Identification and Characterization of Two Ca\(^{2+}\)/CaM-dependent Protein Kinases Required for Normal Nuclear Division in *Aspergillus nidulans* *

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We utilized an expression screen to identify two novel Ca\(^{2+}\)/calmodulin (CaM)-regulated protein kinases in *Aspergillus nidulans*. The two kinases, CMKB and CMKC, possess high sequence identity with mammalian CaM kinases (CaMKs) I/IV and CaM Kα/β, respectively. In vitro CMKC phosphorylates and increases the activity of CMKB, indicating they are biochemical homologues of CaMKII/β and CaMKIV. The disruption of CMKB is lethal; however, when protein expression is postponed, the spores germinate with delayed kinetics. The observed lag corresponds to a delay in the G1-phase activation of the cyclin-dependent kinase NIMX\(^{cd2}\). Disruption of cmkC is not lethal, but spores lacking CMKC also germinate with delayed kinetics and a lag in the activation of NIMX\(^{cd2}\). Analysis of ∆cmkC suggests a role for CMKC in regulating the first and subsequent nuclear division cycles. We conclude that both CMKB and CMKC are required for the proper temporal activation of NIMX\(^{cd2}\) as spores enter the cell cycle from quiescence and suggest that this relationship exists during the G1/S transition of subsequent cell divisions.

The evolutionarily conserved Ca\(^{2+}\)-binding protein calmodulin (CaM)\(^1\) is essential for proliferation in every system analyzed (reviewed in Ref. 1). Similar to mammalian cells, CaM is required during both G1 and G2 in the filamentous fungus *Aspergillus nidulans* (2, 3). To date two Ca\(^{2+}\)/CaM-dependent enzymes have been identified in *A. nidulans*, a Ca\(^{2+}\)/CaM-dependent phosphatase (CnA) and a Ca\(^{2+}\)/CaM-dependent protein kinase (ACMK or CMKA). Similar to CaM, both of these enzymes are required at similar cell cycle transitions as their mammalian homologues, namely CnA is required for G1 progression, and CMKA is essential during G2 (4, 5).

Disruption of cmkA is lethal; however the fungus is viable when the gene is placed under the control of the alcohol dehydrogenase (aclA) promoter (5). When the aclA-mediated CMKA expression is repressed, the strain grows dramatically slower than control strains, and CMKA mRNA and protein are undetectable by Northern and Western analysis, respectively. However, CaM affinity chromatography yielded CaMK activity that represented about 10 percent that present in the wild-type strain. These results suggested that either CMKA repression was not absolute or that *A. nidulans* contains an unidentified additional CaMK.

Pharmacological evidence exists that CaMKs may function not only in G2 but also in the G1 phase of the cell cycle of metazoans. Specifically, the selective CaMK inhibitor, KN-93, has been shown to arrest cells in G1. Although these pharmacological studies have been interpreted as indicating CaMKII activity is required for G1 progression, KN-93 inhibits all three multifunctional CaMKs, CaMKI, II, and IV (6), and therefore inhibition of any of these enzymes could be responsible for the G1 arrest. Additionally, the nature of the KN-93-induced G1 arrest depends upon the cell line and the experimental protocol. The drug arrests HEK cells in G1 with high histone H1 kinase activity, indicating that the arrest is at the G1/S boundary (7). However, NIH-3T3 cells arrest earlier in G1 with low levels of cyclinD1 and an enhanced association of p27\(^{kip1}\) with Cdk2 (8, 9). It is unknown whether the conflicting results are due to cell line differences or represent two independent points at which a CaMK activity is required. Therefore, the putative CMKAs and their role(s) in the G1 phase of the cell cycle have yet to be defined.

Since many Ca\(^{2+}\)/CaM-signaling pathways appear to be functionally conserved between *A. nidulans* and mammalian cells, we have used the filamentous fungus to further our understanding of the essential roles of CaMKs. In the current study we present the characterization of two novel Ca\(^{2+}\)/CaM-dependent protein kinases in *A. nidulans*, CMKB and CMKC. In vitro, the two fungal kinases, CMKB and CMKC, function analogously to the mammalian CaMK cascade members: CaMKI/IV and CaM Kα/β. Disruption of cmkB is lethal, indicating CMKB is required for *A. nidulans* growth. Both retarded expression of CMKB and disruption of cmkC severely delay the nuclear division cycle before the initiation of NIMX\(^{cd2}\) kinase activity. We propose that both CMKB and CMKC are required for the proper temporal activation of NIMX\(^{cd2}\) upon re-entry into the cell cycle from a quiescent state and during the G1/S transition of subsequent nuclear division cycles.

**EXPERIMENTAL PROCEDURES**

*A. nidulans* Strains and Culture Conditions—The *A. nidulans* strains used in this study are: GR5 (AT73; pyrG89; wA2; pyroA4); pAL5#10 (AT73; wA2; pyroA4), JJ25 (AT73; ∆cmkβ; wA2; pyroA4), JJ26 (AT73; ∆cmkC; wA2; pyroA4), JJ27 (AT73; ∆cmkB; wA2; pyroA4). All strains were grown in minimal medium containing 50 mM glucose at a temperature of 37 °C as described by Lu and Means (10). To grow
strains of the pyrG89 or pyroA4 genotype, the media was supplemented with 5 mM uridine and 10 mM uracil or 2 μg/liter pyridoxine hydrochloride, respectively.

Expression Cloning of CMKB and CMKCI—CMKB and CMKCI cDNA were isolated by screening an A. nidulans μgt11 CDNA expression library (obtained from Dr. Mike May) with a protein A-CaM, as described by Stirling et al. (11). The protein A-CaM fusion protein was generated by subcloning a hemagglutinin-tagged CaM as a blunted Afl/III fragment into the bacteriophage EcoRI and BamHI sites of pALP1 (a gift of Dr. Michael Stark), thereby creating pALP-Afl. The protein A-CaM fusion protein was purified from cultures of pALP-Afl by chromatography, following the protocol for purification of bacterially expressed A. nidulans CaM (12). Approximately 4 × 10^5 plaques were screened. Of the approximately 150 positive plaques identified, 40 were randomly chosen for purification. As a secondary screen, these 40 plaques were hybridized with random-primer CDNA probes of A. nidulans CMKA and CnA. Clones negative for CMKA and CnA were plaque-purified, and the plasmids were excised as described by the manufacturer (Stratagene). The inserts were then sequenced by the dideoxynucleotide termination reaction (Amersham Pharmacia Biotech).

Cloning and Expression of CMKB and CMKCI—Both protein kinases were expressed as hexa-histidine-tagged fusion proteins. BamHI and EcoRI sites were added to the CMKB cDNA by polymerase chain reaction, and the resulting product was subcloned into the corresponding sites of pTrecHiB (Invitrogen), generating pTrecCMKB. SacI and KpnI restriction sites were generated on the 5′ and 3′ end of the CMKB cDNA by polymerase chain reaction, and the product was ligated into the corresponding sites of pGEM-7Zf(−) (Promega). The CDNA was subsequently subcloned as a SacI/XhoI fragment into pET30b (Novagen), thus creating pETCMKB. The sequence of both expression vectors was confirmed by sequencing. BLR/DE3/pLysS (Novagen) bacteria were transformed with the expression plasmids, and several colonies were used to inoculate 100 ml of LB and grown overnight at 30 °C. 20 ml of the overnight culture was used to seed 1 liter of LB medium, and the culture was grown at 37 °C to an A_600 nm of 0.6. Protein expression was then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h. After induction, the culture was pelleted by centrifugation and resuspended in 30 ml of lysis buffer (40 mM Tris-HCl, pH 7.5, 100 mM KCl, 100 μg/ml Pefabloc, 10 μg/ml leupeptin, and 10 μg/ml aprotonin). The bacterial pellet was lysed by mild sonication and clarified by centrifugation at 16000 × g for 30 min. The supernatant was incubated with 0.5 ml of nickel nitrilotriacetic acid-agarose resin (Qiagen) with rocking for 1 h at 4 °C. The bound resin was loaded onto a column and washed with 10 ml of lysis buffer followed by 10 ml of lysis buffer supplemented with 50 mM imidazol. The bound protein was eluted in lysis buffer containing 250 mM imidazol. The purified enzymes were stored in 40% glycerol at −80 °C. All point mutations of CMKB were generated by the megaprimer mutagenesis technique and confirmed by sequencing. Glutathione S-transferase-CaMKI and maltose-binding protein were bacterially expressed and purified as described previously (14–16).

Peptide Kinase Assays—All CaM kinases were assayed at 30 °C in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl₂, 200 μM ATP, 0.2 μM [γ-32P]ATP, 1 μM A. nidulans CaM, 1 mM CaCl₂, 0.1% Tween 20. The kinases were assayed using the following concentrations: 100 ng/reaction CMKB, 5 ng/reaction CaMKI, 500 ng/reaction CMCA and CaMKβ8 and 1 μg/reaction crude protein extract. CMKB was assayed using 100 μM ADR1G (LKKLTRARFSQG) as a peptide substrate; 100 μM synapsin site 1 (LRRRLSDANF) was used for CaMKI. The reactions were performed in a 30-μl final reaction volume for 5 min. The kinase reactions were terminated by spotting 20 μl of the reaction onto p31 phosphocellulose filters followed by extensive washing in 75 mM phosphoric acid. The specific activity was determined after liquid scintillation counting of the dried filters. To assay the ability of CMKB and CaMKβ8 to activate the downstream kinases, a 25 μl reaction including both activating and target kinases but without the peptide substrate and [γ-32P]ATP was preincubated at 30 °C for 10 min. The peptide assay was then initiated by the addition of the substrate and [γ-32P]ATP.

Disruption of cmKB and cmkCI—Screening the A. nidulans chromosome-specific library from the Fungal Genetics Stock Center with cDNA probes of both kinases identified cosmid containing the genomic sequences. The genomic clones of CMKB and CMKCI were isolated by polymerase chain reaction of cosmids W4E06 and L4O01, respectively, taking advantage of the same oligonucleotide primers used to isolate the cDNAs. The genomic clones were subcloned into pGEM-7Zf(−) (Promega). To generate the disruption plasmid for cmkCI, an approximately 600-bp ClaI/ScaI fragment of the cmkCI was isolated, blunt-ended, and subcloned into the Smal site of pRG1 (17), thereby generating pCMKBdis. Ligation of an approximately 700 bp of blunt HindIII/SacII fragment of cmkCI into pRG1 generated pCMKCIdis. A. nidulans G89 germ-lings were transformed with the disruption plasmid by electroporation as described by Sanchez and Aguirre (18). The positive transformants were selected by growth on minimal medium in the absence of uridine and uracil to select for the presence of the pyr4 nutritional marker. Transformants of pCMKBdis were maintained as heterokaryon-generating cultures by transfer of mycelia instead of spores. Southern analysis was performed using the vegetatively growing CMKCIa as described by Rasmussen et al. (12). Transformants of pCMKCIdis were streaked three times to ensure strain purity followed by Southern analysis.

pCMKB5′ was generated by blunt end ligation of the BamHI/ScaI fragment of cmkCI into the Smal site of pRG1. A. nidulans G89 germ-lings were transformed with the disruption plasmid and selected according to the protocol followed for disruption of cmkC.

A. nidulans Growth Assays—The nuclear number was determined by growing spores in liquid minimal medium on glass coverslips at a spore concentration of 1 × 10^6 spores/ml. Germ-lings were collected by removing coverslips at various times followed by fixation and staining with the fluorescent dye 4',6-diamidino-2-phenylindole as described by Harris et al. (19).

Antibody Generation and Western Blotting—Rabbit anti-CMKB polyclonal antibodies were generated using keyhole limpet hemocyanin-coupled hexa-histidine-tagged CMKB as the antigen. The coupled protein was injected into rabbits, and antiserum was harvested by standard techniques (20). Western blots were performed using 1:3000 dilution of the anti-CMKB antibody and a similar dilution of IgG-conjugated horseradish peroxidase (Amersham Pharmacia Biotech). Relative CMKB expression was quantified using a Molecular Dynamics PhosphorImager equipped with a densitometer.

Histone H1 Kinase Assays—Extracts for histone H1 kinase assays were prepared as described by Dayton et al. (3). Histone H1 kinase activity was quantified using a Molecular Dynamics PhosphorImager following SDS-polyacrylamide gel electrophoresis separation of the reactions.

RESULTS

Cloning of CMKB and CMKCI—To identify novel CaM-binding proteins in A. nidulans, we used an expression-cloning strategy similar to the modified far-Western technique described by Stirling et al. (11). A primary screen of 4 × 10^6 phage plaques yielded more than 150 positives, 40 of which were chosen randomly for further characterization. To eliminate the cloning of known A. nidulans Ca^2+/CaM-dependent protein kinase (CMKA) and calcineurin (CnA). In this manner, 23 of the 40 positive clones were identified as cmkCI or CnA. Of the remaining 17 plaques, 11 were predicted to encode three independent proteins, two protein kinases and a large coil/coil protein. The six remaining plaques either contained short or unique inserts.

The two protein kinases identified (CMKB and CMKCI) share homology with the protein kinases of the mammalian CaM kinase cascade. The sequence data for both CMKB and CMKCI have been submitted to the DDBJ/EMBL/GenBankTM data libraries.
FIG. 1. Alignments of A. nidulans CMKB and CMKC. A. CMKB aligned with protein kinases of highest sequence identity: C. gloeosporioides CgCMK (AF034963), S. pombe CMK1 (AF073893), rat CaMKI (Q63450), rat CaMKIV (M64757). The site of phosphorylation leading to enzymatic activation by CaMKK is marked by an asterisk. B. CMKC aligned with closely related kinases from other species: rat CaMKKa (A57156), rat CaMKKb (AB018081), C. elegans CaMKK (from cosmID CELC05H8 (U11029)) and S. pombe SspI (D45882). Black boxes indicate identical residues, and gray boxes indicate conservative changes. The alignment was performed using FASTA with shading by BOXSHADE.
The CMKC cDNA is predicted to encode a protein of 518 amino acids. CMKC is most homologous to the family of CaM kinase kinases, sharing 30 and 26% identity with rat CaMKKα and CaMKKβ, respectively. CMKC also retains high sequence homology with Caenorhabditis elegans CeCaMKK (24%) and S. pombe SspI (21%). Thus, based on primary sequence homology, CMKC is a member of a growing family of CaMKKs.

Biochemical Characterization of CMKB and CMKC—Mammalian CaMKα/β activates CaMKI/IV by phosphorylation of a critical activation loop Thr (14, 15). To determine whether CMKB and CMKC are biochemical homologues of CaMKI/IV and CaMKα/β, we analyzed bacterially expressed and purified CMKB and CMKC. As shown in Fig. 2A, recombinant CMKB is dependent upon Ca²⁺/CaM for enzymatic activity. In the presence of Ca²⁺/CaM, the activity of CMKB is approximately 15 nmol/min/mg using ADR1G as a peptide substrate. Preincubation of CMKB with CMKC results in a 73-fold increase in the specific activity of CMKB (Fig. 2B). The increase in protein kinase activity correlates with an increase in the phosphorylation state of CMKB (data not shown). Similar to CaMKs I and IV, mutation of the CMKB activation loop Thr to Ala (T179A) completely abrogates the ability of CMKC to activate CMKB (Fig. 2B) and prevents CMKC-dependent phosphorylation (data not shown). Furthermore, the T179D mutation partially mimics T179 phosphorylation (data not shown). Thus, like mammalian CaMKI and IV, CMKB requires both Ca²⁺/CaM and activation loop phosphorylation for maximal activity.

To assess whether CMKB and CMKC are functionally interchangeable with the mammalian kinases in vitro, we measured the ability of the A. nidulans kinases to activate and be activated by the mammalian cascade members CaMKI and CaMKKβ. As demonstrated in Fig. 3A, recombinant human CaMKI is activated approximately 17-fold by preincubation in the presence of rat CaMKKβ, Ca²⁺/CaM, and ATP. CMKC can functionally mimic CaMKKβ in activating CaMKI 8-fold compared with the unactivated kinase. Reciprocally, rat CaMKKβ weakly activates A. nidulans CMKB (Fig. 3B). Collectively our in vitro biochemical analyses indicate CMKB and CMKC are A. nidulans homologues of the mammalian CaM kinase cascade members CaMKI/IV and CaMKKα/β, respectively.

Disruption of cmkB—To assess the physiological role of cmkB, we disrupted the gene by homologous recombination. The homologous integration of the cmkB disruption vector is diagramed in Fig. 4A. We were unable to isolate any disruption strains despite screening more than 100 transformants (data not shown). Since A. nidulans normally grows as a haploid organism, the disruption of an essential gene results in a non-viable spore; thus, if cmkB is required for proliferation, no viable homologous recombinants would be identified by this method.

![Fig. 2. In vitro protein kinase activities of CMKB and CMKC.](image-url)

![Fig. 3. Activation of CaMKI by CMKC and of CMKB by CaMKKβ.](image-url)
FIG. 4. Generation of *A. nidulans* ΔcmkB. A, representation of the homologous integration of pCMKBdis into the endogenous cmkB locus. The approximate locations of the *Sac*I restriction sites used for the identification of the homologously recombined plasmid are identified. B, diagram of a heterokaryon transformation strategy. The resulting heterokaryon maintains two nuclei, one *pyr*4ΔcmkB*" and the second *pyr*4ΔcmkB". Asexual reproduction produces uninucleate spores that maintain the genotype *pyr*4ΔcmkB*" or *pyr*4ΔcmkB". To determine if cmkB is essential for growth, the spores are grown in YG in the absence of UU; under these conditions neither spore will germinate if cmkB is essential. In the presence of UU, approximately 50% of the spores should germinate, all of which should represent the parental strain. C, Southern analysis of ΔcmkB.
Because we could not generate a viable haploid ΔcmkB strain, we surmised that cmkB is essential for A. nidulans growth and attempted to disrupt cmkB in a heterokaryon. As diagrammed in Fig. 4B, heterokaryons are generated by transforming conidia after the first DNA synthesis or by fusion of two non-viable germinating spores, and the subsequent transformants are maintained as multinucleate mycelia. Strains in which an essential gene is disrupted are forced to grow as heterokaryons because the wild-type nucleus maintains a copy of the essential gene, whereas the disrupted nucleus provides the selectable nutritional marker (absent in wild type). Southern analysis of greater than 75 transformed strains maintained as heterokaryons revealed 3 strains (one of which is depicted in Fig. 4C) that yielded the expected Southern pattern represent...
A. nidulans CaMKs

A. The delayed expression of CMKB and disruption of cmkC result in a delay in the initiation of nuclear division. Percentage of germlings with two or greater nuclei of the nutritionally complemented control, pAL5#10 (circles), dcmkB (squares), and ΔcmkC (triangles) strains. Strains were grown on coverslips, and the nuclear number was determined after staining with 4',6-diamidino-2-phenylindole as described under "Experimental Procedures." Percentages were calculated from more than 500 individual germlings. B. The delay of dcmkB and ΔcmkC growth is not restricted to the first nuclear division cycle. The percentage of germlings with two or greater nuclei that also have four or greater nuclei was calculated for pAL5#10 (circles), dcmkB (squares), and ΔcmkC (triangles). The percentages were calculated from more than 500 individual germlings at each time point. The graph is representative of multiple experiments.

B. FIG. 7. The activation of NIMX<sup>cdc2</sup> is delayed in both dcmkB and ΔcmkC. Histone H1 kinase assays were performed on protein extract from pAL5#10 (black bar), dcmkB (grey bar), and ΔcmkC (white bar) at various times after germination. The graph is representative of three independent experiments.

The endogenous cmkB promoter (dcmkB) (Fig. 5, A and B). As demonstrated in Fig. 5C, the level of CMKB in the control strain (pAL5#10) is low at germination but after 1 h, CMKB begins to accumulate and continues to increase linearly up to 5 h, at which time a peak 5-fold increase in protein level is observed. However, CMKB expression is delayed in dcmkB relative to control by approximately 1 h. Once CMKB begins to accumulate, it does so linearly and reaches a comparable plateau after 6 h of growth. Presumably the CMKB expression in dcmkB is the result of read-through from an upstream promoter.

Using dcmkB, we next examined the physiological consequences of delayed CMKB expression. To determine the effects of altered CMKB expression on the nuclear division cycle of A. nidulans, the number of germlings with two or more nuclei was determined for dcmkB and a nutritionally complemented control (pAL5#10) as a function of time after germination. As demonstrated in Fig. 6A, pAL5#10 begins to undergo the first nuclear division before 6 h, and by 8 h after inoculation, nearly 100% of the control conidia have at least 2 nuclei. In contrast, dcmkB conidia are delayed approximately 2 h in the executing the first nuclear division. Thus, CMKB is required for the proper timing of the initial nuclear division after germination.

To determine whether the delayed expression of CMKB affected only the first nuclear division or also subsequent divisions, we examined the ability of dcmkB to continue with the second nuclear division by scoring the percent germlings that, having undergone one nuclear division, proceed to accomplish subsequent divisions as indicated by the presence of four or more nuclei. As seen in Fig. 6B, by 10 h of growth, all of the pAL5#10 spores have generated germlings with 4 or more nuclei. The absolute timing of initiation of the second nuclear division in dcmkB is delayed approximately 2 h in comparison with the controls, but the rate at which the germlings proceed with the second nuclear division is similar to that of the control. Thus, retarded CMKB expression upon germination affects only the rate of the first nuclear division cycle, not subsequent mitoses.

To more finely probe CMKB function during germination, we investigated whether the lag in dcmkB germination is before activation of NIMX<sup>cdc2</sup>. After germination, the initiation of S-phase is dependent upon and indicated by the activation of the A. nidulans single cyclin-dependent kinase, NIMX<sup>cdc2</sup> (21, 3). We assayed NIMX<sup>cdc2</sup> activity during germination by precipitating the kinase with p13-agarose and measuring the ability of the complex to phosphorylate histone H1 in vitro. As demonstrated in Fig. 7, activity of NIMX<sup>cdc2</sup> in the control strain is first evident after 5 h of growth and increases linearly through

Role of CMKB during Germination—Since CMKB appears to be essential, we generated an A. nidulans strain in which CMKB expression was slightly delayed due to the disruption of the endogenous cmkB promoter (Fig. 5, A and B). As demonstrated in Fig. 5C, the level of CMKB in the control strain (pAL5#10) is low at germination but after 1 h, CMKB begins to accumulate and continues to increase linearly up to 5 h, at which time a peak 5-fold increase in protein level is observed. However, CMKB expression is delayed in dcmkB relative to control by approximately 1 h. Once CMKB begins to accumulate, it does so linearly and reaches a comparable plateau after 6 h of growth. Presumably the CMKB expression in dcmkB is the result of read-through from an upstream promoter.
required for the proper timing of DNA synthesis, we followed only the first but also the subsequent nuclear divisions. cmk for proper timing of the first nuclear division after germination. 20 h post-inoculation. Thus, like CMKB, CMKC is important the germlings fail to undergo a single nuclear division within compared with the control. Surprisingly, approximately 30% of division is dramatically delayed in the absence of CMKC as d cmk nuclei in comparison with the control. The extended duration of the second nuclear division is observed by the reduced rate at which cmkB germlings containing two nuclei accumulate four nuclei in comparison with the control. The extended duration of the second nuclear division in the absence of CMKC indicates that cmkC is required for the proper temporal regulation of not only the first but also the subsequent nuclear divisions.

To determine whether, similar to cmkB, cmkC function is required for the proper timing of DNA synthesis, we followed NIMX cdc2 activation upon germination. Fig. 7 demonstrates that disruption of cmkC severely retards NIMX cdc2 activation by both delaying the onset of activation and dampening its maximal activity. The impaired activation of histone H1 kinase activity correlates with the severe delay in both the initiation of nuclear division and subsequent exponential growth. Thus, we conclude that CMKC plays a non-essential but important role during G1 after germination.

FIG. 8. Generation of ΔcmkC. A, Southern analysis of a representative ΔcmkC. 10 μg of genomic DNA was digested with PvuI and hybridized with a full-length CMKC cDNA probe. A 3.9-kbp band represents the endogenous gene, and the disrupted gene yields 7.0- and 1.9-kbp fragments. B, activation of CMKB by GR5 and ΔcmkC crude protein extracts. CMKB was preincubated with crude protein extracts from either GR5 or ΔcmkC and compared with unactivated CMKB. The values represent the average ± S.E. of three independent experiments.

To determine whether, similar to cmkB, NIMX cdc2 activation is also postponed. Thus, although we cannot rule out the possibility that cmkC plays an important role elsewhere in the nuclear division cycle, we conclude that CMKC is required for the normal temporal regulation of NIMX cdc2 activity before S-phase in A. nidulans.

Generation and Characterization of ΔcmkC—The disruption scheme for cmkC was similar to the original approach utilized for CMKB. In contrast to the pCMKBdis transformation, several strains were identified from the initial transformation of GR5 with pCMKcdis that yielded the Southern blotting pattern predicted for homologous recombination. Fig. 8A shows a representative Southern blot of a single strain in which the cmkC locus has been disrupted. These results indicate cmkC is not required for the viability of A. nidulans.

Since cmkC is not essential for growth of A. nidulans, we first sought to determine whether, in the absence of CMKC, we could detect the activity of other activating kinases in crude protein extract that might functionally complement the loss of cmkC. Fig. 8B demonstrates that crude protein extract from the parental GR5 strain can activate CMKB in vitro, whereas crude protein extract from ΔcmkC cannot. Although we cannot rule out the presence of an insoluble or developmentally regulated activating protein kinase, these results suggest cmkC is the predominant activating kinase of CMKB in A. nidulans.

To assess the requirements for CMKC in the nuclear division cycle, we performed the same experiments as described for ΔcmkC. As demonstrated in Fig. 6A, the timing of first nuclear division is dramatically delayed in the absence of CMKC as compared with the control. Surprisingly, approximately 30% of the germlings fail to undergo a single nuclear division within 20 h post-inoculation. Thus, like CMKB, CMKC is important for proper timing of the first nuclear division after germination. cmkC disruption alters not only the kinetics of the first nuclear division but also subsequent divisions (Fig. 6B). The lag of the second nuclear division is observed by the reduced rate at which ΔcmkC germlings containing two nuclei accumulate four nuclei in comparison with the control. The extended duration of the second nuclear division in the absence of CMKC indicates that cmkC is required for the proper temporal regulation of not only the first but also the subsequent nuclear divisions.

To determine whether, similar to cmkB, cmkC function is required for the proper timing of DNA synthesis, we followed NIMX cdc2 activation upon germination. Fig. 7 demonstrates that disruption of cmkC severely retards NIMX cdc2 activation by both delaying the onset of activation and dampening its maximal activity. The impaired activation of histone H1 kinase activity correlates with the severe delay in both the initiation of nuclear division and subsequent exponential growth. Thus, we conclude that CMKC plays a non-essential but important role during G1 after germination.

DISCUSSION

Using an expression cloning strategy, we identified two novel Ca2+/CaM-dependent protein kinases in A. nidulans. CMKB and CMKC are homologues of the mammalian CaMK cascade members, CaMKI/IV and CaMKα/β, respectively. Although closely related to CaMKI and IV, CMKB shares highest identity with Ca2+/CaM-dependent protein kinases of S. pombe (cmk1) and C. gloeosporioides (CMK), and each of these homologues possesses a Thr within its activation loop at the identical position as CMKB Thr-179, indicating they may also be subject to activating phosphorylation. The closest homologue of CMKC in lower eukaryotes is S. pombe scr1. The identification of CMKB and CMKC and their predicted homologues in other fungal species suggest, similar to other signal transduction systems, the CaMK cascade is evolutionarily conserved from unicellular eukaryotes to mammals.

cmkB is the second essential CaMK identified in any system. The terminal phenotype of ΔcmkB spores indicates CMKB is required for an early step in the nuclear division cycle. Furthermore, we demonstrate that CMKB functions before the S-phase activation of NIMX cdc2, after germination. A. nidulans CMKA, the other essential CaMK, is required for G2 progression (5). Thus, two distinct CaMKs are essential for progression through G1 and G2 in A. nidulans.

The requirement of CMKB in G1 is consistent with previous studies of CaM and CaMks in other systems. The CaM inhibitor W-13 arrests mammalian cell cycle progression in at least two points during re-entry from G0 (22). Furthermore, the general CaM inhibitor, KN-93, blocks cell proliferation before S phase in both cycling cells and those stimulated to enter the cell cycle from G0 (7–9). Our experiments in Aspergillus, which demonstrate that disabling CMKB disrupts entry into the cell cycle, attribute to CMKB, among the CaM kinases, an essential role in the transition of spores from quiescence into S-phase. Experiments are ongoing to determine whether the homologues of CMKB, CaMKS I/IV, are essential targets of CaM during G1 in mammalian cells.

Although CMKC is not essential for proliferation, it does
perform an important role in G1 progression after germination. However, if CMKB and CMKC comprise a linear kinase cascade in vivo, as suggested by both our in vitro biochemical data and by the role of the two kinases at similar points in the cell cycle, it would be predicted that CMKC should also be essential for the nuclear division cycle. There are multiple explanations for this conundrum. The simplest explanation is that other protein kinases can perform the predicted function of CMKC in its absence. Although disruption of CMKC eliminates all activating activity in crude A. nidulans extract, we cannot completely eliminate the possibility that other kinases or CMKC isoforms capable of phosphorylating and activating CMKB are present in the fungus. Another possibility is that CMKB is not the relevant substrate for CMKC in vivo. The mammalian CaMKks have been demonstrated to phosphorylate and activate both protein kinase B and AMP-activated protein kinase (23, 24). Thus it is possible that in vivo CMKC possesses substrates other than CMKB that are important, but not essential, for G1 progression. Alternatively, activation loop phosphorylation of CMKB may not be required for CMKB function in vivo. In vitro, bacterially expressed CaMKI, CaMKIV, and CMKB all possess significant kinase activity that is dramatically enhanced upon activation loop phosphorylation (25, 15). Thus, in the absence of CMKC, it is possible that the reduced but not abolished CMKB activity is sufficient to slowly drive the nuclear division cycle through the G1 in the absence of activation loop phosphorylation. Finally, Hook et al. (26) have recently provided evidence that activation loop phosphorylation may not be required for CaMKI and IV activation but may instead alter their substrate specificity. If this is the case in vivo, the two kinases may form a cascade, but CMKC would not necessarily be essential for proliferation. For example, CMKB may possess an activation-independent substrate essential for G1 progression and an activation-dependent substrate whose phosphorylation is not strictly required for G1 progression but that affects the kinetics of this cell cycle phase. Thus, the fact that CMKC is not essential for the nuclear division cycle in A. nidulans does not eliminate the possibility that CMKB and CMKC form a functional CaMK cascade in vivo.

To investigate if CMKB and CMKC are epistatic, we attempted to functionally complement the absence of CMKC by ectopically expressing CMKB or CMKB T179D in ΔcmkC. CMKB T179D partially mimics T179D phosphorylation; therefore if CMKB and CMKC are members of a kinase cascade, we predicted that overexpression of wild-type CMKB or T179D would rescue the growth delay of ΔcmkC. However, neither enzyme complements the loss of CMKC in ΔcmkC. Furthermore, similar to S. pombe cmk1 (27), ectopic T179D expression lengthens the nuclear division cycle in both wild-type and ΔcmkC strains. Although these experiments failed to make clear whether CMKB and CMKC are components of a linear cascade in vivo, they do demonstrate the importance of the multiple modes of regulation of CMKB. Finally, overexpression of the wild-type enzyme has no effect on the nuclear division cycle, whereas expression of the artificially activated full-length protein is detrimental to growth.

Consistent with our observations in A. nidulans, pharmacological inhibitors of both CaM and CaMK arrest mammalian cells in G1 before cdk activation. KN-93 arrests NIH 3T3 cells with reduced cdk4 and cdk2 activities attributed to a reduction in cyclinD1 levels and an increased association of p2^kip with cdk2/cyclinE (9). The CaM antagonist W13 also inhibits G1 progression in normal rat kidney cells before cdk4 and cdk2 activation, but this CaM inhibition does not appear to affect cyclinD or -E protein levels. Instead Taules et al. (28) demonstrate that in the presence of W13, cdk4/cyclinD1 is exclusively cytoplasmic rather than nuclear. Thus, the authors suggest that CaM directly or indirectly modulates cdk4 activity by regulating its nuclear localization. In Aspergillus, W7 blocks germination before the activation of the S-phase cd/cyclin complex, NIMXcd2/NIMEcyclinB (3). Thus, NIMEcyclinB synthesis and/or the subcellular localization of NIMXcd2/NIMEcyclinB represent attractive targets of the CMKB and CMKC cascade in A. nidulans.

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