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Expression of a constitutive form of calcium/calmodulin dependent protein kinase II leads to arrest of the cell cycle in G_2

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Calcium/calmodulin dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase. We have created a calcium/calmodulin independent form of this enzyme by truncation. Expression of this enzyme fragment in a rabbit reticulocyte lysate yields a constitutive enzyme with specific activity similar to the activated native enzyme. We have established mammalian cell lines that transiently express this constitutive enzyme using the glucocorticoid-inducible mouse mammary tumor virus long terminal repeat. The transient increase in kinase activity results in a complete cessation of cell cycle progression. This block develops as a consequence of a specific arrest of the cell cycle in G_2. During the block, increases in histone H1 kinase activity present in p13 beads or anti-cdc2 immunoprecipitates are seen in parallel with the accumulation of cells at G_2, arguing that the arrest is not due to a failure to activate cdc2 as a histone H1 kinase. These results suggest that other changes in serine/threonine protein phosphorylation besides those involved in activation of cdc2 as a histone H1 kinase may be necessary for proper G_2-M transition.

Key words: CaMKII/cdc2/cell cycle regulation/G_2 arrest/protein phosphorylation

Introduction

Calmodulin (CaM), a primary intracellular calcium receptor, is known to mediate several biological effects that are the result of increases in cytosolic calcium (for review, see Rasmussen and Means, 1989b). Changes in cytosolic calcium occur before nuclear envelope breakdown in sea urchin eggs and mammalian cell lines (Kao *et al.*, 1990; Poenie *et al.*, 1985). In *Saccharomyces cerevisiae* (Uno *et al.*, 1989), *Aspergillus nidulans* (Rasmussen *et al.*, 1990) and a mammalian cell line CHO-K1 (Chafouleas *et al.*, 1982), calmodulin levels increase as cells progress into mitosis. A similar increase in calmodulin content is seen in the temperature sensitive mammalian cell mutant *tsBN2* during the induction of premature chromatin condensation (Nishimoto *et al.*, 1985). In this cell line, the mitotic phosphorylation of histones H1 and H3 that occurs after temperature shift can be blocked by calmodulin antagonists. These results suggest that CaM is involved in the transition from G_2 to M. That calmodulin is necessary at this stage of the cell cycle was also shown in yeast since deletion of the unique CaM gene causes cell cycle arrest at G_2-M. This arrest can be overcome by the inducible expression of a chicken calmodulin gene (Ohya and Anraku, 1989). Calmodulin seems also to be necessary for normal G_2-M progression in mammalian cells as suggested by expression of an antisense construct (Rasmussen and Means, 1989a) or the use of calmodulin antagonists (Sasaki and Hidaka, 1982). However, nothing is known concerning the molecular mechanisms by which CaM acts at this stage of the cell cycle.

Cell cycle progression through G_2-M is regulated by a network of protein kinases and phosphatases. In the last few years, many of the proteins that are involved in G_2-M transition have been identified (for review, see Cyert and Thorner, 1989; Freeman and Donoghue, 1991). A key regulator is the protein kinase cdc2. This enzyme acts as component of the M-phase promotion factor and shows a cell cycle regulated histone H1 kinase activity (for review, see Lewin, 1990; Nurse, 1990). Activation of cdc2 as a histone H1 kinase is regulated by dephosphorylation (Gautier *et al.*, 1989; Morla *et al.*, 1989). In *Schizosaccharomyces pombe* two other protein kinases control mitotic initiation: *wee1*$^+$, which acts as a negative regulator (Russell and Nurse, 1987b) and *nim1*$^+$, which works as an inducer of mitosis (Russell and Nurse, 1987a). In *A.nidulans*, *NIMA* is a protein kinase necessary for entry into mitosis and can cause mitotic induction when overexpressed (Osmani *et al.*, 1988). It is therefore likely that CaM regulates G_2-M progression by affecting protein phosphorylation.

The multifunctional calcium/calmodulin-dependent serine/threonine protein kinase (CaMKII) is a potential target for the effect of CaM on the G_2-M transition due to its broad substrate specificity (reviewed in Colbran *et al.*, 1989; Schulman, 1988). Activation of CaMKII occurs rapidly and transiently after addition of serum or growth factor to cells (Ohba *et al.*, 1991; Ohta *et al.*, 1988) or other events that lead to changes in intracellular calcium (Connelly *et al.*, 1987; MacNicol *et al.*, 1990). This enzyme is widely distributed in mammalian tissues although some isoforms show tissue-specific expression (Fukunaga *et al.*, 1988; Tobimatsu and Fujisawa, 1989). Calcium/CaM dependent protein kinases with properties similar to mammalian CaMKII have been identified in a variety of organisms as diverse as *S.cerevisiae* (Miyakawa *et al.*, 1989), *A.nidulans* (Bartelt *et al.*, 1988), *Neurospora crassa* (Ulloa *et al.*, 1991), and the sea urchin *Lytechinus pictus* (Baitinger *et al.*, 1990).

Several observations suggest that CaMKII may indeed be involved in G_2-M progression. This enzyme has been localized in the interphase nucleus and the mitotic apparatus of mammalian cells (Ohta *et al.*, 1990). Recently, evidence obtained from sea urchin eggs implied that CaMKII is

involved in nuclear envelope breakdown. Baitinger *et al.* (1990) were able to block this process by using antibodies against CaMKII or a synthetic peptide modeled on the autoinhibitory region. Furthermore, microinjection of a cDNA encoding a constitutive form of CaMKII into *Xenopus* oocytes was reported to initiate maturation (Waldmann *et al.*, 1990). These results support a possible role for CaMKII in mediating the effects of CaM on the G_2-M transition. However, the fact that deletion of the two genes that so far have been identified for CaMKII in *S. cerevisiae* is not lethal argues against it (Ohya *et al.*, 1991; Pausch *et al.*, 1991). Nevertheless, both papers suggest that preliminary evidence exists for the presence of a third gene, as is the case for cyclins (Richardson *et al.*, 1989) and other genes that encode regulatory proteins in yeast. In such cases, deletion of all three genes is necessary to obtain a lethal phenotype. It is equally possible that *S. cerevisiae* may differ from other eukaryotes in the involvement of CaMKII in cell cycle progression.

In this study we have generated stable mammalian cell lines that express a constitutive form (i.e. calcium-independent) of CaMKII in an inducible manner. These cell lines have been utilized to characterize how expression of the constitutive kinase affects cell cycle progression. We present evidence that expression of this enzyme leads to cell cycle arrest due to a specific block in G_2. Furthermore, we observed that inhibition of G_2-M progression does not result in a similar inhibition of the activation of cdc2 as a histone H1 kinase. These data support the concept that precise control of multiple serine/threonine protein phosphorylation/dephosphorylation events must be achieved before cells can progress into M.

Results

Construction and characterization of a constitutive form of CaMKII

To generate a constitutive form of CaMKII, we used the mouse cDNA clone encoding the α subunit of brain CaMKII (Hanley *et al.*, 1989). Part of the regulatory domain and the complete association domain (Lin *et al.*, 1987) were removed by truncation at amino acid 291 (Figure 1A). Previous evidence from proteolytic studies (LeVine and Sahyoun, 1987) and from bacterial expression (Kapiloff *et al.*, 1991) indicated that truncation of rat CaMKII in this region would produce a constitutively active enzyme. In our truncation mutant, seven additional amino acids were inserted before the stop codon due to our cloning strategy (see Materials and methods). Figure 1A presents a diagrammatic structure of the wild-type enzyme and compares it with the truncated form. The sequence of the 18 C-terminal amino acids of the mutant enzyme is indicated.

To determine if the truncated enzyme would function in a calcium independent manner, it was expressed in an *in vitro* transcription and translation system (Fong *et al.*, 1989). As a control we expressed the full length wild-type cDNA. As shown in the left panel of Figure 1B translation of the wild-type mRNA in the presence of [^{35}S]methionine yielded the expected 50 kDa protein while the cDNA coding for the putative constitutive form of the enzyme (ctCaMKII) gave rise to a 33 kDa band that corresponds to the predicted molecular size of the truncated enzyme. To assay activity of the expressed kinases we used the GS-10 peptide as a substrate (generously provided by Bruce Kemp; Pearson *et al.*, 1985). The assay was carried out both in the presence and in the absence of Ca^{2+}. Values obtained were corrected for the presence of endogenous kinase activity in the lysate and for efficiency of translation. The calcium-independent activity of the wtCaMKII translation mixture was 0.36 ± 0.11 nmol/min/ml of lysate. An accurate specific activity cannot be calculated due to unknown levels of methionine in each lysate (see Fong *et al.*, 1989). A tenfold increase in kinase activity occurred in the presence of Ca^{2+} resulting in an activity of 3.45 ± 1.0 nmoles/min/ml of lysate (Figure 1B, right panel). In comparison, the ctCaMKII translation mixture gave similar activities in the presence or absence of Ca^{2+} (2.85 ± 0.35 and 3.25 ± 1.7, respectively). These results indicate that the mutant enzyme is constitutive and that its kinase activity is very similar to the wild-type enzyme in the presence of Ca^{2+}.

Expression of ctCaMKII mRNA and ctCaMKII is transiently induced by dexamethasone

The ctCaMKII cDNA was subcloned into the expression vector pMAM-neo. In this plasmid, expression of ctCaMKII is regulated by the mouse mammary tumor virus (MMTV) long terminal repeat. Plasmids were transfected into C127 cells, previously used to overexpress CaM (Rasmussen and Means, 1987). Stable cell lines were isolated by resistance to the antibiotic G418. Southern analysis revealed the presence of the transfected plasmid in all the isolated clones. Those clones that showed a glucocorticoid-dependent expression of ctCaMKII (not shown) were characterized further. To evaluate the temporal expression of ctCaMKII mRNA and ctCaMKII we used the CT11.1 cell line that gave the highest levels of expression. As shown by Northern blot analysis (Figure 2A), a rapid induction of the 2.1 kb mRNA occurred in response to 500 nM dexamethasone (Dex) and peaked at 5 h. Subsequently, mRNA levels began to decrease and had returned to the uninduced level by 18 h. A low basal level of expression occurs in the absence of the inducer but is not detectable at the exposure shown.

Western blot analysis using an α-CaMKII specific antibody detected an analogous transient induction of ctCaMKII that followed the changes seen in ctCaMKII mRNA (Figure 2B). The levels of the 33 kDa ctCaMKII increased gradually from time zero to 6 h where the peak occurred. Again, the protein levels declined progressively returning to basal levels by 21 h. These results suggest that ctCaMKII mRNA and ctCaMKII are relatively short-lived molecules and that, somehow, transcription from the MMTV promoter is not maintained or ctCaMKII mRNA is rapidly degraded, in spite of the continued presence of Dex. Similar observations have been reported in cell lines transfected with another serine/threonine kinase v-*mos* (Hamilton and DeFranco, 1989; Jaggi *et al.*, 1986). Apparently, in the case of v-*mos*, transcription ceases due to alterations in the intracellular partitioning of the glucocorticoid receptor (Qi *et al.*, 1989). A similar mechanism could provide an explanation of the results obtained in our cell lines.

In order to determine if temporal expression of ctCaMKII is reflected by increased kinase activity of CT11.1 cell extracts, cells were harvested at different times after induction and assayed for Ca^{2+}-independent kinase activity. As shown in Figure 2C, the changes in Ca^{2+}-independent activity did parallel the changes in protein. The increase in

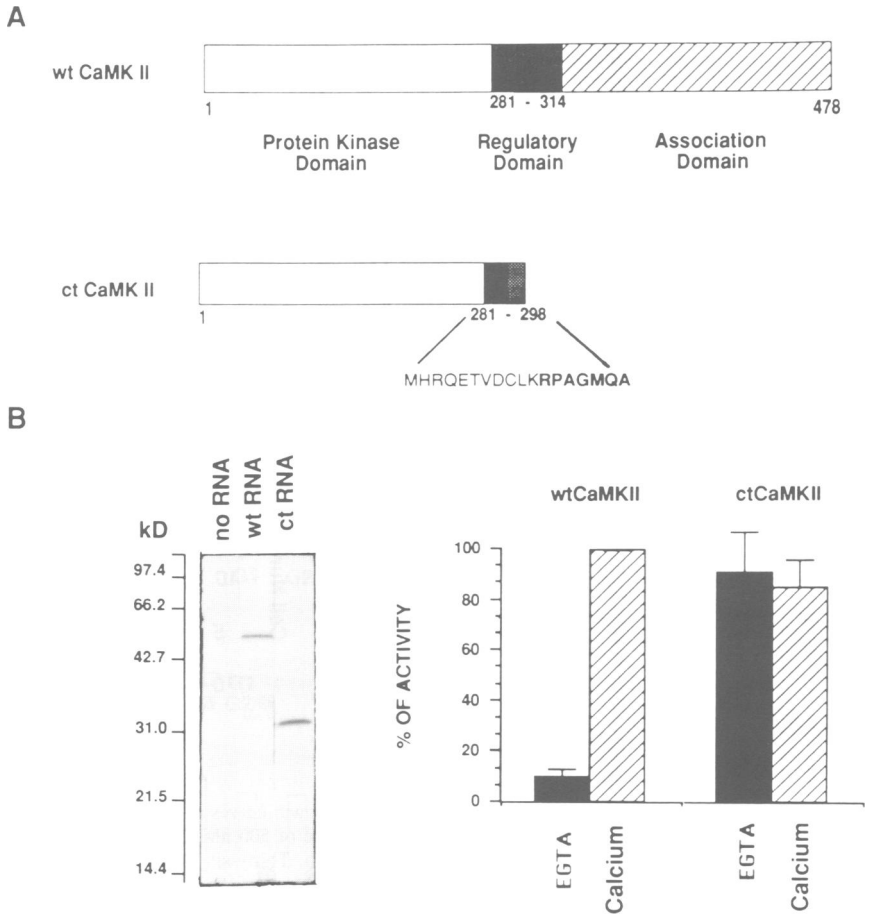

Fig. 1. Structure and *in vitro* characterization of CaMKII wild-type and mutant. (**A**) Diagrammatic representation of the structure of the α subunit of CaMKII showing its major domains and the truncated form of CaMKII (ctCaMKII). The enzyme was truncated at lysine (K) 291. The amino acids present at the COOH-terminus are shown and include the additional amino acids added due to the cloning strategy in bold type. (**B**) Left panel: The wild-type CaMKII cDNA and its truncated form were used in an *in vitro* transcription and translation system (see Materials and methods). The [^{35}S]methionine translation products were subjected to 12% SDS−PAGE and autoradiographed. Right panel: Analysis of kinase activity of the wild-type and mutant kinase translated in the presence of unlabeled methionine. Activity was measured in the presence of Ca^{2+} or in the presence of 2.4 mM EGTA using the synthetic peptide GS-10 as substrate. The experiment was repeated several times with at least four independent translation mixtures. Values represent the average of those obtained in two independent translation reactions (± SD).

activity was also transient, reaching a maximum at 6 h and decreasing thereafter. No changes in kinase activity were observed in the control N2 cells when these cells were exposed to Dex. The maximum levels of Ca^{2+}-independent activity obtained after induction of ctCaMKII were well below the level of CaMKII activity in normal extracts assayed in the presence of Ca^{2+}/CaM. Thus, expression of ctCaMKII does not lead to gross changes in kinase activity. The changes in kinase activity seen in the CT11.1 cells are actually qualitatively but not quantitatively related to the protein levels as detected by Western analysis. We believe that this is due to the conditions of the kinase assay and the use of whole cell extracts which results in high levels of basal activity (i.e. Ca^{2+}-independent activity) in the control or uninduced cells. Nevertheless, these data reveal a temporal association between expression of ctCaMKII mRNA, ctCaMKII and changes in Ca^{2+}-independent kinase activity present in the extracts.

Expression of ctCaMKII causes inhibition of cell cycle progression

Figure 3 shows the effect of Dex on growth properties of a control (N2) and a ctCaMKII expressing cell line (CT11.1). Only a slight inhibition of cell division occurs in the control cell line N2 (transfected with the vector pMAM-neo alone), due to the addition of 500 nM Dex. On the other hand, the same treatment resulted in a profound effect on line CT11.1. This cell line showed complete cessation of cell division for 9 h, between 3 and 12 h after Dex treatment. This halt in cell cycle progression was accompanied by a disappearance of mitotic figures in the cell cultures. Disappearance of mitotic figures was temporary and dependent on the continued presence of Dex. Removal of the Dex any time prior to 8 h shortened the period of arrest. Two other ctCaMKII expressing clonal cell lines showed similar transient growth arrest. In addition, duration of the arrest was dose dependent (data not shown). Together, these data suggest a tight correlation between expression of ctCaMKII and cessation of cell cycle progression.

Cell cycle distribution of dexamethasone treated cells

Expression of the mutant CaMKII could prevent cell division by non-specific toxic effects. Alternatively, cell division could be affected by a more specific effect on cell cycle progression. To determine whether the CT11.1 cells became arrested at a specific phase of the cell cycle, or in a non-specific random fashion, we analyzed the distribution of cellular DNA content as a function of time after Dex

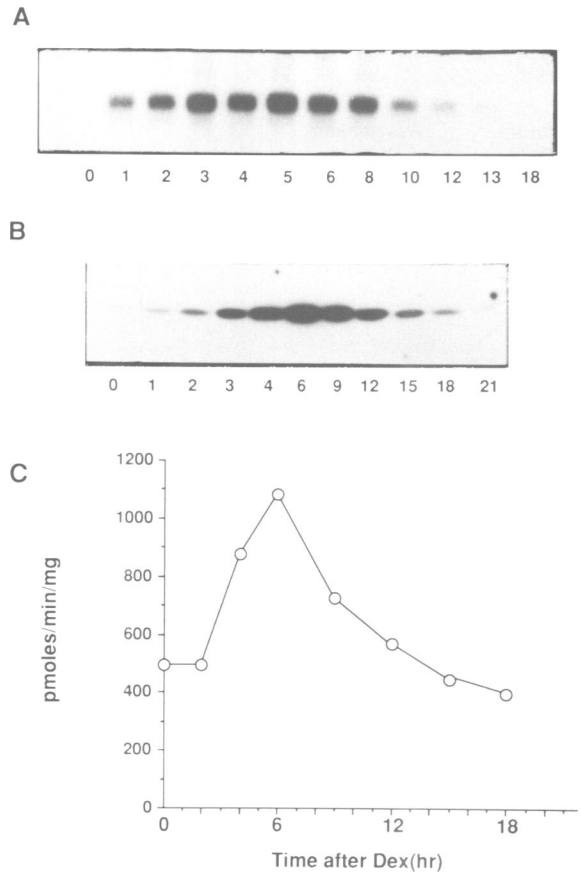

Fig. 2. Transient induction of ctCaMKII mRNA, protein and kinase activity. (**A**) The CT11.1 cells were treated with 500 nM Dex. Cells were harvested at the indicated times, RNA was analyzed by Northern blot analysis using an α-CaMKII specific probe. (**B**) CT 11.1 cells were treated as in (A) and at the designated time cell extracts were prepared and analyzed for ctCaMKII expression by Western blot analysis using anti-CaMKII antibodies. (**C**) Cell extracts were made at different times after treatment of CT11.1 cells with 500 nM Dex. Cell extracts were corrected for the differences in protein concentration and then used in a kinase assay with the GS-10 peptide as substrate.

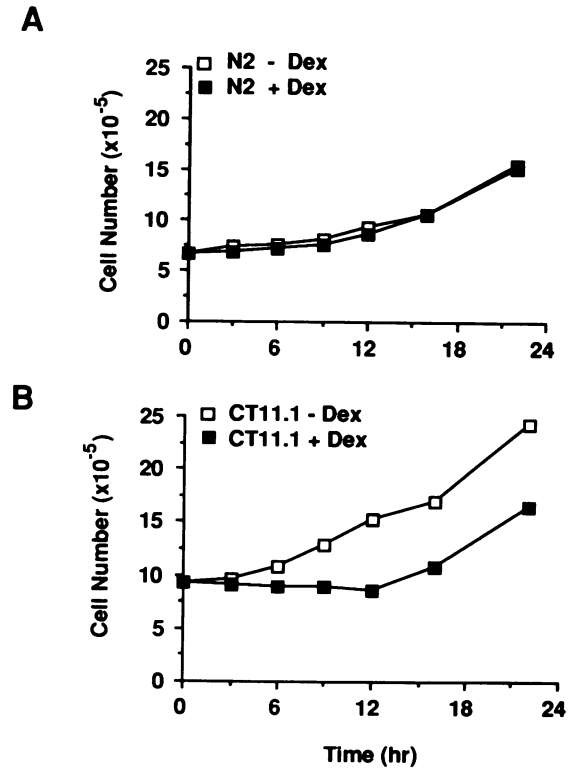

Fig. 3. Growth curves of CT and control cell lines in the presence and absence of 500 nM Dex. Cells were plated at 1.8×10^5 cells per 60 mm dish. Thirty-six hours later the medium was removed and fresh medium containing ethanol (−DEX) or 500 nM Dex in ethanol (+DEX) was added to the dishes. Cells were trypsinized at the indicated times and counted in a Coulter counter. Values represent the average of two dishes from one experiment. Similar results were obtained in other experiments. (**A**) Growth curves of the control cell line N2. (**B**) Growth curves of CT11.1 cells.

treatment. Representative flow microfluorometric analysis (FMF) of the N2 and CT11.1 cells cultured in the presence of hormone is shown in Figure 4A. The control cells (N2) did not exhibit any changes in the FMF pattern with time of treatment. In contrast, substantial changes occurred in the cells expressing ctCaMKII as evidenced by a time dependent increase in cells with a G_2/M content concomitant with a decrease in the number of G_1 cells (Figure 4B). This accumulation was transient and a gradual decrease in G_2/M cells accompanied by an increase in G_1 cells was observed after 15 h. At 24 h most of the cells had exited G_2/M and entered G_1. The highest number of G_2/M cells was obtained from 10−15 h with values between 55 and 85% of the population depending on the cell line and culture conditions. These results reveal that expression of ctCaMKII blocks cell cycle progression in a specific manner and results in an accumulation of cells with a G_2/M DNA content.

Inhibition of cell growth is due to specific cell cycle block at G_2

Since FMF analysis cannot distinguish G_2 from M cells, other methods were required to establish the precise arrest point. We had previously noticed a decrease in mitotic figures during the block which hinted that the arrest was at G_2. To confirm that the blocked cells were in G_2, we evaluated several different criteria that are commonly used to distinguish a mitotic cell from an interphase cell. These criteria include spindle formation, chromosome condensation, absence or presence of a nuclear envelope, and immunostaining with a monoclonal antibody called MPM-2 that is specific for phosphoproteins present predominantly in mitotic cells (Davis *et al.*, 1983).

Microtubules undergo rearrangements during mitosis. Cytoplasmic microtubules depolymerize in prophase and begin to form the spindle (Brinkley *et al.*, 1980). Therefore, microtubule organization can provide a clear distinction between interphase and mitotic cells. To evaluate microtubule organization we chose 10 h of Dex treatment since at this time ~50% of CT11.1 cells had a G_2/M DNA content (Figure 4B). In response to this treatment, very few CT11.1 cells underwent changes normally observed during mitotic progression. As seen in Table I, quantitative analysis of N2 cells revealed 4.9% of cells with mitotic microtubule arrays when stained with anti-tubulin antibodies (MT). In contrast only 0.3% of the arrested CT11.1 cells were in mitosis by this criteria. Figure 5 panels A and B show representative fields of the anti-tubulin stained N2 and CT11.1 cells, respectively. In accord with the mitotic index values, these representative fields have 2 (N2) and zero (CT11.1) mitotic microtubule arrays. The mitotic N2 cells (Figure 5A) are seen to be either in metaphase (M) or late

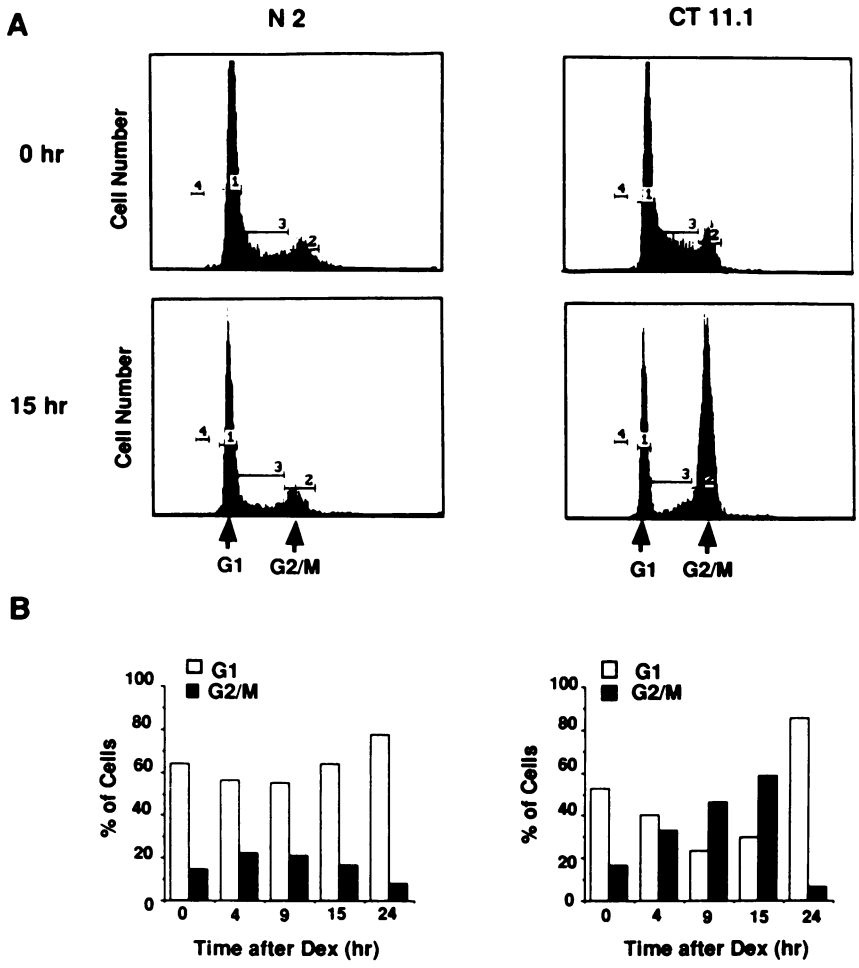

Fig. 4. FMF analysis of N2 and CT11.1 cells during treatment with 500 nM Dex. (**A**) FMF profiles of N2 and CT11.1 cells of uninduced cells and cells treated for 15 h with Dex. (**B**) Graphic representation of % of cells in G_1 and G_2/M phases after addition of hormone calculated from the FMF profiles as shown in (A).

telophase (T). Thus, as evidenced by the absence of spindle formation the block point is in G_2 before any microtubule rearrangements have taken place.

A similar observation was obtained when cells were stained with propidium iodide (PI) (see Table I). Analysis of the control cells N2 showed that 3.9% contained condensed chromatin while only 0.6% of the CT11.1 cells showed this phenotype. Figure 5 panels C and D are representative fields of the PI double-stained cells shown in Figures 5A and 5B corresponding to N2 and CT11.1 cells, respectively. The two N2 mitotic figures exhibit fully condensed (M) and partially condensed chromatin (T). By comparison, the arrested CT11.1 cells show only characteristics of interphase cells: uncondensed chromatin, presence of nuclear envelopes and morphologically distinct nucleoli. These data also support the idea that expression of ctCaMKII leads to G_2 arrest with no visible changes in chromatin condensation.

The MPM-2 staining (MPM-2) of Dex treated cells revealed comparable changes in mitotic index. As indicated in Table I, the mitotic indices from this analysis were 4.0% and 0.4% for N2 and CT11.1 cells, respectively. Representative fields of N2 (Figure 5E) and CT11.1 (Figure 5F) show differences used to assemble the values shown in Table I. The mitotic cells seen in Figure 5E are characterized by a very bright fluorescence while interphase cells reveal a very light (almost undetectable) nuclear staining. No mitotic MPM-2 immunoreactivity is apparent in any of the cells present in the representative field of CT11.1 cells (Figure 5F). Again, this outcome implies that the arrest is in G_2 and that the appearance of MPM-2 antigens is blocked in the arrested cells. The MT, PI and MPM-2 characteristics of cells at other time points during

Table I. Mitotic index after dexamethasone treatment[a]

	N2			CT11.1		
Stain	Cells (#)	M cells	% in M	Cells (#)	M cells	% in M
MT[b]	1081	53	4.9	1022	3	0.1
PI[c]	1013	40	3.9	1016[d]	6	0.6
MPM-2[e]	1068	43	4.0	1002	4	0.4

[a]Cells grown on coverslips were treated with 500 nM Dex for 10 hr.
[b]Cells were stained for tubulin. Mitotic cells were scored following the criteria established by Brinkley and co-workers (1980).
[c]Cells were stained with propidium iodide (30 μg/ml). Cells with partially or fully condensed chromatin were counted as mitotic.
[d]Some cells showed a zone of very bright fluorescence surrounding the nucleus in addition to the cytoplasmic array. Multiple short microtubules occupied the nuclear area.
[e]Immunofluorescence was done using the mitotic specific MPM-2 antibody (Davies *et al.*, 1983). Mitotic cells were distinguished by fluorescence intensity.

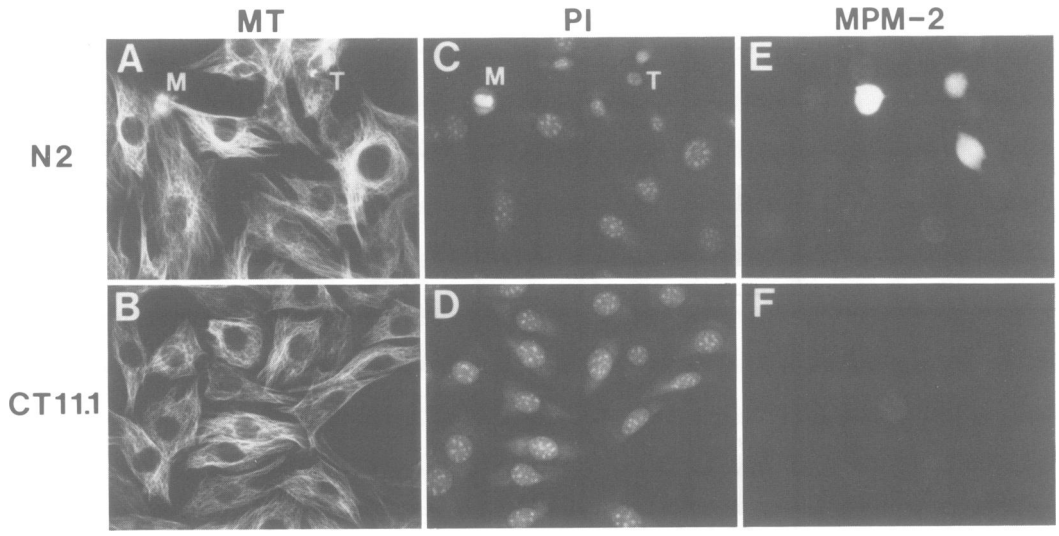

Fig. 5. Characterization of dexamethasone arrested CT11.1 cells as G_2 cells. Cells were grown on coverslips and treated with 500 nM Dex for 10 h. At this time, at least 50% of the cells had a G_2/M DNA content (Figure 4B). Individual coverslips were processed for immunofluorescence with anti-tubulin antibody or the MPM-2 monoclonal antibody. Some coverslips were double stained with propidium iodide. Magnification used was 400x. The quantification of these analyses is shown in Table I. (**A and B**): immunofluorescence with anti-tubulin antibodies of N2 cells and CT11.1 cells respectively. (**C and D**): propidium iodide fluorescence of N2 cells and CT11.1 respectively. (**E and F**): MPM-2 imunofluorescence of N2 and CT11.1 cells respectively. M, metaphase; T, telophase.

the G_2/M arrest were similar to those described. Taken together, these observations argue that expression of ctCaMKII results in a specific block in G_2 which precedes early mitotic changes.

G_2 arrest is not due to inhibition of histone H1 kinase activity

A commonly used criterion for evaluation of the G_2/M transition is the activation of cdc2 kinase. This activation can be assessed using histone H1 as a substrate following precipitation of the kinase from cell extracts using either suc-1-beads (p13 beads) (Arion *et al.*, 1988) or antibodies to p34 cdc2 (Draetta *et al.*, 1989; Gould and Nurse, 1989; Krek and Nigg, 1991). Because p13 beads or PSTAIR antibodies can precipitate kinases in addition to p34 cdc2 (Paris *et al.*, 1991) we undertook a comparative analysis of H1 kinase activity present in immunoprecipitates derived from p13 beads or an affinity-purified antibody to a 25 amino acid synthetic peptide analog of the carboxyl terminus of murine p34 cdc2 (Samiei *et al.*, 1991). After optimization of conditions, changes in H1 kinase activity in response to Dex were very similar when comparing results obtained using the two reagents. Because activities were somewhat higher and more reproducible using p13 beads, this was the predominant method used in subsequent experiments. Figure 5 illustrates the changes in histone H1 kinase activity in the control cells N2 (top panel) and the ctCaMKII expressing cells CT11.1 (bottom panel) after treatment with Dex. No major changes in histone H1 kinase activity are observed after Dex treatment of N2 cells. As expected from these results, the mitotic index (MI) of N2 cells remained practically constant throughout the experiment (MI = 8). Very different results were obtained during Dex treatment of CT11.1 cells (Figure 5, bottom panel). There was a gradual and transient increase in histone H1 kinase activity. The levels of activity increased > 10-fold by 14 h and started to decline by 16 h; at 24 h the levels were similar to those seen at the start of the experiment. These changes did not

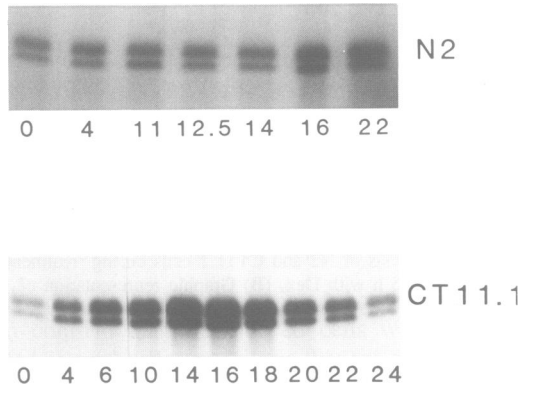

Fig. 6. Changes in p13-precipitable histone H1 kinase activity after treatment with Dex. Cells where treated with 500 nM Dex at the start of the experiment. At the indicated times, samples of N2 cells (**top panel**) and CT11.1 cells (**bottom panel**) were harvested and processed for histone H1 kinase activity.

correlate with those seen in the mitotic index as between 4 and 12 h the MI values were practically constant and low (i.e. 1.1% at 4 and 12 h and even lower in between). Therefore, these results indicated that ctCaMKII was not causing a G_2 arrest by blocking cdc2 activation as a histone H1 kinase.

The results obtained could be explained if the observed increases in histone H1 kinase were due to partial activation of cdc2 and full activation is necessary for G_2-M progression. To study this possibility, the changes in histone H1 kinase observed during the G_2 arrest were compared with those of control cells arrested in M phase with fully active cdc2. In order to block progression of cells in M phase, cultures were treated with the microtubule depolymerizing drug, nocodazole (Noc) at 5.5 h. This treatment has previously been used by Draetta and Beach (1988) to assess the fully active cdc2 kinase present in metaphase cells. Figure 7 presents analyses of changes in

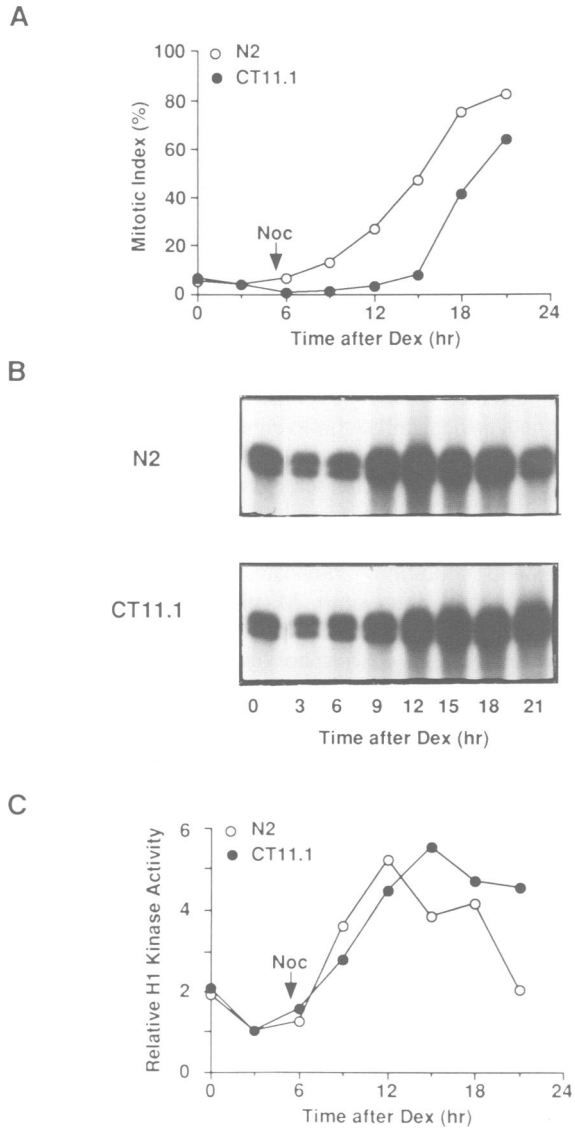

Fig. 7. Changes in mitotic index and histone H1 Kinase activity of N2 and CT11.1 cells during treatment with dexamethasone. Medium from N2 and CT11.1 cells (except $t = 0$) was collected and made 500 nM Dex at 0 h. Nocodazole was added to 0.4 μg/ml 5.5 h. after the addition of Dex. Cells were harvested by trypsinization at the indicated times and washed thoroughly. An aliquot was taken to determine the mitotic index and the rest was processed for histone H1 kinase activity. (**A**) Comparison of the mitotic index of N2 and CT11.1 cells. The chromosome mitotic index was evaluated for each sample. (**B**) Histone H1 kinase activity of the same samples as in (A). The results represent the histone H1 kinase activity following p13 precipitation of 80 μg of total protein. (**C**) The radiolabeled histone H1 bands from (B) were excised and quantified. The peak of histone H1 kinase activity for N2 cells (12 h) gave a value of 95.6 c.p.m./min/μg protein while for CT11.1 (15 h) the value was 71.5.c.p.m./min/μg.

mitotic index (panel A) and H1 kinase activity (panels B and C) in control (N2) and ctCaMKII expressing cells (CT11.1) as a function of time after Dex treatment. At the beginning of the experiment (time 0), both cell lines had similar chromosomal mitotic indices (MI) and similar histone H1 kinase activities. Dex treatment resulted in a reduction of the MI of CT11.1 cells to almost zero by 6 h (Figure 7A). No further appreciable changes in the MI were evident until 15–18 h. Between these times, a sharp increase in MI occurred. These changes in MI took place at times when

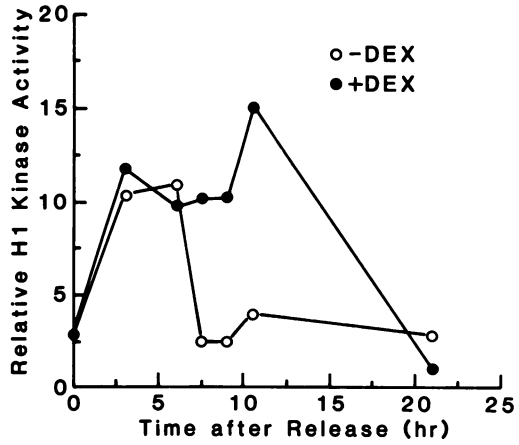

Fig. 8. Histone H1 kinase activity after release from G_1/S block. CT11.1 cells were arrested at G_1/S by the double thymidine block. At different times after release of the block, cells that were incubated in the presence or absence of 5×10^{-7} M Dex were harvested and analyzed for histone H1 kinase activity. Autoradiographs were scanned and values were normalized using the lowest level as 1.

the mitotic wave is seen during Dex treatment of CT11.1 cells in the absence of nocodazole. By comparison, a slight increase in MI of N2 cells was observed by 6 h which continued to rise until 18–21 h. Figure 7 (panel B) reveals very similar temporal changes in H1 kinase activity in N2 and CT11.1 cells. After an initial decrease over the first few hours, kinase activity continually increases, reaches maximal values by 12 (N2) or 15 h (CT11.1) and then declines (Figure 7, panel C). Whereas changes in MI and H1 kinase are closely coupled in N2 cells, this is not the case in CT11.1 cells; rather, H1 kinase activity rises considerably in CT cells at times when the MI is close to zero. Even at 15 h when H1 kinase activity is maximal, the MI is only slightly above the uninduced values (MI = 8.0 at $t = 15$ and 6.5 at $t = 0$). The decrease in H1 kinase activity at the later time points takes place in both cell lines and could be due to long-term deleterious effects of nocodazole. The decrease in kinase activity occurred concomitantly with the appearance of fragmented nuclei in the mitotic cells. Thus, no further increases in H1 kinase activity are seen when CT11.1 cells accumulate in M. Moreover, the changes in histone H1 kinase activity observed for the G_2 arrested CT11.1 cells are comparable to those occurring in the M-phase arrested N2 cell line. Because of the questionable specificity of p13 beads, the experiment was repeated using a p34 cdc2 antibody (Samiei *et al.*, 1991). Very similar temporal changes in H1 kinase activity were found in both N2 and CT11.1 cells (data not shown). Therefore, these data suggest that full activation of the p13-binding histone H1 kinase activity had occurred in the G_2 arrested cells. These results also show that changes in histone H1 kinase activity do not necessarily parallel changes in MI. These observations imply that the G_2 block caused by overexpression of ctCaMKII is independent of activation of histone H1 kinase.

If the ctCaMKII-mediated arrest allows full activation of cdc2 to occur normally, any changes in the kinetics of cdc2 activation as a histone H1 kinase should not be affected by expression of ctCaMKII. To alter the kinetics of cdc2 activation, cells were synchronized at the G_1/S boundary by a double thymidine block. In this way a transient increase in histone H1 kinase activity should be observed when cells

go through G_2-M. The levels of histone H1 kinase activity obtained at different times after release from the G_1/S block are shown in Figure 8. High levels of histone H1 kinase activity were detected between 3 and 6 h after G_1/S release in the presence or absence of Dex. After 6 h in the uninduced cells, histone H1 kinase activity decreased and remained low at the later points assayed. Strikingly, histone H1 kinase activity remained at high levels in the ctCaMKII expressing cells even 10 h after the G_1/S release. By 21 h the histone H1 kinase activity was low again, as would be expected from the fact that most of the cells have progressed through M phase by this time. In the uninduced cells, maximum levels of histone H1 kinase are close to 4-fold higher than those seen at time zero or at 9 h. By contrast, at 10 h the values in induced cells were 5-fold and 15-fold greater than those seen at zero or 21 h, respectively.

The data presented herein support the idea that levels of ctCaMKII can modulate progression through G_2 by mechanisms that do not affect the activation of cdc2 as a histone H1 kinase. The accumulation of cdc2-associated histone H1 kinase during this G_2 arrest suggests that other regulatory events besides activation of cdc2 as a histone H1 kinase are necessary for normal G_2-M transition.

Discussion

The experiments described herein reveal that the inducible but transient expression of a constitutively active form of CaMKII results in a specific cell cycle block in G_2. To our knowledge, this is the first report of a specific G_2 arrest due to expression of a mammalian serine/threonine protein kinase. The G_2-arrested cells contain a fully active cdc2-associated histone H1 kinase. Thus, our results also suggest that whereas activation of cdc2 as a histone H1 kinase is necessary for movement of mammalian cells from G_2 to M, this event may not be sufficient. The implication is that multiple protein phosphorylation/dephosphorylation pathways must interplay in order for cells to progress from G_2 to M.

The results presented here might seem to contradict several previous studies on the effects of CaMKII on cell cycle progression (see Introduction). Those reports suggested that CaMKII could exert a positive effect on G_2-M transition while the results shown here indicate, instead, an inhibitory role. An explanation for this apparent contradiction would be if a CaMKII dependent phosphorylation event was necessary for G_2 progression but was followed by a requisite dephosphorylation that also preceeded, and was necessary for the transition from G_2 into M. Continual presence of the active form of ctCaMKII could shift the phosphorylation/dephosphorylation equilibrium of these putative reactions in a way that favors G_2 and therefore cells could not proceed into M. This scheme would involve a necessary transient activation of CaMKII during G_2-M progression which is in accord with what is known about activation of CaMKII by Ca^{2+}/CaM. Expression of a constitutive enzyme would abolish the transient nature of CaMKII activation and by maintaining high levels of CaMKII activity affect progression through G_2-M.

Our studies clearly show that a transient increase of a constitutively active CaMKII causes a transient cell cycle block in G_2. Strikingly, this block is very tight, as almost 85% of the cells can accumulate in G_2 within 12 h after addition of Dex to asynchronously growing cultures. This indicates close to 100% efficiency of the block since the doubling time of these cells is normally about 15 h. Preliminary evidence using cells synchronized at the G_1/S boundary supports the idea that the block is the result of ctCaMKII affecting a G_2 specific function as cells that are going through G_2 are still efficiently blocked from entering into M by expression of ctCaMKII.

The primary control of G_2-M progression in eukaryotic cells is widely believed to involve regulation of cdc2 and mitotic cyclins (Lewin, 1990; Nurse, 1990). Our initial prediction was that the activity of ctCaMKII somehow prevented the dephosphorylation and/or activation of cdc2. Such a result would be consistent with several studies designed to determine the events that result in G_2 arrest. For example, the G_2 arrest induced by an inhibitor of topoisomerase II is characterized by suppression of the histone H1 kinase activity in the G_2 blocked cells (Lock and Ross, 1990; Roberge *et al.*, 1990). Similarly, the tyrosine phosphatase inhibitor vanadate was reported to block reversibly the entry into M phase of cells that were synchronized by refeeding after serum starvation by preventing tyrosine dephosphorylation of cdc2 (Morla *et al.*, 1989). Two temperature sensitive mammalian cell lines FT210 (Th'ng *et al.*, 1990) and *ts85* (Matsumoto *et al.*, 1980) arrest at G_2 at the restrictive temperature. Both lines showed low levels of histone H1 kinase at the restrictive temperature, although only FT210 has a temperature sensitive *cdc2* gene product.

We were surprised to find high levels of cdc2-associated histone H1 kinase activity at times when the mitotic index was close to zero. Multiple independent experiments led us to conclude that the p13-precipitable histone H1 kinase activity was actually increasing in parallel with the number of G_2 arrested cells. The expression of ctCaMKII was able to override normal cell cycle progression and impede transition into M in spite of the presence of active cdc2 as a histone H1 kinase. Initially, we considered the possibility that we were accumulating cells with only partially active cdc2. For example, the cells could be accumulating active cdc2−cyclin A complex while cdc2−cyclin B complex was not being fully activated thus causing the G_2 arrest. Such a result was found in studies on the *S.cerevisiae* mutant *cdc28-1N* (Surana *et al.*, 1991). In this temperature sensitive mutant, cells at the non-permissive temperature arrest at G_2/M with high levels of p13-precipitable histone H1 kinase. The authors explained their results by arguing that in this mutant the complexes formed between cdc2 and cyclins CLB1 and CLB2 were defective while those with cyclins CLB3 and CLB4 were normal. The activation of the cdc2 kinase present in the two latter complexes explained the high levels of histone H1 kinase observed but full activation of all the complexes was needed for normal G_2/M progression. This scenario cannot explain our data as the levels of histone H1 kinase in the G_2 arrested cells were similar to those seen in nocodazole arrested cells (Figure 7) or in a partially synchronized G_2/M population (Figure 8). Moreover, the morphology of the ctCaMKII-arrested cells differs from the *cdc28-1N* arrested cells in that none of the changes associated with M-phase have occurred (Figure 5).

The results presented here suggest the presence of other regulatory molecules that can affect transition from G_2-M without perturbing the histone H1 kinase activity of cdc2.

In addition, these data imply that other regulatory events may be necessary for the initiation of M. These events could either converge downstream of cdc2 activation as a histone H1 kinase or proceed in a separate but parallel manner. It is possible that ctCaMKII may be affecting MPF activity *in vivo* by perturbing its localization, interaction with other molecules, induction of an inhibitor, or other steps that may occur independent of its activation as histone H1 kinase. Some examples of possible independent events have recently been suggested by Jessus *et al.* (1990) and Gautier and Maller (1991).

Our results could also be explained if CaMKII affects a pathway that is independent of cdc2 but also necessary for G_2-M transition. Osmani *et al.* (1991) have shown that in *A.nidulans* the NIMA protein kinase acts independently from the histone H1 kinase activity of cdc2 to drive cells into M. Both kinases are necessary for entry into M but neither is sufficient for transition from G_2 to M in the absence of the other. In the mutant *nim*A5, the arrested cells block at G_2 with tyrosine dephosphorylated cdc2 and a corresponding high level of p13-precipitable histone H1 kinase. In another mutant *nim*T23, NIMA protein kinase is fully activated but cdc2 histone H1 kinase is not during the G_2 arrest. Thus, in this organism, two apparently parallel pathways of activation occur during normal G_2-M progression. Preliminary evidence from this laboratory (K.P.Lu, S.A.Osmani and A.R.Means, unpublished observations) show that both Ca^{2+} and calmodulin are required to activate NIMA in *A. nidulans* cells and that *A. nidulans* CaMKII can phosphorylate NIMA *in vitro*. Therefore, CaMKII could affect the NIMA pathway and lead to G_2 arrest with high levels of cdc2-associated histone H1 kinase. A mammalian NIMA homolog has recently been cloned (Ben-David *et al.*, 1991). This should allow direct analysis of whether a pathway similat to the one in *A. nidulans* exists in mammalian cells and, if so, whether its disruption could explain the G_2 arrest observed in the present study.

Studies done in mammalian cells have also suggested that other cdc2-independent events are necessary for G_2-M progresssion. Lamb *et al.* (1991) reported that co-injection of cdc2-cyclin and cAMP-dependent protein kinase inhibitor (PKI) into rat fibroblasts induced nuclear envelope breakdown whereas the cdc2−cyclin complex alone was not sufficient. These results led the authors to propose that a distinct but necessary event mediated through cAMP-dependent protein kinase (PKA) was required for G_2-M transition. If this is the case then ctCaMKII might phosphorylate similar or analogous substrates to those of PKA that block progression into M. Certainly, these two protein serine/threonine kinases exhibit overlapping substrate specificity and influence similar processes (Jefferson and Schulman, 1991; Sheng *et al.*, 1991). Therefore, we cannot rule out the possibility that the G_2 arrest is due to the phosphorylation of a PKA substrate. Alternatively, ctCaMKII activity could lead to activation of PKA. Overexpression of the catalytic subunit of cAMP-dependent protein kinase in mammalian cells does not seems to cause major detrimental effects on growth (Brown *et al.*, 1990; Uhler and McKnight, 1987). The fact that previous attempts to make stable cell lines expressing a constitutively active CaMKII were unsuccessful (Yamauchi *et al.*, 1989) supports the idea that the G_2 arrest documented herein is due specifically to constitutive expression of CaMKII and not to a PKA-like effect. Whether this proves to be the case or not, elucidation of the molecular events involved in the generation of the G_2 block due to unscheduled protein phosphorylation(s) by ctCaMKII should provide new insights into the regulation of G_2-M progression in mammalian cells.

Materials and methods

Construction of mutant CaMKII and expression vector

The construct pGem-wtCaMKII containing the complete cDNA for the mouse brain α subunit (Hanley *et al.*, 1989) was used to generate the truncation mutant. Truncation was done by religating the DNA obtained from *Hin*cII and partial *Xmn*I digestion. All the procedures used were according to Maniatis *et al.* (1982). The new plasmid designated pGem-ctCaMKII was sequenced to confirm that no errors were incorporated during the ligation. Seven additional amino acids encoded by the polylinker site in pGem were added after the truncation site. The *Eco*RI−*Pvu*II fragment of pGem-ctCaMKII was blunt-ended and subcloned into the blunt-ended *Nhe*I site of pMAM-neo (Clontech), resulting in plasmid pM-ctCaMKII. Plasmids for transfection were purified by CsCl gradient followed by extraction with phenol, phenol−chloroform (1:1) and ethanol precipitation.

Expression and kinase assay of in vitro translated CaMKII

Plasmids pGem-wtCaMKII and pGem-ctCaMKII were linearized before *in vitro* transcription with a Riboprobe Gemini kit (Promega). Transcripts were analyzed by electrophoresis and then used in a rabbit reticulocyte lysate system to generate the respective proteins. All these procedures were carried out as described by the manufacturer (Promega). The reactions were done in the presence of [^{35}S]methionine to quantify and characterize the translation products. Parallel unlabeled translation reactions were used for kinase assays. Translation mixtures were aliquoted, frozen in liquid nitrogen and stored at −70°C until ready to assay. The aliquot to be assayed was thawed and diluted 5-fold with 1 mg/ml BSA, 50 mM PIPES (pH 7.0), 0.5 mM dithiothreitol (DTT). Standard assay conditions included 50 mM PIPES (pH 7.0), 10 mM magnesium acetate, 1 mg/ml BSA, 50 μM ATP (2.5 Ci/mmol), 1 μM calmodulin, 1 mM DTT, 1.5 μM protein kinase A inhibitor (PKI) and 25 μM GS-10 peptide (PLRRTLSVAA) containing either 0.48 mM Ca^{2+} or 2.4 mM EGTA in a final volume of 50 μl. Reactions were started by addition of lysate and incubated at 30°C. At least three time points were taken per reaction tube. Aliquots of each assay were spotted on P-81 Whatman paper and processed as described by Roskoski (1983).

Isolation of established cell lines

The plasmids pMAM-neo and pM-ctCaMKII were used for transfection of C127 cells following the protocol described by Chen and Okayama (1987). Twenty micrograms of DNA were added per 100 mm dish. Cells were trypsinized 24 h after the end of transfection and plated at 1×10^3 or 1×10^4 cells per 100 mm dish. After an additional 16 h of incubation at 37°C, G418 (Gibco) was added to a final concentration of 400 μg/ml. Cells were fed every five days. G418-resistant colonies were picked on the third week and expanded. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS; Hyclone) and 200 μg/ml of G418.

Cytoplasmic RNA isolation and analysis

Cytoplasmic RNA was extracted from cell cultures as described (Rasmussen *et al.*, 1987). RNA yields were determined by spectrophotometric analysis. The RNA samples were electrophoresed on a 1.2% agarose−formaldehyde−MOPS gel. After electrophoresis the gel was stained with acridine orange to evaluate RNA integrity and to assure that loading of each lane was equal. The RNA was transferred to Biotrans nylon membranes (ICN) by capillary action using 10×SSC. Membranes were air-dried and baked at 68°C for 4 h. Prehybridization, hybridization and washes were done as described by Rasmussen *et al.* (1987). The ^{32}P-labeled CaMKII probe corresponding to the 1.3 Kb *Eco*RI−*Cla*I fragment of CaMKII was made using the random primer method (Feinberg and Vogelstein, 1983).

Cell extracts for CaMKII Western blot and kinase assays

Cell monolayers were washed twice with ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). Cells were scraped into PBS and pelleted at 1500 r.p.m. for 5 min at 4°C. Cell pellets were resuspended in extraction buffer containing 50 mM PIPES (pH 7.0), 10% glycerol, 1 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Triton X-100 was added to the cell suspension to a final

concentration of 0.1%. Tubes were mixed gently, incubated on ice for 10 min, and centrifuged at 13 000 r.p.m. for 15 min at 4°C. Supernatant fluids were aliquoted, frozen in liquid N_2 and stored at −70°C until assay.

For Western blot analysis 60 μg of total soluble protein were run per lane on a 12% SDS−PAGE and transferred to Immobilon-P (Millipore). The filter was blocked for 1 h in Tris-buffered saline (TBS), pH 7.5, containing 5% non-fat dry milk. Subsequently, the filter was incubated for 3 h in a 1:2000 dilution of an α-CaMKII affinity purified rabbit polyclonal antibody obtained from Dr A.Czernik (Yoko *et al.*, 1991). After five washes with TBS−Tween (0.01%) the filter was incubated for 1 h in 5 ml TBS containing 1.25 μCi of [^{125}I]protein A (ICN). Five more washes of 5 min each were done before autoradiography.

For CaMKII assays cell extracts were corrected for differences in protein content. Protein concentration was determined using the Bio-Rad protein assay kit. Equal amounts of cell extract were added per kinase reaction. The assay conditions were identical to those described below.

Cell cycle analysis

For flow microfluorometric analysis (FMF) cells were harvested by trypsinization, pelleted gently and resuspended in 2 ml of Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS). Cells were fixed by the gradual addition of 5 ml of 95% ethanol while vortexing. After 30 min at room temperature, cells were placed at 4°C. Before FMF analysis, cells were collected by centrifugation and stained by addition of 1 ml of a 50 μg/ml propidium iodide (PI) solution. Twenty microliters of a 5 mg/ml solution of RNase A were added subsequently. Flow cytometric analysis was carried out using a Coulter EPICS Profile flow cytometer. At least 5000 cells were counted per analysis.

For mitotic index determination, trypsinized cells were collected by centrifugation. Cells were fixed by dropwise addition of methanol−glacial acetic acid (3:1). Fixative was changed once before staining. Cells were stained with 2.0% aceto-orcein and pressed firmly onto microscope slides. Cells with condensed chromatin or with a disrupted nuclear envelope were scored as mitotic. Nocodazole was used at a final concentration of 0.4 μg/ml from a 4 mg/ml stock solution in dimethylsulfoxide (DMSO).

Indirect immunofluorescence

Cells were grown to 60−80% confluency on coverslips. The coverslips were removed from the dish, dipped 10 times in stabilizing buffer (PEM) (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 4% polyethylene glycol) and lysed for 90 s in 0.5% Triton X-100. After several dips in stabilizing buffer, cells were fixed in 3% formaldehyde in PEM + 0.75% DMSO for 20−30 min. For staining of MPM-2 antigens, cells were fixed first and then lysed using the same solutions and protocol. Coverslips were washed exhaustively with Dulbecco's PBS (pH 7.2) before inverting them over a small drop of a 1:800 MPM-2 (compliments of Dr P.Rao; Davis *et al.*, 1983) or 1:25 anti-tubulin antibody dilution (Cedar Lane). Coverslips were incubated at 37°C for 1 h in a humidified incubator after which they were rinsed extensively with Dulbecco's PBS. Coverslips were then inverted over a fluoresceine labeled anti-mouse IgG antibody and incubated at 37°C for 45 min. Finally, after thorough washes, cells were rinsed in distilled water and mounted on microscope slides. In some cases, cells were double stained with propidium iodide by immersing them in 30 μg/ml PI for 1 min and rinsing them well before the distilled water step. Photographs were taken through a 40× objective on a Zeiss Axiophot photomicroscope.

Preparation of cell extracts for p13-precipitation of cdc2/cyclin complexes and histone H1 kinase assays

Cells were harvested by treatment with HBSS (5 mM EDTA, 5 mM EGTA) or by trypsinization followed by two washes with HBSS. Both procedures gave similar yields of p13-precipitable histone H1 kinase activity. Cell pellets were resuspended in lysis buffer [50 mM Tris−HCl (pH 7.5), 0.25 M NaCl, 0.1% Triton X-100, 5 mM EDTA, 15 mM EGTA, 1 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml tosyl phenylalanine chloromethyl ketone, 10 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor) and incubated on ice for 15 min after which they were centrifuged at 13 000 *g* for 5 min. Aliquots were taken for protein assays and the rest of the supernatant fluid was frozen in liquid N_2.

Commercially available p13−agarose beads (Oncogene Science) were used to precipitate cdc2-associated histone H1 kinase (Arion *et al.*, 1988). A comparison was made of H1 kinase assays after p13-precipitation or after immunoprecipitation with an antiserum to the carboxyl-terminal region of murine cdc2 (generously provided by Dr S.Pelech, Samiei *et al.*, 1991). The yield of histone H1 kinase activity was somewhat greater using p13 beads (see Draetta *et al.*, 1989) although similar increases in histone H1 kinase were seen in Dex treated CT11.1 cells using both methods. The experiment shown in Figure 7B was reproduced using both methods with very similar results (data not shown). Equal amounts of cell extracts were added to a 1:10 solution of p13 beads in lysis buffer. The mixture was incubated at 4°C with rotation for 2 h and then centrifuged for 1 min. Histone H1 kinase assay was carried out as described by Morla *et al.* (1989). The p13−agarose beads were washed three times with kinase assay buffer [50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM DTT] before resuspension in 20 μl of kinase assay buffer containing 80 μg/ml histone H1 (Boehringer Mannheim). After 5 min at 30°C, the assay was initiated by addition of [γ-^{32}P]ATP to a final concentration of 2 μM (4×10^4 μCi/μmol). Reactions were stopped after 10 min at 30°C by addition of 8.5 μl 4×SDS sample buffer. Samples were boiled and analyzed by 12% SDS−PAGE and autoradiography. Histone H1 bands were excised and counted for Cerenkov radiation.

Double thymidine block

The double thymidine method (Bhuyan and Groppi, 1989) was used for synchronization of cells at G_1/S. Medium was collected from dishes, made 2 mM thymidine (100× stock) and placed back into dishes. Cells were treated with 2 mM thymidine for 12 h. After this period, cells were washed two to three times with prewarmed DMEM (without FBS). Fresh, warm complete medium was added to the cells and incubated at 37°C for 9 h. Again, at this time, medium was collected, made 2 mM thymidine and poured back into dishes. After a further incubation for 12 h, cells were washed and refed as before. In some cases, the zero time point dishes were left in the incubator and did not receive the last series of washes. They were processed immediately after the other dishes were placed back into the incubator. At different times after the release from the double thymidine block, cells were harvested and analyzed for mitotic index or histone H1 kinase as necessary.

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