Expression of a Constitutively Active Ca\textsuperscript{2+}/Calmodulin-dependent
Kinase in Aspergillus nidulans Spores Prevents Germination and
Entry into the Cell Cycle*

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The unique gene for Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMK) has been shown to be essential in Aspergillus nidulans. Disruption of the gene prevents entry of spores into the nuclear division cycle. Here we show that expression of a constitutively active form of CaMK also prevents spores from entering the first S phase in response to a germinating stimulus. Expression of the constitutively active kinase induces premature activation of NIMA<sup>-cyclin B</sup>/NIMX<sup>-dc2</sup> in G<sub>2</sub>/G<sub>1</sub>. As NIMX<sup>-dc2</sup> is present in spores, the elevation of maturation promotion factor activity may be secondary to the early production of NIMA<sup>-cyclin B</sup> or post-translation modification of maturation promotion factor. The expression of the constitutively active CaMK also results in the appearance of NIMA kinase activity within 1 h of the germination signal. These results support the contention that the activities of maturation promotion factor and NIMA are coincidentally regulated in A. nidulans and suggest that the unscheduled appearance of one or both of these activities may be sufficient to prevent A. nidulans spores from entering DNA synthesis.

Both extracellular Ca\textsuperscript{2+} and intracellular calmodulin (CaM) are essential for the nuclear division cycle and growth of Aspergillus nidulans (1, 2). Disruption of the unique CaM gene is lethal, and cells arrest with one or two nuclei and with absent or very short germ tubes (2). Analysis of a strain made conditional for the expression of CaM revealed a requirement for this regulatory protein for both G<sub>S</sub> and G<sub>M</sub> transitions (1). Two Ca<sup>2+</sup>-dependent targets of CaM have also been shown to be essential in A. nidulans, namely the catalytic or A subunit of the protein phosphatase 2B (3), also called calcineurin, and a multifunctional protein kinase (CaMK) (4). Disruption of the CaMK gene arrested spores with a single nucleus and with no germ tube extension, whereas when calcineurin is held low, spores proceed through the first DNA synthesis before arresting and hyphal growth as well as septation occur in the absence of nuclear division. A strain conditional for the expression of CaMK was created, and, whereas the nuclear division cycle and growth were markedly slowed, both proceeded (4). This was found to be due to a low amount of expression of the CaMK gene in repressing medium that resulted in about 10% the normal level of CaMK. Thus, it was difficult to determine the mechanism responsible for the markedly slowed growth.

Insight into the potential role of CaMK in cell cycle progression in other systems has been obtained from the expression of constitutively active forms of the enzyme. The transient expression of a constitutively active CaMK using the mouse metallotheonine viral promoter caused a G<sub>2</sub> arrest in C127 mouse cells (5). When samples from arrested cells were assayed for histone H1 kinase (H1 kinase) activity following p15<sup>ras1</sup> affinity purification, the arrest was found to be associated with elevated H1 kinase activity. Similarly, a constitutive form of rat brain Ca<sup>2+</sup>/CaM-dependent kinase IIα (CaMKIIα) expressed in Schizosaccharomyces pombe causes a G<sub>2</sub> arrest, although H1 kinase activity was reported to remain low in this case (6).

Because the disruption of the CaMK gene in A. nidulans arrests spores prior to nuclear division and germ tube formation, the present studies were undertaken in order to examine the potential role of CaMK in spore germination. We have demonstrated that truncation of the A. nidulans CaMK at Ile<sup>292</sup> generates a constitutively active Ca<sup>2+</sup>/CaM-independent enzyme. The expression of this enzyme in germinating spores causes arrest before entry into the first S phase prior to germ tube extension and this arrest is completely reversible up to 9 h. The arrest is accompanied by prematurity elevated H1 kinase activity and NIMA kinase activity. In contrast the expression of a truncated inactive kinase has no phenotypic consequences. The data suggest a role for CaMK-dependent phosphorylation and appropriate dephosphorylation upon germination for entry into S phase.

EXPERIMENTAL PROCEDURES

Strains—Strains were R153 (ua3; pyroA4), GR5 (A773; pyrG89, wA2; pyroA4), S026 (nimT23; pyrG89; wA2; biA1; pabaA1), SO6 (nimA5; pyrG89; uA2; cndE16; scD12; ya2; choA1; chaA1), and SO7 (wA3; nimA5), as well as SWJ-32 (nimG10; pyrG89; uA21; choA1) and SWJ-310 (nimR21; pabaA1) (both kindly provided by Steven W. James of Gettysburg College, Gettysburg, PA).

Site-directed Mutagenesis—The plasmid p4b-11, which contains the full-length A. nidulans CaMK cDNA, was kindly provided by Dr. Diana C. Bartelt of St. Johns University (7). The cDNA fragment was introduced into the EcoRI site of M13mp8 and used for mutagenesis by the method of Olsen and Eckstein (8) following the Amersham protocol. The inactive kinase was made by mutating Lys<sup>50</sup> to Met using the oligonucleotide 5′-CAGGATAATCATTACGGCAAC-3′. The truncated kinase was created by introducing a stop codon at Ile<sup>292</sup> using the oligonucleotide 5′-GCAATGTACGCTCAGATCTCGGG-3′.
Expression of Mutant Enzymes in Vitro and Determination of Kinase Activity—The wild type and mutant CaMK cDNAs were introduced into pGEM3Zf(−) (Promega) between the KpnI and SalI sites. One μg of each purified plasmid was linearized with SalI and transcribed by the method of Melton et al. (9) using T7 RNA polymerase. In vitro translation reactions were performed with 1 μg of CaMK transcript in a 50-μl reaction containing 33 μl of reticulocyte lysate and 20 μCi of [35S]Met according to the Promega protocol. In order to correct for the efficiency of translation of the different cDNAs, the reaction mix was subjected to SDS-polyacrylamide gel electrophoresis and the radiolabeled bands were excised from the gel and counted in a liquid scintillation counter. Endogenous kinase activity in reticulocyte lysate was subtracted from each sample, and the activity was normalized for the efficiency of translation using 1 μCi of translation product for each Met residue in the various protein sequences. Kinase activity was measured in the presence of 35 mM Na-Hepes, pH 7.8, 10 mM MgOAc2, 50 μM ATP, 1 μCi/μl tube [γ-32P]ATP (4 Ci/mmol), 50 μM GS-10 peptide (PLRRTLS-VA) containing either 1 μM CaCl2 and 1 μM A. nidulans CaM or 2 μl EGTA in a final volume of 50 μl. The reaction mixture was incubated for 2 min at 30 °C, 20 μl of the mixture was spotted onto P-81 filter paper and immediately washed with 75 mM H3PO4 and radioactivity incorporated into the peptide was quantified using a liquid scintillation counter (10).

A. nidulans Medium—A. nidulans strains were grown in minimal medium (10 mM urea, 7 mM KCl, 2 mM MgSO4, and 1 mM H1 histone, 80 μM hystidine, and 1 mg/ml Pefabloc (Boehringer Mannheim) for 5 min on ice. The H1 kinase assays were performed following p13′′−1 affinity purification with p13 agarose (Oncogene Science, Uniondale, NY) from 100 μg of cell extract as described by Booher et al. (16). Briefly, affinity-purified samples were washed three times in H buffer and once in KAB buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol). The samples were assayed at 30 °C for 10 min in KAB buffer containing 125 μg/ml H1 histone, 80 μM ATP, and 2.5 μCi/μl tube [γ-32P]ATP. Reactions were stopped by addition of 5 × Laemmli sample buffer and separated on a 12% acrylamide gel containing SDS. NIMA kinase activity was determined as described by Lu and Means (17) with the following modifications. 1) NIMA protein was immunoprecipitated from 200 μg of extract protein using an antibody generated to the COOH-terminal 17 amino acids of A. nidulans NIMA. The antibody was generously provided by Kun Ping Lu and Tony Hunter (The Salk Institute, La Jolla, CA). 2) Assays contained 100 mM microcytis (Calbiochem) and 10 μM protein kinase A inhibitor (Sigma). In addition, 100 μM PLM-(54–72) (DEEGTRFSRRELSTRRR) was used as a peptide substrate.

Western Analysis—Western blots were performed by standard methods (18). A. nidulans CaMK was measured using affinity-purified (19) rabbit polyclonal antibody to CaMK (4), followed by incubation with 125I-protein A. Bands were quantified using a Molecular Dynamics PhosphorImager equipped with a densitometer.

**RESULTS**

Changes in CaMK Expression during Growth of A. nidulans—Since our previous work had demonstrated that CaMK is essential for germination (4), we examined changes in CaMK protein expression in germinating spores. Wild type GR5 spores were isolated and grown for 0–6 h in MMD/A, and, at the times indicated in Fig. 1A, aliquots were frozen. Extracts were prepared and CaMK protein quantified by Western blot analysis. Fig. 1A demonstrates that CaMK protein is present at low levels in A. nidulans spores and accumulates as the spores germinate. Fig. 1B shows the densitometric quantitation of the autoradiograms from Fig. 1A, and a 15-fold increase in CaMK over the 6-h time course. Germination of spores in minimal medium requires 3–4 h for entry into the first S phase (4, 14, 20) and 5–6 h for completion of the first mitosis. This suggests that CaMK increases as spores germinate in a manner similar to that found for CaM, which increases 4-fold within 4 h of...
germination (2). In contrast, once the cells are exponentially growing, there is no cell cycle-dependent change in CaMK protein levels as shown in Fig. 1C. SO26 (nimT23)<sub>4</sub> cells were grown 6 h in enriched medium (YG) at 32 °C. The cells were arrested in G<sub>2</sub> at the non-permissive temperature for 4 h and released into fresh YG at the permissive temperature. Aliquots were harvested at 5- or 10-min intervals for 90 min. In YG medium one complete cell cycle occurs within 95 min (14). In addition, these extracts were assayed for CaMK activity in the presence of excess Ca<sup>2+</sup>/CaM and no changes were observed. Equivalent CaMK protein expression was also observed throughout a 150-min time course following release from the SO6 (nimA5)<sub>4</sub> G<sub>2</sub> arrest in YG medium. Similar results were obtained with release of the GR5 strain from hydroxyurea (S phase) arrest. Collectively these experiments demonstrate that CaMK protein is present at a constant level throughout the normal cell cycle. Since CaM levels vary during the A. nidulans cell cycle (21), the physiologically relevant CaMK activity is likely to be regulated by changes in the Ca<sup>2+</sup> and/or CaM concentration, rather than at the level of transcription or translation of the kinase.

Construction and Characterization of Constitutively Active and Inactive Forms of A. nidulans CaMK—Fig. 2A is a schematic representation of the domain structure of A. nidulans CaMK compared to rat brain CaMKIIα. The latter enzyme can be made independent of Ca<sup>2+</sup>/CaM by truncation at Leu-290 or rendered inactive by mutation of Lys-42, which is involved in ATP binding (22). By analogy a constitutive form of A. nidulans CaMK was created by removing the putative CaM-binding domain by truncation at amino acid Ile-292 (CaMKct). An inactive construct was created by changing Lys-50 to Met (K50M). This mutation was made in both the wild type and truncated enzymes as depicted in Fig. 2A. The mutants were expressed in an in vitro transcription and translation system to verify that the K50M mutation produced an inactive enzyme and the amino acid 292 truncation produced a constitutive enzyme. The activity of the translated proteins was determined using the GS-10 peptide as a substrate as shown in Fig. 2B. The assay was carried out in the presence of Ca<sup>2+</sup>/CaM or in the presence of EGTA. The translation mixture from wild type CaMK (CaMKwt) cDNA showed Ca<sup>2+</sup>/CaM-dependent phosphorylation of GS-10. The reaction velocity was 3.74 × 10<sup>−8</sup> mol of phosphate/ml of lysate/min in the presence of Ca<sup>2+</sup>/CaM and 0.14 × 10<sup>−8</sup> mol/ml of lysate/min in the presence of EGTA. However, the translation mixture of CaMKct contained similar kinase activity either in the presence of Ca<sup>2+</sup>/CaM or EGTA (Ca<sup>2+</sup>/CaM, 5.01 × 10<sup>−8</sup> mol/ml of lysate/min; EGTA, 6.80 × 10<sup>−8</sup> mol/ml of lysate/min). Thus the specific activity of the Ca<sup>2+</sup>/CaM-independent form was at least as high as the Ca<sup>2+</sup>/CaM-dependent activity of the wild type enzyme. The translation mixture containing CaMKwtK50M or CaMKctK50M did not show significant kinase activity above that in the translation mixture without added mRNA. These results demonstrate that truncation of A. nidulans CaMK converted the enzyme to a Ca<sup>2+</sup>/CaM-independent form, whereas the point mutation, K50M, abolished kinase activity whether in the context of the full-length or truncated protein.

Generation of A. nidulans Strains Conditional for the Expression of Mutated CaMK cDNAs—In order to be able to regulate the expression the mutant CaMK cDNAs in A. nidulans, the cDNAs for CaMKct and CaMKctK50M were independently subcloned into the A. nidulans expression vector, pAL5 (12). The pAL5 vector contains the alcA promoter and the histone

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**Fig. 2. Generation and characterization of A. nidulans CaMK mutants.** A, diagrammatic representation of CaMK, constitutively active CaMK, and mammalian CaM<sub>KI</sub>s. The top diagram shows the putative major domains of wild type CaMK (CaMKwt). In order to generate a constitutive form of CaMK (CaMKct), the putative regulatory domain was removed by truncation at Ile-292 (CaMKct, middle). To obtain kinase-inactive mutants, the Lys-50 residue was changed to Met (CaMKwtK50M and CaMKctK50M). The structure of mammalian CaM<sub>KI</sub>s is shown for comparison (bottom). B, protein kinase activity of wild type and mutant CaMK cDNAs generated by in vitro transcription and translation. Aliquots of the translation reaction were analyzed for kinase activity in the presence of Ca<sup>2+</sup>/CaM (solid bars) or EGTA (open bars) using GS-10 peptide as a substrate. Panel is representative of two experiments performed in duplicate. C, constitutive CaMK activity is inducible in the alcCaMKct-4 strain. The alcCaMKct-4 spores were grown to early log phase (8–12 h) in repressing liquid medium. The mycelia were collected on Miracloth, washed with sterilized water, and transferred to fresh repressing (solid symbols) or inducing (open symbols) medium. The mycelia were harvested at the indicated times after the medium change and frozen in liquid nitrogen. Ten μg of extract protein was assayed in the presence of EGTA (circles) or Ca<sup>2+</sup>/CaM (triangles) using autocamtide-2 as a substrate. Data are representative of two experiments performed in duplicate.

H2A 3′-terminator to target integration of the construct to a non-essential but homologous site and allow the expression of heterologous cDNAs. The plasmid also contains the pyr4 gene from *Neurospora crassa* to provide a nutritional marker, which complements the *pyrG89* mutation and allows growth in the
absence of exogenous pyrimidines (11). The plasmids were used for the transformation of the GR5 strain carrying the pyrG89 mutation. The pure haploid strains were analyzed by Southern blot analysis. The strains were designated as follows: alcCaMKct-4, conditional for the expression of CaMKct cDNA; and alcCaMKctK50M-4, conditional for the expression of CaMKctK50M cDNA. The number of copies of the cDNA integrated into the genome was assessed by measuring the ratio of the intensity of the 0.83-kb band of the transgene and the 2-kb band of the wild type. As there is only one CaMK gene in A. nidulans (23), the signal from the 2.0-kb endogenous CaMK gene was used as an internal standard for a single copy signal. The estimated copy number predicted in this manner is indicated with a hyphen after the name of the strain. In inducing medium (minimal medium containing tryptone and glucose, or MMG/T) CaMKct mRNA rapidly increases 10-fold within 1 h in alcCaMKct mycelia. In de-repressing conditions (minimal medium containing glycerol, or MMG), a moderate 2.5-fold increase of CaMKct mRNA, was detected between 30 min and 1 h. In contrast, in repressing conditions (minimal medium containing glucose, or MMD), there was no significant CaMKct mRNA production. These changes in mRNA are not shown but were measured as a prerequisite to examining changes in CaMK activity. Fig. 2 shows the inducible constitutive CaMK activity in alcCaMKct-4 cell lysates using autocamtide-2 as a peptide substrate, because it was found in preliminary studies to be the best and most specific substrate for A. nidulans CaMK. The alcCaMKct-4 spores were inoculated into MMD and grown for 8–12 h. The germings were transferred to fresh MMD or MMG/T and harvested at the times indicated in Fig. 2C. Extracts were prepared and assayed in the presence of EGTA to detect only the CaMKct activity or in the presence of Ca2+/CaM to detect the total CaMK activity. As shown in Fig. 2C, switching from repressing to inducing medium produced a 3-fold increase in autocamtide-2 phosphorylation above the normal level of Ca2+/CaM-dependent enzyme activity within 1 h that is maintained for at least 4 h (open circles compared to closed triangles). Addition of Ca2+/CaM to the extract of alcCaMKct-4 produced even greater CaM kinase activity (open triangles compared to open circles). Since we do not know the level of Ca2+ in vivo, it is safe to presume that the endogenous level of CaM kinase activity is something between these values. Therefore, the activity produced represents an overexpression of CaM kinase activity. As expected, there is no significant Ca2+/CaM-independent kinase activity in cell lysates from cells continuously grown in MMD (closed circles). Furthermore, no CaMK activity is induced in the alcCaMKctK50M-4 strain, although the induction of the CaMKct and CaMKctK50M proteins was verified as early as 1 h after transfer to inducing medium by Western blot analysis.  

Overexpression of CaMKct Inhibits Germination of A. nidulans Spores—In order to assess the effect of overexpression of CaMKct cDNAs, transformant spores were germinated on MMD and MMG/T solid medium. The wild type strain R153 and the GR5 and alcCaMKctK50M-4 strains grew equally well on inducing or repressing plates (data not shown). In contrast the alcCaMKct-4 strain did not grow on inducing plates. Fig. 3 shows representative germings from GR5, alcCaMKct-4, and alcCaMKctK50M-4 after 12 h of growth in inducing or repressing liquid cultures. Induction of CaMKctK50M has no pheno- typic effect on either nuclear division or germ tube extension (Fig. 3F) compared to the GR5 control (Fig. 3B). On the other hand, expression of CaMKct inhibits both nuclear division and germ tube extension (Fig. 3D). These results indicate that expression of Ca2+/CaM-independent CaMK severely inhibits the growth of A. nidulans in a manner that requires an active protein kinase. The effect of repression or overexpression of CaMKct on A. nidulans growth was also evaluated by monitoring the changes in total dry weight. There was no difference in the dry weight of GR5 and alcCaMKct-4 strains grown in repressing medium, confirming the results seen in Fig. 3. However, in inducing medium, there was virtually no increase in the dry weight of alcCaMKct, whereas the dry weight of GR5 showed a linear increase with time.  

Fig. 3. Overexpression of CaMK in A. nidulans inhibits growth. Spores from alcCaMKct-4, alcCaMKctK50M-4, and GR5 were grown in inducing or repressing liquid medium at 37 °C for 12 h, harvested, fixed, and stained with DAPI. A–F, GR5 repressing (A) or inducing (B), alcCaMKct repressing (C) or inducing (D), and alcCaMKctK50M repressing (E) or inducing (F). A–F, original magnification, ×1000. Bar represents 10 μm. G, growth inhibition due to overexpression of CaMKct is reversible. Spores of alcCaMKct-4 were germinated in inducing medium for 3, 6, 9, or 12 h at 37 °C then shifted to repressing medium, time 0 on the graph. After incubation for the indicated times, spores were harvested, fixed, and stained with DAPI. The number of nuclei in each cell was determined. A minimum of 100 germings was analyzed at each time point.
Overexpression of CaMKct prevents entry into S phase.

In order to determine more specifically the point of arrest due to overexpression of CaMKct, DNA synthesis was measured by quantifying the incorporation of [3H]adenine into DNA. The alcCaMKct-4 spores were germinated in MMD or MMG/T in the presence of [3H]adenine. After the indicated times, aliquots were collected and the incorporation of [3H] into the DNA fraction was quantified. Data are representative of three independent experiments performed in duplicate.

The incorporation of [3H]adenine into DNA in the alcCaMKct-4 nucleipercell is shown for each time point in Fig. 4. Arrowheads above the graph show approximate times of successive S phases and mitoses based on quantifying the number of DNA fractions. Interestingly in panel 6 the DNA content of the SWJ-32 (nimG<sup>G</sup>) strain released into W7 at the permissive temperature does not change compared to that in panel 3 in the absence of W7 at the non-permissive temperature. These results suggest that CaM is required after the NIMA<sup>Y</sup> temperature-sensitive arrest point for entry into S phase. Panel 5 verifies that progression from a G2 arrest through mitosis and into a new round of DNA synthesis also requires CaM. As controls, panels 7–9 show the cycling of the nimR, nima, and nimG germlings, respectively, 2 h after release from the non-permissive temperature.

Overexpression of CaMKct in A. nidulans Spores Causes Premature Activation of Histone H1 Kinase and NIMA Kinase Activities—In order to investigate the mechanism responsible for the complete arrest of spores prior to entry into the first S phase, which results from the overexpression of CaMKct, we evaluated changes in two protein kinases known to be required for progression through the nuclear division cycle in A. nidulans. First, the H1 kinase activity in spores and mycelia grown for up to 6 h in MMG/T was determined following p13<sup>aff</sup> α affinity purification. As can be seen in Fig. 6A, the presence of the constitutive CaMK caused a premature elevation in H1 kinase activity as early as 1 h after exposure to inducing medium. This increase in H1 kinase activity was similar to that seen at 4 h in the alcCaMKctK50M-4 strain, which corresponds to the time of entry into S phase (Fig. 4). In addition, when alcCaMKct-4 spores are grown in MMD, the increase in H1 kinase activity is very similar to the activity seen in the alcCaMKctK50M-4 strain grown in MMD or MMG/T at each time point between 0 and 6 h. This demonstrates that the increase in H1 kinase activity is the result of the expression of CaMKct. Similar results were obtained using an anti-NIMA<sup>Y<sub>c</sub></sup> antibody to precipitate NIMA<sup>Y<sub>c</sub></sup>, the A. nidulans homologue of p34<sup>cdc2</sup>, followed by the kinase assay. The antibody was kindly provided by Steve Osmani of the Weis Research Institute, Danville, PA. (24) Thus the increase in H1 kinase activity caused by overexpression of CaMKct appears to be due to unscheduled activation of NIMA<sup>Y<sub>c</sub></sup>, which is required for both G<sub>1</sub> and G<sub>2</sub> in A. nidulans (24).

Panel B of Fig. 6 shows the changes in the activity of the mitotic Ser/Thr protein kinase, NIMA (25, 26), in the same extracts. NIMA was immunoprecipitated with an antibody to the COOH-terminal 17 amino acids of NIMA. The immunoprecipitates were assayed using PLM (DEEETFTSSIRRL-STRRR) for the substrate peptide. This peptide is the best substrate characterized for NIMA (27) and is a very poor substrate for A. nidulans CaMK. The data demonstrate that NIMA activity is low in spores and increases between 4 and 6 h as cells enter mitosis in the alcCaMKctK50M-4 strain. However, in the alcCaMKct-4 strain NIMA, activity is elevated by 1 h and remains elevated over the 6-h time course. Thus as was the case for NIMA<sup>Y<sub>c</sub></sup>, the expression of CaMKct results in premature activation of NIMA to the level usually restricted to the G<sub>2</sub> phase of the nuclear division cycle.

Panel C of Fig. 6 shows the changes in the activity of the mitotic Ser/Thr protein kinase, NIMA (25, 26), in the same extracts. NIMA was immunoprecipitated with an antibody to the COOH-terminal 17 amino acids of NIMA. The immunoprecipitates were assayed using PLM (DEEETFTSSIRRL-STRRR) for the substrate peptide. This peptide is the best substrate characterized for NIMA (27) and is a very poor substrate for A. nidulans CaMK. The data demonstrate that NIMA activity is low in spores and increases between 4 and 6 h as cells enter mitosis in the alcCaMKctK50M-4 strain. However, in the alcCaMKct-4 strain NIMA, activity is elevated by 1 h and remains elevated over the 6-h time course. Thus as was the case for NIMA<sup>Y<sub>c</sub></sup>, the expression of CaMKct results in premature activation of NIMA to the level usually restricted to the G<sub>2</sub> phase of the nuclear division cycle.

<sup>3</sup>C. Rasmussen, personal communication.
**DISCUSSION**

*A. nidulans* CaMK like other multifunctional CaMKs can be made Ca²⁺/CaM-independent (CaMKct) by truncation prior to the regulatory domain. *A. nidulans* CaMKct expressed in rabbit reticulocyte lysate had similar activity in the presence of Ca²⁺/CaM or EGTA to the expressed wild type CaMK in the presence of Ca²⁺/CaM. The truncated CaMKct was expressed in *A. nidulans* with the inducible alcA promoter and caused germinating spores to arrest prior to S phase. Previous studies have documented that the overexpression of a constitutive CaMK causes arrest in *G2* phase and prevents entry into the proliferative cycle from the dormant state of conidia. This arrest is accompanied by elevated histone H1 and NIMA kinase activities. The H1 kinase activity is at least partly due to NIMXcdc2 as increased activity is immunoprecipitable with an antibody to the NIMXcdc2 protein (24).

The mechanism explaining the arrest with elevated H1 kinase activity is not clear. It is possible that the presence of CaMKct leads to the phosphorylation of a component of the NIMXcdc2/cyclin B complex, resulting in activation of the cyclin-dependent kinase (CDK). Alternatively CaMKct may prevent an obligatory dephosphorylation step resulting in the induction of a checkpoint irrespective of the state of CDK activity. Finally CaMKct may lead to phosphorylation of NIMXcdc2 on an inappropriate site resulting in a non-productive activation of H1 kinase. It has been shown that decreased extracellular Ca²⁺ or intracellular CaM causes a G₁ arrest and prevents activation of NIMXcdc2 and NIMA (20). Thus some Ca²⁺/CaM-dependent event, perhaps phosphorylation by CaMK, is important for the activation of NIMXcdc2 and NIMA in *A. nidulans*. One possibility to consider is the fact that the highly conserved activating phosphorylation site of p34cdc2, Thr-161, exists in the *A. nidulans* NIMXcdc2 sequence and is located in a consensus CaMK phosphorylation site, making it a potential target for CaMK (24). However, our preliminary data indicate that CaMK does not phosphorylate NIMXcdc2 immunoprecipitated from *A. nidulans* extracts. Another model for activation of p34cdc2 has been suggested (28), in which the target for CaMK may be the Tyr phosphatase Cdc25, which is responsible for the activation of p34cdc2/cyclin B by dephosphorylation of Thr-14 and Tyr-15. In theory initial phosphorylation of Cdc25 causes a low level of activation of the Cdc25 phosphatase, which can then dephosphorylate and activate p34cdc2/cyclin B. The activation of p34cdc2/cyclin B causes a positive feedback to further phosphorylate and activate Cdc25. This model is supported by the recent observation that CaMKII can phosphorylate and increase the activity of Cdc25 in vitro. Thus NIMTcdc2, the *A. nidulans* Cdc25 homologue, is perhaps a more likely target for CaMK.

One explanation for the premature increase of NIMA activity is that the elevated NIMXcdc2 activity causes the activation or stabilization of this protein kinase. The carboxyl-terminal non-catalytic domain of NIMA has multiple p34cdc2 consensus phosphorylation sites. O’Connell et al. (29) have shown that this phosphorylation is not required for the activity of NIMA; however, they suggest that the phosphorylation of these sites may stabilize or help localize NIMA. Perhaps the elevated NIMA activity is simply a consequence of the G₁/S arrest and prematurely elevated NIMXcdc2 activity. Consistent with this possibility, either a G₁ arrest or a mitotic arrest has been shown to result in elevated NIMA kinase activity (30–32).

Our results show that spores contain a low level of NIMXcdc2, however, NIMEcyclin B was not detectable in spores by Western blot analysis. The H1 kinase activity increases severalfold between 3 and 4 h, the time that corresponds to the initiation of germination and the arrest of proliferation. This suggests that CaMK may be involved in the regulation of H1 kinase activity.

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**FIG. 5.** The CaM inhibitor W7 causes arrest prior to S phase but after nimA⁺ and nimG⁺ in *A. nidulans*. Spores from the SWJ-310 (nimR⁺; panels 1, 4, and 7), SO7 (nimA⁺; panels 2, 5, and 8), and SWJ-32 (nimG⁺; panels 3, 6, and 9) strains were grown in MMD at the non-permissive temperature (44°C) for 4 h to arrest the cells in G₁, G₂, and G₁, respectively (panels 1, 2, and 3). The germlings were then divided into two flasks. To one flask 250 μM W7 was added prior to transferring to the permissive temperature for 2 h (panels 4–6). The second flask was transferred directly to the permissive temperature for 2 h (panels 7–9).
of the first S phase. Therefore, it is possible that the synthesis of NIME\textsubscript{cyclin B} could regulate the entry into DNA synthesis. Indeed, overexpression of NIME\textsubscript{cyclin B} increases pre-maturation promotion factor levels in \textit{A. nidulans} (33). Apparently, \textit{A. nidulans} contains a single cyclin-dependent kinase (NIMX\textsubscript{cdc2}) that is required both for \textit{G}_{1} and \textit{G}_{2} progression (24). It may also be the case that a single B-type cyclin is required (NIME\textsubscript{cyclin B}). The \textit{nimB} gene was originally defined as a temperature-sensitive mutation that caused a block in \textit{G}_{2} (14, 33, 34), whereas \textit{nimG} defined a temperature-sensitive mutation that arrested cells in \textit{G}_{1} (Fig. 5, panel 3). Both \textit{nime} and \textit{nimG} have been shown to be the result of mutations in the same gene, that for the \textit{A. nidulans} equivalent of cyclin B (33).\textsuperscript{3} The possibility that a single cyclin-CDK complex might regulate progression into both S and M phases in \textit{A. nidulans} is strengthened by the intriguing observations that in \textit{S. pombe} mutants have been generated in which a single B-type cyclin can regulate the unique p34\textsuperscript{cdc2} to promote both S phase and mitosis (35–37). Furthermore, analysis of DNA content by flow cytometry suggests that there is a CaM requirement for \textit{G}_{2}/S progression distal to the arrest point caused by the mutation in cyclin B present in the \textit{nime} strain.

Why would premature H1 kinase activity lead to arrest prior to entry into S phase? The study of replication and the prevention of re-replication may shed some light on this issue. Screens for mutations which allow re-replication in \textit{S. pombe} have resulted in the isolation of mutations in \textit{cdc2} and in \textit{cdc13} (the cyclin partner of \textit{cdc2} in \textit{S. pombe}) (38, 39). This evidence makes a strong case for the role of the cyclin-CDK complex in preventing re-replication during G\textsubscript{2}. Su et al. (40) have proposed a model whereby active cyclin-CDK complexes, which are present throughout S and \textit{G}_{2} phases, prevent reinitiation by preventing further assembly of functional replication origins. In mitosis cyclin is degraded and CDK inactivated, allowing entry into the \textit{G}_{1} phase and the ability to assemble a replication origin for a new round of DNA synthesis. Therefore, re-replication is coupled to progression through mitosis. There is reason to believe this model also applies to other organisms in addition to \textit{S. pombe}. For example mutations in \textit{Drosophila} cyclin A led to endoreduplication in normally mitotic cells (41), and in cultured rat fibroblasts treatment with Tyr kinase inhibitors to inactivate cyclin B/CDK induces DNA rereplication (42). Finally CDKs have been shown to inhibit assembly of pre-replication centers in \textit{Xenopus} oocyte extracts (43). If \textit{A. nidulans} spores do not contain functional replication origins, this model would predict that the premature H1 kinase activity caused by CaMKct prevents assembly of origins of replication and therefore prevents DNA synthesis. Furthermore, this model would also predict that the effects of CaMKct should be reversible as we have shown to be the case. The removal of CaMKct would allow the inactivation of H1 kinase and permit the formation of origins of replication and subsequent entry into S phase. The fact that this occurs with a lag of 2–3 h would provide time for the destruction of CaMKct protein and assembly of replication complexes. Taken together it seems likely that the arrest occurs because some unknown protein(s) is phosphorylated by CaMKct and must be dephosphorylated to allow entry into the cell cycle. Further understanding of these experiments awaits the elucidation of the \textit{in vivo} substrate(s) for \textit{A. nidulans} CaMK and CaMKct.

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