Inhibition of G₂/M Progression in *Schizosaccharomyces pombe* by a Mutant Calmodulin Kinase II with Constitutive Activity

Colin Rasmussen and Grace Rasmussen

Intracellular signaling by the second messenger Ca²⁺ through its receptor calmodulin (CaM) regulates cell function via the activation of CaM-dependent enzymes. Previous studies have shown that cell cycle progression at G₁/S and G₂/M is sensitive to intracellular CaM levels. However, little is known about the CaM-regulated enzymes involved. Protein phosphorylation has been shown to be important for cell-cycle regulation. Because CaM regulates several protein kinases, and at least one protein phosphatase, our studies are focusing on the roles of these enzymes within the cell cycle. As an initial approach to this problem, cDNAs encoding either normal or mutant calcium/calmodulin kinase II (CaMKII) have been expressed in *Schizosaccharomyces pombe*. The results show that overexpression of a constitutively active mutant CaMKII caused cell-cycle arrest in G₂. Arrest was associated with a failure to activate the p34/cdc2 protein kinase. Expression of the mutant CaMKII in strains of *S. pombe* with altered timing of mitosis revealed that this effect is not mediated either by cdc25⁺ or wee1⁺, suggesting that CaMKII may regulate G₂/M progression by another mechanism.

**INTRODUCTION**

The Ca²⁺ receptor protein calmodulin (CaM) is an important regulator of intracellular signaling in eukaryotic cells. Several studies suggest that Ca²⁺ and CaM are involved in controlling the G₂/M transition in eukaryotic cells (Lu and Means, 1993). Increased Ca²⁺ levels are associated with nuclear envelope breakdown and chromatin condensation (Poenie *et al.*, 1985; Twigg *et al.*, 1988). We and others have shown that CaM is essential for cell-cycle progression at multiple points in the cell cycle including G₂/M (Davis *et al.*, 1986; Takeda and Yamamoto, 1987; Rasmussen and Means, 1989; Rasmussen *et al.*, 1990). Calmodulin antagonist drugs or the expression of CaM antisense RNA block progression from G₂ into M-phase (Sasaki and Hidaka, 1981; Rasmussen and Means, 1989). However, little is known about the molecular basis for this requirement or the specific biochemical pathways regulated by CaM-dependent enzymes.

Protein phosphorylation/dephosphorylation is important in the control of cell-cycle progression. Because CaM is known to regulate several different protein kinases and at least one protein phosphatase (Means, 1988), it is possible that the role for CaM and CaM-regulated enzymes in cell-cycle regulation involves promotion of the phosphorylation state of key cell cycle regulatory proteins. Consistent with this prediction, previous studies have suggested that the multifunctional CaM-dependent protein kinase II (CaMKII) is required for the G₂/M transition. Monoclonal antibodies directed against the 50 kDa α-CaMKII isoform blocked nuclear envelope breakdown in sea urchin eggs, as did a peptide encompassing the autoinhibitory domain of this enzyme (Baitinger *et al.*, 1990). However, the role of CaMKII is not as straightforward as suggested by these earlier studies, because expression of a truncated version of CaMKII encompassing the catalytic domain caused transient cell-cycle arrest in mouse C127 cells (Planas-Silva and Means, 1992). However, this result must be interpreted carefully, because the expressed enzyme lacked the regulatory and multimerization domains present in the native enzyme. Consequently, the block...
to cell-cycle progression could have been because of alterations in either the substrate specificity or subcellular localization of the truncated enzyme, as compared to normal CaMKII.

The central regulator of G2/M progression is the p34/cdc2 protein kinase (Doree, 1990; Nurse, 1990). p34/cdc2 activity is regulated by its phosphorylation state. Phosphorylation of Tyr-15 is inhibitory while dephosphorylation activates the kinase (Gould and Nurse, 1989). Two competing pathways control p34/cdc2 activity (Russell and Nurse, 1986, 1987a,b). The product of the cdc25 gene is a protein phosphatase (Dunphy and Kumagai, 1991; Gauthier et al., 1991; Kumagai and Dunphy, 1991; Lee et al., 1992) that dephosphorylates Tyr-15, whereas the products of the mik1 and wee1 genes are the Tyr-15 kinases (Featherstone and Russell, 1991; Lundgren et al., 1991). The activity of wee1 and mik1 are in turn regulated by at least one additional kinase, nim1 (also called cdr1), whose role is to inhibit wee1/mik1 (Parker et al., 1993; Wu and Russell, 1993).

Recent studies suggest ways in which CaMKII could be involved in G2/M progression. This could either be via modification of cdc25 phosphatase activity (Patel et al., 1992; Gabrielli, Piwnica-Worms, Rasmussen, unpublished data) or by activation of the NIMA protein kinase, a gene first identified in Aspergillus nidulans (Lu et al., 1993b). NIMA is a mitotic inducer that functions parallel to, and in concert with, p34/cdc2 to control G2/M progression in A. nidulans. Temperature-sensitive mutations in NIMA block cells in G2 even though p34/cdc2 is dephosphorylated and H1 kinase activity increases (Osmani et al., 1991). Although CaM appears to be required for NIMA activation, the effect is indirect and the enzyme that is immediately responsible for the activation of NIMA is unknown (Lu et al., 1993b). Finally, it has been shown that a truncated CaMKII can release Xenopus oocytes from metaphase II arrest, apparently by inducing the degradation of cyclin, with the concomitant inactivation of p34/cdc2 histone H1 kinase activity (Lorca et al., 1993). Together, these studies point to an important role for CaMKII in G2/M control.

Because previous studies have used truncated versions of CaMKII or been performed in model systems where genetic analysis is not possible, it has been difficult to assess the role of CaMKII relative to known biochemical pathways that regulate cell proliferation. The goal of this study was to examine the effect of expressing either normal or mutant forms of CaMKII in the fission yeast Schizosaccharomyces pombe as an approach to understanding the role of this enzyme in cell-cycle control. The mutant form has a single amino acid change (T286 to D286) previously shown to produce an enzyme independent of CaM and constitutively active but that retains the functional domains of the native enzyme (Waldmann et al., 1990). The results of this study indicate that overexpression of normal CaMKII has no effect on cell growth and division rates in exponentially growing cells. In contrast, expression of the mutant CaMKII resulted in a G2 arrest. Arrest was also associated with a failure to activate p34/cdc2 histone H1 kinase activity, and p34/cdc2 was maintained in a tyrosine-phosphorylated state. Expression of CaMKII in cdc2 mutants that have altered responses to either the cdc25+ or wee1+ proteins also caused cell cycle arrest, suggesting that the constitutively active CaMKII does not act through either cdc25+ or wee1+ or directly to regulate p34/cdc2. These studies suggest that another pathway functions to delay mitosis in S. pombe in response to increased CaMKII activity.

MATERIALS AND METHODS

Strains and Media

The S. pombe strains used in these studies are listed in Table 1. All strains were grown in standard minimal medium (Moreno et al., 1991). Uracl and adenine were added at 75 µg/ml, leucine at 250 µg/ml. Thiamine-HCl (Sigma, St. Louis, MO) was prepared in Milli-Q water as a 1 mM stock, sterile-filtered, and added to growth medium at a final concentration of 2 µM. All cultures were grown at 25°C.

CaMKII Constructs

A cDNA encoding the 50-kDa isof orm of mouse CaMKII was used to construct the expression plasmids used in this study (Hanley et al., 1989). An Nde I site was placed at the initiator Met codon by polymerase chain reaction (PCR). cDNA was the fragment containing the NIMA protein kinase, a gene first identified in Aspergillus nidulans (Lu et al., 1993b). NIMA is a mitotic inducer that functions parallel to, and in concert with, p34/cdc2 to control G2/M progression in A. nidulans. Temperature-sensitive mutations in NIMA block cells in G2 even though p34/cdc2 is dephosphorylated and H1 kinase activity increases (Osmani et al., 1991). Although CaM appears to be required for NIMA activation, the effect is indirect and the enzyme that is immediately responsible for the activation of NIMA is unknown (Lu et al., 1993b). Finally, it has been shown that a truncated CaMKII can release Xenopus oocytes from metaphase II arrest, apparently by inducing the degradation of cyclin, with the concomitant inactivation of p34/cdc2 histone H1 kinase activity (Lorca et al., 1993). Together, these studies point to an important role for CaMKII in G2/M control.

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S. pombe Transformation

S. pombe were transformed with plasmid DNA by the method of Okazaki et al. (1990). Cells were allowed to recover for 1 h after heat shock then plated onto leucine plates to recover leucine prototrophs. Primary transformant colonies were picked after 4 d, and cells were streaked to single colonies twice more then tested for expression of the plasmid-borne CaMKII by Western blot after 24 h in medium lacking thiamine.
Mouse CaMKII in S. pombe

Western Blotting
Total protein lysates of S. pombe were made as previously described (Boohr et al., 1989). Protein concentrations were determined using the BioRad (Richmond, CA) protein assay kit that is based on the method of Bradford (1976). Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and transferred to Immobilon-P by the method of Towbin et al. (1979). CaMKII was detected using a monoclonal antibody specific for the 50-kDa mouse CaMKII α isoform (H. Schulman, Stanford University). The primary antibody was localized by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL) according to the procedures recommended by the manufacturer. Exposures were for 10 s at room temperature using XAR film (Kodak, Rochester, NY). Anti-cdc2 antisera was directed against the conserved PSTAIR domain of cdc2 and was the kind gift of S. Pelch (University of British Columbia, Vancouver). cdc2 antibody was localized using 125I-Protein A (ICN Radiochemicals, Irvine, CA). Anti-phosphorytrosine antisera was obtained commercially (UBI, Lake Placid, NY) and was localized by ECL as above.

Calmodulin Overlay
A bacterial expression plasmid containing an A. nidulans CaM CDNA ligated 3' to the temperature-inducible Trp promoter was used to synthesize CaM in Escherichia coli. Protein production was induced by incubation of exponentially growing cultures at 42°C for 90 min, and CaM was purified by phenyl-Sepharose affinity chromatography (Means et al., 1991) and ion exchange chromatography using Q-Sepharose Fast Flow. Purified CaM was labeled with 125I-Bolton-Hunter reagent as described (Means et al., 1991), and labeled protein was separated by gel filtration on PD-10 columns (Pharmacia, Piscataway, NJ). For Calmodulin overlay, total protein was resolved by SDS-PAGE and transferred to Immobilon-P membranes as described for Western blotting. Overlays were performed as described using 10 ng/ml 125I-CaM (Pausch et al., 1991).

CaM Kinase Assays
CaM kinase assays were performed as follows based on a previously described method (Planas-Silva and Means, 1992). Protein extracts were made by breaking cells with glass beads in CaMKII homogenization buffer (50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [HEPES] pH 7.5, 10% glycerol, 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid [EGTA], 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% Triton X-100). Extracts were then centrifuged at 10,000 × g for 10 min and the supernate retained. Protein concentrations were determined by Bradford assay and adjusted to 1 mg/ml in homogenization buffer. For each kinase assay, 40 μl of extract was used in a 50-μl reaction consisting of 20 mM HEPES pH 7.5, 1 mM dithiothreitol (DTT), 50 μM ATP, 10 mM MgCl₂, 1 μM calmodulin, 2.5 μCi [γ-32P]-ATP, either excess Ca²⁺ (1 mM; for Ca²⁺/CaM-dependent activity) or EGTA (2 mM; for Ca²⁺/CaM-independent activity), and 20 μM syntide-2 peptide as substrate (Life Technologies, Grand Island, NY). Reactions were started by addition of the extract to the kinase buffer. Reactions were incubated for 5 min at 30°C after which 10 μl was spotted onto P-81 phosphocellulose paper, immersed into 75 mM phosphoric acid to stop the reaction, and then washed three more times for 5 min each wash in 75 mM phosphoric acid (Roskoski, 1985). After washing filters were air dried, and activity was quantified by liquid scintillation counting. The data are expressed as pmol PO₄ incorporated/min/mg total protein.

Cytotoxic Procedures
For staining of nuclei with 4,6-diamidino-2-phenylindole (DAPI), the procedure of Moreno et al. (1991) was used. Photographs were taken with a Zeiss Axioskop (Thornwood, NY) equipped for epifluorescence. All photographs were printed at the same final magnification.

p34/cdc2 Histone H1 Kinase Assays
Histone H1 kinase assays were performed as described (Ducommun and Beach, 1990). Protein extracts were made from S. pombe cells by breakage with glass beads in extraction buffer (25 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 15 mM EGTA, 0.1% Triton X-100, 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 10 μg/ml L-1-toxylamide-2-phenethylchloromethyl, 0.1 mM NaF, 60 mM β-glycerophosphate, 15 mM p-nitrophenolphosphate, 0.1 mM Na-orthovanadate). p34/cdc2 was purified from bacterial extracts expressing the S. pombe p34 cDNA as previously described (Brizuela et al., 1987) using a strain generously provided by Dr. David Beach, Howard Hughes Medical Institute, Cold Spring Harbor Laboratory. Immuno-2 (Fisher Scientific, Pittsburgh, PA) microtiter plate wells were coated with 2 μg pure p34 in 0.1 M carbonate buffer overnight at 4°C. The wells were then blocked with 3% bovine serum albumin in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl). Before addition of protein extracts, the wells were washed three times with 300 μl TBS. Samples of 200 μg per well of total protein extract were added and incubated for 5 h at 4°C to allow binding of cdc2 kinase to the immobilized p34. Wells were then washed four times, for 2 min each wash, with TBS and 0.1% Triton X-100 to remove unbound proteins, and then washed once with kinase assay buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM DTT, and protease and phosphatase inhibitors as used in the extraction buffer). Kinase assays were performed by adding 100 μl of a cocktail containing 8.3 μg histone H1 (Boehringer, Indianapolis, IN), 2.5 μCi γ-32P-ATP in kinase buffer. Reactions were allowed to proceed for 60 min at 30°C and were quantified by spotting 10 μl of the reaction on Whatman (Clifton, NJ) 3MM paper followed by trichloroacetic acid (TCA) precipitation and washing as described (Ducommun and Beach, 1990). Activity was quantified by liquid scintillation counting of the TCA-precipitated sample. Nonspecific background was measured using wells not coated with p34, but otherwise treated identically, and was subtracted from each sample. All determinations were done in triplicate and standard errors calculated.

p34/cdc2 Tyrosine Phosphorylation
Total protein extracts (200 μg) were incubated in p13-Sepharose-coated microtiter plates as described above for p34/cdc2 assays. After incubation, the wells were washed four times with TBS + 0.1% Triton X-100, bound protein was solubilized by the addition of SDS-PAGE sample buffer, and the microtiter plate was incubated in a boiling water bath for 5 min. Proteins were electrophoresed and transferred to Immobilon-P filters as described above, and p34/cdc2 phosphotyrosine content was determined immunologically using an anti-P-

<table>
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<tr>
<th>Table 1</th>
<th>Strains used in this study</th>
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<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>SP-32</td>
<td>h-, cdc2-25</td>
</tr>
<tr>
<td>SP-130</td>
<td>h-, ade6-210, leu1-32</td>
</tr>
<tr>
<td></td>
<td>h-, cdc2-1w</td>
</tr>
<tr>
<td></td>
<td>h-, cdc2-3w</td>
</tr>
<tr>
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<td>h-, cdc2-3w, leu1-32, [pREP4.1-CII(D286)]</td>
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Shown are the strains used in this study. SP strains were obtained from Dr. David Beach, Cold Spring Harbor Laboratory: pFYC strains were from the CSH fission yeast molecular genetics course; and the designated strains were obtained from Dr. Vietsim Simanis, ISREC. YEG strains have been produced in our laboratory at the University of Alberta.
Tyr monoclonal antibody (UBI). The antibody was localized using the ECL procedure (Amersham) as described above.

RESULTS
Calmodulin kinase II isoforms have three major functional domains, the catalytic, regulatory, and multimerization domains (Schulman and Hanson, 1993). The enzyme exists in vivo as a dodecamer of ~600 kDa molecular weight with multimerization dependent on the C-terminal region. The regulatory region consists of overlapping substrate inhibitory and CaM-binding domains (Payne et al., 1988). Upon binding Ca\(^{2+}\)/CaM, CaMKII autophosphorylates at several residues including threonine-286, which lies amino-terminal to the substrate inhibitory domain (Hanson et al., 1989). This autophosphorylation renders the kinase active and independent of CaM. Mutation of threonine-286 to aspartic acid (D286) has been shown to produce an enzyme with constitutive kinase activity 35% of the level induced by CaM activation of the normal enzyme. In contrast, normal CaMII activity in the absence of CaM is <1% of the stimulated activity (Waldmann et al., 1990).

Previous studies indicated that a truncated CaMKII encompassing the catalytic domain can cause G\(_2\) cell-cycle arrest in mouse C127 cells (Planas-Silva and Means, 1992). However, as had been suggested by the authors of that study, the truncated enzyme might have significantly different properties than native CaMII. In this study, two forms of mouse CaMKII \(\alpha\) were expressed in fission yeast, either the normal enzyme or a mutant with constitutive activity produced by mutation of the autophosphorylation site required for CaM-independent activity (T286) to aspartic acid. As all the functional domains of the protein have been conserved in the mutant, in addition to possessing CaM-independent activity, this enzyme can be further stimulated by Ca\(^{2+}\)-CaM (Waldmann et al., 1990).

Normal (CaMKII-T286) or mutant (CaMKII-D286) cDNAs were ligated in the nmt promoter−containing expression plasmid pREP4.1 (Figure 1), and the resulting plasmids were transformed into S. pombe. In the presence of thiamine, expression from the nmt promoter is repressed, whereas in thiamine-free medium, high levels of expression are obtained from this promoter (Maundrell, 1990). pREP4.1 that contains an attenuated version of the original nmt promoter was used as the high levels of expression obtained with the wild-type nmt promoter can sometimes produce nonspecific effects on cell growth in S. pombe. From each transformation four colonies were selected and initially characterized for expression of the plasmid borne CaMKII cDNAs by Northern blot. It was found that nearly identical levels of mRNA expression were obtained among all the isolates examined. One isolate from each was retained for further study. The strain expressing normal CaMKII-T286 was designated CK-T, whereas the strain expressing the mutant CaMKII-D286 was designated CK-D.

Calmodulin overlay, Western blot, and in vitro analysis of CaMKII activity were used to assess expression of normal and mutant CaMKII in S. pombe. CK-T and CK-D cells were grown for 24 h in medium with or without 2 \(\mu\)M thiamine. For overlay and Western blotting total protein was extracted, equal amounts were resolved by SDS-PAGE and transferred to Immobilon-P membrane, and the filters were probed either with \(^{125}\)I-CaM or a monoclonal antibody specific for the 50 kDa CaMKII isoform. The CaM overlay showed that in the absence of thiamine, a 50-kDa CaM-binding protein, the expected size for CaMKII, was expressed in both strains (Figure 2A). Binding of \(^{125}\)I-CaM to the 50-kDa protein was Ca\(^{2+}\) dependent (unpublished observations). Endogenous S. pombe CaM-binding proteins are likely not observed as we used \(^{125}\)I-labeled A. nidulans CaM that we have previously shown binds mouse CaMKII (Rasmussen et al., 1990) but may not bind S. pombe proteins sufficiently well to allow detection in the CaM overlay under the conditions used. However, these data demonstrate that both the normal and mutant forms of CaMKII retain the ability to bind CaM when expressed in S. pombe. Western blot analysis using a monoclonal antibody specific for murine CaMKII \(\alpha\) confirmed that the protein is expressed in each strain (Figure 2B). The results also indicate that similar levels...
Figure 2. Expression of mouse CaMKII in S. pombe. CaMKII expression was characterized in S. pombe strains transformed either with the pREP4.1-CKII (T286) or pREP4.1-CKII(D286) plasmids. Cultures were started from stationary phase precultures and grown for 24 h in minimal medium with (+) or without (−) 2 μM thiamine. Protein extracts, SDS-PAGE, and transfer to Immobilon-P filters were as described in MATERIALS AND METHODS. Blots were then processed either for (A) CaM overlay using 125I-CaM or (B) Western blot analysis using a monoclonal antibody directed against the 50-kDa α CaMKII isoform. For the CaM overlays, blots were processed in buffers containing Ca2+ or EGTA to demonstrate Ca2+-dependent binding to the 125I-CaM.

of protein are expressed in both the CK-T and CK-D strains, and that the expressed proteins appear full length.

To determine if mouse CaMKII was active in S. pombe extracts, CaMKII assays were performed. After 24 h in minimal medium with or without thiamine, protein extracts were made as described in MATERIALS AND METHODS, and CaMKII activity was assayed by phosphorylation of a peptide substrate (syntide-2). Because mouse CaMKII becomes independent of Ca2+/CaM after autophosphorylation, or in the case of the D286 mutant has constitutive activity, assays were performed both in the absence or presence of free Ca2+. The results show that a slight increase (~40%) in CaM-independent activity is observed in cells expressing the normal form of CaMKII (Figure 3, top), likely representing the normal activation of a fraction of the mouse CaMKII present. In contrast, Ca2+/CaM-independent activity was fourfold higher in cells expressing the D286 mutant CaMKII, as compared with the activity observed in cells not expressing the kinase (Figure 3, top). Total CaMKII levels were expressed in the two strains as indicated by the levels of CaM-stimulated activity observed in the CK-T and CK-D strains grown in the absence (inducing conditions) of thiamine (Figure 3, bottom). CaM-independent activity in CK-D cells expressing the altered CaMKII was ~17% of the total CaM-stimulated activity, similar to earlier studies (Waldmann et al., 1990).

In summary, active mouse CaMKII can be expressed in S. pombe, and the mutant form of CaMKII displays constitutive kinase activity as expected.

Cells were next examined microscopically to determine if expression of the normal or mutant kinases had any obvious phenotypic effects on S. pombe cells. CK-T and CK-D cells were grown for 24 h ± thiamine, fixed, and then stained with the fluorescent dye DAPI. In the CK-T strain, no significant differences were observed as a consequence of expressing normal CaMKII (Figure 4, A and B). In contrast, expression of the mutant CaMKII in the CK-D strain produced elongated cells with a single nucleus located at the midpoint of the cell (Figure 4, C and D). These cells are phenotypically similar to cdc mutants that block cell cycle progression but allow continued growth.

To analyze the effect of CaMKII expression on cell growth and division, cells were grown in minimal medium either in the presence or absence of thiamine, and cell numbers were determined over time. As shown, expression of normal CaMKII had no effect on cell division rates in S. pombe (Figure 5A). In contrast, expression of the D286 mutant resulted in a blockage of cell division, consistent with the cellular phenotype we had observed (Figure 5B). The kinetics of arrest were consistent with the known expression kinetics of the nmt promoter (Maundrell, 1990). Because expression of the mutant CaMKII caused cell-cycle arrest, cells were examined to determine where in the cell cycle blockage occurred. Flow cytometric analysis showed that these

Vol. 5, July 1994 789
cells have a DNA content indicative of cells arrested in G₂ (Figure 6).

It has been previously shown that p34/cdc2 histone H1 kinase activity becomes elevated in late G₂ before entry into M-phase, with full activation of kinase activity during M-phase (Booher et al., 1989). Therefore, we examined histone H1 kinase activity to determine if the cell cycle arrest might be associated with a failure to activate the p34/cdc2 kinase. Using the procedure of Ducommun and Beach (1990), p34/cdc2 histone H1 kinase activity was assayed in cells expressing both the normal and mutant forms of CaMKII (Figure 7). The results indicate that expression of normal CaMKII had no effect on p34/cdc2 kinase activity as expected from the lack of effect on cell division. In CK-D cells arrested in G₂ by expression of the mutant CaMKII, p34/cdc2 H1 kinase activity was only increased slightly (25%). In contrast, cdc25-22 cells arrested at the restrictive temperature for 4.25 h had p34/cdc2 kinase activity twofold that of exponentially growing control cells, whereas 20 min after release from the cdc25-22 induced arrest histone H1 kinase levels were sevenfold higher than control levels. This result suggests that the CaMKII(D286)-induced cell-cycle arrest occurs in G₂ before the activation of p34/cdc2.

To better characterize this G₂ arrest, several approaches were taken. First, the levels and tyrosine phosphorylation of p34/cdc2 were examined in control cultures and in cells expressing either normal or mutant

<table>
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<th>Strain/growth condition</th>
<th>Relative length</th>
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<tbody>
<tr>
<td>CK-T + thiamine</td>
<td>1.00</td>
</tr>
<tr>
<td>CK-T - thiamine</td>
<td>1.07</td>
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<tr>
<td>CK-D + thiamine</td>
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Cell lengths were measured from the photographs in Figure 4. Length was normalized relative to the CK-T strain grown in the presence of thiamine.
CaMKII. As above, total protein was extracted from control cultures and those expressing either the normal or mutant forms of CaMKII. p34/cdc2 levels were examined by Western blot. To assess tyrosine phosphorylation of p34/cdc2, total protein was added to microtiter wells coated with p13 and the p34/cdc2 allowed to bind overnight. The wells were then washed to remove unbound protein, and the p13-bound proteins were solubilized with SDS-PAGE sample buffer. The solubilized proteins were then resolved by SDS-PAGE and P-Tyr present in p34/cdc2 analyzed by Western blot using a monoclonal antibody specific for P-Tyr. As can be seen, p34/cdc2 was at similar levels in both strains under both sets of growth conditions (Figure 8A) and was tyrosine phosphorylated (Figure 8B), consistent with the histone H1 kinase assay results. These data also suggest that expression of the mutant CaMKII causes cell-cycle arrest in G2 before the point when p34/cdc2 undergoes cdc25-dependent tyrosine dephosphorylation that leads to the activation of histone H1 kinase activity.

To address the question of whether CaMKII affects p34/cdc2 activity, the CaMKII-(D286) mutant was expressed in mutant S. pombe strains with altered timing of mitosis. Two cdc2 mutant alleles, cdc2.1w and cdc2.3w, affect the ability of either wee1+ or cdc25+ to regulate p34/cdc2 (Nurse and Thuriaux, 1980; Fantes, 1981). Each strain was transformed with the pREP4.1-CMKII(D286) plasmid, and a representative isolate was selected. The effect of expressing the mutant CaMKII on cell division was then examined. As shown, expression of the mutant CaMKII caused cell-cycle arrest in both the cdc2.1w and cdc2.3w strains (Figure 9A). The effect on cell proliferation was virtually indistinguishable from that observed in cells with a normal cdc2 gene. In addition, the same elongated cell phenotype was observed (Figure 9B). This result suggests that cell-cycle arrest induced by the mutant CaMKII is likely not mediated through either cdc25 or wee1. These data suggest that CaMKII most likely inhibits G2/M progression by another pathway and not by direct regulation of p34/cdc2 activity. Alternatively, CaMKII might act via the mik1 protein kinase or another kinase that has a function redundant to that of the wee1 kinase (Lundgren et al., 1991) or through phosphorylation of multiple substrates, in which case a simple epistatic relationship would not be observed. The present data indicate that CaMKII does not directly regulate p34/cdc2 activity in S. pombe.

**DISCUSSION**

The results of this study demonstrate that expression of a mutant form of mouse CaMKII with constitutive activity in S. pombe causes cell-cycle arrest in G2. Associated with cell-cycle arrest is a failure to activate the p34/cdc2 H1 kinase activity, a process that normally...
occurs late in G₂ in *S. pombe* (Booher et al., 1989). In contrast, expression of the normal CaM-dependent form of CaMKII had no apparent effects on exponentially growing *S. pombe*. These studies indicate that substrates for CaMKII exist in *S. pombe* that are involved in the negative regulation of cell proliferation.

Previous work has implicated CaMKII as a necessary component of the biochemical mechanism that is involved in G₂/M and mitotic progression, playing a positive role in cell-cycle progression at these times. Inhibition of CaMKII in sea urchin eggs either by injection of anti-CaMKII antibodies or peptide inhibitors of CaMKII prevented nuclear envelope breakdown, suggesting that CaMKII is required for the G₂/M transition (Baitinger et al., 1990). Injection of a cDNA encoding the D286 mutation into stage VI *Xenopus* oocytes initiated maturation as indicated by migration of the germinal vesicle but did not lead to nuclear envelope breakdown (Waldmann et al., 1990), consistent with the negative regulatory role for CaMKII we have observed in *S. pombe*.

Calmodulin-dependent phosphorylation of a 62-kDa protein in sea urchin embryos has been associated with breakdown of the mitotic apparatus (Dinsmore and Sloboda, 1988). However, the identity of the 62-kDa protein or the specific CaM-dependent protein kinase responsible for its phosphorylation are not known. In *A. nidulans* CaM has been shown to be required for the G₂/M transition and the activation of the NIMA protein kinase (Lu et al., 1992, 1993b), but the mechanism through which CaM affects NIMA activity has yet to be elucidated. One possibility is that CaMKII might be an upstream component of a protein kinase cascade that ultimately regulates the NIMA kinase, because NIMA appears to be activated by phosphorylation but is not directly phosphorylated by CaMKII (Lu et al., 1993a).

In contrast to these earlier studies, overexpression of constitutively active CaMKII appears to negatively regulate progression at the G₂/M boundary. In mouse C127 cells expression of a truncated form of mouse CaMKII was associated with a transient blockage of cell-cycle progression in G₂ (Planas-Silva and Means, 1992). In these studies it was not clear if this was physiologically relevant because only the N-terminal half of the kinase was expressed. Thus, potentially critical regulatory regions of the protein including the CaM-binding and multimerization domains were lacking. Because CaMKII exists in vivo as a 600-kDa dodecamer, this altered form of the kinase, which would be a 30-kDa monomer, could have markedly different substrate specificity and/or different cellular distribution as compared to native CaMKII. The results of this study show that a constitutively active mutant form of CaMKII, differing only by a single amino acid, has the same effect on cell cycle progression in yeast. Because the mutant CaMKII used in this study differed from the wild-type version of the enzyme by only a single amino acid, it is expected that the constitutive mutant would have both normal substrate specificity and subcellular localization.

The results of this study in combination with earlier work suggest that CaMKII plays both positive and negative regulatory roles at the G₂/M transition of the cell cycle. Recent in vitro studies have suggested that CaMKII might play a role in the activation of the cdc25 protein phosphatase, the enzyme responsible for dephosphorylating Y15 in p34/cdc2, a step that activates the p34/cdc2 kinase before entry into M-

**Figure 7.** p13-associated histone H1 kinase activity in CaMKII expressing strains. p34/cdc2-associated histone H1 kinase assay was determined as described in MATERIALS AND METHODS. Relative activities are expressed as a percentage of the value obtained for exponentially growing CK-T cells grown in the presence of 2 μM thiamine to repress CaMKII expression. Samples are as indicated in the accompanying legend. CK-T and CK-D cells were grown for 24 h either in the presence or absence of thiamine. The cdc25 samples are cdc25-22 cells grown at 25°C (cdc25), cdc25-22 cells arrested for 4 h at the restrictive temperature (cdc25 arrest), and cdc25-22 cells arrested for 4 h at the restrictive temperature then returned to permissive conditions for 20 min (cdc25 release). cdc25-22 cells 20 min after release are predominantly in M-phase and have the highest levels of p34/cdc2 H1 kinase activity found during the cell cycle (Booher et al., 1989).

**Figure 8.** cdc2 and phosphotyrosine-associated cdc2 levels. (A) cdc2 Western blot in total protein extracts. Western blotting of total protein extracts was performed as described. Samples were CK-T (T) or CK-D (D) cells grown for 24 h either in the presence (+) or absence (−) of 2 μM thiamine. Expression is expected in the absence of the nmt promoter repressor thiamine. Blots were probed with an anti-P-Tyr antibody. (B) Phosphotyrosine content of p13-associated cdc2. p34/cdc2 was isolated from p13-coated microtiter wells as described, resolved by SDS-PAGE, and Western blotted for phosphotyrosine using an anti-P-Tyr antibody. Samples were CK-T (T) or CK-D (D) cells grown for 24 h either in the presence (+) or absence (−) of 2 μM thiamine.
Figure 9. Genetic analysis of CaMKII effects on cell division. To determine whether the effect of the mutant CaMKII is mediated via wee1 or cdc25, cdc2-1w and cdc2-3w cells were transformed with the pREP4.1-CaMKII(D286) plasmid, and growth in the presence or absence of thiamine was examined. For the effect of the mutant CaMKII on growth of cdc2w strains, cells were grown for 24 h at 25°C with or without thiamine. (A) Cell density in cultures expressing the kinase (+ thiamine) are expressed as a percentage of the cell density obtained when the same strain was grown in the presence of thiamine (i.e., not expressing the plasmid-borne CaMKII gene). Also included are the values for the CK-T and CK-D strains (which have a normal cdc2 gene) grown under identical conditions. (B) Photomicrographs of cdc2-1w/CK-D and cdc2-3w/CK-D cells. Cells were grown in the presence (+ thiamine) or absence (- thiamine) of 2 μM thiamine-HCl as described in MATERIALS AND METHODS. The top panels show cdc2-1w cells containing the pREP4.1-CaMKII(D286) plasmid, whereas the bottom panels are cdc2-3w cells containing the pREP4.1-CaMKII(D286) plasmid.

The nature of the negative regulation of cell proliferation by constitutively active CaMKII is more complex. The data presented in this study suggest that constitutive CaMKII activity suppresses G2/M progression at a point before the activation of p34/cdc2. In theory, CaMKII could act either directly on p34/cdc2 or indirectly on some other cell-cycle regulator that ultimately feeds into the p34/cdc2 pathway. It has recently been shown in metaphase II-arrested Xenopus eggs that a truncated constitutively active form of CaMKII inactivates p34/cdc2 by inducing the degradation of cyclin B (Lorca et al., 1993). However, we have examined the levels of cdc13 protein (the S. pombe cyclin B homologue) levels in the CK-T and CK-D strains both in the presence and absence of thiamine and see no difference in cyclin B levels (unpublished observations). Thus, the effect of constitutive CaMKII activity in cycling cells appears to be different than that observed in metaphase-arrested Xenopus eggs. This may indicate that CaMKII regulates p34/cdc2 via cdc25 or wee1/mikl, direct regulators of p34/cdc2. However, our preliminary results (Parker, Piwnica-Worms, Rasmussen, unpublished data) indicate that CaMKII does not phosphorylate the human homologues of either the wee1 or cdc1 proteins or p34/cdc2-cyclin B. Finally, the genetic experiments in the present study suggest that the negative effect of CaMKII on cell-cycle progression is not mediated by either cdc25 or wee1. Thus, our results suggest that another pathway, regulated by CaMKII, inhibits G2/M progression in fission yeast. Whatever the mechanism through which CaMKII inhibits G2/M progression our data would indicate that it does not appear to directly involve p34/cdc2, although activation of p34/cdc2 may be downstream of the pathway that is affected by CaMKII.

Although the targets of CaMKII are presently unknown, we do not think that this negative regulation is mediated by the S. pombe NIMA homologue. Previous work in A. nidulans has shown that ts mutants of NIMA kinase arrest in G2, but that p34/cdc2 is both tyrosine dephosphorylated and active as a histone H1 kinase.
Weinert, bleomycin observed. It is possible that CaMKII may be acting through the mik1 protein kinase or that multiple substrates may be involved, in which case suppression of the growth arrest phenotype in the cdc2-1w strain would not have been observed.

A potential hypothesis suggested by the results of this study is that the negative regulation of G2/M progression by constitutively active CaMKII could be part of a G2 checkpoint mechanism. Cell-cycle checkpoints ensure that mitosis only occurs when the genome is completely replicated and undamaged (Hartwell and Weinert, 1989). It has been known for some time that CaM is required for DNA repair. Treatment of cells with CaM antagonists inhibits repair of DNA damaged by bleomycin (Chafouleas et al., 1984). Thus, if CaMKII was involved in DNA repair, elevated CaMKII activity might be associated with the DNA repair in progress. If this were the case, constitutively elevated CaMKII levels would be expected to cause arrest at the G2 checkpoint, upstream of the p34/cdc2 activation point. Because arrest at the G2 checkpoint is associated with tyrosine phosphorylation of p34/cdc2 (Smythe and Newport, 1992), our observation that CaMKII(D286)-expressing cdc25-arrested cells contain tyrosine phosphorylated and inactive p34/cdc2 is consistent with this interpretation. Further studies are necessary to pinpoint the target or targets that CaMKII affects to regulate G2/M progression in eukaryotic cells. The strains created in the course of this study should allow us to use a genetic approach to isolate suppressors of the growth arrest phenotype and thus potentially define critical targets of CaMKII in S. pombe.

ACKNOWLEDGMENTS

The authors thank Dr. David Beach (Howard Hughes Medical Institute, Cold Spring Harbor Laboratory) for S. pombe strains and the cdc13 antiserum. Dr. Viesturs Alderton (International Science and Engineering Research Council, Switzerland) for the cdc2a strains, and Dr. Steve Pelech (University of British Columbia) for the cdc2 antisera. The pREP4.1 plasmid was obtained from Dr. P. Romanow (NIH). This work was supported by grants from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research to C.R. C.R. is a Scholar of the Alberta Heritage Foundation for Medical Research and Medical Research Council, Canada.

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