Calcium/Calmodulin-dependent Phosphorylation and Activation of Human Cdc25-C at the G2/M Phase Transition in HeLa Cells*

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The human tyrosine phosphatase (p54<sup>cdc25-c</sup>) is activated by phosphorylation at mitosis entry. The phosphorylated p54<sup>cdc25-c</sup> in turn activates the p34-cyclin B protein kinase and triggers mitosis. Although the active p34-cyclin B protein kinase can itself phosphorylate and activate p54<sup>cdc25-c</sup>, we have investigated the possibility that other kinases may initially trigger the phosphorylation and activation of p54<sup>cdc25-c</sup>. We have examined the effects of the calcium/calmodulin-dependent protein kinase (CaM kinase II) on p54<sup>cdc25-c</sup>. Our in vitro experiments show that CaM kinase II can phosphorylate p54<sup>cdc25-c</sup> and increase its phosphatase activity by 2.5-3 fold. Treatment of a synchronous population of HeLa cells with KN-93 (a water-soluble inhibitor of CaM kinase II) or the microinjection of AC3-I (a specific peptide inhibitor of CaM kinase II) results in a cell cycle block in G₂ phase. In the KN-93-arrested cells, p54<sup>cdc25-c</sup> is not phosphorylated, p34<sup>cdc2</sup> remains tyrosine-phosphorylated, and there is no increase in histone H1 kinase activity. Our data suggest that a calcium-calmodulin-dependent step may be involved in the initial activation of p54<sup>cdc25-c</sup>.

Calcium, an intracellular second messenger, is known to be required for cells to traverse the cell cycle checkpoints at G₁/S, at entry into mitosis (G₂/M phase), and at mitosis exit (reviewed in Refs. 1–3, 4, and 5). A number of studies have shown that this requirement for calcium during cell division is mediated by calmodulin (CaM), an intracellular calcium-binding protein (6, 7). Studies in several species have shown that the level of calmodulin protein rises 2-fold as cells progress through the G₁/S phase checkpoint (8–10). The results of several studies also suggest that calcium/CaM may also be involved in the G₂/M phase transition in mammalian cells (reviewed in Ref. 2). Transient increases in intracellular calcium can be detected as cells undergo nuclear envelope breakdown and chromatin condensation (11, 12) and both calcium and CaM are localized to the centrosomal region of the mitotic apparatus (13–15). The level of the calmodulin protein is also reported to increase 2-fold when mammalian tsBN2 cells are induced to undergo premature chromatin condensation upon incubation at the restrictive temperature (16). In addition, the reduction of intracellular calmodulin levels using calmodulin antisense RNA (17) or the use of calmodulin antagonists (18) is reported to block entry of the cells into mitosis and imply a role for CaM at the G₂/M phase checkpoint.

However, the immediate targets of calcium/CaM at the G₂/M phase checkpoint remain largely unknown. Studies in sea urchin eggs and Xenopus oocytes suggest the target to be a multifunctional calcium/calmodulin-dependent serine/threonine protein kinase (CaM kinase II). CaM kinase II is a member of a family of multifunctional calcium/CaM-dependent protein kinases, which include CaM kinases Ia, Ib, and IV and which have broad substrate specificity and wide distribution in mammalian tissue (Ref. 18; reviewed in Refs. 19 and 20). CaM kinases with properties similar to the mammalian CaM kinase II have been found in a number of species (21–23) including the sea urchin (24). Inhibition of the sea urchin CaM kinase II using either anti-CaM kinase antibodies or an inhibitory peptide was found to block nuclear envelope breakdown (24). In addition, it is reported that microinjection of a CDNA encoding a calcium/CaM-independent form of CaM kinase II induced maturation in Xenopus oocytes (25). These observations together with the finding that mammalian cells contain nuclear isoforms of CaM kinase II (26) and that the enzyme is localized in the nucleus during interphase and in the mitotic apparatus of dividing cells (27) suggest that CaM kinase II may be a positive regulator of the G₂/M phase transition.

The targets of the Ca<sup>2+</sup>/CaM/CaM kinase II second messenger system must ultimately be the cell cycle control proteins. Of these, the p34<sup>cdc2</sup> protein kinase is generally acknowledged to be the key mediator of the G₂/M phase transition in all eucaryotic cells (reviewed in Refs. 28 and 29). The active mitotic kinase (MPF, or mitosis-promoting factor) is a dimer comprising a catalytic subunit, p34<sup>cdc2</sup>, and a regulatory subunit, a B-type cyclin (30–32). Cyclins are a class of proteins that are synthesized during the interphase of each cell cycle and rapidly degraded at the end of each mitosis (reviewed in Ref. 33). The activity of the p34<sup>cdc2</sup> protein kinase depends not only on its association with cyclin B but also on its phosphorylation state. Three major sites of phosphorylation have been identified, corresponding to Thr<sup>14</sup>, Tyr<sup>15</sup>, and Thr<sup>161</sup> in the human p34<sup>cdc2</sup> protein (34, 35). Phosphorylation of either Thr<sup>14</sup> or Tyr<sup>15</sup> inhibits p34<sup>cdc2</sup> kinase activity (36, 37), while phosphorylation of the Thr<sup>161</sup>
In this study, we show that Cdc25-C is phosphorylated during M phase in mammalian cells and that this phosphorylation can be inhibited by KN-93, a water-soluble, cell-permeable inhibitor of CaM kinase II. Treatment of HeLa cells with either KN-93 or AC3-I, a specific peptide inhibitor of CaM kinase II, inhibits progression of the cells through the G1/M phase checkpoint. We also show that in vitro the Cdc25-C protein is a substrate for CaM kinase II and that the phosphorylated enzyme has a 2.5–3-fold greater tyrosine kinase activity when compared with the nonphosphorylated enzyme.

MATERIALS AND METHODS
Cell Culture, Synchronization, and Cell Extracts—HeLa cells were cultured in Dulbecco's minimum essential medium (MEM) (Life Technologies, Inc., Paisley, UK) supplemented with 10% fetal calf serum (Life Technologies), as described in Ref. 53. Briefly, after isopropyl β-D-thiogalactopyranoside induction, the bacterial pellet was resuspended in 10 vol of cold (4 °C) TEN (50 mM Tris-HCl, pH 7.4, 1 mM MgCl2, and 0.5 mM EDTA, 0.3 mM NaCl), and the bacteria was lysed by the addition of 10 ml of lysozyme (5 mg/ml) and incubation for 30 min at 4 °C. 1 ml of 10% Nonidet P-40 was then added, and the incubation continued for another 10 min. The lysate was diluted with 75 ml of 1.5 M NaCl, 12 mM MgCl2, and incubated with Dnase I (0.2 mg/ml) for 60 min at 4 °C. The lysate was then sonicated for 30 s at 4 °C and centrifuged at 12,500 × g for 30 min. The pellet was resuspended in 40 ml of cold (4 °C) TEN, mixed with 1 ml of lysozyme (10 mg/ml), and incubated on ice for 10 min. 1 ml of 10% Nonidet P-40 was added to the lysate before centrifugation at 15,000 × g for 15 min. The pellet, containing mostly inclusion bodies, was resuspended in 10 ml of 6 M urea, 25 mM dithiothreitol, 10 mM Tris-HCl, pH 8. The suspension was boiled for 5 min at 100 °C, cooled to 37 °C, and centrifuged for 10 min at 10,000 × g. The supernatant was recovered, warmed to 37 °C, and diluted into 100 ml of warm (37 °C) 50 mM Tris-HCl, pH 8, 10% glycerol, 5 mM glutathione (reduced), 0.5 mM glutathione (oxidized), 0.4 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin. After overnight incubation at 37 °C, the solution was centrifuged for 30 min at 14,000 × g. The supernatant was recovered and dialyzed against 5 × 50 ml of Tris-HCl, pH 8, 1 M dithiothreitol, 10% glycerol. The E. coli Cdc25-C fusion protein was recovered from Escherichia coli and purified using glutathione-Sepharose chromatography, as described in Ref. 48.

Protein Kinase and Protein Phosphatase Assays—Phosphorylation of Cdc25-C by CaM kinase II was performed in a final volume of 20 μl containing 50 mM Pipes, pH 7.4, 10 mM MgCl2, 100 μM EDTA, 100 μM CaCl2, 0.3 μg of calmodulin, 100 μM ATP, and 5 μg of recombinant CaM kinase II (Amersham Pharmacia Biotech), 2.5 μg of CaM kinase II, and 50–200 ng of CaM kinase II. The reaction was incubated at 37 °C for 5 min before determination of the reaction by the addition of 20 μl of 2× sample buffer. Phosphorylation of Cdc25-C by the purified p34<sup>Cdc2</sup>-protein kinase was performed in a final volume of 25 μl containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 80 μM β-glycerophosphate, 6 mM EGTA, 10 μM ATP, 10 μM [γ-<sup>32</sup>P]ATP, 2.5 μg of Cdc25-C, and 1 μl of Cdc2 kinase. The reaction was incubated at 30 °C for 10 min before terminating by the addition of 25 μl of 2× sample buffer. Phosphorylation of Cdc25-C by CaM kinase II was assayed using a p34<sup>Cdc2</sup>-cyclin B complex isolated from sea urchin embryos (40 min after fertilization) using p13<sup>kinase</sup>-Sepharose beads as described previously (54). The p13 precipitates were washed three times with assay buffer (50 μM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM dithiothreitol) before the addition of Cdc25-C. The tubes were incubated for 20 min at 25 °C, washed three times with buffer C, and assayed for H1 kinase activity as described (58). H1 kinase activity was quantitated by excising the histone H1 bands from the dried gels, dissolving in NCS tissue solubilizer (Amersham Pharmacia Biotech), and scintillation counting, or by phosphor image analysis. Alternatively, the p13 beads were washed three times in wash buffer, resuspended in 2 volumes of 2× sample buffer, and used for Western blotting to determine the tyrosine phosphorylation state of p34<sup>Cdc2</sup>. The H1 kinase activity of the p34<sup>Cdc2</sup>-immunoprecipitates from HeLa cells was assayed as described by Gu et al. (53).
Phosphopeptide and Phosphoamino Acid Analysis of Cdc25-C—The phosphopeptide and phosphoamino acid analysis of Cdc25-C following phosphorylation by CaM kinase II was performed as described previously (57).

Drug Treatment of HeLa Cells—KN-93 was dissolved as a 10 mM stock in sterile distilled water (KN-92 was dissolved in MeSO, 10 mM stock solution) and stored at −20 °C. KN-93 (10 μM) and KN-92 (10 μM) were added to HeLa cells (approximately 50% confluent cultures) 3 h after release from the aphidicolin-thymidine block. Samples were collected at 2–3-h intervals over an 18–22-h period. After washing the cells in cold (4 °C) PBS containing 0.4 mM EDTA, the cells were either lysed in lysis buffer or fixed in 1 ml of cold (−20 °C) methanol (70%). The fixed cells were washed two times in PBS and incubated in the dark for 60 min with PBS containing propidium iodide (Sigma) and RNase I (type 1-AS; Sigma). Analysis of the DNA content was performed using an EPICS Elite cell sorter (Coulter Electronics, Luton, UK). Okadic acid (potassium salt; Affinity) was dissolved in MeSO at a concentration of 2 mM. Okadacid (0.5 μM) was added to HeLa cells 3 h after release from an aphidicolin-thymidine block. The cells were scraped from the plates and washed in cold (4 °C) PBS containing 0.4 mM EDTA and 0.5 μM okadacid. The cell pellet was lysed in 3 volumes of lysis buffer.

Microinjection of HeLa Cells—HeLa cells were cultured on sterile 22 × 22-mm coverslips etched with a cross (to aid relocation of microinjected cells). The cells were blocked and released from G/S phase as described above. The peptide was microinjected into the cells between 4 and 6 h after release from the G/S phase arrest. The cells were injected around the cross essentially as described by Grassman et al. (59). The AC3-I peptide was dissolved in 0.5× PBS (pH 7.2) and was used at a needle concentration of 10 μM. To facilitate identification of the microinjected cells, a fluorescent dye, tetramethylrhodamine isothiocyanate-dextran (0.5 mg/ml, M, 3000; Molecular Probes, Inc., Eugene, OR) was incorporated into the peptide solution. The control peptide, AC3-C, was prepared similarly and used at a needle concentration of 10 μM. Approximately 50–100 cells were injected in five separate experiments with approximately 0.1–1.0 picoliters of peptide (1–10% of cell volume as estimated in Ref. 60). Both peptides were microinjected into the cytoplasm. Using this technique, 85–90% of the microinjected cells remained viable as assessed by trypan blue exclusion. After microinjection the cells around the etched cross were photographed on a Nikon TMS light microscope (×10 objective) using a Nikon F-801s camera. Cells were then returned to a 37 °C incubator for 24 h. After 24 h, the cells were again photographed around the etched cross and then fixed with 3.7% formaldehyde (in PBS). Coverslips were mounted in 90% glycerol, 10% PBS, and tetramethylrhodamine isothiocyanate-dextran-positive cells were counted under a Zeiss Axioskop epifluorescence microscope.

Results—The protease inhibitors aphidicolin and thymidine were purchased from Sigma (Poole, UK). All other chemicals were of “Analar” grade and were purchased from BDH unless indicated otherwise.

Results—CaM Kinase II Phosphorylates and Activates Cdc25-C in Vitro—The recombinant human Cdc25-C protein was purified from bacterial inclusion bodies and renatured. As shown in Fig. 1A (left lane) a major 54-kDa band could be detected on a Coomassie-stained gel. The 54-kDa protein, when Western blotted, immunoreacted with a polyclonal antibody to the human Cdc25-C protein (see Fig. 1A, right lane). To determine the tyrosine phosphatase activity of the renatured, purified Cdc25-C protein, we incubated it with the sea urchin p34cdc2-cyclin B complex (isolated from embryos 40 min postfertilization) bound to p13sep-Sepharose beads. We have shown previously that at 40 min postfertilization the sea urchin p34cdc2 is phosphorylated on tyrosine and that cyclin B is present in the complex (54, 61). As shown in Fig. 1, B and C, Cdc25-C (0.01–1.25 mg/ml) caused a dose-dependent dephosphorylation of p34cdc2 on tyrosine, confirming the results of an earlier study (44).

To determine if Cdc25-C was a substrate for CaM kinase II in vitro, we treated the purified Cdc25-C protein (1 μg) with varying concentrations of purified, rat brain CaM kinase II (25–200 ng). The samples were subsequently resolved by 10% SDS-PAGE, electroblotted onto a nylon membrane, and analyzed by autoradiography. Our results (shown in Fig. 2A, top) indicate that a 54-kDa protein (Cdc25-C) was strongly phosphorylated by CaM kinase II in a calcium/calmodulin-dependent manner to a high stoichiometry (1.2 mol of phosphate/mol of Cdc25-C). In the absence of Cdc25-C, only weak phosphorylation was observed, which we presume represents the known autophosphorylation of the α- (51 kDa) and β- (66 kDa) subunits of CaM kinase II (19). The nylon membrane from the preceding experiment was then immunoprobed with an anti-cdc25-C antibody and visualized using a colorimetric detection assay as described under “Materials and Methods.” The result of the Western blot (shown in Fig. 2A, bottom) indicates that the mobility of the Cdc25-C protein in the SDS gel was retarded...
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following phosphorylation by CaM kinase II. A slight upshift of Cdc25-C was seen (see Fig. 2A, bottom, lane 3) following phosphorylation by 100 ng of CaM kinase II. The upshift was readily apparent (see Fig. 2A, bottom, lane 4) when 200 ng of Cdc25-C kinase II was used; the mobility of Cdc25-C was increased by approximately 9 kDa, from 54 to 63 kDa.

We performed both phosphopeptide mapping and phosphoamino acid analysis of Cdc25-C protein after phosphorylation by CaM kinase II. The phosphopeptides present in the digest were excised from the dried gel, digested with trypsin, and hydrolyzed with HCl. The products were then resolved by electrophoresis by on cellulose TLC plates followed by autoradiography. The mobility of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards is shown. C, CaM kinase II phosphorylates and activates Cdc25-C. The recombinant Cdc25-C protein was phosphorylated by CaM kinase II (100 ng) and then incubated with the sea urchin p34\textsuperscript{cdc2}–cyclin B complex, which was isolated from sea urchin egg extracts using p13\textsuperscript{cyclin B}–Sepharose beads. We then determined if there was any increase in the histone H1 kinase activity of the p34\textsuperscript{cdc2}–cyclin B complex following incubation with the phosphorylated and nonphosphorylated Cdc25-C protein. As shown in Fig. 2C, we obtained a significant and consistent increase (approximately 2.5–3-fold) in the histone H1 kinase activity with the phosphorylated Cdc25-C protein compared with the nonphosphorylated form. Incubation of the CaMK II-phosphorylated Cdc25-C with vanadate (1 mM) inhibited activation of the p34\textsuperscript{cdc2}–cyclin B complex as assessed by its histone H1 kinase activity. This result indicates that in vitro the Cdc25-C protein has enhanced phosphatase activity after phosphorylation by CaM kinase II.

To eliminate the possibility that the phosphorylation of

FIG. 2. A, phosphorylation of Cdc25-C by CaM kinase II \textit{in vitro}. Top, recombinant Cdc25-C protein (1 μg) was incubated with Ca\textsuperscript{2+} (100 μM), calmodulin (100 μM), CaM kinase II (25–200 ng), and 30 μM [γ-\textsuperscript{32}P]ATP (lanes 1–4), without Ca\textsuperscript{2+} (lane 5) or without the Cdc25-C protein (lane 6). The samples were electrophoresed on a 10% polyacrylamide gel and blotted onto nylon membrane. The autoradiogram of the blotted proteins is shown. Bottom, the blot from A was then immunoprobed with the Cdc25-C antibody and developed using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Molecular mass standards (kDa) are shown shown on the right. B, phosphopeptide and phosphoamino acid analysis of Cdc25-C phosphorylated by CaM kinase II \textit{in vitro}. The recombinant Cdc25-C protein (1–2 μg) was phosphorylated by CaM kinase II (100 ng) as described under “Materials and Methods.” Left, two-dimensional phosphopeptide analysis of phosphorylated, recombinant, renatured Cdc25-C. Phosphopeptides were electrophoresed (pH 3.5) in the first dimension (\textit{horizontal}) followed by chromatography in the second dimension (\textit{vertical}). Left inset, two-dimensional tryptic phosphopeptide map of the Cdc25-C glutathione S-transferase fusion protein phosphorylated with CaM kinase II (100 ng). Right, phosphoamino acid analysis of recombinant, renatured Cdc25-C phosphorylated by CaM kinase II. The phosphorylated Cdc25-C band was excised from the dried gel, digested with trypsin, and hydrolyzed with HCl. The products were then resolved by electrophoresis on cellulose TLC plates followed by autoradiography. The mobility of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards is shown. C, CaM kinase II phosphorylates and activates Cdc25-C. The recombinant Cdc25-C protein was phosphorylated by CaM kinase II (100 ng) and then incubated with the sea urchin p34\textsuperscript{cdc2}–cyclin B complex bound to p13\textsuperscript{cyclin B}–Sepharose beads, either in the absence or presence of vanadate (1 mM). The activity of the p34\textsuperscript{cdc2}–cyclin B complex was assayed using histone H1 as substrate. Each bar shows the mean ± S.E. of four experiments. Left, phosphorylation of the Cdc25-C fusion protein by CaM kinase II. Lane 1, a Western blot of the Cdc25-C fusion protein using an antibody to the human protein; Lane 2, phosphorylation of the Cdc25-C fusion protein (2 μg) with 25 ng of CaM kinase II; lane 3, phosphorylation of the Cdc25-C fusion protein (2 μg) with 25 ng of CaM kinase II in the absence of Ca\textsuperscript{2+} or CaM. Right, phosphorylation of the 54-kDa, recombinant Cdc25-C protein by the purified starfish p34\textsuperscript{cdc2}–cyclin B protein kinase. Lane 1, Cdc25-C plus p34\textsuperscript{cdc2} kinase; lane 2, histone H1 plus p34\textsuperscript{cdc2} kinase; lane 3, Cdc25-C plus CaM kinase II.
Cdc25-C by CaM kinase II was fortuitous, possibly the result of incorrect protein folding during renaturation, we used a soluble form of the human Cdc25-C protein. We purified the human Cdc25-C glutathione S-transferase fusion protein from bacteria using glutathione-Sepharose affinity chromatography as described previously (45). A Western blot of the purified fusion protein with a polyclonal anti-Cdc25-C antibody (Fig. 2D, left, lane 1), indicated the presence of two major polypeptides with molecular masses of 82 and 54 kDa. The 82-kDa band represents the full-length Cdc25-C fusion protein, while the 54-kDa band represents a degradation product of Cdc25-C that has also been described in a previous study (48). To test if the soluble Cdc25-C fusion protein was a substrate for CaM kinase II, we incubated 2 μg of the purified protein (a mixture of the 82- and 54-kDa polypeptides) with 25 ng of CaM kinase II in the presence of [γ-32P]ATP, resolved the proteins by 10% SDS-PAGE, and analyzed protein phosphorylation by autoradiography. Both the 54- and the 82-kDa polypeptides were phosphorylated to a high level by CaM kinase II (Fig. 2D, left, lane 2). Phosphorylation of the polypeptides was Ca2+/CaM-dependent, since no significant phosphorylation was observed when both were excluded from the incubation mixture (Fig. 2D, left, lane 3).

We also performed phosphopeptide mapping and phosphoamino acid analysis of the 82-kDa Cdc25-C fusion protein after phosphorylation by CaM kinase II (200 ng). The two-dimensional tryptic peptide map of the Cdc25-C fusion protein indicated that two major acidic peptides (comparable with peptides 1 and 2 in the 54-kDa protein; see Fig. 2B, inset) were generated, along with two minor acidic peptides (comparable with peptides 3 and 4 in the 54-kDa protein; see Fig. 2B and compare with Fig. 2B, inset). The two minor peptides in the Cdc25-C fusion protein, however, were not phosphorylated as efficiently as the two minor peptides (labeled 3 and 4) in the 54-kDa Cdc25-C protein. Phosphoamino acid analysis of the labeled peptides indicated that they were phosphorylated exclusively on serine residues (data not shown). Since both the renatured Cdc25-C protein and the Cdc25-C fusion protein were both found to be phosphorylated by CaM kinase II, the remainder of the experiments described in this paper were performed with the renatured 54-kDa protein.

We also determined whether the Cdc25-C protein could be phosphorylated by either cAMP-dependent protein kinase or protein kinase C. Neither of these kinases phosphorylated Cdc25-C (data not shown). In HeLa cells, the phosphorylation and activation of Cdc25-C is believed to be mediated by the p34cdc2 kinase present in mitotic cell extracts (48). We have confirmed and extended this observation and shown that Cdc25-C is phosphorylated by a pure preparation of starfish p34cdc2 protein kinase (see Fig. 2D, right).

**Fig. 3. KN-93 arrests HeLa cells with a G2/M DNA content.** A, flow cytometry DNA profiles of control HeLa cells (left) or HeLa cells treated with 10 μM KN-93 (right). B, flow cytometry DNA profiles of HeLa cells (18 h after release from the aphidicolin/thymidine cell cycle block) treated with Me2SO alone (left), 10 μM KN-92 (middle), or 10 μM KN-93 (right). C, p34cdc2 is phosphorylated on tyrosine in KN-93-arrested HeLa cells. p34cdc2 was immunoprecipitated from either a synchronous culture of HeLa cells at various intervals after release from an aphidicolin/thymidine block or from cells arrested in G2 phase with KN-93 (10 μM). One-half of each sample was immunoblotted with an anti-phosphotyrosine antibody (top) and the other half with the p34cdc2 antibody (bottom). Samples from left to right are as follows: G1/S phase blocked cells, S phase cells, G2 phase cells, nocodazole-arrested cells in M phase, and KN-93 (10 μM)-arrested cells.

In a separate experiment, we treated a synchronous population of cells with either 10 μM KN-92 (2-[N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), an inactive analogue of KN-93 (up to 30 μM KN-92 has no effect on CaM kinase II, cAMP-dependent protein kinase, or protein kinase C),2 for comparison with 10 μM KN-93, or with an equivalent volume of Me2SO alone (control cells). Flow cytometric analysis of the DNA content of the KN-92-treated, KN-93-treated, and control cells (Fig. 3B) indicated that 15 h after the addition of the drugs, both the control and the KN-92-treated cells had completed one cell division cycle, whereas 59% of the KN-93-treated cells were found at G2/M with a 4n DNA content. This result provides additional evidence of the specificity of the KN-93-induced G2 arrest and demonstrates a role for CaM kinase II in regulating the G2/M phase transition in HeLa cells.

To determine the tyrosine phosphorylation state of p34cdc2 in the KN-93-arrested HeLa cells, we immunoprecipitated p34cdc2 with a G2/M DNA content was observed.

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and immunoblotted it with either an anti-phosphotyrosine antibody or an anti-Cdc2 antibody. The results (Fig. 3C) indicate that p34cdc2 was phosphorylated on tyrosine in the KN-93-arrested cells and at a level comparable with that seen in G2 phase cells. This result suggests that KN-93 causes G2 phase arrest by inhibiting activation of the Cdc25-C tyrosine phosphatase.

Since KN-93 was found to arrest HeLa cells specifically at the G2/M phase transition, we wanted to determine if CaM kinase II was present in this cell line. Its existence in HeLa cells had not been reported previously. The presence of CaM kinase II in HeLa cells was determined by assessing the phosphorylation of autocamtide-2 (24), a specific, synthetic peptide substrate of CaM kinase II. By comparing the specific activity of the purified enzyme (from rat brain) with that in HeLa cells, our data (not shown) indicate that CaM kinase II comprises 0.003% of total cell protein.

**FIG. 4.** KN-93 inhibits phosphorylation of Cdc25-C at mitosis. A (top), immunoprecipitation and immunoblot analysis of Cdc25-C from a synchronous culture of HeLa cells. Samples from left to right are as follows: cells arrested at the G1/S phase boundary, S phase cells, G2 phase cells, M phase cells, nocodazole-blocked (50 ng/ml) cells at M phase, and in cells treated with both KN-93 (10 μM) and nocodazole (50 ng/ml) treated cells. Center, KN-93 inhibits dephosphorylation of p34cdc2 on tyrosine. p34cdc2 was isolated from a synchronous culture of HeLa cells using an antibody to the human protein. One half of each sample was immunoblotted with an anti-phosphotyrosine antibody, and the other half was immunoblotted with an anti-Cdc2 antibody. The results (Fig. 3C) indicate that p34cdc2 was phosphorylated on tyrosine in the KN-93-arrested cells and at a level comparable with that seen in G2 phase cells. This result suggests that KN-93 causes G2 phase arrest by inhibiting activation of the Cdc25-C tyrosine phosphatase.

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**KN-93 Inhibits the Phosphorylation of Cdc25-C in HeLa Cells**—We treated a synchronous population of HeLa cells with 10 μM KN-93 and looked at the phosphorylation of Cdc25-C using its relative mobility on SDS-polyacrylamide gels as an indication of its phosphorylation state. Using a polyclonal antibody against the human protein, we immunoprecipitated and then immunoblotted Cdc25-C from HeLa cell extracts. Fig. 4A (top) shows that in interphase cells the antibody detected a doublet (54–56 kDa), a finding consistent with previous reports (41). These bands were not detected in the control experiment, where duplicate HeLa cell extracts were immunoprecipitated with a preimmune serum and then immunoblotted with the Cdc25-C antibody (data not shown). In cells blocked at M phase for 14 h with nocodazole (50 ng/ml), the Cdc25-C protein is seen to shift to a higher molecular mass (60 kDa, Fig. 4A, top). We were unable to detect hyperphosphorylation of the Cdc25-C protein (as assessed by its mobility on SDS-PAGE) in M phase cells collected by mitotic shake-off (Fig. 4A, top). Indeed, hyperphosphorylation has not been reported
under these conditions (48). Since the hyperphosphorylation of Cdc25-C was only observed in the nocodazole-treated cells, we examined the effect of KN-93 on Cdc25-C phosphorylation in the presence of nocodazole. We added both KN-93 (10 μM) and nocodazole (50 ng/ml) to a synchronous population of HeLa cells and determined the state of Cdc25-C phosphorylation 14 h after the addition of the two inhibitors. As Fig. 4A (top) shows, we did not observe any change in the mobility of the Cdc25-C protein in cells treated with both nocodazole and KN-93, while cells treated with nocodazole alone showed Cdc25-C hyperphosphorylation. This result indicates that there is a calcium/calmodulin kinase-dependent step in the phosphorylation of Cdc25-C in HeLa cells, probably mediated by CaM kinase II or a CaM kinase II-like enzyme.

We also examined the tyrosine phosphorylation state of p34\textsuperscript{cdc2} in the KN-93 blocked HeLa cells. Using a polyclonal anti-human cdc2 antibody, we immunoprecipitated p34\textsuperscript{cdc2} from HeLa cell extracts prepared at different stages of the cell cycle. One half of the immunoprecipitate was then immunoblotted with an anti-phosphotyrosine antibody, and the other half was immunoblotted with the anti-human Cdc2 antibody. Our results show that in the KN-93-arrested cells, p34\textsuperscript{cdc2} was present in a tyrosine-phosphorylated state (see Fig. 4A, center) and that the level of phosphotyrosine was comparable with that detected in p34\textsuperscript{cdc2} during the G1 phase of the cell cycle (see Fig. 4, A, center, and B, left). Although we have not tested it formally, we presume that the p34\textsuperscript{cdc2} kinase also remains inactive in the KN-93-blocked cells. By way of comparison, in the control cells p34\textsuperscript{cdc2} was gradually phosphorylated on tyrosine during interphase (Fig. 4A, center) and dephosphorylated in M phase. The tyrosine dephosphorylation of p34\textsuperscript{cdc2} was more apparent in the nocodazole-arrested cells than in M phase cells collected by mitotic shake-off (see Fig. 4A, center). The results of the immunoblot with the p34\textsuperscript{cdc2} antibody indicated that a uniform level of p34\textsuperscript{cdc2} protein was present in the immunoprecipitates (see Fig. 4, A, bottom, and B, right).

To confirm the specificity of KN-93 in vivo, we wanted to eliminate the possibility that the G2/M phase block induced by KN-93 did not result from a direct inhibition of Cdc25-C phosphatase activity or from inhibition of cyclin B synthesis. To determine if KN-93 was inhibiting the tyrosine phosphatase activity of Cdc25-C, we assayed its activity in vitro in the presence and absence of KN-93 (10 μM). The Cdc25-C protein was incubated with its substrate, the inactive, tyrosine-phosphorylated sea urchin p34\textsuperscript{cdc2}-cyclin B complex (isolated on p13\textsuperscript{p13}-Sepharose beads). Following the incubation, the phosphatase activity was determined by immunoblotting with an anti-phosphotyrosine antibody. The results of this experiment, shown in Fig. 4C (top and bottom), indicate that KN-93 had no effect on the tyrosine phosphatase activity of Cdc25-C (compare lanes 2 and 3). However, incubation of Cdc25-C in the presence of 1 mM vanadate, an inhibitor of tyrosine phosphatases (63), completely inhibited its enzyme activity (lane 4). In the presence of vanadate, the level of tyrosine phosphorylation of p34\textsuperscript{cdc2} was comparable with that of control samples (lanes 1 and 5), which were not treated with Cdc25-C. To test the effect of KN-93 on the synthesis of cyclin B, we used a monoclonal antibody to human cyclin B1 to immunoprecipitate and then immunoblot cell extracts from KN-93-blocked cells and from G2 and G1 phase cells. The results, shown in Fig. 4D (top and bottom), indicate that the cyclin antibody detected a 54-kDa protein and that the cyclin B1 protein was present in the KN-93-arrested cells (lane 3) at a level comparable with that detected in the G2 phase cells (lane 1). G1/S cyclin B levels are shown for comparison (lane 2). This result led us to conclude that KN-93 does not inhibit cell cycle progression by inhibiting the synthesis of cyclin B1.

**Fig. 5. Okadaic acid induces activation of the p34\textsuperscript{cdc2} kinase in HeLa cells.** Top, the p34\textsuperscript{cdc2} kinase was immunoprecipitated, using an anti-p34\textsuperscript{cdc2} antibody, from a synchronous population of HeLa cells following the addition of okadaic acid (0.5 μM). The histone H1 kinase activity associated with the immunoprecipitated p34\textsuperscript{cdc2} was assayed as described under "Materials and Methods." Each bar represents the mean ± S.E. of three experiments. Bottom, immunoblot analysis of p34\textsuperscript{cdc2}, immunoprecipitated from OA-treated HeLa cells, using an anti-phosphotyrosine antibody. Lane 1, control cells without OA at 0 min; lane 2, 30 min after the addition of OA; lane 3, 90 min after the addition of OA; lane 4, cells at 90 min without OA; lane 5, cells with OA plus KN-93 at 90 min.
KN-93 did not inhibit either the OA-induced increase in the histone H1 kinase activity of p34\(^{cdc2}\) (Fig. 5, top) or the OA-induced tyrosine dephosphorylation of p34\(^{cdc2}\) (see Fig. 5, bottom, lane 5). These data demonstrate that KN-93 has no direct effect on histone H1 kinase activity in vitro.

A Peptide Inhibitor of CaM Kinase II (AC3-I) Arrests HeLa Cells in G2—The finding that KN-93 caused a G2/M phase block in HeLa cells prompted us to test whether a specific peptide inhibitor of CaM kinase II would also arrest HeLa cells in G2. HeLa cells were arrested at G2/S phase using an aphidicolin block. After release from the block (4–6 h later) the cells were microinjected with either AC3-I or AC3-C was 311. 24 h after microinjection, the number of cells microinjected with AC3-I was 294 and that with AC3-C was 527. B. light microscopy photographs of HeLa cells microinjected with either AC3-I (1 \(\mu\)M) or the control peptide AC3-C (1 \(\mu\)M). The cells were photographed just after microinjection (0 h), and the same field of cells was photographed 24 h after microinjection. All the cells shown in each field were microinjected. Scale bar, 10 \(\mu\)m.

Trypan blue exclusion indicated that the majority of the microinjected cells (>95%) remained viable 24 h postinjection. In contrast, the cells microinjected with the control peptide (AC3-C) continued to replicate, as seen by a 73% increase in cell number 24 h after microinjection.

We also determined the selectivity of AC3-I for CaM kinase II over protein kinase C, CaM kinase I, and CaM kinase IV. For CaM kinase II, the IC\(_{50}\) (using 10 \(\mu\)M autocamtide-2 as substrate) was found to be approximately 3 \(\mu\)M. For protein kinase C (using myelin basic protein as substrate) the IC\(_{50}\) was approximately 500 \(\mu\)M, or 100-fold less effective. We have found that AC3-I had no effect on the activity of either CaM kinase IV (at concentrations of up to 100 \(\mu\)M using GS-10 as substrate) or CaM kinase I (at concentrations of up to 200 \(\mu\)M using 50 \(\mu\)M site 1 peptide as substrate (66)), whereas it inhibited CaM kinase II activity with an IC\(_{50}\) of 1 \(\mu\)M (using 3 \(\mu\)M autocamtide-3 as substrate). Thus, AC3-I has a 100-fold selectivity for CaM kinase II over either protein kinase C or CaM kinase IV and at least 200-fold over CaM kinase 1.

DISCUSSION

The results we have obtained in this study suggest that CaM kinase II or a CaM kinase II-like activity is required at the G2/M phase transition in HeLa cells. This result is consistent with previous studies, which also indicate a requirement for CaM kinase II at mitosis entry in sea urchin embryos (24) and Xenopus oocytes (25). The target(s) of CaM kinase II at the G2/M phase transition, however, have not been identified. We
show here that one possible target may be the Cdc25-C protein. A current model for the activation of the p34<sup>cdc2</sup>-cyclin B mitotic kinase (Ref. 48 and Fig. 7) requires the presence of a small amount of phosphorylated, active Cdc25-C enzyme to trigger the activation of the p34<sup>cdc2</sup>-cyclin B protein kinase. Once activated, the p34<sup>cdc2</sup>-cyclin B protein kinase engages a positive feedback loop in which it phosphorylates and further activates Cdc25-C, which, in turn, increases activation of the p34<sup>cdc2</sup>-cyclin B protein kinase (48). It has been shown in HeLa cells that the Cdc25-C enzyme is phosphorylated and activated by the p34<sup>cdc2</sup> protein kinase (45). While we do not disagree with this result and indeed have reproduced it, our data suggest that CaM kinase II may function as a trigger to initiate the phosphorylation and activation of Cdc25-C.

We have studied the regulation of Cdc25-C enzyme activity by CaM kinase II using both in vitro and in vivo methods. Our in vitro results indicate that the 54-kDa Cdc25-C recombinant protein, isolated from the bacterial inclusion bodies, is a substrate for CaM kinase II. A possible criticism regarding the use of the renatured, recombinant Cdc25-C protein is that its phosphorylation by CaM kinase II may result from the exposure of non-specific phosphotyrosine sites in an incompletely or incorrectly folded protein. To address this criticism, we have shown that the soluble Cdc25-C fusion protein can also be phosphorylated by CaM kinase II. The similarity of the phosphopeptide maps, of the renatured Cdc25-C protein and the Cdc25-C fusion protein, suggest that CaM kinase II phosphorylates the same sites in both proteins, although our data indicate that CaM kinase II phosphorylates sites in the renatured protein more efficiently. That Cdc25-C is a substrate for CaM kinase II is also consistent with the finding that the homologue of Cdc25-C in the fungus Aspergillus nidulans, a product of the nim<sup>T<sup>cdc2</sup></sup> gene, can also be phosphorylated by CaM kinase II in vitro (2). The human Cdc25-C protein contains four CaM kinase II substrate consensus sequences (RXXS/T; Ref. 67), the potential phosphorylation sites being serine 38, serine 216, serine 449, and serine 451 (56). Our phosphoamino acid analysis data shows that the Cdc25-C protein is specifically phosphorylated on serine residues only and is consistent with the phosphorylations being within the four putative CaM kinase II consensus sequences present within the human Cdc25-C protein. This result provides further evidence for the phosphorylation of CaM kinase II-specific sites in the Cdc25-C protein.

Both the human and the Xenopus Cdc25-C enzymes are known to be phosphorylated at multiple sites (48, 68), although the phosphorylation sites that regulate Cdc25-C activity in vivo have not all been identified, perhaps one reason being the reported low abundance of the Cdc25-C protein in mammalian cells (48). More is known about the kinase(s) that may regulate the activity of the Cdc25-C enzyme in vitro. Protein kinases able to phosphorylate the Xenopus Cdc25-C protein include p34<sup>cdc2</sup>-cyclin A, p34<sup>cdc2</sup>-cyclin B, Cdk2-cyclin A, Cdk2-cyclin E (51, 68), and the serine/threonine protein kinase, Plx1 (69), whereas only p34<sup>cdc2</sup>-cyclin B (and not p34<sup>cdc2</sup>-cyclin A) is able to phosphorylate the human Cdc25-C enzyme (48). Phosphorylation of both the human and the Xenopus Cdc25-C enzyme enhances its tyrosine phosphatase activity and also retards its mobility on SDS-PAGE, by between 13 kDa (human Cdc25-C; Ref. 48) and 30 kDa (Xenopus Cdc25-C; Ref. 49). A negative regulator of Cdc25 activation has also recently been identified. Chk1, a serine/threonine protein kinase (70, 71), is activated in response to DNA damage and has been shown to phosphorylate and inactivate both the fission yeast and human Cdc25 enzyme. It is unclear why multiple protein kinases phosphorylate the Cdc25 enzyme. One possibility is that phosphorylation by a specific protein kinase which enhances binding of Cdc25 to proteins such as Pin1 (72) and 14-3-3 (73) may serve to regulate its activity by altering its subcellular localization (72).

Both p34<sup>cdc2</sup> and Plx1 can phosphorylate and activate the Xenopus Cdc25 enzyme (69). However, it is clear from phosphopeptide mapping data (69) that other, unidentified protein kinase(s) are also able to phosphorylate the Cdc25 enzyme. In this study, we have demonstrated that the phosphorylation of Cdc25-C by CaM kinase II results in a 2.5–3-fold greater tyrosine phosphatase activity when compared with the nonphosphorylated enzyme. The relatively modest increase in the ac-
tivity of the CaM kinase II-phosphorylated Cdc25-C in our assay may be a reflection of the fact that we have used a substrate bound to p13aucl-Sepharose beads instead of soluble p34cdc2 (where a 10-fold increase in the activity of the phosphorylated Cdc25-C enzyme has been reported (49) or an artificial substrate such as p-nitrophenyl phosphate (where a 4–5-fold increase in the activity of the phosphorylated cdc25-C enzyme has been reported (48)). Our data are consistent with a previous study that also reported a 3–4-fold activation of the phosphorylated Cdc25-C enzyme when assayed using immobilized p34cdc2-cyclin B as substrate (68) and are consistent with the reported modest increase in HeLa cells between interphase and mitosis (84). We have also shown that the phosphorylation of Cdc25-C by CaM kinase II, as by p34cdc2 (48, 68), retards the electrophoretic mobility of the protein on SDS-polyacrylamide gels.

Similar phosphorylation and activation of the Xenopus Cdc25 protein by CaM kinase II in vitro has also been reported (51). Izumi and Maller (51) concluded that Cdc25 was unlikely to be a physiological substrate for CaM kinase II, since high concentrations of the calcium chelator EGTA, present in the Xenopus extracts, would apparently inhibit any Ca2+/CaM-dependent enzymatic activity. However, we note that in Xenopus extracts prepared in the presence of high concentrations of EGTA, a calcium-dependent activation and inactivation of the p34cdc2-cyclin B protein kinase can be demonstrated (74). The existence of local transient increases in Xenopus egg extracts (74) is consistent with the finding that p34cdc2-insensitive phosphorylation site mutants of the Xenopus Cdc25 protein show small but detectable shifts in electrophoretic mobility in both mitotic and microcystin-treated extracts (51). Our data are therefore in accord with the observation that another kinase (in addition, that is, to the p34cdc2-cyclin B, p34cdc2-cyclin A, Cdc2-cyclin A, and Cdk2-cyclin E protein kinases) is able to phosphorylate and activate the Cdc25-C tyrosine phosphatase (51, 68).

Our in vitro experiments have been aimed at determining the physiological role of CaM kinase II in the activation of the human Cdc25-C enzyme. Using a functional assay of CaM kinase II activity (the phosphorylation of a synthetic peptide substrate, autocomtide-2) we have demonstrated the presence of this enzyme in HeLa cells. Autocomtide-2 is a specific substrate for CaM kinase II and, unlike syntide-2, the autocomtide series of peptide substrates is not phosphorylated by either CaM-dependent protein kinase or protein kinase C (75). There is a possibility that other multifunctional CaM kinases (types I and IV), which may be present in HeLa cells, also contribute toward the phosphorylation of autocomtide-2. However, this is unlikely, since CaM kinase IV phosphorylates autocomtide-2 poorly (76), while CaM kinase I has a substrate specificity that is similar to CaM-dependent protein kinase rather than CaM kinase II (77, 78). These observations suggest that, in HeLa cells, it is CaM kinase II that is primarily responsible for the phosphorylation of autocomtide-2.

To inhibit the HeLa cell CaM kinase II, we used KN-93, a specific, water-soluble inhibitor of CaM kinase II that is not prone to nonspecific, hydrophobic interactions (IC50 0.37 M; Ref. 62). The addition of KN-93 to a synchronous culture of Xenopus egg extracts, would apparently inhibit any Ca2+/CaM-dependent enzymes, as well as CaM kinase II, were inhibited (81). KN-93 is a water-soluble inhibitor of CaM kinase II, and at the concentrations used in this study it is reported to inhibit cAMP-dependent protein kinase, protein kinase C, myosin light chain kinase, and a calcium-dependent phosphodiesterase by less than 10% (62). KN-93 is known to inhibit both CaM kinase I and IV (IC50 values of 2.7 and 50 M, respectively).2 Therefore, it is possible that the KN-93-induced G2 phase arrest of HeLa cells may also result from the inhibition of CaM kinase I activity. To exclude a role for either CaM kinase I or IV at the G2/M phase transition, we microinjected AC3-I, a specific peptide inhibitor of CaM kinase II (65), into a synchronized population of HeLa cells. Upon microinjection in G2 phase cells, AC3-I, but not AC3-C, blocked entry into M phase. This result demonstrates that the AC3-I-induced cell cycle cycle arrest is most likely to be mediated by the inhibition of CaM kinase II, although our microinjection study does not allow us to monitor the phosphorylation state of Cdc25-C in these cells. The inference is that Cdc25-C is not hyperphosphorylated and hence not activated in the AC3-I-arrested cells. Since our peptide inhibitor data complement the KN-93 data, it seems clear that a multifunctional Ca2+/CaM-dependent protein kinase, almost certainly CaM kinase II, is required for Cdc25-C activation.

We have additionally demonstrated the specificity of the KN-93-induced G2/M phase block by showing that it does not inhibit either the synthesis of cyclin B, the regulatory subunit of the p34cdc2 protein kinase, or the tyrosine phosphatase activity of the Cdc25-C enzyme. The inability of KN-93 to inhibit the OA-induced activation of p34cdc2 indicates that OA can bypass the calcium-dependent regulatory step for mitosis entry in mammalian cells as it does in early sea urchin embryos, where OA-induced mitosis is insensitive to calcium chelators and a peptide inhibitor of CaM kinase II (82). OA is a phosphatase inhibitor. The identity of the phosphatase whose inhibition bypasses upstream controls and leads to mitosis entry is not known. It may inhibit the protein phosphatase 2A-like INH phosphatase activity (83). Inhibition of Cdc25-C-directed phosphatase activity may lead to activation of Cdc25 and thus p34cdc2 protein kinase activity via the positive feedback mechanism postulated by Hoffman and co-workers (48), eliminating the need for CaM kinase-dependent phosphorylation of Cdc25. OA treatment might also lead directly to enhanced activating phosphorylation of p34cdc2 itself.

We propose a hypothetical model (see Fig. 7) for the activation of the p34cdc2-cyclin B protein kinase at mitosis entry that is consistent with our data in this study. The first step involves activation of a multifunctional Ca2+/CaM-dependent protein kinase (CaM kinase II) as a result of an increase in Cao, which...
initiates the phosphorylation and activation of Cdc25-C. In the second step, the phosphorylated Cdc25-C enzyme triggers activation of the p34\(^{cdcl}\) protein kinase, which, in accordance with the published data (48, 68), phosphorylates and activates additional Cdc25-C enzyme in an autoamplification loop. An autocatalytic model of this sort predicts that CaMK II-dependent Cdc25-C phosphorylation will be difficult to test by measurement of Cdc25-C phosphorylation in vivo, since the major part of Cdc25-C phosphorylation will be due to p34\(^{cdcl}\). We note that recent studies have used cell lines overexpressing Cdc25-C (presumably because Cdc25-C is a low abundance protein) for phosphopeptide map analysis of Cdc25-C (73), although it is unclear whether all of the sites phosphorylated on the overexpressed protein are the same as those phosphorylated on the native protein. Our data, therefore, indicate that CaMK II may be the enzyme that triggers the phosphorylation and activation of Cdc25-C at mitosis entry in mammalian cells.

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