmed by plating cultures onto SC-M/C solid media. Ten mice were used per strain and were monitored for 6 weeks after injection.

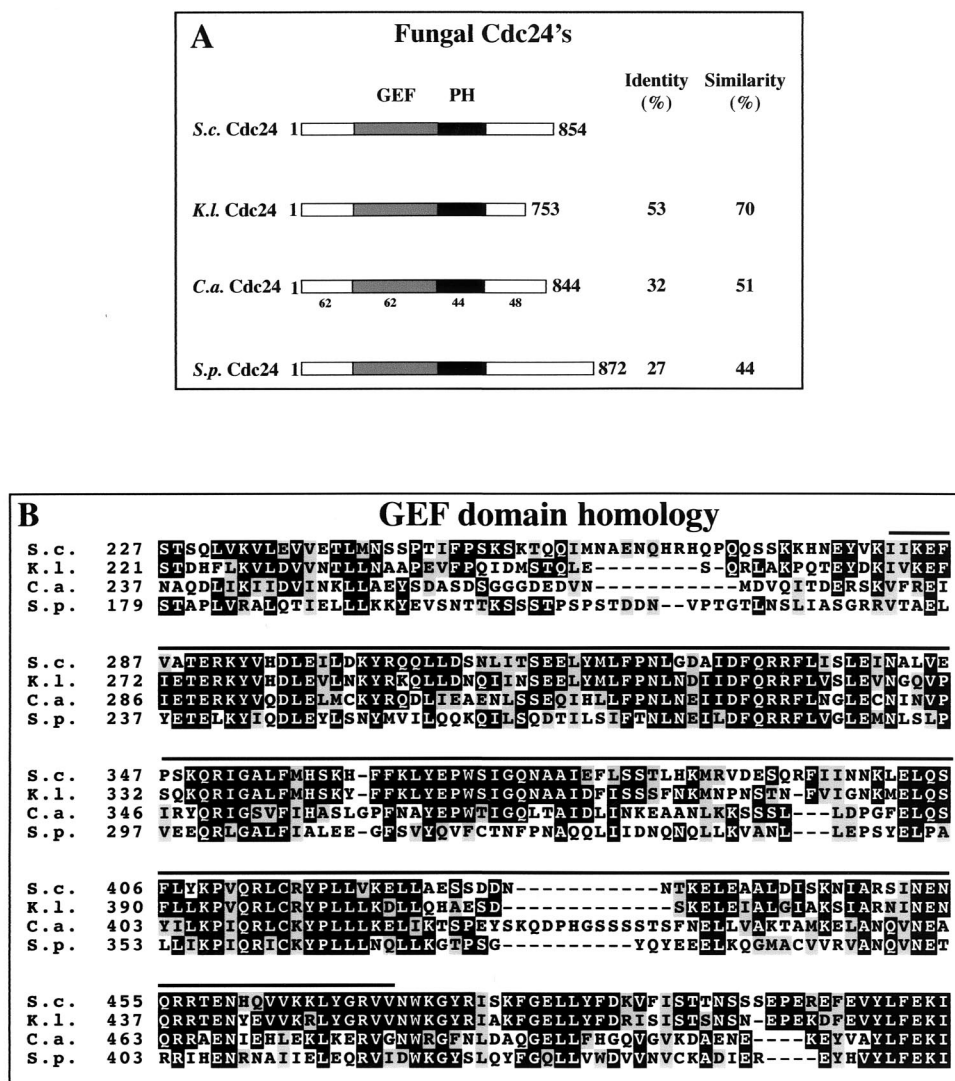


FIG. 1. Comparison of fungal Cdc24 guanine nucleotide exchange factors. (A) Protein sequence comparison of fungal Cdc24s. Percentage identity and similarity of *Saccharomyces cerevisiae* (*S.c.*), *Kluyveromyces lactis* (*K.l.*), *Candida albicans* (*C.a.*), and *Schizosaccharomyces pombe* (*S.p.*) Cdc24 proteins. The GenBank accession number for *C. albicans* CDC24 is AY208122. The guanine nucleotide exchange factor (GEF) and plekstrin homology domains are shown in gray and black, respectively. Numbers below the schematic representation of *C. albicans* Cdc24 indicate the percent similarity of each region with *Saccharomyces cerevisiae* Cdc24. Alignments were carried out by using the BLAST algorithm (1). (B) Protein sequence alignment of fungal guanine nucleotide exchange factor domain. Residues boxed in black and in gray are identical and similar to *S.c.* Cdc24, respectively. The black line above the sequences represents the *S. cerevisiae* Cdc24 GEF domain. *S.c.*, *S. cerevisiae*; *K.l.*, *K. lactis*; *C.a.*, *C. albicans*; *S.p.*, *S. pombe*.

**RESULTS**

**Identification of *C. albicans* Cdc24 homolog.** Since attempts to isolate *CaCDC24* by complementation of an *S. cerevisiae* *cdc24* temperature-sensitive mutant were unsuccessful, degenerate oligonucleotides based on conserved amino acid sequences in *S. pombe*, *K. lactis*, and *S. cerevisiae* Cdc24s were used to PCR amplify two different regions of *CDC24* from a *C. albicans* genomic library in pRS202. Exact-match oligonucleotides were then used to screen this library for a clone containing a partial *CDC24* sequence. The remaining portion of *CDC24* was isolated by PCR amplification of the library. The sequence of the *C. albicans* *CDC24* ORF revealed three bases that were different from the *CDC24* sequence Contig6-

1854 (Stanford *C. albicans* genome sequencing project; <http://www-sequence.stanford.edu/group/candida>), the first change resulting in a methionine at amino acid 735 and the latter two changes being silent. *C. albicans* Cdc24 is 32% identical to its budding yeast counterpart, displaying 43% identity within the guanine nucleotide exchange factor domain (Fig. 1). A BLAST search of the human genome sequence with the *C. albicans* Cdc24 sequence revealed a number of human guanine nucleotide exchange factors with characteristic exchange factor domains that were  $\leq 28\%$  identical to the exchange factor domain. The overall sequence identity of *C. albicans* Cdc24 with different human exchange factors, such as RhoGEF KIAA1209 and Cdc42 GEF hPEM-2, is ca. 8%. In contrast, Cdc42 is



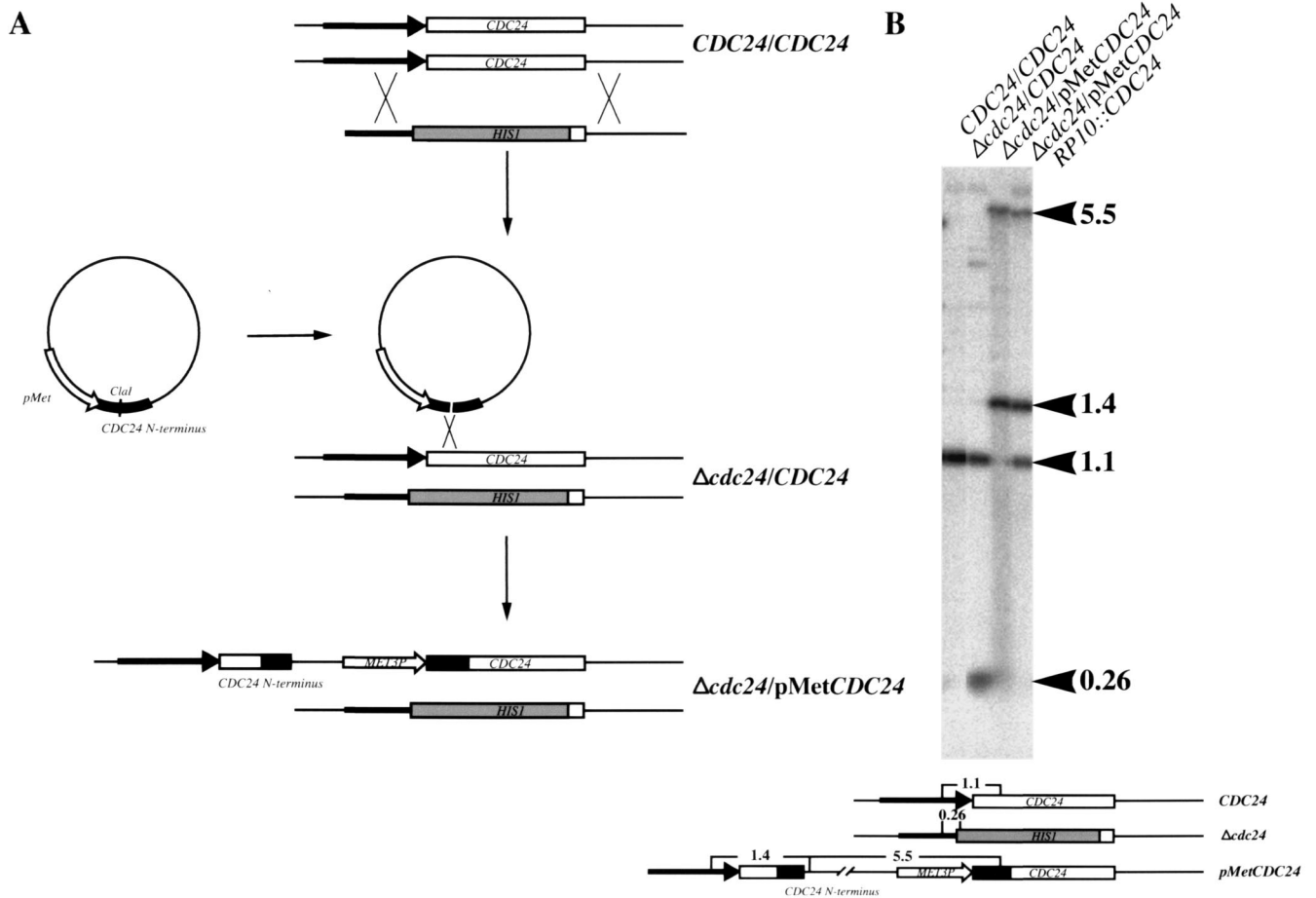


FIG. 2. Construction and Southern analysis of *C. albicans cdc24* mutant strains. (A) Schematic representation of *CDC24* strain construction. (B) Southern blot analysis of *CDC24* mutant strains. Five micrograms each of total genomic DNA from the indicated strains was digested with *MfeI* and analyzed by Southern blotting. Blots were probed with a radiolabeled 1.1-kb *CaCDC24 MfeI* fragment. The size of the fragments is indicated in kilobases.

highly conserved in many eukaryotes, with *C. albicans* Cdc42 having 88 and 77% identity to *S. cerevisiae* and human Cdc42, respectively.

**Construction and analysis of *cdc24* mutant strains.** In order to examine the function of *CDC24* in *C. albicans* vegetative and hyphal growth, strains were constructed in which one copy of the *CDC24* ORF was replaced by the *HIS1* marker and the *MET3* promoter was integrated immediately 5' of the ATG of the second copy of *CDC24* (Fig. 2A). To confirm that any defects observed with the  $\Delta cdc24/pMetCDC24$  strain were due to the loss of *CDC24* function, an additional copy of *CDC24* was reintroduced into this strain at the *RP10* locus. Correct  $\Delta cdc24/CDC24$ ,  $\Delta cdc24/pMetCDC24$ , and  $\Delta cdc24/pMetCDC24/ RP10::CDC24$  integrants were identified and confirmed by PCR (not shown) and Southern analysis (Fig. 2B).

To compare the regulation of *CDC24* expression via the *MET3* promoter and its own promoter, we examined the levels of epitope-tagged Cdc42 in cells grown in different conditions. The *MET3* promoter is fully repressed in the presence of both millimolar methionine (Met) and cysteine (Cys) and yet permits expression at lower Met and Cys levels (6) (Fig. 3A). We compared the amount of Cdc42 expressed from the *MET3*

promoter in permissive (–M/C), repressive (+2.5 mM M/C) and intermediate (YEPD; which contains 1 mM methionine) conditions. Using a *GFP CDC24* fusion (data not shown) and a triple-HA epitope-tagged version of *CDC24* behind the *MET3* promoter, two to three times less Cdc42 was observed when cells were grown on YEPD medium compared with SC medium lacking Met and Cys (Fig. 3B, lane 6 versus lane 4). These results indicate that the Met (1 mM) and Cys concentrations present in YEPD medium are sufficient for partial repression of the *MET3* promoter. The addition of 2.5 mM Met and Cys to SC media completely blocked *CDC24* expression (lane 5). Together, these results indicate that the *MET3* promoter constructs can be used to assess the function of Cdc42 in invasive hyphal growth. We next compared the expression level of *CDC24* behind the *MET3* and its own promoter (Fig. 3C). The level of 3xHACdc42 expressed from the *MET3* promoter was ~3-fold lower than that observed with the *CDC24* promoter in cells grown in YEPD (Fig. 3C, lanes 8 and 10). However, we observed similar levels of 3xHACdc42 with the two different promoters in cells grown in synthetic medium lacking Met and Cys (Fig. 3C, lanes 7 and 9). As illustrated in Fig. 3B and C, the 3xHACdc42 fusion migrated as a double band on SDS-PAGE,

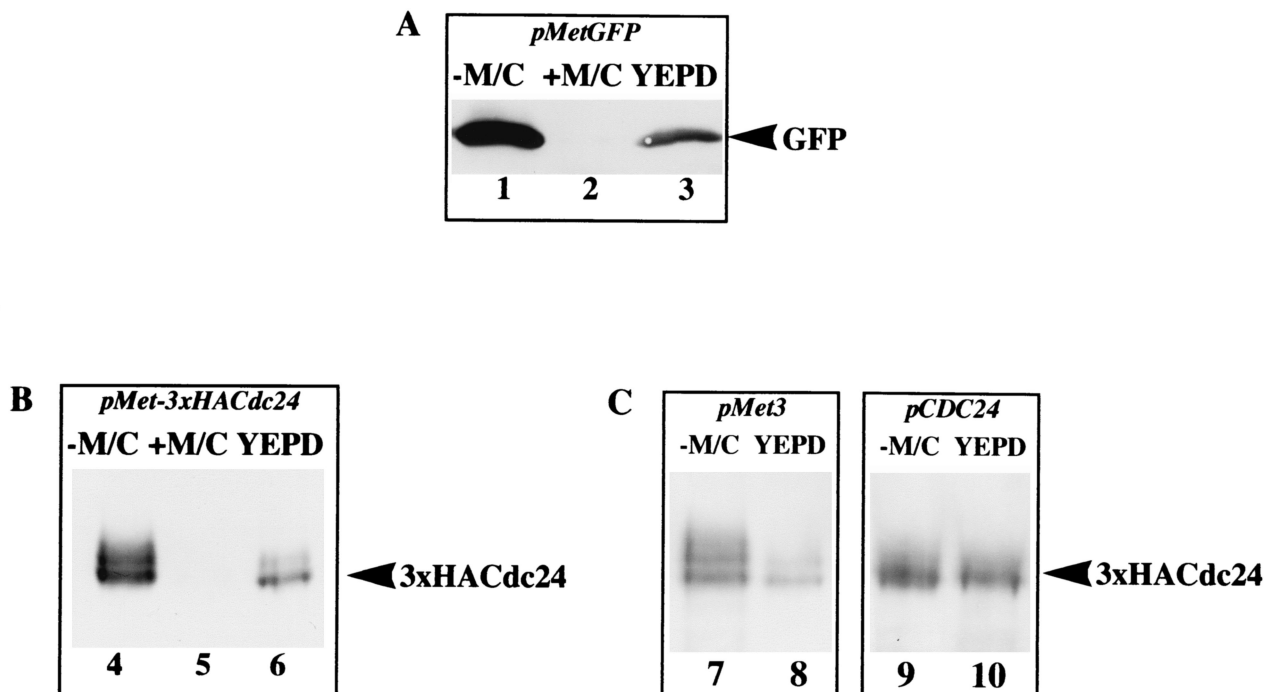


FIG. 3. Regulated expression of the *MET3* promoter. (A) Cells carrying *MET3* promoter GFP grown in SC medium lacking Met and Cys (lane 1), in SC medium containing 2.5 mM Met and Cys (lane 2), or in YEPD (lane 3) were lysed by agitation with glass beads and analyzed by SDS-PAGE, followed by immunoblotting, and then probed with anti-GFP polyclonal sera (30). (B) *CDC24/pMet3xHACdc24* cells were analyzed as described for panel A with anti-HA monoclonal sera (lanes 4 to 6). (C) Cells expressing 3xHACdc24 under the control of the *MET3* promoter (left panel) or *CaCdc24* promoter (right panel) grown in SC medium lacking Met and Cys (lanes 7 and 9) and YEPD (lanes 8 and 10) were analyzed as described for panel A. Similar amounts of protein were loaded in each lane, as judged by Ponceau red staining of the blots.

suggesting that this protein is phosphorylated, similar to Cdc24 in *S. cerevisiae* (3, 12).

Using the same approach, we constructed strains in which one copy of *C. albicans CDC42*, *BUD1*, or *BEM1* was deleted and the *MET3* promoter was integrated immediately 5' of the ORF of the sole copy of each gene.

**Cdc24 is required for vegetative growth.** Initially, we compared the growth of wild-type,  $\Delta cdc24/CDC24$   $\Delta cdc24/pMetCDC24$ , and  $\Delta cdc24/pMetCDC24/RP10::CDC24$  strains in repressive conditions. Although the wild-type and heterozygote strains grew normally,  $\Delta cdc24/pMetCDC24$  cells were inviable (Fig. 4A). Reintroduction of a copy of *CDC24* into  $\Delta cdc24/pMetCDC24$  cells restored growth on synthetic medium containing 2.5 mM Met and Cys. This recovery of growth indicates that the *pMetCDC24* does not behave in a dominant fashion, a finding consistent with normal growth of *CDC24/pMetCDC24* strains (data not shown). Strains with regulated expression of *CaCDC42*, *CaBUD1*, and *CaBEM1* were also examined (Fig. 4B).  $\Delta cdc42/pMetCDC42$  cells were also inviable under repressive conditions, as previously reported (41), whereas the  $\Delta bud1/pMetBUD1$  cells grew normally, similar to a homozygous disruptant (44), and  $\Delta bem1/pMetBEM1$  cells grew poorly. *C. albicans* strains with regulated expression of *CDC24*, *CDC42*, *BUD1*, and *BEM1* all grew on rich YEPD medium (data not shown). These results suggest that the level of Cdc24 or Cdc42 in this condition is sufficient for vegetative growth.

**The Cdc24/Cdc42 GTPase module is required for invasive**

**growth.** We next examined the ability of the various strains to invade solid agar. Strains were grown on YEPD plates containing FCS and incubated at 30°C. Under these conditions, all strains initially grew as colonies on the surface of the agar. Figure 5A illustrates that wild-type and  $\Delta cdc24/CDC24$  colonies were substantially filamentous and invaded the agar substrate extensively. Strikingly, the  $\Delta cdc24/pMetCDC24$  strain did not become hyphal and invade the solid surface (Fig. 5A). The invasive hyphal growth defects of this strain could be due to a suboptimal level of Cdc24 in YEPD FCS medium which, although sufficient for growth of the yeast form, is insufficient for hyphal growth. Consistent with this notion, reintroduction of a copy of *CDC24* into  $\Delta cdc24/pMetCDC24$  cells suppressed the hyphal growth defect to a level similar to that of the wild type or heterozygote (Fig. 5A). As illustrated in Fig. 5B,  $\Delta cdc42/pMetCDC42$  cells are also unable to invade an agar substrate, in contrast to  $\Delta bem1/pMetBEM1$  cells, which grow in an invasive hyphal fashion. Colonies of  $\Delta bud1/pMetBUD1$  cells were intermediate between these two extremes, forming invasive filaments slower than the wild-type and  $\Delta bem1/pMetBEM1$  cells, a finding consistent with a previous study (44). The invasive hyphal growth defects of both  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  were independent of the stimuli used; similar results were observed with YEPD alone (data not shown) or on medium completely lacking Met and Cys (synthetic media containing extensively dialyzed FCS) at 30°C (Fig. 5C and D), which nonetheless triggered invasive hyphal growth of wild-type strains. Together, these results suggest that

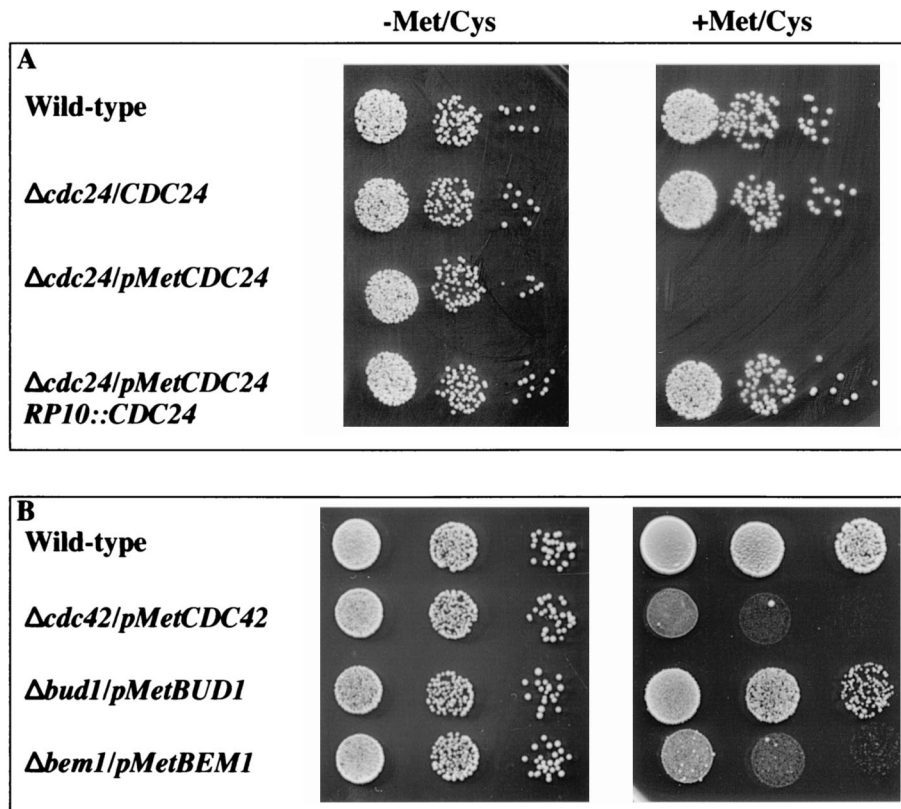


FIG. 4. *C. albicans* Cdc24 and Cdc42 are essential for normal vegetative growth. (A and B) Indicated strains were grown to exponential phase in SC medium lacking methionine and cysteine. Serial dilutions were spotted onto SC plates lacking or containing 2.5 mM of methionine and cysteine, and the plates were incubated at 30°C for 2 to 4 days.

$\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  cells are defective in invasive hyphal growth either due to a reduced level of the respective protein or due to an inability to appropriately regulate *CDC24* and *CDC42* expression in response to stimuli, which trigger hyphal growth. The comparison of the expression level of *CDC24* behind *CDC24* promoter and Met promoter in cells grown in synthetic medium (Fig. 3C) indicates that it is unlikely that the absolute level of Cdc24 is responsible for the hyphal growth defect, suggesting that this defect is rather the result of *CDC24* ectopic expression.

**Cdc24 and Cdc42 are required for initiation of hyphal growth.** A defect in invasive hyphal growth could be due to either an inability of yeast-form cells to hyperpolarize and form germ tubes, i.e., initiate hyphal growth, or to an inability to maintain this hyphal polarized growth. To distinguish between these possibilities, we analyzed the behavior of cells in liquid YEPD medium containing FCS at 37°C and counted the number of cells ( $n > 200$ ) with germ tubes. After 3 h in the presence of 50% FCS, >90% of wild-type cells had elongated germ tubes (Fig. 6A and B). Strikingly, only 0.5% of  $\Delta cdc24/pMetCDC24$  cells had observable germ tubes (Fig. 6A). This germ tube formation defect was not seen with  $\Delta cdc24/CDC24$  or  $\Delta cdc24/pMetCDC24/ RP10::CDC24$  cells, which both had >90% of cells with germ tubes.  $\Delta bud1/pMetBUD1$  and  $\Delta bem1/pMetBEM1$  strains formed germ tubes similar to wild-type cells (40 and 70% cells with germ tubes, respectively) in contrast to  $\Delta cdc42/pMetCDC42$  cells, which had the same defect

than  $\Delta cdc24/pMetCDC24$  cells (Fig. 6B). Despite the defect in germ tube formation,  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  cells nonetheless grew in YEPD FCS, as indicated by the presence of budded cells. However, the size of cells from each strain was larger than with the wild type, as previously reported for a *PCK1*-regulated *cdc42* strain (41). No further increase of the percentage of  $\Delta cdc24/pMetCDC24$  or  $\Delta cdc42/pMetCDC42$  cells with germ tubes was observed after 5 h (data not shown), suggesting that this defect was not due to a slower morphological transition.

Since the germ tube formation defect of  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  cells in YEPD FCS media could be due to a reduced level of these two proteins, the behavior of these cells was observed in medium lacking Met and Cys containing dialyzed FCS at 37°C. A similar defect in germ tube formation was observed in this medium (Fig. 6C), with only 10 and 1% of cells with germ tubes for  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  strains, respectively, compared to 65% for the wild-type. No further increase of the percentage of  $\Delta cdc24/pMetCDC24$ ,  $\Delta cdc42/pMetCDC42$ , or wild-type cells with germ tubes was observed after 5 h (data not shown). Closer examination revealed that the cell size of the two mutant strains was now similar to the wild-type cells and that a portion of the buds were elongated and had a pseudohyphal appearance. Our results suggest that  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  strains are defective in initiation of cell polarization, which precedes hyphal formation, in YEPD FCS

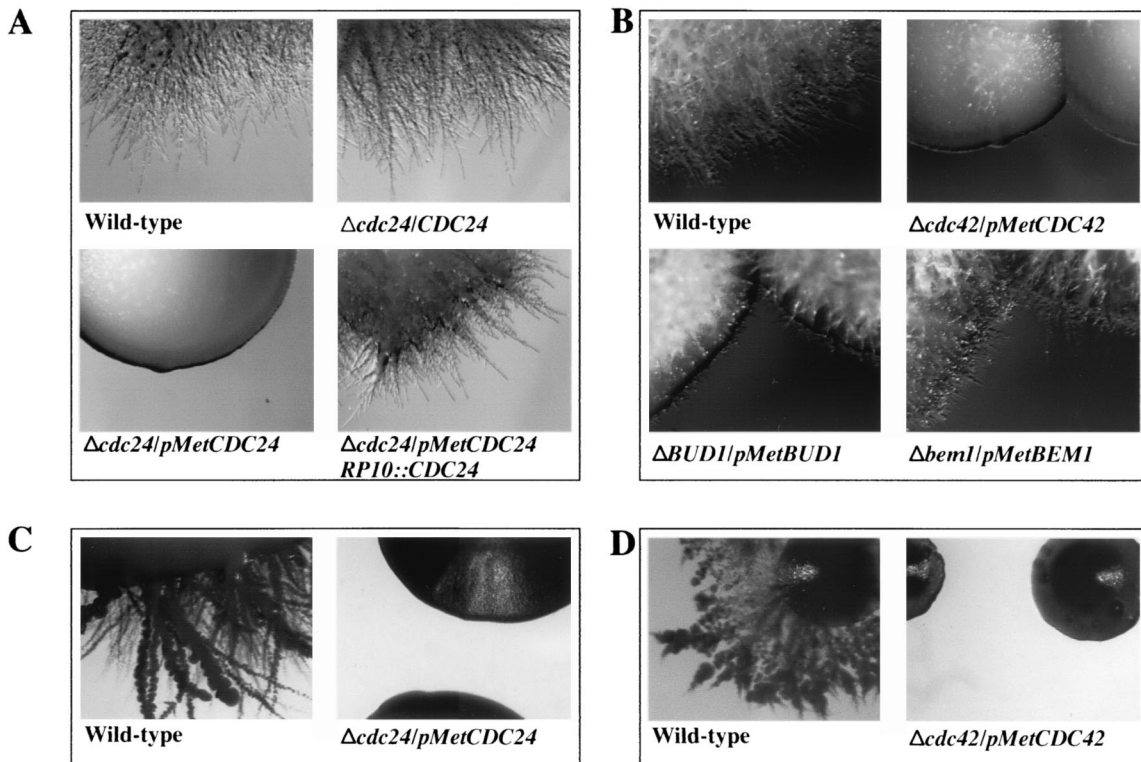


FIG. 5. *C. albicans* Cdc24 and Cdc42 are essential for invasive hyphal growth in response to serum. The indicated strains were grown in SC medium lacking methionine and cysteine, and serial dilutions were spotted onto YEPD plates containing FCS (A and B) or plates with SC medium that lacked methionine and cysteine but containing DFCS (C and D). Plates were then incubated for 5 (A, C, and D) or 3 (B) days at 30°C.

media. However, in SC DFCS media lacking Met and Cys, in which a higher level of Cdc24 was observed,  $\Delta cdc24/pMetCDC24$  cells appeared to initiate polarization but were still unable to maintain this directional growth. Both  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  strains, although able to undergo vegetative, yeast-form growth, were unable to form hyphae in response to serum.

**The Cdc24/Cdc42 GTPase module is necessary for virulence.** To assess the role of these proteins in *C. albicans* virulence, we tested these strains in a murine model for systemic infection. Mice were injected with a  $URA^+$  wild-type strain (15) and the four  $URA^+ HIS^+$  strains  $\Delta cdc24/pMetCDC24$ ,  $\Delta cdc42/pMetCDC42$ ,  $\Delta bud1/pMetBUD1$ , and  $\Delta bem1/pMetBEM1$ . Mice injected with the wild-type strain resulted in 50% mortality after 5 days, whereas even after 40 days the  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  strains did not result in mouse mortality (Fig. 7A). Mice injected with either of the other two strains,  $\Delta bud1/pMetBUD1$  and  $\Delta bem1/pMetBEM1$ , survived longer than those injected with the wild-type control; 50% of the former group died after 9 days, and 30% of the latter group died after 16 days. To rule out the possibility that the avirulence of  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  strains is due to their arginine auxotrophy,  $ARG4$  was reintroduced into the  $\Delta cdc24/pMetCDC24$  strain. As a control, a strain prototrophic for  $URA^+$ ,  $HIS^+$ , and  $ARG^+$  was used. The results illustrated in Fig. 7B demonstrate that the  $HIS1$  and  $ARG4$  markers do not affect virulence in a murine model of systemic infection, since 50% of mice injected with either

strain ( $URA^+$  or  $URA^+/HIS^+/ARG^+$ ) died after 6 days. The reintroduction of  $ARG4$  into the  $\Delta cdc24/pMetCDC24$  cells had no effect on the avirulence of this strain, whereas the reintroduction of  $CDC24$ , under the control of its own promoter, resulted in a strain with a virulence identical (50% mice mortality after 5 days) to that of the wild type. In addition, the virulence of the heterozygote strain was examined and was also similar to that of the wild-type strain (50% mouse mortality after 7 days), indicating that cells with only one copy of  $CDC24$  are pathogenic. These results suggest that the Cdc24/Cdc42 GTPase module is necessary for virulence, although we cannot exclude the possibility that the reduced virulence of  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  strains is in part due to an impaired growth rate of these strains in mice.

## DISCUSSION

Cdc24, the Cdc42 exchange factor, is required for viability and invasive hyphal growth in *C. albicans*. A strain in which the only functional copy of  $CaCDC24$  is under the control of the regulatable  $MET3$  promoter was used to examine the consequences of complete shutoff of expression, reduced expression, and ectopic expression. Complete repression on methionine- and cysteine-containing media resulted in inviable cells, indicating that the CaCdc24 is required for vegetative growth. Under conditions of reduced  $CaCDC24$  expression, such as in YEPD medium, cells grew vegetatively; they were, however, larger than wild-type cells. In synthetic media lacking methio-



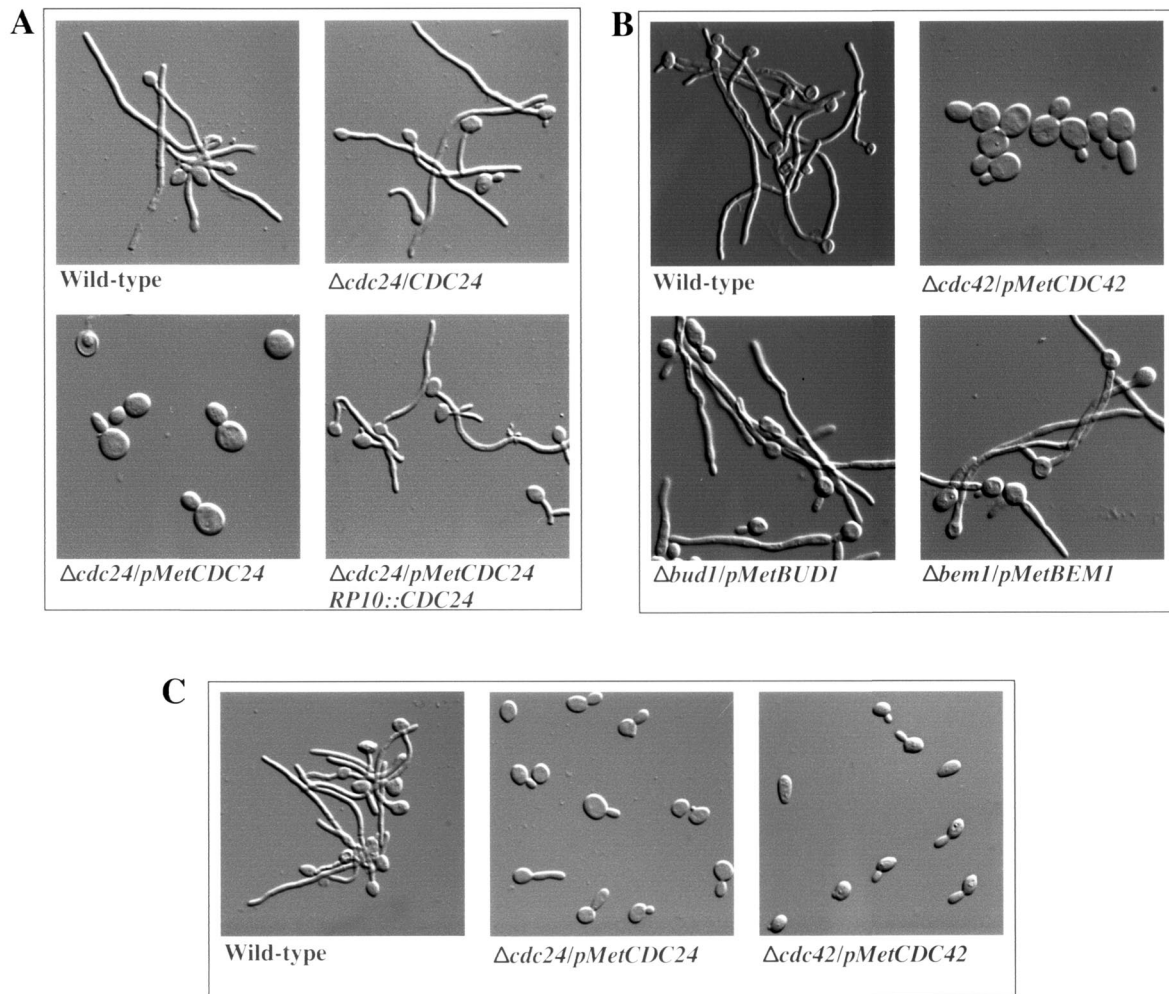


FIG. 6. *C. albicans* Cdc24 and Cdc42 are required for germ tube formation. (A and B) The indicated strains were grown in YEPD, pelleted, and resuspended in YEPD, and then an equal volume of FCS was added. Cells were incubated at 37°C for 3 h, fixed with formaldehyde, and imaged. (C) The indicated strains were treated and analyzed as described for panels A and B, except that they were grown in SC medium lacking methionine and cysteine and then resuspended in the same medium containing an equal volume of DFCS. The percentages of cells with germ tubes were determined ( $n > 200$ ).

nine and cysteine, where little difference was observed in *CaCDC24* expression under the control of the *MET3* promoter or its own promoter, vegetative growth was comparable to that of a wild-type strain. *C. albicans* yeast-form cells undergo a transition to invasive hyphal filaments in response to serum and/or elevated temperature. Strikingly,  $\Delta cdc24/pMetCDC24$  cells were defective for invasive hyphal growth and germ tube formation in a media permissive for vegetative growth that contained dialyzed serum.

This defect in invasive hyphal growth in conditions permissive for vegetative growth was also observed for a similarly constructed *CaCDC42* strain, suggesting that regulation of Cdc24 and/or Cdc42 activity is required for invasive hyphal growth. In contrast, strains in which the only functional copy of *CaBUD1* or *CaBEM1* is under the control of the regulatable *MET3* promoter formed invasive hyphal filaments on agar containing media and germ tubes in liquid media. These results show that the defects observed with regulated *CaCDC24* or *CaCDC42* strains were specific to this GTPase module and not

a common feature of polarity establishment proteins. Furthermore, by using a mouse model for systemic infection, these strains were avirulent, suggesting that their defects in invasive hyphal growth result in reduced pathogenicity.

In *S. cerevisiae*, Bud1 is required for pseudohyphal growth (11, 40), as this G protein is necessary for unipolar budding and therefore filament formation. Although *CaBUD1* is not essential for invasive hyphal growth, its reduced expression resulted in a decrease in hyphal growth and pathogenicity, a finding consistent with a previous report in which a  $\Delta bud1/\Delta bud1$  *C. albicans* strain exhibited modest defects in the yeast-to-hyphal transition and virulence (44). The role of Bem1 in fungal pseudohyphal and hyphal growth is less clear. In *S. cerevisiae*, *BEM1* is necessary for polarity establishment and has recently been shown to be required for pseudohyphal growth (22). In the dimorphic yeast *Yarrowia lipolytica*, cells deleted for *BEM1* are viable but have cell polarity defects (13). This mutant, though unable to form hyphae in liquid and solid media, nevertheless forms pseudohyphae to a reduced extent on agar



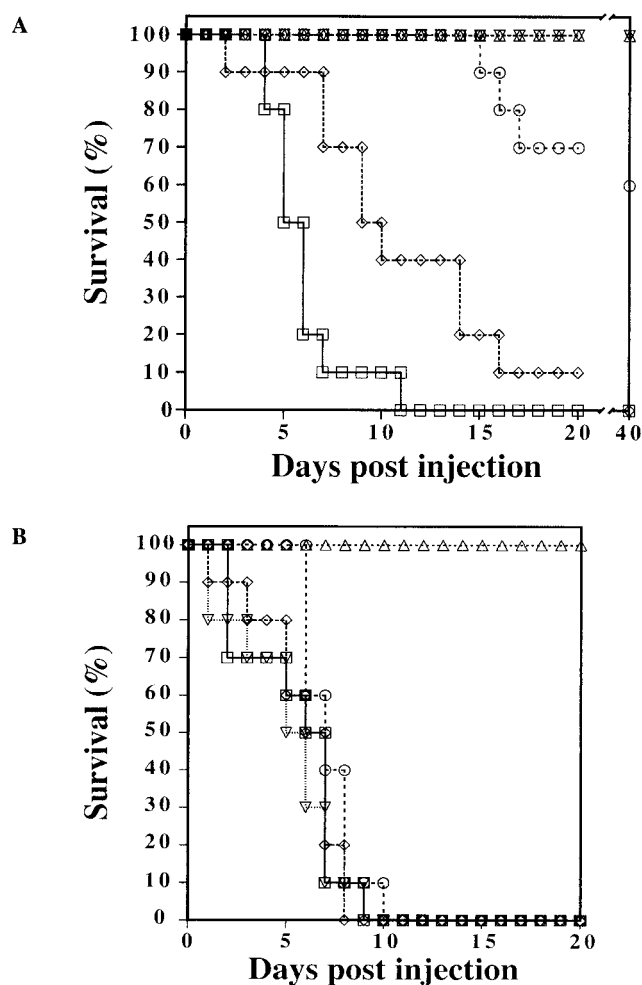


FIG. 7. *C. albicans*  $\Delta cdc24/pMetCDC24$  and  $\Delta cdc42/pMetCDC42$  strains are avirulent. (A)  $\Delta cdc24/pMetCDC24$  ( $\Delta$ ),  $\Delta cdc42/pMetCDC42$  ( $\nabla$ ),  $\Delta bud1/pMetBUD1$  ( $\diamond$ ), and  $\Delta bem1/pMetBEM1$  ( $\circ$ ) strains ( $10^6$  cells) were injected into the tail vein of CD1 mice ( $n = 10$ ) and monitored for survival over 40 days. A  $URA^+$  wild-type ( $\square$ ) strain was used as a control. (B) Prototrophic ( $HIS^+ URA^+ ARG^+$ ) versions of wild-type ( $\diamond$ ),  $\Delta cdc24/CDC24$  ( $\circ$ ),  $\Delta cdc24/pMetCDC24$  ( $\Delta$ ), and  $\Delta cdc24/pMetCDC24/RP10::CDC24$  ( $\nabla$ ) strains and a  $URA^+$  wild-type ( $\square$ ) were injected in mice as described in panel A, and mouse mortality was monitored for 20 days.

plates. In contrast, although our results indicate that  $\Delta bem1/pMetBEM1$  cells grow poorly under Met and Cys repressive conditions, the cells form hyphae in solid and liquid media under conditions of partial repression (YEPD medium with FCS) or ectopic expression (SC-M/C medium with DFCS). Despite this ability to form invasive hyphal filaments, this strain displays a substantially reduced virulence in a mouse model.

Our results show that the level or activity of CaCdc42 and CaCdc24 required for yeast-form growth is likely to be different from that necessary for germ tube and subsequent filament formation.  $\Delta cdc24/CDC24$  and  $CDC24/pMetCDC24$  strains form hyphae on YEPD in response to serum, identical to a wild-type strain, indicating that a two- to threefold reduction in Cdc24 level does not affect the dimorphic transition. Strikingly,

a  $\Delta cdc24/pMetCDC24$  strain with  $\sim 6$ -fold reduction in Cdc24 levels is defective both for germ tube formation and invasive hyphal growth. These results indicate either that a minimal level of Cdc24 is required for invasive hyphal growth or that  $CDC24$  expression is precisely regulated via its own promoter.

Previously, it was demonstrated that transcript levels of  $CDC42$  in *C. albicans* (25) and another pathogenic fungus, *Penicillium marneffei* (4), increased upon germ tube formation; however, it remains unclear whether such an increase is associated with the hyphal state. The function of Cdc42 in fungal hyphal growth by using dominant-active and dominant-negative mutants has been difficult to infer. For example, a dominant-active form of Cdc42 represses hyphal growth in the zoopathogenic fungus *Wangiella dermatitidis*; however, a  $cdc42$  deletion strain formed hyphae normally (45). In *P. marneffei*, dominant-active or dominant-negative forms of Cdc42 were normal for dimorphic switching, forming hyphae similar to wild-type cells (4). In *C. albicans*, expression of a dominant-active or -negative form of  $cdc42$  modifies cell morphology but, even so, hyphal structures were observed (41). Our results show that *C. albicans* strains ectopically expressing  $CDC42$  are unable to form invasive hyphal filaments, suggesting a central role of the Cdc42 GTPase module in the dimorphic switch. Both a MAP kinase cascade (defined by  $CaCPH1$  requirement) and a cAMP signaling pathway (defined by  $CaEFG1$  requirement) are necessary for the *C. albicans* yeast-to-hyphal transition (2, 5, 9, 19, 21, 26, 38, 39). Recent studies suggest that both pathways require  $CaRAS1$  (10, 18). Perhaps CaRas1 forms a complex with CaCdc24 and CaCdc42, as is the case in *S. pombe* (7), and this complex is required for signaling to the MAP kinase cascade via the  $STE20$  homolog  $CaCST20$  and the cAMP-dependent pathway via adenylyl cyclase ( $CaCYR1$ ). In such a scenario, CaCdc24 and CaCdc42 might be required for the coordinated activation of MAP kinase and cAMP-dependent signaling pathways. The deleterious effects of overexpression of dominant active Cdc42 in *C. albicans* are suppressed by deletion of the  $STE20$  homolog  $CaCST20$ , suggesting that this PAK family kinase is the primary effector of Cdc42 in this pathogenic fungus (41). Our results indicate that a specific regulation of Cdc24/Cdc42 activity is required for invasive hyphal growth and suggest that these proteins are required for the pathogenicity of *C. albicans*.

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