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Ca^{2+}/Calmodulin-dependent Kinase Is Essential for both Growth and Nuclear Division in *Aspergillus nidulans*

Jennifer S. Dayton and Anthony R. Means*

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

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The calmodulin gene has been shown to be essential for cell cycle progression in a number of eukaryotic organisms. In vertebrates and *Aspergillus nidulans* the calmodulin dependence also requires calcium. We demonstrate that the unique gene encoding a multifunctional calcium/calmodulin-dependent protein kinase (CaMK) is also essential in *A. nidulans*. This enzyme is required both for the nuclear division cycle and for hyphal growth, because spores containing the disrupted gene arrest with a single nucleus and fail to extend a germ tube. A strain conditional for the expression of CaMK was created. When grown under conditions that resulted in a 90% decrease in the enzyme, both nuclear division and growth were markedly slowed. The CaMK seems to be important for progression from G_2 to mitosis.

INTRODUCTION

Our previous studies in *Aspergillus nidulans* have shown that both Ca^{2+} and calmodulin (CaM) are essential for progression of the nuclear division cycle and hyphal growth (Rasmussen *et al.*, 1990; Lu *et al.*, 1992). Disruption of the unique CaM gene is lethal (Rasmussen *et al.*, 1990), and the creation of a strain conditional for the expression of CaM showed that this regulatory protein was required for both the G_2/M and G_1/S transitions (Lu *et al.*, 1992). Execution of both checkpoints also required Ca^{2+} consistent with previous studies in mammalian cells (Whitaker and Patel, 1990; Lu and Means, 1993b). These observations suggested that at least one essential target of CaM must exist in *A. nidulans* that also required Ca^{2+}.

The Ca^{2+}/CaM-dependent protein phosphatase calcineurin was found to be unique and essential in *A. nidulans* (Rasmussen *et al.*, 1994). Disruption of the gene for the catalytic or A subunit was lethal, and morphological examination suggested that arrest of the nuclear division cycle might have occurred in G_1. Thus, another Ca^{2+}-dependent CaM target was postulated to be responsible for the requirement of Ca^{2+}/CaM in G_2.

* Corresponding author: Department of Pharmacology, Duke University Medical Center, Box 3813, Durham, NC 27710.

The only other Ca^{2+}/CaM-dependent enzyme whose cDNA has been cloned from *A. nidulans* is a protein kinase. The purified protein is monomeric; however, it phosphorylates a number of substrates in common with mammalian CaMKIIα (Bartelt *et al.*, 1988). Kornstein *et al.* (1992) cloned the cDNA, which was only 29% identical to CaMKIIα, and, on the basis of Southern blot analysis, suggested that the gene was unique.

Precedent exists for a specific role of CaMKII in cell cycle progression in other organisms. Microinjection of a weakly constitutively active form of rat brain CaMKIIα into activated *Xenopus* oocytes arrested at metaphase of meiosis II, stimulated germinal vesicle breakdown and resumption of meiosis (Waldmann *et al.*, 1990). In contrast, microinjection of antibodies to CaMKIIα or autoinhibitory peptides that serve as a competitive substrate antagonist of the endogenous form of the enzyme inhibited nuclear envelope breakdown in response to a Ca^{2+} signal (Baitinger *et al.*, 1990). Finally, Lorca *et al.* (1993) demonstrated that microinjection of a constitutively active truncated form of CaMKIIα induces cyclin degradation and $p34^{cdc2}$ inactivation in the absence of Ca^{2+}, providing proof that this enzyme is the essential target for Ca^{2+}/CaM required to escape cytostatic factor-induced metaphase arrest. More indirect evidence has impli-

cated CaMKII as important for the G_2/M transition. Overexpression of a constitutively active form of CaMKIIα in both mouse C127 cells (Planas-Silva and Means, 1992) and *Schizosaccharomyces pombe* (Rasmussen and Rasmussen, 1994) results in a G_2 arrest. Thus the existing evidence pointed to a role for an ordered phosphorylation/dephosphorylation reaction initiated by CaMKII in the regulation of entry into and progression through mitosis/meiosis.

The present studies were undertaken to determine whether the unique CaMK is required for cell cycle progression in *A. nidulans*. We have demonstrated that disruption of the CaMK gene produces spores that are unable to germinate and initiate hyphal growth or to complete mitosis. Thus CaMK is essential in *A. nidulans*. Furthermore, decreasing CaMK protein levels by 90% causes a slowing of hyphal growth and a slowing of the nuclear division cycle in G_2.

MATERIALS AND METHODS

Strains

The following strains were used in this work: R153 (*wA3*; *pyroA4*), GR5 (A773; *pyrG89*; *wA2*; *pyroA4*), A767 (pyrG89; *nicA2*), and SO26 (*nim*T23; *pyrG89*; *wA2*; *biA1*; *paba*A1).

Medium

Aspergillus nidulans strains were grown in minimal media containing (in mM): 50 glycerol (MMG), 50 glycerol plus 100 threonine (MMG/T), or 50 dextrose with (MMD/A) or without (MMD) 100 sodium acetate. Agar (2%) was added for solid media, 5 mM uridine and 10 mM uracil were added for *pyrG89* strains, 8 μM nicotinamide for *nicA2* strains, and 5 μM pyridoxine for *pyroA4* strains. Enriched medium (YG) contained 0.5% yeast extract and 2% glucose.

Transformation of A. nidulans

The conditional strain was created by homologous recombination of the plasmid palcCaMK into the CaMK gene locus. To control for the effects of repression and induction of CaMK, we generated an inactive CaMK strain by nonhomologous integration of the plasmid palcK50M. An inactive CaMK construct was created by changing Lys 50 to Met (K50M; Dayton, Sumi, Nanthakumar, and Means, unpublished results). A CaMK cDNA fragment was introduced into the *Eco*RI site of M13 mp8 and used for mutagenesis by the method of Olsen and Eckstein (1990) with the oligonucleotide 5'CAGGATAATCATTACCGCAAC3'. After verification that the K50M mutation generated an inactive kinase, this mutant cDNA was ligated into the pAL5 *A. nidulans* expression vector behind the alcohol dehydrogenase promoter. The resulting palcK50M plasmid also contains the *pyr4* nutritional marker from *Neurospora crassa* and a histone H2A gene 3' terminator to allow expression of the cDNA. After transformation of the GR5 strain as described by Lu and Means (1993a) with palcCaMK or palcK50M, the resulting transformant strains, alcCaMK and alcK50M, were selected on MMG plates without uridine or uracil and were cloned by streaking to single colonies three times.

The disruption was accomplished by homologous recombination, using the plasmid pACaMK dis and maintaining each colony as a heterokaryon by transferring mycelia from the transformation plate to dialysis membrane on new selective plates. Two different approaches that used the pACaMK dis plasmid produced a similar phenotype. The GR5 strain was transformed with pACaMK dis and plated onto MMD plates without uridine or uracil. In addition, GR5 and A767 protoplasts were transformed together with pACaMK dis and plated onto MMD plates without uridine, uracil, pyridoxine, or nicotinamide to force heterokaryon growth. These heterokaryon colonies were easily recognizable because they grew with regions of white and green (GR5 is white, and A767 is the wild-type green color).

Southern and Northern Analyses

Genomic DNA and total RNA were extracted from dried mycelia as described by Lu and Means (1993a). Genomic DNA was digested overnight with the indicated restriction enzymes at 37°C, separated by 1% agarose gel electrophoresis, and transferred to a nylon membrane (Zeta-probe; Bio-Rad, Richmond, CA) with 10× SSC. Total RNA (10–20 μg) was separated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Zeta-probe; Bio-Rad) with 20× SSC (Maniatis, 1989). Filters were probed with a ^{32}P-labeled *A. nidulans* CaMK gene fragment from the ATG initiation codon to the *Bam*HI site located 1280 bp downstream in 500 mM $NaPO_4$, pH 7.2, and 7% SDS at 65°C for Southern blots and 68°C for Northern blots (Church and Gilbert, 1984). The β-tubulin (*ben*A) gene probe was the generous gift of Greg May (Baylor College of Medicine, Houston, TX).

Determination of Kinase Activity in Cell Lysates

The germlings were harvested on Miracloth, washed with water, and frozen in liquid nitrogen. Frozen samples were ground with mortar and pestle for 5 min on ice in 0.5 ml of homogenizing buffer (35 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid [HEPES]-Na, pH 7.8, 10% glycerol, 50 mM NaCl, 1 mM $MgCl_2$, 5 mM ethylene glycol bis(β-aminoethyl)ether-*N,N,N',N'*-tetra-acetic acid [EGTA], 1 mM dithiotreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The homogenate was clarified by centrifugation at 16,000 × *g* and at 4°C for 10 min, and the protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). CaMK activity was determined in assay buffer including 35 mM HEPES, pH 7.8, 10 mM $Mg(OAc)_2$, 1 mM $CaCl_2$, 1 μM CaM, 10 μM protein kinase A inhibitor peptide (Sigma, St. Louis, MO), 100 nM microcystin (Calbiochem, La Jolla, CA), 50 μM ATP, and 1 μCi per tube of γ-^{32}P-ATP (Amersham, Arlington Heights, IL). The assays were performed with 10 μg of total cell extract and 50 μM of ADR1(41) (LKKLTRRASFSGQ) as a peptide substrate for 2 min at 30°C (Roskoski, 1983). Because this peptide can also be phosphorylated by PKA, 10 μM PKI, the peptide inhibitor of PKA, was added in all assays (Cherry *et al.*, 1989).

Determination of Cell Growth

Dry weight was measured as described by Lu and Means (1993a). Radial colony growth was determined as described by McGoldrick *et al.* (1995).

Determination of Cell Cycle Kinetics

The number of nuclei per germling was determined after staining samples with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI; Lu and Means, 1993a). DNA synthesis was quantified by [^{3}H]-adenine incorporation according to the method of Bergen and Morris (1983).

RESULTS

Disruption of CaMK Gene in A. nidulans Is Lethal

To determine whether CaMK is essential in *A. nidulans*, we disrupted the CaMK gene in a heterokaryon. A schematic representation of the pCaMK dis plasmid and the predicted disruption of the CaMK locus is

shown in Figure 1A. It was created by inserting the *Pst*I (bp 263–1215 of the gene) fragment of the *A. nidulans* CaMK gene into the polylinker of the pRG1 vector (Waring *et al.*, 1989). pRG1 is a pUC9-based vector containing the *Neurospora crassa pyr*4 gene, (encoding orotidine-5′-phosphate decarboxylase) a selectable marker that complements the *pyrG*89 mutation in *A. nidulans*. This circular plasmid DNA was used to transform the GR5 and A767 strains that carry the *pyrG*89 mutation. The successful transformation confers pyrimidine auxotrophy, allowing colony growth in the absence of uridine and uracil. The homologous recombination event should change the 2-kb *Bam*HI fragment to one that is ~8.0 kb. Figure 1B demonstrates the method used to evaluate the growth of the resulting heterokaryon colonies.

The transformation results in many nonhomologous recombination events; thus ~200 putative heterokaryon colonies were analyzed. The samples were grown 3–4 d on dialysis membrane on selective plates, harvested, frozen, and lyophilized for DNA isolation. Approximately 10–15% did not grow enough to be analyzed by Southern blot, and only three contained both the wild-type and disrupted CaMK genes. These disrupted heterokaryon colonies grew very slowly. The Southern blot of DNA from one heterokaryon colony (dis#1) is shown in lane 1 of Figure 1C with two bands, the wild-type (2 kb) and the disrupted band (~8.0 kb). The wild-type GR5 band pattern is shown in lane 2.

Spores from dis#1 were inoculated into enriched selective medium (YG) and enriched nonselective medium (YGUU) and incubated for 10 h. In the presence of uridine and uracil, 65% of the conidia germinate normally, representing the *pyr*4$^-$CaMK$^+$ nuclei. A representative photograph (Figure 2A) shows the growth of the *pyr*4$^-$CaMK$^+$ nuclei after 10 h in YGUU. The remaining 35% of the conidia do not extend germ tubes and contain a single nucleus, representing the *pyr*4$^+$CaMK$^-$ nuclei (Figure 2B). In the absence of uridine and uracil neither the *pyr*4$^-$CaMK$^+$ nuclei nor *pyr*4$^+$CaMK$^-$ nuclei send out germ tubes or undergo nuclear division. The fact that the heterokaryons are viable but generate conidia, which do not germinate in the absence of uridine and uracil, demonstrates that the CaMK gene disruption is a lethal mutation.

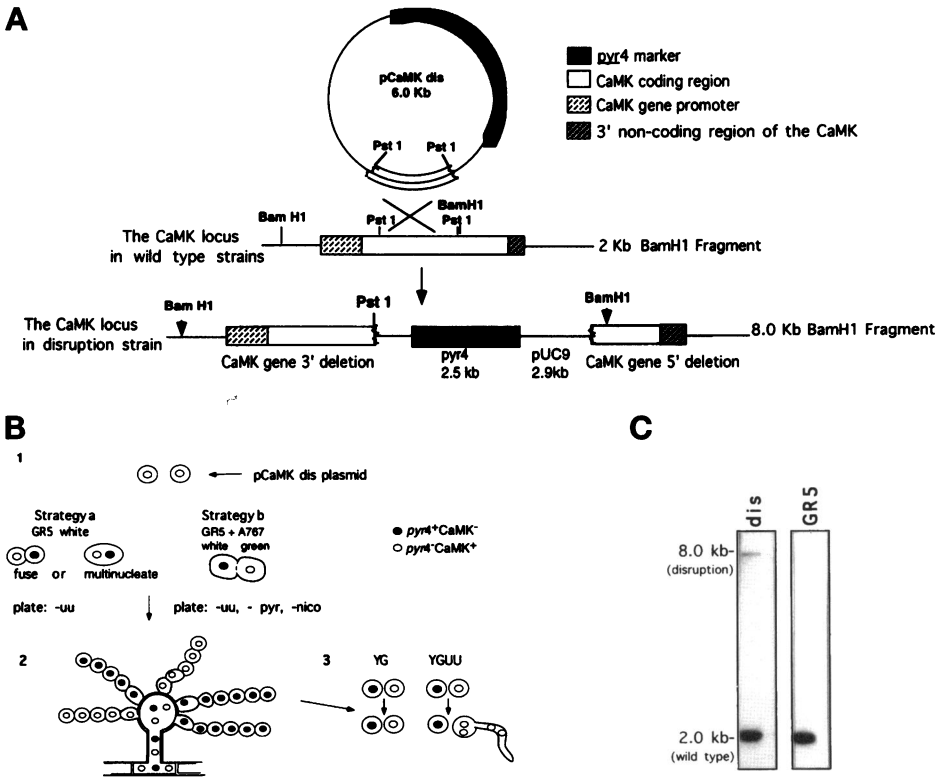

Figure 1. Construction of *A. nidulans* CaMK disruption. (A) A schematic representation of the predicted result of homologous recombination in the CaMK locus to disrupt the wild-type gene and generate a truncated gene without the 3′ end and a copy with the promoter and first 250 bp deleted. (B) Diagram of heterokaryon transformation and growth in *A. nidulans*. GR5 protoplasts or GR5 plus A767 protoplasts were transformed with the pACAMK dis plasmid (1). Because both GR5 and A767 are *pyr*G89, neither can grow in the absence of uridine and uracil (UU). If one nucleus of a protoplast with two nuclei is transformed with pACAMK dis or if two protoplasts, one with a transformed nucleus and one with wild-type nucleus, fuse, the protoplast will no longer require UU for growth because of the *pyr*4 gene in the plasmid (left side of diagram). Alternatively, on the right side, nutritional selection will force the fusion of a GR5 protoplast with an A767 protoplast, and one of the nuclei must contain the disruption and the *pyr*4 gene. The resulting heterokaryon from either strategy (2) will sporulate, producing uninucleate conidia that are either *pyr*4$^-$CaMK$^+$ or *pyr*4$^+$CaMK$^-$. When the conidia are germinated (3) in selective medium without uridine and uracil, the *pyr*4$^-$CaMK$^+$ conidia will not germinate, and the *pyr*4$^+$CaMK$^-$ conidia will demonstrate the phenotype of the disruption of CaMK, whereas in nonselective medium containing uridine and uracil the *pyr*4$^-$CaMK$^+$ conidia will germinate normally, and the *pyr*4$^+$CaMK$^-$ conidia will demonstrate the phenotype of the disruption of CaMK. (C) Southern blot analysis of mycelia grown from one disrupted heterokaryon colony. Genomic DNA was restricted with *Bam*HI and hybridized with a probe beginning at the ATG and ending with the *Bam*HI site ~1280 bp 3′ in the gene.

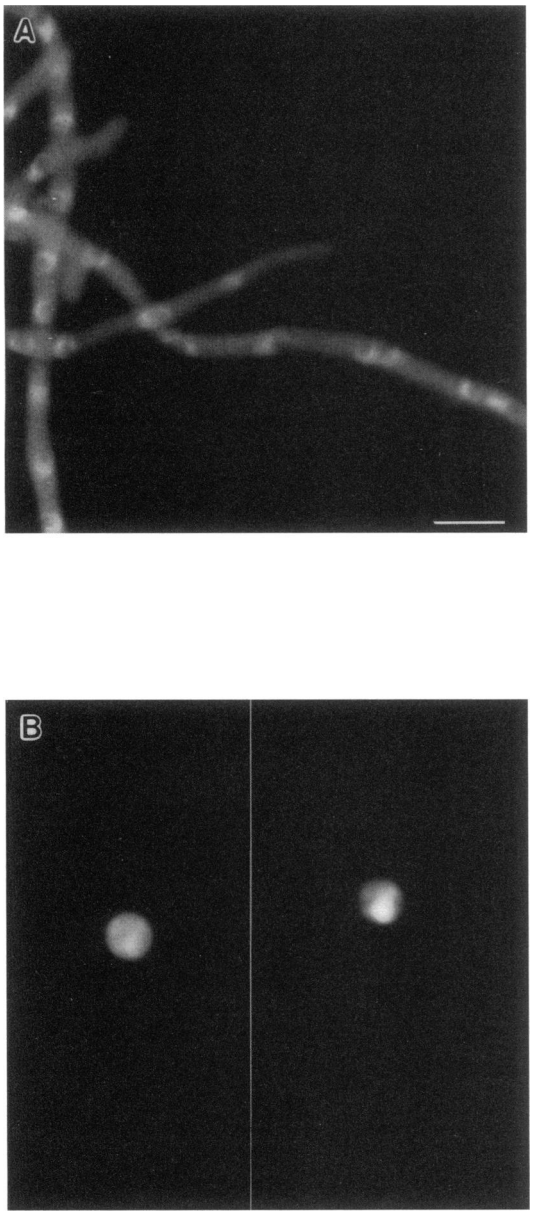

Figure 2. Effect of the CaMK gene disruption on germination in liquid cultures. Conidia from one colony of disrupted heterokaryons were germinated for 10 h in nonselective medium (YGUU). After incubation the germlings were harvested, fixed, stained with DAPI, and photographed. (A) A representative *pyr4*$^-$CaMK$^+$ germling and (B) two representative *pyr4*$^+$CaMK$^-$ germlings. Scale, ×1000 (A and B). Bar, 10 μm.

Construction and Characterization of an A. nidulans Strain Conditional for the Expression of Wild-Type CaMK

The point of arrest caused by the disruption of CaMK could not be analyzed further by [^{3}H]-adenine incorporation because so few spores were recovered from the small colonies. Furthermore, flow cytometry cannot be performed on *A. nidulans* spores because of background fluorescence from the spore coat. Thus to examine the specific roles performed by CaMK in cell cycle progression of *A. nidulans*, we created a strain conditional for its expression. Figure 3A shows a diagram of the plasmid DNA construct, the wild-type CaMK locus, and the predicted result of homologous recombination of the vector DNA into the CaMK locus. The 5′ 800 bp regulated by the wild-type CaMK promoter is predicted to be inactive because it is truncated ~300 bp before the conserved arginine, which marks the end of the catalytic domain of calmodulin-dependent kinases (Payne *et al.*, 1988; Soderling, 1990). A second strain, alcK50M, was used as a control in these studies. The alcK50M strain is conditional for the expression of an inactive CaMK in the presence of an intact wild-type CaMK locus. The inactive CaMK was generated by mutation of Lys 50 in the ATP binding region to Met. This construct was expressed in rabbit reticulocyte lysates and verified to produce an inactive protein before creation of the alcK50M strain (our unpublished results). The alcohol dehydrogenase promoter used in both strains is regulated by the carbon source in the culture medium (Waring *et al.*, 1989). Minimal medium containing glucose (D) and/or acetate (A) represses transcription from the promoter, minimal medium containing glycerol (G) allows a basal, low level of transcription, and minimal medium containing glycerol and threonine (G/T) induces transcription.

Homologous recombination was verified by Southern blot, as illustrated in Figure 3B. Lane 1 shows the 2 kb wild-type CaMK *Bam*HI/*Kpn*I fragment obtained when restricted GR5 DNA is probed with the 5′ 1280 bp, beginning at the ATG and ending at the *Bam*HI site of the CaMK gene. Homologous recombination deletes a *Bam*HI site and inserts a *Kpn*I site at the 3′ end of the alcohol dehydrogenase promoter, creating a 6.7-kb fragment and a 1.3-kb fragment. Lane 2 shows a representative clone with homologous recombination designated alcCaMK. Lane 3 shows the wild-type 2-kb fragment and the integration of the transgene K50M mutant cDNA, which creates a 0.83-kb cDNA fragment in the alcK50M strain. The regulation of the CaMK mRNA is demonstrated in Figure 3C. The parental GR5 strain shows similar levels of CaMK mRNA in all three carbon sources. The alcCaMK strain shows a low level of CaMK mRNA detectable in derepressing medium (G) similar to that in the GR5 strain. In the presence of inducing medium (T) the mRNA is induced, and in the presence of repressing medium (D) the mRNA is undetectable. The Northern blot was stripped and reprobed with a *Bst*EII restriction fragment of the *A. nidulans* β-tubulin (*ben*A) gene (May, 1989) to verify that equivalent total RNA was present in each lane.

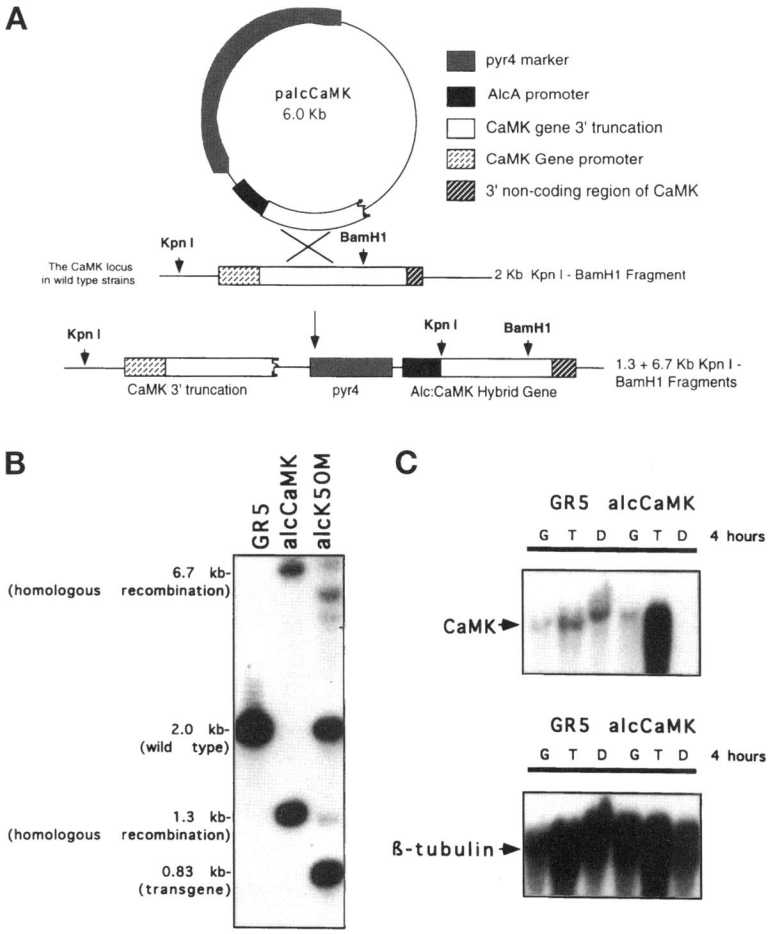

Figure 3. Construction of an *A. nidulans* CaMK conditional strain. (A) The predicted result of homologous recombination of palcCaMK into the *A. nidulans* CaMK locus to disrupt the wild-type gene and generate a gene regulated by the alcohol dehydrogenase promoter. The first 834 bp (ATG to *Hin*cII site) of the CaMK gene were ligated into the pAL3 *A. nidulans* expression vector behind the alcohol dehydrogenase promoter. The vector also contains the *pyr4* selectable marker from *Neurospora crassa*. (B) Southern blot analysis of alcCaMK and alcK50M mutant conditional strains shows homologous recombination in alcCaMK and integration of the transgene in the alcK50M mutant. (C) Northern blot analysis of 10 μg of total RNA isolated from GR5 and alcCaMK strains grown in derepressing (G), inducing (T), or repressing (D) medium verifies regulation of CaMK mRNA with changing carbon sources.

The changes in mRNA are recapitulated in the CaMK protein expression and enzyme activity. Western blot analysis demonstrated that the CaMK protein was induced and repressed in the presence of T and D, respectively (our unpublished observations). Table 1 shows that the changes in mRNA and protein observed in different carbon sources correspond to the predicted changes in activity. Spores were grown for 4 h in liquid cultures containing MMD/A, MMG, or MMG/T, and extracts were prepared from frozen samples. The induction of CaMK in the alcCaMK strain for 4 h causes an 18-fold increase in Ca^{2+}/CaM-dependent kinase activity, using the ADR1 peptide as the substrate, with only slightly elevated kinase activity in the presence of EGTA. In contrast, extracts from alcCaMK grown in repressing or derepressing medium have no detectable Ca^{2+}/CaM-dependent kinase activity above that in the presence of EGTA. As expected, the alcK50M control strain has similar levels of Ca^{2+}/CaM-dependent kinase activity in repressing, derepressing, and inducing medium. The activity present in alcK50M is also similar to the activity in the parental GR5 strain grown in glucose. The fact that there is very little activity in the alcK50M extracts grown in inducing conditions demonstrates that the protein made is inactive. The small variability in CaMK activity that exists in the alcK50M strain in the three carbon sources may be a real effect of the ALCR inducer on the wild-type CaMK promoter; however, the CaMK promoter has not been characterized.

Table 1. CaMK activity in *A. nidulans* extracts

Strain and growth conditions	Ca^{2+}/CaM nmol · min^{-1} · mg^{-1} extract	EGTA nmol · min^{-1} · mg^{-1} extract
alcCaNK D/A	0.31 ± 0.08	0.26 ± 0.01
alcCaMK glycerol	0.28 ± 0.06	0.29 ± 0.00
alcCaMK G/T	17.70 ± 0.00	0.95 ± 0.05
alcK50M D/A	1.78 ± 0.00	0.54 ± 0.08
alcK50M glycerol	0.70 ± 0.08	0.36 ± 0.03
alcK50M G/T	0.75 ± 0.07	0.37 ± 0.02
GR5 D/A	1.08 ± 0.06	0.43 ± 0.00

Repression of the alcCaMK Gene Slows Growth and Progression of the Nuclear Division Cycle

Initially, growth of the alcCaMK and alcK50M strains was examined on agarose plates and in liquid medium. In inducing medium, growth of these strains was similar to each other and to a w^+ strain, R153 (Figure 4 and our unpublished observations). Although growth of the alcCaMK was significantly reduced in repressing medium during the first 24 h after germination relative to the other two strains, growth did occur on plates, measured by radial colony diameter, or in liquid, measured by dry weight. This phenotype is less severe than in cells containing the disrupted gene because the alcA promoter, although highly repressed in glucose, must produce some CaMK mRNA. As shown in Table 2, Ca^{2+}/CaM-dependent protein kinase activity can be recovered by CaM affinity chromatography from extracts prepared from the alcCaMK strain grown in either derepressing or repressing medium. This activity was 10 and 7.6%, respectively, relative to that present in the w^+ R153 strain. Figure 4 shows the difference in the appearance of the DAPI-stained germlings in the alcCaMK and alcK50M strains grown in liquid medium for 9 h (A–D) or 12 h (E). The growth of the alcK50M is more rapid in glucose (Figure 4A) than in glycerol plus

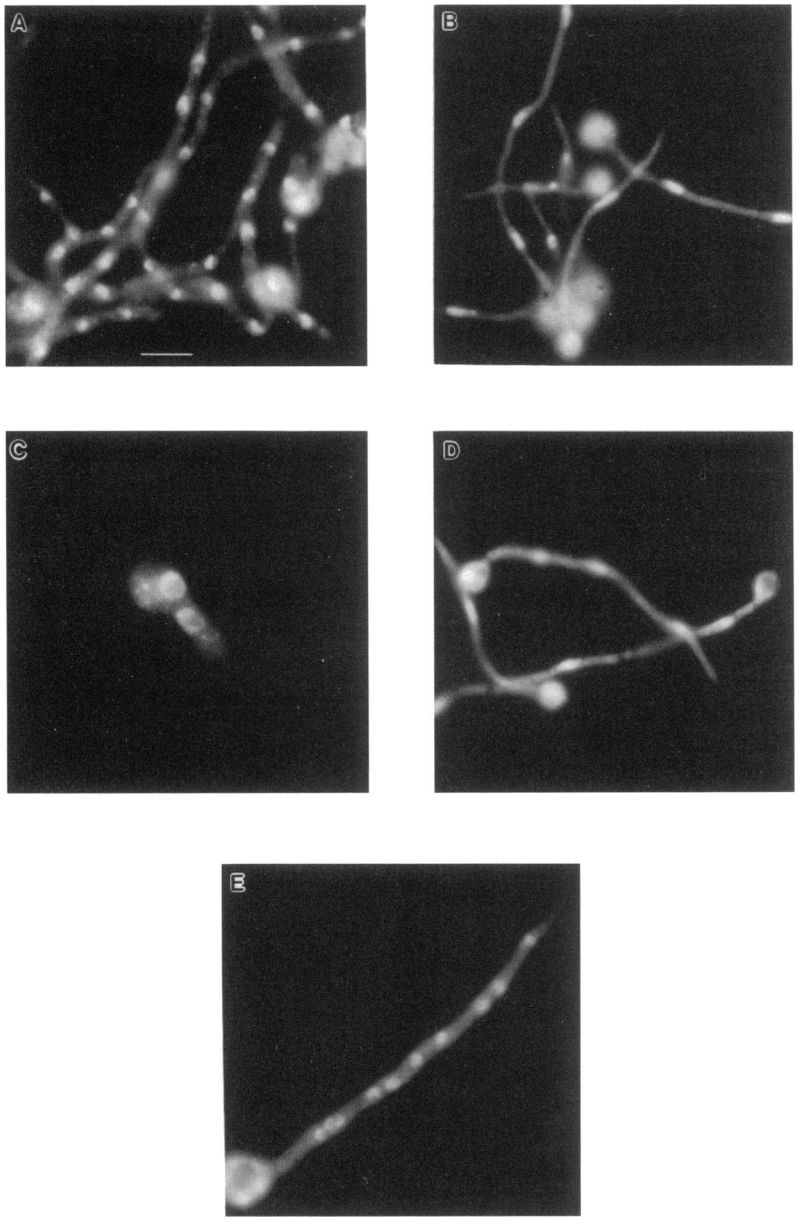

Figure 4. Effects of repression or overexpression of CaMK on growth and nuclear distribution in *A. nidulans*. AlcCaMK or alcK50M were inoculated into repressing or inducing medium. After incubation, mycelia were harvested, fixed, stained with DAPI, and photographed. (A) alcK50M 9 h in MMD/A; (B) alcK50M 9 h MMG/T; (C) alcCaMK 9 h MMD/A; (D) alcCaMK 9 h MMG/T; (E) alcCaMK 12 h MMD/A. Scale, ×1000 (A–E). Bar, 10 μm.

Table 2. CaMK activity recovered from CaM-sepharose chromatography

Strain and conditions	Activity from CaM-sepharose (mU/mg)	Percentage of wild type (%)
R153, MMD/A	0.328	100
alcCaMK, MMG	0.035	10.6
alcCaMK, MMD/A	0.025	7.6

threonine (Figure 4B). Both samples, however, show extended germ tubes and from 4 to >8 nuclei per germling. In contrast, alcCaMK grown in glucose (Figure 4C) for 9 h shows very small or no germ tube extension and 2 or 4 nuclei per germling. The growth of alcCaMK in glycerol plus threonine (Figure 4D) is indistinguishable from that of alcK50M (Figure 4B), ~40- to 50-μm germ tubes containing approximately four nuclei. After 12 h growth in glucose, the alcCaMK strain (Figure 4E) has extended germ tubes, and the number of nuclei has increased significantly. The nuclei often look close together, suggesting there may be some effect on nuclear migration.

To determine where the nuclear division cycle of these germlings was slowed, we measured [^{3}H]-adenine incorporation into DNA. AlcCaMK or alcK50M spores were inoculated into MMD/A at 37°C containing [^{3}H]-adenine. At the indicated times aliquots were harvested, and incorporation into DNA was measured (Bergen and Morris, 1983). Compared with the w^+ R153 strain germinated in the presence of hydroxyurea (HU), the alcK50M strain shows incorporation starting between 4 and 5 h after initiation of germination (Figure 5A). This corresponds to the first S phase, and the further rapid increases after 7 h correspond to the second and subsequent nonsynchronous rounds of DNA synthesis. The results of similar experiments with the w^+ R153 strain in the absence of HU corroborate those obtained with the alcK50M strain. The alcCaMK strain grown in repressing medium also shows similar incorporation between 4 and 5 h of germination but markedly slowed incorporation during subsequent S phases. Thus the repression of CaMK does not alter entry into the first cell cycle upon germination. We next examined the change in the number of nuclei per germling with time. Figure 5B demonstrates that nuclear division is slowed by the repression of CaMK. After 9 h of incubation, the alcCaMK cells have ~3 nuclei per cell, whereas alcK50M cells have >10. These results reveal that the 90% decrease in CaMK slows progression through G_2 of the nuclear division cycle.

DISCUSSION

Disruption of the unique CaMK gene in *A. nidulans* is lethal. The frequency of this recombinatorial event was very low, suggesting the possibility that disruption could result in a partially dominant lethal mutation. That is, the wild-type CaMK protein would not be able to function fully for both the transformed and untransformed nuclei within a heterokaryon. This suggestion could explain the difficulty in recovering

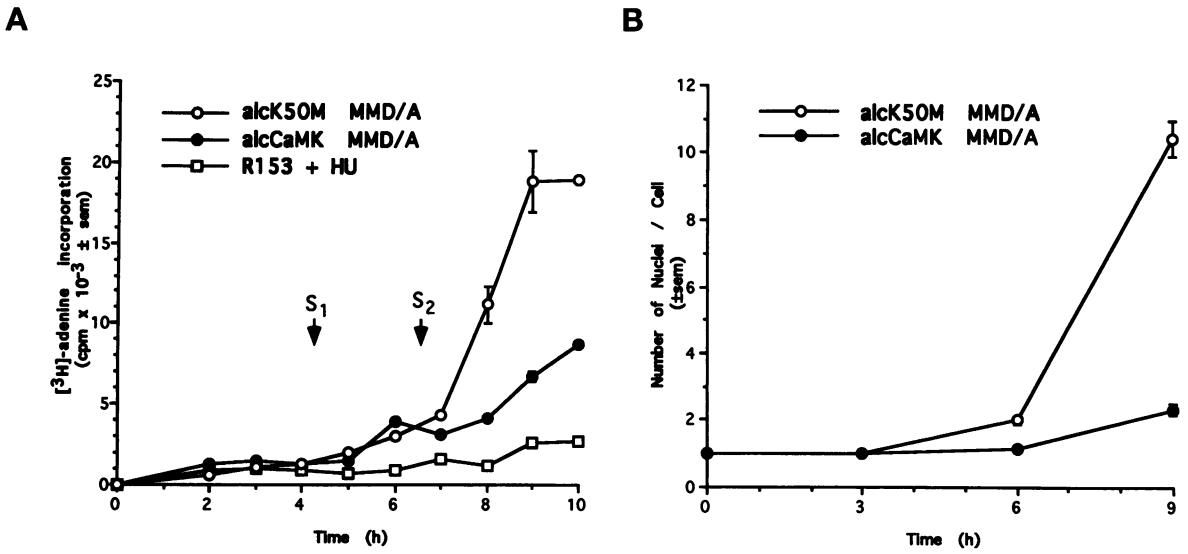

Figure 5. Effects of repression or overexpression of CaMK on DNA synthesis and nuclear division in *A. nidulans*. (A) Repression of CaMK slows the incorporation of [^{3}H]-adenine into DNA. This representative experiment was performed in duplicate. (B) Repression of CaMK slows nuclear division. At least 100 germlings were analyzed for each point. Data are representative of two experiments.

viable heterokaryon carrying the gene disruption. Additional support for this possibility is that the few disrupted heterokaryon that were recovered grew very slowly and produced few spores. In contrast, the frequency of homologous recombination of the palcCaMK plasmid was closer to 20%, and other groups have reported frequencies between 30 and 50% (Miller *et al.*, 1985; Osmani *et al.*, 1988). The fact that the conditional strain did contain some CaMK (10% of normal levels) might be why this strain could be manipulated so readily.

The terminal phenotype of the CaMK null cells is more severe than that in cells in which either the CaM or the CnA gene has been disrupted (Rasmussen *et al.*, 1990, 1994). In both latter instances disrupted spores complete the first nuclear division cycle before arresting. For the CaM case, the movement into and through the first nuclear division cycle is presumably a consequence of the CaM stored in the spore. Interestingly, however, hyphal growth as measured by germ tube extension is minimal. Perhaps the CaM requirement for nuclear division is less than that for growth or determination of cell polarity before germ tube extension at this early developmental stage. To explain the phenotype in cells in which the CnA gene has been disrupted, one could conclude either that entry into DNA synthesis does not require this enzyme or that, similar to the case for CaM, sufficient CnA is present in spores to allow one nuclear division cycle to occur. Because morphological analysis of germlings from the CnA null strain suggested that the block was early in the nuclear division cycle, possibly in G_1 (Rasmussen *et al.*, 1994), the latter possibility seems most likely. Supporting this contention is the observation that germination of w^+ spores in the presence of an anticalmodulin drug, W7, arrests cells with a 1 N content of DNA (our unpublished observations). Collectively, the data show that CaM is required for entry into S phase and completion of mitosis. Based on the fact that both CnA and CaMK are essential genes in *A. nidulans*, it seems possible that they may mediate the requirements for CaM at different stages of the nuclear division cycle.

Indeed, the results from the conditional strain in this study indicate that CaMK is important for progression through the G_2/M transition. The use of KN93, an inhibitor of CaMK, has suggested a role for CaMK in the movement of quiescent (G_0) HeLa cells and NIH-3T3 fibroblasts to the first DNA synthesis (Rasmussen and Rasmussen, 1995; Tombes *et al.*, 1995). In addition, KN93 was shown to arrest HeLa cells in G_2 (Patel, Philipova, Moss, Schulman, Hidako, and Whitaker, 1995). Our studies add support to the latter study with KN93, because we have shown that a 90% reduction in CaMK in *A. nidulans* slows the rate of cell cycle progression such that only one round of nuclear division occurs in the time normally required for three to four rounds of nuclear division in the control strain. The decrease in CaMK does not affect the initial S phase transition, although subsequent DNA synthesis is decreased. Perhaps this effect is not due to an S phase requirement for CaMK but to slowing of progression through the G_2/M transition. On the other hand, on the basis of our data we cannot rule out the possibility that the low level of CaMK present in the conditional strain is necessary and sufficient for some phosphorylation event essential for the initial progression from G_0 into the proliferative cycle.

The repression of CaMK in the conditional strain of *A. nidulans* did cause a slowing of cellular growth. The slowed growth occurred on agar plates or in liquid cultures. The delayed growth seen over time appeared morphologically normal. A single germ tube was extended from the swollen spore, and normal septa were formed. In addition, normal conidiophore structures formed, although more slowly than in control strains. Indeed, the phenotype of the cells containing the disrupted gene shows that CaMK is necessary for both the nuclear division cycle and hyphal growth. The growth of *A. nidulans* is not regulated coordinately with nuclear division as treatment with hydroxyurea arrests nuclear division in S phase, although the spores continue to germinate and grow hyphae (Harris *et al.*, 1994). In addition, the *nim*T23 strain, which arrests nuclei in G_2 at the restrictive temperature, continues to grow despite the arrest of nuclear division. The spores containing a disrupted CaMK gene arrest very early in germination before germ tube extension or nuclear division. Because, in normal cells, germination results in a 15-fold increase in CaMK (our unpublished results), it is possible that the spore store of this enzyme is insufficient for the germination process. Thus these data also indicate that CaMK is important for the normal cellular growth of *A. nidulans* irrespective of the effects on nuclear division and suggest the possibility that the target for these two processes could be different.

The repression of the alcA promoter in *A. nidulans* is tight; however, it is not complete. This observation has been made by others (Waring *et al.*, 1989). In this study we attempted to quantify the leakiness of the alcA promoter by enriching extracts for CaMK with CaM-sepharose chromatography. We have shown that the recovered CaMK activity after 16 h of growth in repressing conditions is 8–10% of that recovered from a control strain. Because the repression of the alcA promoter is mediated by a competition between the CREA repressor and the ALCR inducer (Kulmburg *et al.*, 1992; Mathieu and Felenbok, 1994), over time there may be differences in the balance of these two transcription factors and/or ability for the cells to compensate for the repression of the alcohol dehydrogenase promoter. These factors may contribute to the apparent decrease in the inhibition of growth with

time even in repressing medium. Consistent with this possibility, inhibition of both growth on plates and in liquid culture is most marked at earlier time points. In the case of CaMK, leakiness of the alcA promoter was beneficial because it allowed further investigation into the nature of the role of the enzyme, which the disruption did not.

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REFERENCES

Baitinger, C., Alderton, J., Poenie, M., Schulman, H., and Steinhardt, R.A. (1990). Multifunctional Ca^{2+}/calmodulin-dependent protein kinase is necessary for nuclear envelope breakdown. J. Cell Biol. *111*, 1763–1773.

Bartelt, D.C., Fidel, S., Farber, L.H., Wolff, D.J., and Hammell, R.L. (1988). Calmodulin-dependent multifunctional protein kinase in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA *85*, 3279–3283.

Bergen, L., and Morris, N.R. (1983). Kinetics of the nuclear division cycle of *Aspergillus nidulans*. J. Bacteriol. *156*, 155–160.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. *72*, 248–254.

Cherry, J.R., Johnson, T.R., Dollard, C., Shuster, J.R., and Denis, C.L. (1989). Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. Cell *56*, 409–419.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991–1995.

Harris, S.D., Morrell, J.L., and Hamer, J.E. (1994). Identification and characterization of *Aspergillus nidulans* mutants defective in cytokinesis. Genetics *136*, 517–532.

Kornstein, L.B., Gaiso, M.L., Hammell, R.L., and Bartelt, D.C. (1992). Cloning and sequence determination of a cDNA encoding *Aspergillus nidulans* calmodulin-dependent multifunctional protein kinase. Gene *113*, 75–82.

Kulmburg, P.K., Judewicz, N., Mathieu, M., Lenouvel, F., Sequeval, D., and Felenbok, B. (1992). Specific binding sites for the activator protein, ALCR, in the alcA promoter of the ethanol regulon of *Aspergillus nidulans*. J. Biol. Chem. *267*, 21146–21153.

Lorca, T., Cruzlegui, F.H., Fesquet, D., Cavadore, J., Mery, J., Means, A., and Doree, M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. Nature *366*, 270–273.

Lu, K.P., and Means, A.R. (1993a). Conditional mutants for studying functions of calmodulin in *Aspergillus nidulans*. Methods Mol. Genet. *2*, 255–274.

Lu, K.P., and Means, A.R. (1993b). Regulation of the cell cycle by calcium and calmodulin. Endocr. Rev. *14*, 1–20.

Lu, K.P., Rasmussen, C.D., May, G.S., and Means, A.R. (1992). Cooperative regulation of cell proliferation by calcium and calmodulin in *Aspergillus nidulans*. Mol. Endocrinol. *6*, 365–374.

Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

May, G.S. (1989). The highly divergent β-tubulins of *Aspergillus nidulans* are functionally interchangeable. J. Cell Biol. *109*, 2267–2274.

Mathieu, M., and Felenbok, B. (1994). The *Aspergillus nidulans* CREA protein mediates glucose repression of the ethanol regulon at various levels through competition with the ALCR-specific transactivator. EMBO J. *13*, 4022–4027.

McGoldrick, C.A., Gruver, C., and May, G.S. (1995). *myo*A of *Aspergillus nidulans* encodes an essential myosin I required for secretion and polarized growth. J. Cell Biol. *128*, 577–587.

Miller, B.L., Miller, K.Y., and Timberlake, W.E. (1985). Direct and indirect gene replacements in *Aspergillus nidulans*. Mol. Cell. Biol. *5*, 1714–1721.

Olsen, D.B., and Eckstein, F. (1990). High-efficiency oligonucleotide-directed plasmid mutagenesis. Proc. Natl. Acad. Sci. USA *87*, 1451–1455.

Osmani, S.A., Pu, R.T., and Morris, N.R. (1988). Mitotic induction and maintenance by overexpression of a G_2-specific gene that encodes a potential protein kinase. Cell *53*, 237–244.

Payne, M.E., Fong, Y., Ono, T., Colbran, R.J., Kemp, B.E., Soderling, T.R., and Means, A.R. (1988). Calcium/calmodulin-dependent protein kinase II: characterization of distinct calmodulin binding and inhibitory domains. J. Biol. Chem. *263*, 7190–7195.

Planas-Silva, M., and Means, A.R. (1992). Expression of a constitutive form of calcium/calmodulin-dependent protein kinase II leads to arrest of the cell cycle in G_2. EMBO J. *11*, 507–517.

Rasmussen, C., Garen, C., Brining, S., Kincaid, R.L., Means, R.L., and Means, A.R. (1994). The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. EMBO J. *13*, 2545–2552.

Rasmussen, C.D., Means, R.L., Lu, K.P., May, G.S., and Means, A.R. (1990). Characterization and expression of the unique calmodulin gene of *Aspergillus nidulans*. J. Biol. Chem. *265*, 13767–13775.

Rasmussen, C., and Rasmussen, G. (1994). Inhibition of G_2/M progression in *Schizosaccharomyces pombe* by a mutant calmodulin kinase II with constitutive activity. Mol. Biol. Cell *5*, 785–795.

Rasmussen, G., and Rasmussen, C. (1995). Calmodulin-dependent protein kinase II is required for G_1/S progression in HeLa cells. Biochem. Cell Biol. *73*, 201–207.

Roskoski, R. (1983). Assays of protein kinase. Methods Enzymol. *99*, 3–6.

Soderling, T.R. (1990). Protein kinases. Regulation by autoinhibitory domains. J. Biol. Chem. *265*, 1823–1826.

Tombes, R.M., Grant, S., Westin, E.H., and Krystal, G. (1995). G_1 cell cycle arrest and apoptosis are induced in NIH-3T3 cells by KN93, an inhibitor of CaMK-II (the multifunctional Ca^{2+}/CaM kinase). Cell Growth Differ. *6*, 1–8.

Waldmann, R., Hanson, P.I., and Schulman, H. (1990). Multifunctional Ca^{2+}/calmodulin-dependent protein kinase made Ca^{2+} independent for functional studies. Biochemistry *29*, 1679–1684.

Waring, R.B., May, G.S., and Morris, N.R. (1989). Characterization of an inducible expression system in *Aspergillus nidulans* using alcA and tubulin-coding genes. Gene *79*, 119–130.

Whitaker, M., and Patel, R. (1990). Calcium and cell cycle control. Development *108*, 525–542.