Molecular Biology of the Cell
Vol. 6, 199–213, February 1995

Control of the Cdc2/Cyclin B Complex in *Xenopus* Egg Extracts Arrested at a G2/M Checkpoint with DNA Synthesis Inhibitors

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Submitted November 18, 1994; Accepted January 11, 1995
Monitoring Editor: Tim Hunt

Proliferating eukaryotic cells possess checkpoint mechanisms that block cell division in the presence of unreplicated or damaged DNA. Using cell-free extracts from *Xenopus* eggs, we have investigated the mechanisms underlying the inability of a recombinant Cdc2/cyclin B complex to induce mitosis in the presence of incompletely replicated DNA. We found that the activities of the kinases and phosphatases that regulate the major phosphorylation sites on Cdc2 (e.g., tyrosine 15, threonine 14, and threonine 161) are not altered significantly under conditions where *Xenopus* extracts remain stably arrested in interphase due to the presence of the replication inhibitor aphidicolin. However, at threshold concentrations, a Cdc2/cyclin B complex containing a mutant Cdc2 subunit that cannot be phosphorylated on either tyrosine 15 or threonine 14 displays a markedly reduced capacity to induce mitosis in the presence of aphidicolin. This observation indicates that the replication checkpoint in *Xenopus* egg extracts functions without the inhibitory tyrosine and threonine phosphorylation of Cdc2. We provide evidence that the checkpoint-dependent suppression of the Cdc2/cyclin B complex involves a titratable inhibitor that is regulated by the presence of unreplicated DNA.

INTRODUCTION

In dividing eukaryotic cells, a diverse family of cyclin-dependent kinases (Cdks) directs the progression of the cell cycle by regulating the accurate replication of the genome during S-phase and the faithful segregation of the chromosomes at mitosis (M-phase). The M-phase of the cell cycle is controlled by maturation promoting factor (MPF), which contains a B-type cyclin and the Cdc2 protein kinase. MPF acts by phosphorylating a wide variety of substrates that collaborate in the execution of mitotic processes such as chromosome condensation, nuclear envelope breakdown, and spindle assembly (Nurse, 1990; Solomon, 1993; reviewed in Dunphy, 1994).

The action of MPF must be controlled both temporally and spatially in a very stringent manner. This strict regulation is imparted by a number of checkpoint mechanisms that operate during the preparation for mitosis (reviewed in Hartwell and Weinert, 1989; Murray, 1992; Sheldrick and Carr, 1993). A key question in the cell cycle field is how these checkpoint mechanisms control the activation and/or action of Cdks such as MPF so that cell cycle transitions occur only when all essential prerequisites have been fulfilled. In the case of M-phase, these requirements include the absence of unreplicated or damaged DNA, the attainment of an adequate cell size for division, and the accumulation of the various proteins necessary for spindle assembly and other mitotic processes.

The activation of MPF is controlled by an intricate series of phosphorylation reactions on the Cdc2 subunit (reviewed in Fleig and Gould, 1991; Draetta, 1993; Nigg, 1993; Pines, 1993; Solomon, 1993). Specifically, phosphorylation of threonine residue 161 (T161) is absolutely required for catalytic activity, but the effect of this phosphorylation is overridden during interphase by concomitant inhibitory phosphorylations on tyrosine 15 (Y15) and threonine (T14). The tyrosine phosphorylation and subsequent dephosphorylation

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of Cdc2 are mediated by the Wee1 and Cdc25 proteins, respectively (Russell and Nurse, 1986, 1987; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). In turn, the activities of both the Wee1 and Cdc25 proteins are controlled by upstream kinase/phosphatase networks, which themselves are highly regulated so that tyrosine phosphorylation of Cdc2 is strongly favored during interphase and dephosphorylation is rapidly triggered at mitosis (Solomon *et al.*, 1990; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Clarke *et al.*, 1993; Tang *et al.*, 1993; Mueller *et al.*, 1995).

It has appeared highly plausible that the phosphorylation of Cdc2 on one or more of its key residues (e.g., Y15, T14, T161) would play a pivotal role in cell cycle checkpoint mechanisms. For this reason, the role of Cdc2-specific tyrosine phosphorylation in the operation of the replication checkpoint has been examined in a number of systems, including fission yeast, budding yeast, and *Xenopus* eggs (Enoch and Nurse, 1990; Lundgren *et al.*, 1991; Amon *et al.*, 1992; Enoch *et al.*, 1992; Smythe and Newport, 1992; Sorger and Murray, 1992; Mueller *et al.*, 1995). However, these studies have arrived at apparently contradictory conclusions. Similarly, the roles of phosphorylation on T14 and T161 in the replication checkpoint have not been resolved conclusively.

The *Xenopus* egg system offers many advantages for the study of cell cycle–regulatory mechanisms. In particular, the progression of the cell cycle can be reconstituted in cytoplasmic extracts from *Xenopus* eggs, and the replication checkpoint can be triggered in vitro by addition of DNA synthesis inhibitors such as aphidicolin (Dasso and Newport, 1990; Murray, 1991). Normally, activated egg extracts synthesize cyclin B, produce active MPF, and undergo mitosis in approximately 1 h (Murray and Kirschner, 1989). However, the addition of aphidicolin results in a prolonged arrest in interphase (Dasso and Newport, 1990; Kumagai and Dunphy, 1991; Smythe and Newport, 1992; Kornbluth *et al.*, 1992, 1994). To date, the analysis of the biochemical mechanisms underlying this checkpoint has been hampered somewhat by the fact that aphidicolin-containing egg extracts arrest properly only with endogenously synthesized cyclin B. Surprisingly, recombinant, bacterially expressed cyclin B induces mitosis even in the presence of unreplicated DNA (Smythe and Newport, 1992).

In this paper, we have developed an experimental system in which *Xenopus* egg extracts containing exogenously added, baculovirus-expressed cyclin B and Cdc2 proteins arrest properly in interphase due to the addition of aphidicolin. Accordingly, we have been able to comprehensively assess the effect of unreplicated DNA upon the phosphorylation of Cdc2 protein. The principal finding of these studies is that a Cdc2 mutant that cannot undergo phosphorylation on Y15 or T14 still responds to the replication checkpoint. We present evidence for a titratable inhibitor that interacts with the Cdc2/cyclin B complex in checkpoint-arrested extracts.

MATERIALS AND METHODS

Production of Histidine-tagged Cyclin B and ΔCyclin B

The human cyclin B1 cDNA (Pines and Hunter, 1989) was digested with *Nco*I and *Bam*HI and ligated with *Nco*I/*Bgl*II-digested pVL1393-HIS6 (kindly provided by D. Morgan, University of California at San Francisco, San Francisco, CA), thereby creating a baculovirus transfer vector encoding the full-length cyclin B with the extra amino acids MSHHHHHHGS at the N-terminal end. Truncated, histidine-tagged human cyclin B1 lacking the N-terminal 87 amino acids of the natural protein (Δcyclin B) was prepared by digesting the cDNA with *Kpn*I, blunt-ending with T4 DNA polymerase, and ligating with an *Nco*I linker (CAGCCATGGCTG). The resulting construct was cloned into pVL1393-HIS6 using the *Nco*I and *Eco*RI sites. Recombinant baculoviruses encoding cyclin B and Δcyclin B were prepared by standard methods (Desai *et al.*, 1992; Coleman *et al.*, 1993). Sf9 insect cells (60 ml at a density of 2×10^6 cells/ml) were infected with the baculoviruses for 48 h, washed twice in ice-cold Tris buffered-saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl), and lysed in HBS (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl) containing 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin, chymostatin, and leupeptin. The presence of EGTA in the lysis buffer greatly reduced nonspecific binding to the nickel-IDA beads during the purification of the histidine-tagged proteins (see below). The lysate was centrifuged at 4°C for 20 min at 10 000 rpm in the Sorvall HB-4 rotor. The resulting supernatant was incubated with 1 ml nickel-IDA Sepharose Fast Flow (Pharmacia, Piscataway, NJ) at 4°C for 30 min. The IDA Sepharose beads were charged with nickel chloride according to the instructions of the manufacturer. The cyclin-coated, nickel-IDA beads were washed successively with 50 volumes of HBS containing 0.5% NP-40 and 50 volumes of HBS. The washed beads were aliquoted, frozen in liquid nitrogen, and stored at −80°C.

Production of Wild-Type and Mutant Xenopus Cdc2 Proteins in Sf9 Insect Cells

Plasmids encoding various *Xenopus* Cdc2 mutant proteins (T161A, T14A, Y15F, T14AY15F, and T14AY15FT161A; Solomon *et al.*, 1992) were digested with *Nco*I and *Ban*II. The resulting *Nco*I/*Ban*II fragments were ligated into pVL1393 encoding wild-type *Xenopus* Cdc2 (pVL1393-Cdc2; Coleman *et al.*, 1993), which had been digested previously with *Nco*I and *Ban*II. This procedure created plasmids encoding various Cdc2 mutants that lacked the hemagglutinin tag provided by the original plasmids (Solomon *et al.*, 1992). The Cdc2-N133A mutant (containing the hemagglutinin tag) was prepared by in vitro mutagenesis using the kit from Amersham and the oligonucleotide GAAACCTCAGGCCCTCCTCATCG. The mutated cDNA was cloned into pVL1393-Cdc2 that had been digested with *Nco*I and *Pst*I. Recombinant baculoviruses, prepared as described above, were used to infect Sf9 insect cells. At 48 h post-infection, the insect cells were washed twice with ice-cold Tris buffered saline and resuspended in one-tenth of the original vol of a lysis buffer that contained 10 mM HEPES-KOH (pH 7.5), 10 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin, chymostatin, and leupeptin. The cells were lysed by dounce homogenization, NaCl was added to a final concentration of 150 mM, and the lysate was centrifuged at 4°C for 20 min at 10 000 rpm

in the Sorvall HB-4 rotor. The supernatant was frozen in liquid nitrogen and stored at −80°C.

Preparation of Various Cdc2/Cyclin B Complexes

Nickel-IDA agarose beads (25 μl) containing either cyclin B or Δcyclin B were incubated with 800 μl of Cdc2-containing Sf9 cell lysate for 20 min either at 22°C in the presence of 0.5 mM ATP and 10 mM $MgCl_2$ or at 4°C in the absence of ATP and $MgCl_2$. The beads were washed four times with HBS and eluted in 50 μl of 200 mM imidazole in HBS. The isolated Cdc2/cyclin complex was used on the same day it was prepared because it was not stable upon freezing and thawing.

Preparation of Xenopus Egg Extracts

In all cases, CSF extracts from unactivated *Xenopus* eggs were prepared as described previously (Murray, 1991; Kumagai and Dunphy, 1992; Tang *et al.*, 1993). Typically, the extracts were activated by the addition of 0.4 mM $CaCl_2$ in the presence of 100 μg/ml cycloheximide (which prevents endogenous cyclin B synthesis). Demembranated *Xenopus* sperm nuclei (up to 1000 per μl of egg extract) were routinely added at the same time as $CaCl_2$. Recombinant cyclin B and Δcyclin B proteins (20–60 nM) were added at 50 min post-activation when nuclear assembly around the sperm chromatin was complete. Depending upon their final concentration, the cyclin proteins induced mitosis in 30–60 min. The entry into mitosis was monitored by visualizing nuclear envelope disassembly and chromosome condensation by phase contrast and fluorescence microscopy (Kumagai and Dunphy, 1991; Murray, 1991). To block the extracts in interphase caused by the presence of unreplicated DNA (Dasso and Newport, 1990; Kornbluth *et al.*, 1992), we added 100 μg/ml aphidicolin (dissolved in DMSO at 10 mg/ml) to extracts containing 1000 sperm nuclei per μl. Control extracts lacked aphidicolin, and contained 0–1000 sperm nuclei per μl and 1% DMSO. In our experience, control extracts containing 0, 200, or 1000 nuclei per μl entered mitosis at comparable times because these extracts can carry out the efficient replication of 4000 or more nuclei per μl in less than 60 min. The aphidicolin-induced block to mitosis typically persisted for 120–150 min after the addition of recombinant cyclin B or Δcyclin B. To overcome this block, caffeine (5 mM) or okadaic acid (3 μM) could be added in addition to aphidicolin.

In Vitro Phosphorylation of Cdc2 on T161

Nickel-IDA beads (10 μl) containing cyclin B were mixed with Sf9 insect cell lysates containing either wild-type *Xenopus* Cdc2 (200 μl vol) or the Cdc2-N133A mutant (450 μl vol), and incubated at 4°C for 20 min. The beads were centrifuged, the supernatant was removed, and the beads were mixed with 50 μl of a fresh Cdc2-containing lysate (either wild-type or mutant). The beads were then incubated for 20 min at 22°C in the presence of 0.2 mCi ^{32}P-ATP, 50 μM nonradioactive ATP, and 10 mM $MgCl_2$ to allow the phosphorylation of *Xenopus* Cdc2 on T161 due to the action of a Cdk-activating kinase (CAK) present in Sf9 cell lysates. No detectable phosphorylation of T14 or Y15 occurs under these conditions. After the incubation, the beads were washed with HBS (five times) and eluted in 20 μl of HBS containing 200 mM imidazole.

Assay for Total Cdc2-specific Tyrosine Kinase Activity

The Δcyclin B protein (40 nM, final concentration) was added to cycloheximide-containing interphase extracts 50 min after activation with calcium. At various times thereafter, 100 μl aliquots were removed, chilled on ice, and then mixed with 20 μl of nickel-IDA agarose beads. The beads were incubated with the extract for 10 min on ice, then washed successively with HBS containing 0.1 mM sodium vanadate (once) and HBS alone (twice). Bead-associated proteins were then eluted with sodium dodecyl sulfate (SDS) gel sample buffer, electrophoresed in 10% polyacrylamide gel, and finally subjected to immunoblotting with anti-phosphotyrosine or anti-*Xenopus* Cdc2 antibodies. For the Cdc2-specific tyrosine kinase assays presented in this paper, we did not find it necessary to include sodium vanadate as an inhibitor of Cdc25 activity, which is low during interphase. However, similar results were obtained in the presence of sodium vanadate (our unpublished observation). Also, for the tyrosine kinase assays described in this paper, we verified that the aphidicolin-treated extracts remained arrested in interphase for the duration of the experiment.

Radiolabeling of Xenopus Cdc2 on Y15

The Cdc2/cyclin B complex (8 μl vol) was prepared as described above and mixed with the *Xenopus* Wee1 protein (10 ng; Mueller *et al.*, 1995) in 40 μl of 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 1 mM DTT, and 40 μCi of ^{32}P-ATP. After a 30-min incubation at 22°C, the reaction was terminated by incubating for an additional 20 min at 37°C (which results in the thermal inactivation of the *Xenopus* Wee1 protein). The Cdc2/cyclin B complex was re-isolated with 15 μl of nickel-IDA beads and eluted in 40 μl of 200 mM imidazole in HBS.

^{35}S-Labeling of the Xenopus Cdc2 Protein in Sf9 Insect Cells

Sf9 insect cells (10 ml at 2×10^6 cells per ml) were infected with the *Xenopus* Cdc2-encoding baculovirus for 40 h in complete TNM-FH medium (Invitrogen, San Diego, CA). The cells were rinsed twice with methionine-free Grace's medium (Life Technologies, Inc., Gaithersburg, MD), and incubated for 7 h in 2.5 ml of the same medium containing 10% dialyzed fetal calf serum and 0.5 mCi of ^{35}S-Translabel (ICN, Cleveland, OH). The cells were lysed and a ^{35}S-Cdc2/cyclin B complex was prepared as described above.

^{32}P-Labeling of the Cdc2/Cyclin B Complex in Xenopus Egg Extracts

A CSF extract (300 μl vol) was activated in the presence of 100 μg/ml cycloheximide by the addition of 0.4 mM $CaCl_2$ and incubated for 50 min at 22°C with 2 mCi of ^{32}P-orthophosphate (ICN). At this time, 20 nM of cyclin B was added and the incubation was continued for 10 min. The extract was chilled on ice, mixed with nickel-IDA beads (10 μl), incubated for 30 min at 4°C under constant agitation, and spun in a microfuge E (Beckman Instruments, Palo Alto, CA) for 5 sec. The beads were washed as follows: once with extraction buffer (80 mM sodium β-glycerolphosphate, pH 7.3, 20 mM EGTA, and 15 mM $MgCl_2$) containing 0.5% NP-40, 1 mM sodium vanadate, 1 μM microcystin, and 10 μM each of phosphotyrosine, phosphothreonine, and phosphoserine; five times with extraction buffer containing 0.5% NP-40; twice with HBS; and finally twice with HBS containing 25 mM imidazole. Bound proteins were eluted in HBS containing 250 mM imidazole, electrophoresed in a 10% polyacrylamide gel, and visualized with x-ray film. Bands corresponding to the Cdc2 and cyclin B proteins were processed for phosphoamino acid analysis and tryptic phosphopeptide mapping as described previously (Boyle et al., 1991).

Purification of the Cdc2-AF/ΔCyclin B Complex by Gel Filtration Chromatography

Nickel-IDA beads (50 μl) containing Δcyclin B were mixed with Sf9 cell lysate (1.2 ml) containing the *Xenopus* Cdc2-AF mutant protein. The beads were incubated for 30 min at 4°C under constant agitation, centrifuged, and mixed with an additional 1.2 ml of the mutant Cdc2-containing lysate. At this point, the beads were incubated for 20 min at 22°C in the presence of 0.5 mM ATP and 10 mM $MgCl_2$. Finally, the beads were washed four times with HBS and eluted in

50 μl of HBS containing 300 mM imidazole. The eluted complex was chromatographed using a SMART system (Pharmacia) on a Superdex 200 PC column equilibrated in 10 mM HEPES-KOH (pH 7.5) containing 150 mM NaCl. Fractions (50 μl vol) were collected at 4°C. Under these conditions, free Δcyclin B not associated with Cdc2 migrated as an oligomer in the void volume, whereas the dimeric Cdc2/cyclin B complex eluted at a position corresponding to ~100 kDa. A complex between Δcyclin B and the Cdc2-T14AY15FT161A mutant was prepared in a similar manner.

Miscellaneous

H1 kinase assays were performed as described previously (Dunphy and Newport, 1989). [γ-^{32}P]ATP (3000 Ci/mmol) for kinase assays was purchased from ICN. Affinity-purified antibodies against the bacterially expressed *Xenopus* Cdc2 protein (Milarski *et al.*, 1991) were prepared by established procedures (Kumagai and Dunphy, 1992). Antibodies against human cyclin B1 and phosphotyrosine were obtained from Upstate Biotechnology (UBI, Lake Placid, NY). Immunoblotting was performed as described previously (Coleman *et al.*, 1993) with ^{125}I-protein A (ICN) or ^{125}I-sheep anti-mouse antibodies (Amersham, Arlington Heights, IL). In some cases, SDS gel electrophoresis was performed with 10% polyacrylamide gels containing 0.4% PDA (piperadine diacrylamide; Bio-Rad, Richmond, CA) instead of 0.26% bisacrylamide to enhance the separation of various phosphorylated forms of the Cdc2 protein. Detection and quantitation of immunoblots was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Preparation of Recombinant Cyclin B that Responds to the Replication Checkpoint in the Xenopus System

To study the mechanism by which unreplicated DNA inhibits mitosis in *Xenopus* egg extracts, we attempted to establish an experimental system in which an exogenously added, recombinant cyclin B or Cdc2/cyclin B complex would be prevented from inducing M-phase in the presence of a DNA synthesis inhibitor (e.g., aphidicolin). We found that cyclin B and Cdc2 proteins that had been expressed in baculovirus-infected Sf9 insect cells were suitable for this purpose. For the studies reported in this paper, we prepared histidine-tagged versions of both the full-length human cyclin B1 and a truncated, nondegradable mutant cyclin B1 that lacks a destruction box (Pines and Hunter, 1989; Glotzer *et al.*, 1991). These proteins, which we will refer to as cyclin B and Δcyclin B, were purified by a modified procedure for nickel-IDA agarose chromatography that greatly reduced nonspecific binding to the nickel beads (see MATERIALS AND METHODS). Using this improved protocol, it was possible to obtain highly purified cyclin B or Δcyclin B in a single step (Figure 1A, lanes c and d). In parallel, we prepared in Sf9 cells the intact *Xenopus* Cdc2 protein and a variety of mutant Cdc2 proteins that lack key phosphorylatable residues, including the Cdc2-T14AY15F mutant (Cdc2-AF) in which T14 and Y15 have been mutated to alanine and phenylalanine, respectively (Solomon *et al.*, 1992; see MATERIALS AND METHODS). The intact and mutant forms of Cdc2 were purified from insect cell lysates by virtue of their ability to bind to cyclin B-coated nickel agarose beads.

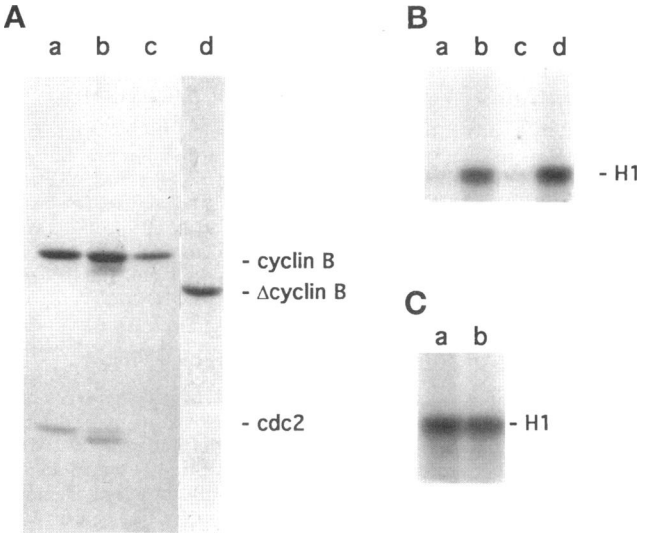

Figure 1. Preparation of baculovirus-expressed cyclin B and Cdc2 proteins. (A) Purification of the Cdc2/cyclin B complex. Nickel-IDA beads containing human cyclin B1 (lane c) were added to Sf9 insect cell lysates containing the intact *Xenopus* Cdc2 protein in the presence of 0.5 mM ATP and 10 mM $MgCl_2$ at 22°C (lane b) or in the absence of ATP and $MgCl_2$ at 4°C (lane a). The beads were washed with HBS and eluted with 150 mM imidazole as described in MATERIALS AND METHODS. Lane d depicts Δcyclin B that was purified similarly with nickel-IDA beads. The eluted complexes were subjected to gel electrophoresis and Coomassie blue staining. (B) H1 kinase activity of the recombinant Cdc2/cyclin B complex. The Cdc2/cyclin B (lanes a and b) and Cdc2/Δcyclin B (lanes c and d) complexes that were prepared in the absence (lanes a and c) or presence (lanes b and d) of ATP were assayed for H1 kinase activity as described in MATERIALS AND METHODS. (C) The H1 kinase activity of a complex between cyclin B and either wild-type *Xenopus* Cdc2 (lane a) or the Cdc2-AF mutant (lane b) was determined as in panel B.

We prepared either the inactive or active Cdc2/cyclin B complex for the studies described in this paper. Fortuitously, Sf9 insect cells contain an endogenous CAK that efficiently phosphorylates *Xenopus* Cdc2 on T161 if binding to cyclin is allowed to occur at 22°C in the presence of $MgCl_2$ and ATP (see Desai *et al.*, 1992). As shown in Figure 1, a highly purified Cdc2/cyclin B complex in which the Cdc2 subunit was phosphorylated on T161, as judged by a downward mobility shift during gel electrophoresis, was highly active as an H1 kinase (Figure 1A, lane b; Figure 1B, lane b). Control experiments with the Cdc2-T161A mutant confirmed that this down-shifting was due to phosphorylation on T161 (our unpublished observation). In contrast, when the Cdc2/cyclin B complex was allowed to form at 4°C, it lacked T161 phosphorylation and was inactive (Figure 1A, lane a; Figure 1B,

lane a). Using the same protocol with isolated Δcyclin B (Figure 1A, lane d), we also purified either the inactive or active Cdc2/Δcyclin B complex (Figure 1B, lanes c and d). Finally, for many experiments in this paper, we prepared a complex between cyclin B and the Cdc2-AF mutant, which displayed a level of H1 kinase activity similar to the wild-type Cdc2/cyclin B complex (Figure 1C, lanes a and b).

Both recombinant cyclin B and Δcyclin B, either alone or in a complex with Cdc2, induced mitosis efficiently, as judged by their ability to elicit both nuclear envelope breakdown and stimulation of Cdc2-associated H1 kinase activity (Figure 2, A and C, closed circles; Figure 2B, lanes a-e). The minimal effective concentrations of cyclin B and Δcyclin B for mitotic induction were 20 nM and 16 nM, respectively (see Kobayashi *et al.*, 1991). In contrast, neither the full-length nor the Δcyclin B proteins at equivalent or considerably higher concentrations (up to 60 nM) could induce mitosis in extracts that contained the DNA synthesis inhibitor aphidicolin. In such extracts, the recombinant cyclins failed to trigger both nuclear disassembly and activation of Cdc2, typically for at least 120 min after addition to the extract (Figure 2, A and C, closed triangles; Figure 2B, lanes f-j). As a control, we added caffeine, a drug that is known to override the inhibition of mitosis by unreplicated DNA in both tissue culture cells and *Xenopus* egg extracts (Schlegel and Pardee, 1987; Dasso and Newport, 1990). We observed that caffeine could overcome the inhibitory effect of aphidicolin (Figure 2A, open triangles). Likewise, the addition of 3 μM okadaic acid could reverse the aphidicolin-induced block to mitosis (our unpublished observation).

In summary, the recombinant cyclin B and Δcyclin B proteins that we prepared for this paper induce mitosis efficiently at physiological concentrations. Both cyclin proteins are stable during interphase, and the Δcyclin B protein is also stable during mitosis (see Figure 2A, closed circles). As expected, the full-length cyclin B protein is degraded during mitosis (see Figure 2C, closed circles), indicating that the presence of the histidine tag does not interfere with ubiquitin-mediated proteolysis. Most importantly, neither cyclin protein can induce mitosis in the presence of unreplicated DNA, indicating that these polypeptides obey a replication checkpoint that operates in the cell cycle extracts.

Total Cdc2-specific Tyrosine Kinase Activity in Checkpoint-arrested Extracts

Using both the recombinant cyclin B and Cdc2 proteins, we set out to assess the effect of unreplicated DNA upon the regulation of the enzymes that control phosphorylation at key residues on the Cdc2 protein. These baculovirus-expressed proteins readily come

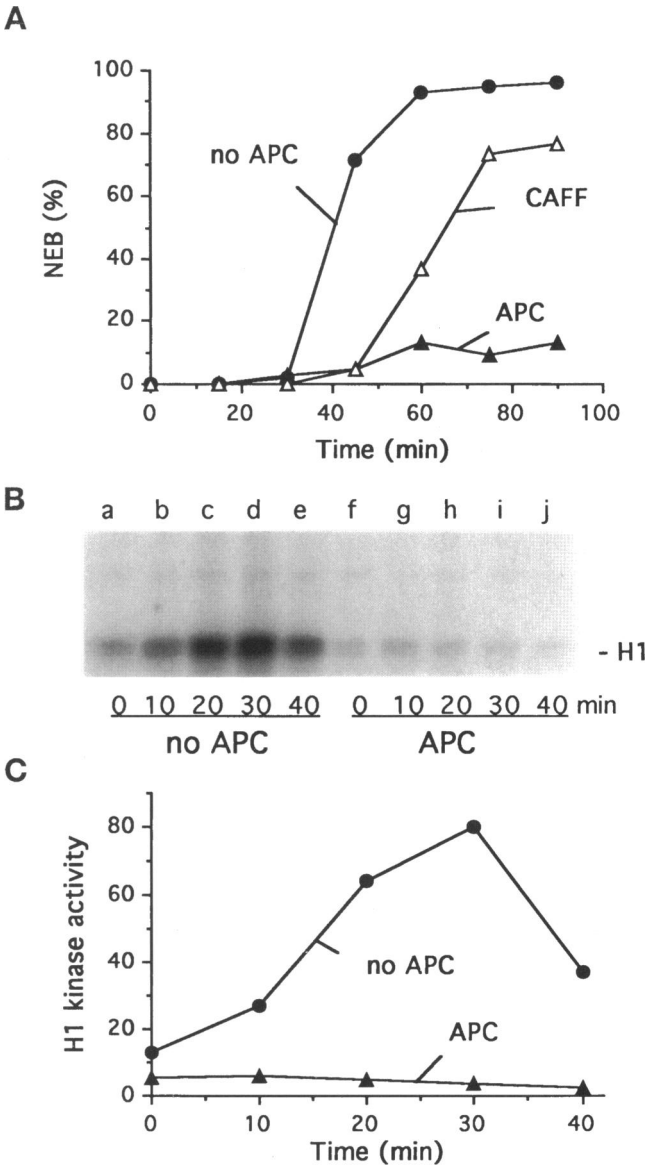

Figure 2. The recombinant cyclin B and Cdc2 proteins cannot induce mitosis in the presence of unreplicated DNA. (A) Purified Δcyclin B (32 nM) was added to cycloheximide-containing interphase egg extracts in the presence of: 200 demembranated *Xenopus* sperm nuclei per μl of extract (closed circles); 1000 nuclei per μl and 100 μg/ml aphidicolin (closed triangles); or 1000 nuclei per μl, 100 μg/ml aphidicolin, and 5 mM caffeine (open triangles). At the indicated times after the addition of cyclin (t = 0), the percentage of nuclear envelope breakdown (NEB) in the extracts was determined. APC, aphidicolin; CAFF, caffeine. (B) The purified, inactive Cdc2/cyclin B complex (32 nM) was added to interphase extracts containing 200 nuclei per μl (lanes a-e) or 1000 nuclei per μl and 100 μg/ml aphidicolin (lanes f-j). Aliquots were removed for H1 kinase assays at the following times after the addition of the complex: 0 min (a, f); 10 min (b, g); 20 min (c, h); 30 min (d, i); and 40 min (e, j). (C) Quantitation of the data in panel B (in arbitrary units).

under the control of the replication checkpoint, so it was feasible to prepare reliable substrates for these Cdc2 phosphorylation assays.

The phosphorylation of Cdc2 on tyrosine 15 plays an important role in mitotic control in a variety of organisms (Russell and Nurse, 1987; Dunphy and Newport, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989; Solomon *et al.*, 1990; Krek and Nigg, 1991; McGowan and Russell, 1993). To measure total Cdc2-specific tyrosine kinase activity in the *Xenopus* egg system, we added recombinant Δcyclin B to egg extracts, isolated the resulting Cdc2/Δcyclin B complex at successive intervals by chromatography on nickel-IDA agarose, and finally measured tyrosine phosphorylation of Cdc2 by immunoblotting with anti-phosphotyrosine antibodies (Figure 3, A and B). There was a linear increase with respect to time in the phosphotyrosine content of Cdc2 as the Cdc2/cyclin B complex formed in control interphase extracts (Figure 3A, lanes d-g; Figure 3B, open circles). However, the rate of Cdc2-specific tyrosine phosphorylation did not change in the presence of aphidicolin (Figure 3A, lanes h-k; Figure 3B, closed circles). Moreover, the presence of caffeine in addition to aphidicolin also had no effect on Cdc2-specific tyrosine kinase activity (Figure 3A, lanes l-o; Figure 3B, closed triangles). In contrast, the tyrosine phosphorylation of Cdc2 was greatly diminished in M-phase extracts (Figure 3A, lanes a-c; Figure 3B, open triangles), indicating that this assay was suitable for detecting the expected reduction of total Cdc2-specific tyrosine kinase activity at mitosis (So-

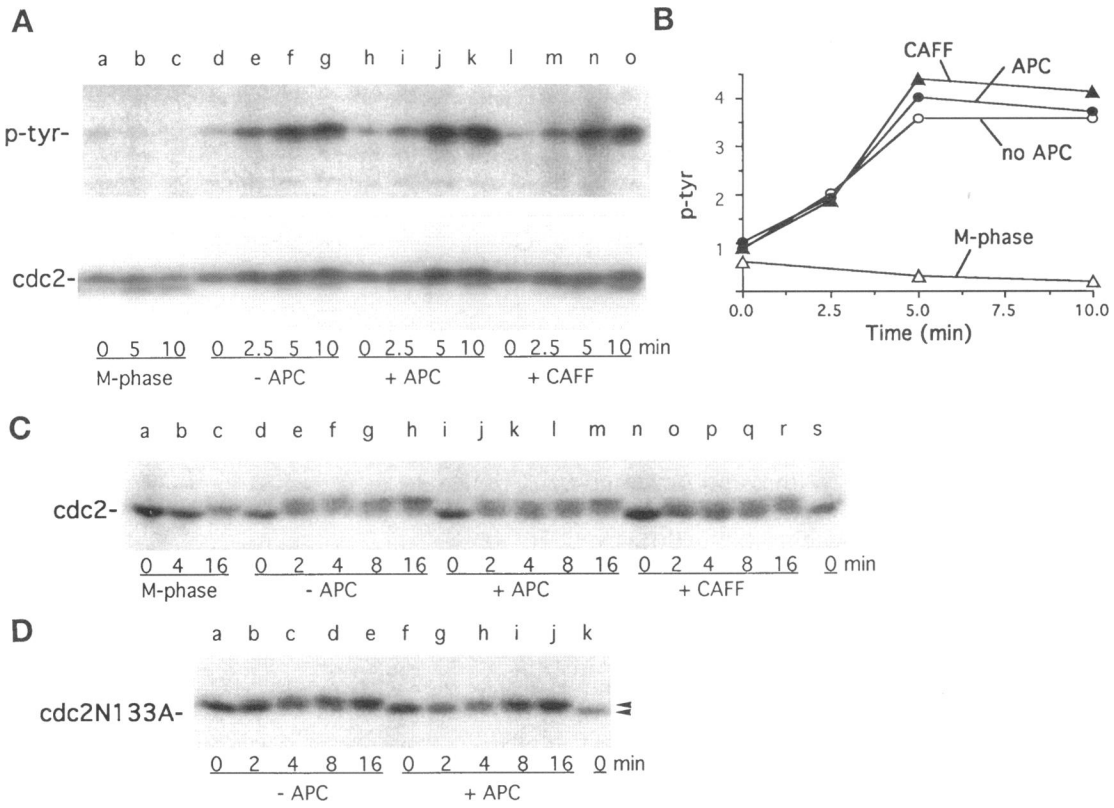

Figure 3. Cdc2-specific tyrosine kinase activity in aphidicolin-containing *Xenopus* egg extracts. (A) The Δcyclin B protein (32 nM) was added either to M-phase extracts (lanes a-c) or to interphase extracts containing: no nuclei (lanes d-g); 1000 nuclei per μl and 100 μg/ml aphidicolin (lanes h-k); or 1000 nuclei per μl, 100 μg/ml aphidicolin, and 5 mM caffeine (lanes l-o). At the indicated times (in minutes), the resulting Cdc2/Δcyclin B complexes were isolated by nickel-IDA chromatography and subjected to immunoblotting with either anti-phosphotyrosine (top panel) or anti-Cdc2 (bottom panel) antibodies. (B) Quantitation of the phosphotyrosine content of the Cdc2 protein from the data in panel A (in arbitrary units). The data were normalized to the amount of Cdc2 protein associated with cyclin at each time point, as determined by immunoblotting with anti-Cdc2 antibodies. (C) The active Cdc2/cyclin B complex was radiolabeled with ^{32}P on T161 as described in MATERIALS AND METHODS, and added to the following extracts: M-phase (lanes a-c); interphase lacking aphidicolin (lanes d-h); interphase containing aphidicolin (lanes i-m); or interphase containing aphidicolin and caffeine (n-r). At the indicated times, aliquots were removed for electrophoresis and phosphorimaging. Lane s depicts the unincubated complex. (D) A complex between cyclin B and the kinase-negative Cdc2-N133A mutant that was labeled on T161 was prepared as in panel C, and added to extracts that either lacked (lanes a-e) or contained (lanes f-j) aphidicolin. Aliquots were removed at the indicated times and processed as described in panel C. Lane k depicts the unincubated complex.

lomon *et al.*, 1990; Smythe and Newport, 1992; Tang *et al.*, 1993; Mueller *et al.*, 1995).

To obtain an independent measure of Cdc2-specific tyrosine kinase activity, we also added a pre-formed Cdc2/cyclin B complex to the *Xenopus* extracts. In particular, we prepared a Cdc2/cyclin B complex that was specifically labeled with ^{32}P on T161 as described in MATERIALS AND METHODS. Upon the addition of this complex to egg extracts, the labeled Cdc2 protein underwent phosphorylation on both Y15 and T14, as judged by an upward mobility shift that could be detected after gel electrophoresis (Figure 3C). As a control, we demonstrated that the Cdc2-AF mutant did not undergo this phosphorylation-dependent mobility shift (our unpublished observation). Using this method, we determined that neither unreplicated DNA nor caffeine affected the rate at which the labeled Cdc2 protein was converted to its tyrosine phosphorylated form (Figure 3C, lanes d-s; note that tyrosine kinase activity is reduced in M-phase, lanes a-c). Finally, to rule out the possibility that the addition of small amounts of active Cdc2/cyclin B complex might perturb the accurate measurement of tyrosine kinase activity, we also prepared a complex of cyclin B and the kinase-negative Cdc2-N133A mutant (Connell-Crowley *et al.*, 1993). The Cdc2 subunit of this complex was radiolabeled with ^{32}P on T161, and then added to interphase extracts in the absence and presence of aphidicolin (Figure 3D). Consistent with the results described above, there was no apparent effect of unreplicated DNA upon the rate at which this catalytically-inactive Cdc2 protein was converted to its tyrosine-phosphorylated form (Figure 3D, compare lanes a-e with lanes f-j). In conclusion, using three different assay methods, we found that total Cdc2-specific tyrosine kinase activity was unaffected by the presence of unreplicated DNA.

Total Cdc2-specific Tyrosine Phosphatase Activity in Checkpoint-arrested Extracts

Previous studies in which the C-type Cdc25 protein was immunoprecipitated from *Xenopus* extracts and assayed for Cdc2-specific tyrosine phosphatase activity indicated that the presence of unreplicated DNA did not affect the steady state activity of Cdc25 during interphase (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). However, it is conceivable that these assays did not detect other potential classes of the Cdc25 protein besides the C-type that might be important for mitotic induction (Galaktionov and Beach, 1991). Therefore, we felt that it would be worthwhile to perform in situ measurements of total Cdc2-specific tyrosine phosphatase activity in *Xenopus* extracts using the baculovirus-expressed Cdc2/cyclin B complex as the substrate. For this purpose, we prepared a Cdc2/cyclin B complex that was specifically labeled with radioactive phosphate on Y15 by treatment with a *Xenopus* Wee1-like tyrosine kinase (see MATERIALS AND METHODS). Upon addition of this radiolabeled complex to

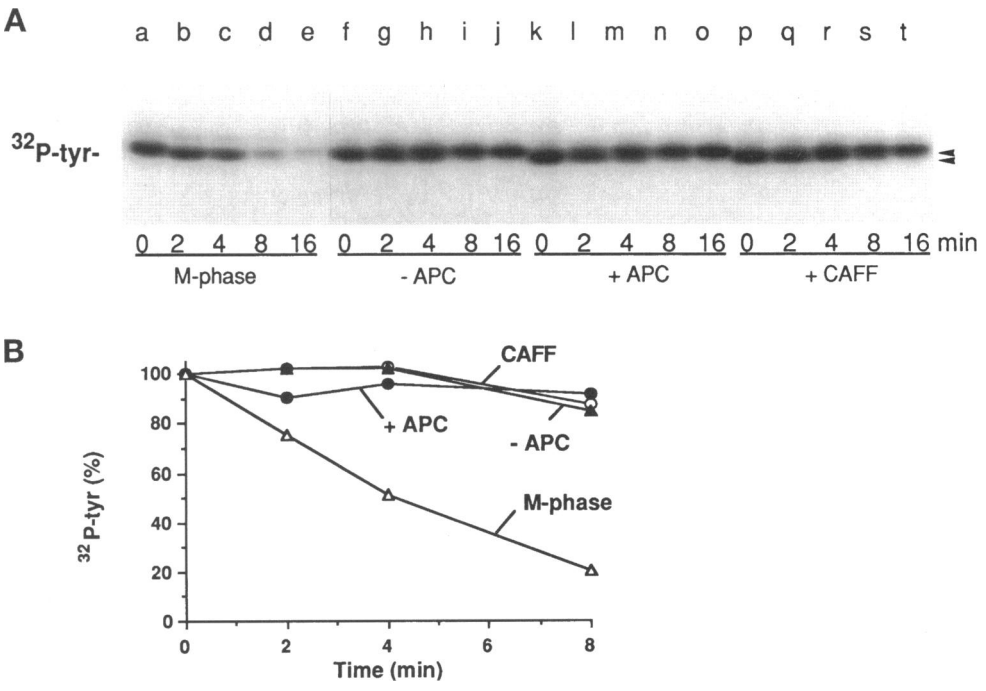

Figure 4. Rates of total Cdc2-specific tyrosine phosphatase activity in cycling and checkpoint-arrested *Xenopus* egg extracts. (A) A complex between cyclin B and recombinant *Xenopus* Cdc2 protein that was radiolabeled with ^{32}P specifically on Y15 by treatment with *Xenopus* Wee1 tyrosine kinase was purified as described in MATERIALS AND METHODS. The ^{32}P-labeled Cdc2/cyclin B complex was added to M-phase extracts (lanes a-e) or interphase extracts containing: no nuclei (lanes f-j); 1000 nuclei per μl of egg extract and 100 μg/ml aphidicolin (lanes k-o); or 1000 nuclei per μl, 100 μg/ml aphidicolin, and 5 mM caffeine (lanes p-t). At the indicated times (in minutes), aliquots were removed and electrophoresed in a 10% polyacrylamide-PDA gel. The arrowheads denote the Y15-singly phosphorylated (bottom) and T14Y15-doubly phosphorylated (top) forms of the Cdc2 protein. (B) Quantitation of the rates of tyrosine dephosphorylation of the ^{32}P-labeled Cdc2 protein shown in panel A.

Xenopus extracts, we observed that the ^{32}P-phosphate on Y15 was relatively stable in interphase extracts (Figure 4A, lanes f-t; Figure 4B), as would be expected (Solomon *et al.*, 1990; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). In contrast, the Y15 site was dephosphorylated with a rapid half-time of approximately 4 min in M-phase extracts (Figure 4A, lanes a-e; Figure 4B, open triangles), consistent with earlier studies. However, the rate of tyrosine dephosphorylation during interphase was essentially identical in extracts that either lacked or contained aphidicolin (Figure 4A, lanes f-o; Figure 4B), or contained both caffeine and aphidicolin (Figure 4A, lanes p-t; Figure 4B). Therefore, using a recombinant Cdc2/cyclin B complex that comes under the control of a replication checkpoint in *Xenopus* extracts, we detected no direct modulation of total Cdc25-mediated tyrosine phosphatase activity in the presence of unreplicated DNA.

During these experiments, we observed that the Cdc2 protein that was radiolabeled solely on Y15 underwent an additional phosphorylation upon incubation in the interphase extracts, which could be observed as an upward mobility shift during SDS gel electrophoresis (Figure 4A). Because this shift did not occur if the T14A mutant of Cdc2 was exchanged for the wild-type Cdc2 protein (our unpublished observation), we attribute this mobility shift to phosphorylation on T14. Significantly, by this assay method, we could observe no difference in the rate of T14 phosphorylation between extracts that contained aphidicolin and comparable extracts that either lacked aphidicolin or contained both caffeine and aphidicolin (Figure 4A, lanes f-t; the top and bottom arrows denote the T14/Y15 doubly phosphorylated and Y15 singly phosphorylated forms of Cdc2, respectively). This experiment suggests that T14-specific kinase activity is not up-regulated in the presence of unreplicated DNA, at least as measured by the ability of this kinase to act upon Cdc2 that had been phosphorylated previously on Y15.

Rates of T161 Phosphorylation and Dephosphorylation in Checkpoint-arrested Extracts

It is well established that phosphorylation of Cdc2 on T161 is essential for its catalytic activity. To examine the possibility that phosphorylation of T161 might be affected in the presence of unreplicated DNA, we prepared a complex between cyclin B and the ^{35}S-labeled T14AY15F mutant of Cdc2 that can be phosphorylated only on T161 (Figure 5). Upon addition of this complex to *Xenopus* egg extracts, the phosphorylation of the radiolabeled Cdc2 protein on T161 could be detected as a downward mobility shift during gel electrophoresis (Figure 5A). Because the dephosphorylation of T161 occurs very slowly in interphase extracts (Lee *et al.*, 1994; see Figure 6), this assay detects the rate of T161 phosphorylation selectively. We observed that T161 phosphorylation in *Xenopus* extracts occurred rapidly with a half-time of approximately 1 min, but this rate did not vary depending upon the presence of unreplicated DNA or caffeine (Figure 5A,

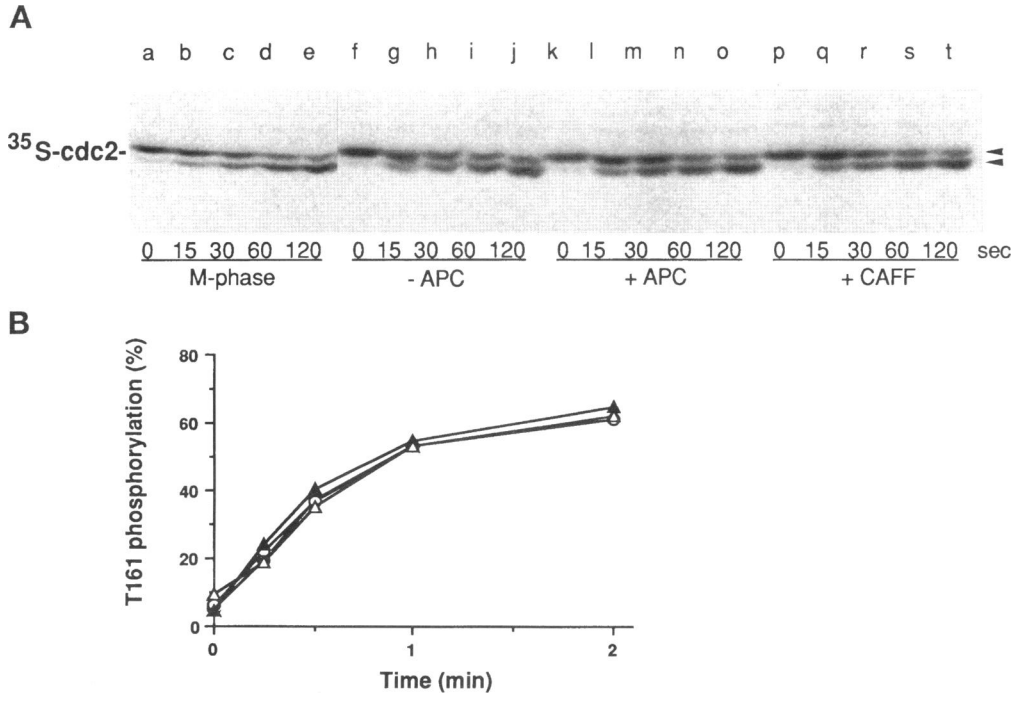

Figure 5. The rate of T161 phosphorylation is not affected in aphidicolin-treated extracts. (A) A complex between cyclin B and ^{35}S-labeled Cdc2-AF protein was purified as described in MATERIALS AND METHODS. The ^{35}S-labeled complex was added to M-phase extracts (lanes a-e) or to interphase extracts containing: no nuclei (lanes f-j); 1000 nuclei per μl of extract and 100 μg/ml aphidicolin (lanes k-o); or 1000 nuclei per μl, 100 μg/ml aphidicolin, and 5 mM caffeine (lanes p-t). At the indicated times (in seconds), aliquots were subjected to SDS electrophoresis in a 10% polyacrylamide-PDA gel. (B) Quantitation of the data in A. Symbols are the same as in Figure 4.

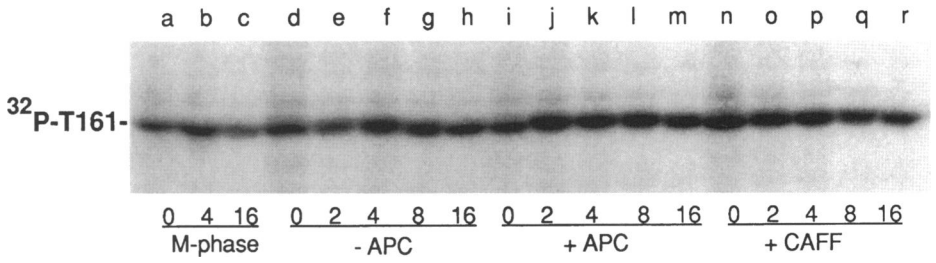

Figure 6. The rate of T161 dephosphorylation is not accelerated in *Xenopus* egg extracts arrested in S-phase. A complex between cyclin B and the Cdc2-AF protein that was radiolabeled with ^{32}P on T161 was purified as described in MATERIALS AND METHODS. The ^{32}P-labeled complex was added either to M-phase extracts (lanes a-c) or to interphase extracts containing: no nuclei (lanes d-h); 1000 nuclei per μl of egg extract and 100 μg/ml aphidicolin (lanes i-m); or 1000 nuclei per μl, 100 μg/ml aphidicolin, and 5 mM caffeine (lanes n-r). At the indicated times (in minutes), aliquots were removed for electrophoresis in a 10% polyacrylamide-PDA gel.

lanes f-t; Figure 5B). Moreover, consistent with earlier reports (Solomon *et al.*, 1992; Brown *et al.*, 1994; Lee *et al.*, 1994), the rate of T161 phosphorylation was the same during both interphase and mitosis (Figure 5A, lanes a-j; Figure 5B).

Acting in opposition to CAK is an unknown phosphatase that dephosphorylates T161. To measure the activity of the T161-specific phosphatase, we prepared a ^{32}P-labeled Cdc2/cyclin B complex in which Cdc2 was phosphorylated specifically on T161 as described in MATERIALS AND METHODS. Upon the addition of this substrate to interphase egg extracts, the ^{32}P-labeled phosphate group was quite stable whether or not the extracts contained unreplicated DNA (Figure 6). Specifically, the phosphate levels on Cdc2 remained relatively constant for at least 16 min in interphase extracts that either lacked or contained aphidicolin (Figure 6, lanes d-m). Likewise, the presence of caffeine in addition to aphidicolin had no effect on T161 dephosphorylation (Figure 6, lanes n-r). In contrast, there was significant dephosphorylation of T161 in an M-phase extract by the latest time point (16 min; Figure 6, lane c), which corresponded to the time by which substantial degradation of cyclin B had occurred (our unpublished observation). Therefore, although this assay method can readily detect dephosphorylation of T161 when cyclin is degraded in mitotic extracts (Lorca *et al.*, 1992), the presence of unreplicated DNA has no effect upon this phosphatase activity. Collectively, these results suggest that neither phosphorylation nor dephosphorylation of T161 can account for the inability of Cdc2 to induce mitosis in the checkpoint-arrested extracts.

Unreplicated DNA Inhibits the Capacity of the Cdc2-AF Mutant to Induce Mitosis

The above results indicate that the inability of Cdc2/cyclin B to trigger mitosis in the presence of a DNA synthesis inhibitor cannot be explained solely on the basis of the stimulation or inhibition of the enzymes that regulate phosphorylation at known sites on the Cdc2 protein. Moreover, we demonstrated by tryptic phosphopeptide mapping that the overall phosphorylation pattern of Cdc2 was very similar in the presence and absence of aphidicolin (Figure 7, compare panels A and B), arguing against the involvement of novel phosphorylation sites. One prediction of these findings is that a Cdc2/cyclin B complex in which the Cdc2 subunit cannot be phosphorylated on Y15 or T14 also would be unable to induce mitosis in the presence of incompletely replicated DNA. To test this possibility, we prepared a complex between Δcyclin B and the Cdc2-AF protein as described above, except with the modification that the complex was purified further by gel filtration chromatography on a Superdex 200 PC column. This procedure allowed us to prepare a complex containing equimolar amounts of the Δcyclin and Cdc2-AF proteins that migrated with an apparent size of 100 kDa on the gel filtration column (see silver-stained gel of a typical preparation in Figure 8A). For these experiments, we prepared the catalytically active complex by allowing T161 phosphorylation of Cdc2 to occur before purification of the complex. In addition, we used only the Δcyclin B protein in these experiments because of a poor recovery of the full-length cyclin B/Cdc2 complex from the Superdex column.

As expected, the purified Δcyclin B/Cdc2-AF complex induced mitosis efficiently in cycloheximide-containing extracts that lacked aphidicolin (Figure 8C, closed squares in panels a, b, and c). The Δcyclin B/Cdc2-AF complex was very potent as a mitotic inducer: concentrations of 4 nM or greater were sufficient to trigger nuclear disassembly in the egg extracts. For comparison, it was necessary to add 16 nM of the isolated Δcyclin B protein to elicit mitosis.

In parallel, we added the Δcyclin/Cdc2-AF complex to aphidicolin-containing extracts. Significantly, there was a pronounced delay in the rate at which the Cdc2-AF protein could induce mitosis in these extracts relative to the control extracts, which lacked aphidicolin (Figure 8C, closed circles in panels a, b, and c). This aphidicolin-dependent delay varied with the amount of added Δcyclin/Cdc2-AF complex. At the threshold concentration that was sufficient to induce mitosis in a control extract, there was no nuclear en-

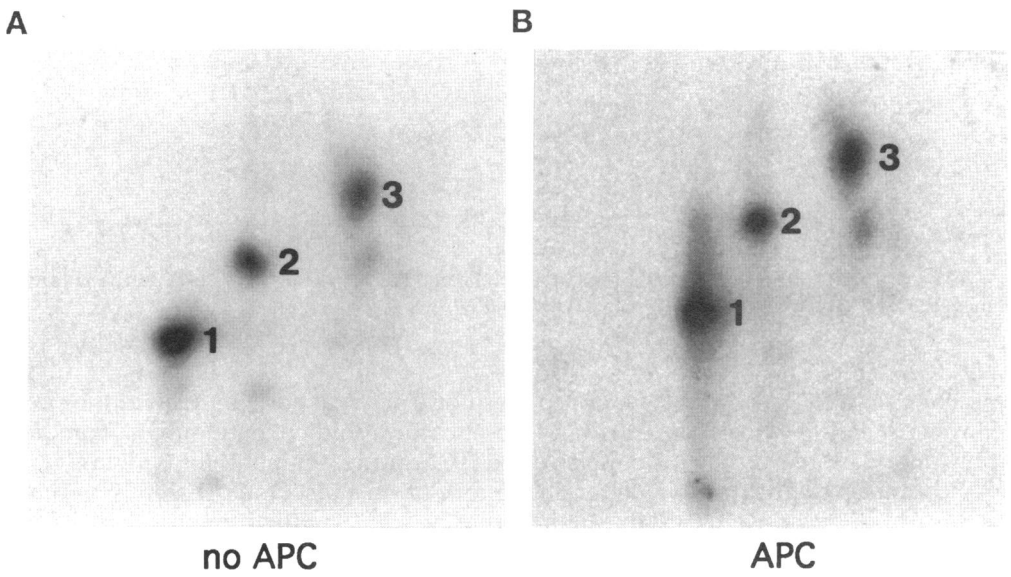

Figure 7. Phosphopeptide maps of the Cdc2 protein in the absence and presence of unreplicated DNA. The cyclin B protein (32 nM) was added to interphase extracts that had been pre-incubated with ^{32}P-orthophosphate. The extracts contained either no added nuclei (A) or 1000 nuclei per μl and 100 μg/ml aphidicolin (B). The peptides are phosphorylated on: T14 and Y15 (spot 1); Y15 alone (spot 2); or T161 (spot 3).

velope breakdown in the aphidicolin-containing extract, even after 120 min. At an intermediate concentration of Δcyclin/Cdc2-AF complex, there was only partial nuclear envelope breakdown (~30 to 40% of total) at 120 min, whereas nuclear disassembly was complete by 60 min in the control extract. Interestingly, the nuclei that did not break down in this incubation (60–70% of total) showed no indication that active Cdc2 might be present. In particular, the chromatin displayed no signs of mitotic condensation, and the nuclear envelope appeared fully intact by phase contrast microscopy (our unpublished observation). Finally, at the highest concentration of added complex in this experiment, there was still an obvious delay of approximately 20 min in the onset of mitosis in the aphidicolin-treated extract. In control experiments with ^{35}S-labeled Cdc2-AF protein, we showed that the Cdc2-AF protein remained stably associated with Δcyclin B under these conditions (Figure 8B, lanes c and d). Moreover, the Cdc2-AF protein in the aphidicolin-containing extract was present mainly in the T161-phosphorylated form (Figure 8B, lane d), as was the case in the control extract (Figure 8B, lane c).

To further characterize the reduced capacity of the Cdc2-AF protein to elicit mitosis in the presence of unreplicated DNA, we utilized drugs that override the replication checkpoint. We observed that the addition of caffeine could abrogate the inhibitory affect of aphidicolin upon the ability of the Cdc2-AF/Δcyclin B complex to trigger nuclear envelope disassembly (Figure 9A). Similarly, 3 μM okadaic acid could reverse the effect of aphidicolin upon the Cdc2-AF protein (our unpublished observation).

Next, we examined the H1 kinase activity of the Cdc2-AF/Δcyclin B after re-isolation from the extracts containing unreplicated DNA. For this purpose, we added the complex to extracts in the absence and presence of aphidicolin, re-isolated the complex with nickel-IDA beads, and measured Cdc2-associated H1 kinase activity (Figure 9B). Under these particular conditions, the Cdc2-AF/Δcyclin B complex isolated from the aphidicolin-containing extracts displayed an H1 kinase activity comparable to complexes that had been incubated either in the absence of aphidicolin or in the presence of both aphidicolin and okadaic acid (Figure 9B, lanes d-i). One interpretation of this observation is that the Cdc2-AF/Δcyclin B complex might have dissociated from an inhibitor during re-isolation of the soluble complex from the aphidicolin-containing extracts.

To evaluate this possibility, we asked whether a catalytically inert Cdc2/cyclin B complex could override the aphidicolin-dependent block to mitosis by acting as a competitor for the putative inhibitor (Figure 10). For this purpose, we utilized the Cdc2-T14AY15FT161A mutant (Cdc2-AFA), which cannot be phosphorylated on T14, Y15, and T161. In particular, we purified a complex between this triple mutant of Cdc2 and the Δcyclin B protein on a Superdex 200 PC column. Then, we added this mutant complex to an aphidicolin-containing extract that lacked cycloheximide and hence accumulated an endogenous, inactive Cdc2/cyclin B complex. The triply-mutated Cdc2 must always remain inactive as a kinase, so it normally cannot induce mitosis. However, upon addition to the aphidicolin-treated extracts, the recombinant Cdc2-AFA/Δcyclin B complex (10 nM) efficiently reversed the inhibition of mitosis (Figure 10, compare open and closed cir-

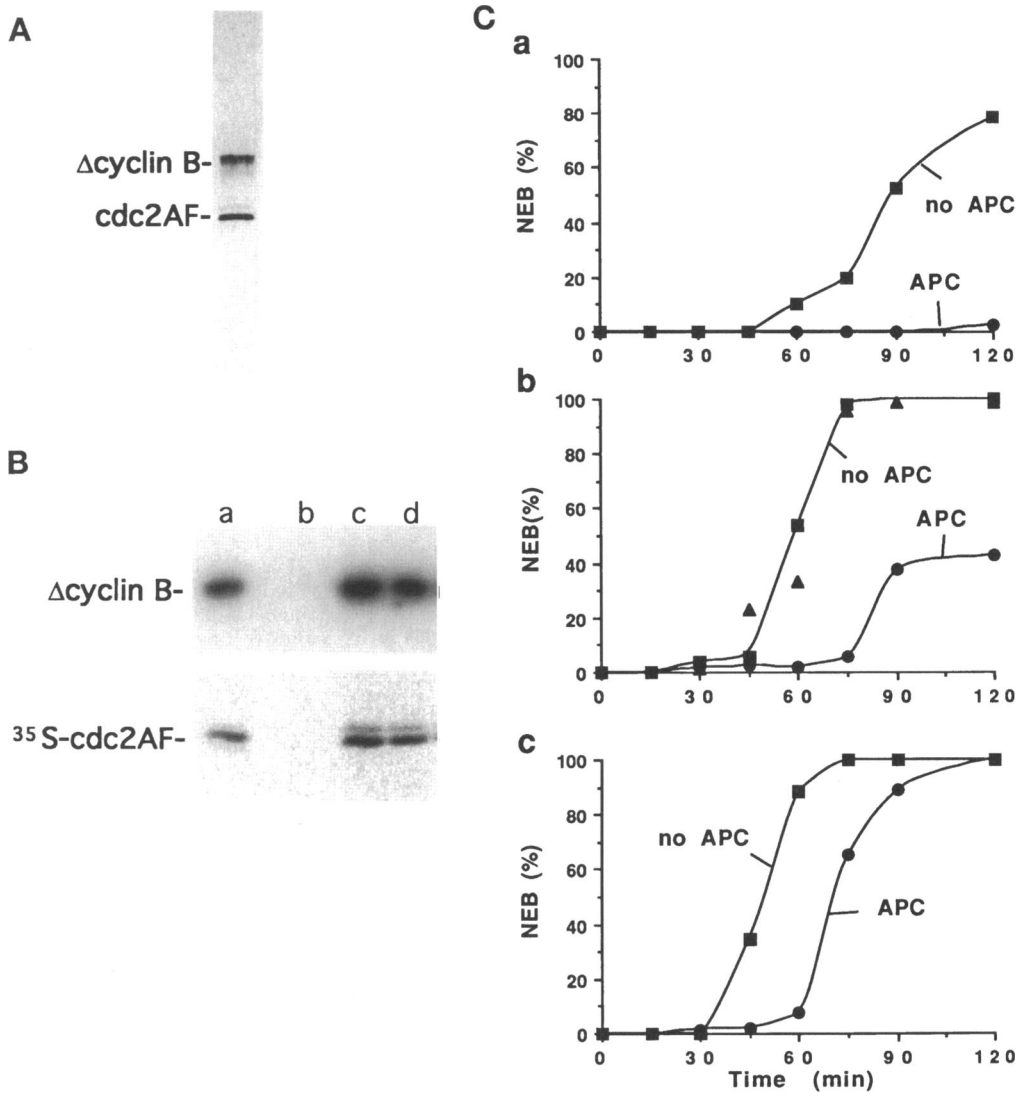

Figure 8. The Cdc2-AF mutant cannot induce mitosis efficiently in aphidicolin-containing extracts. (A) A complex between Δcyclin B and the Cdc2-AF mutant was purified by gel filtration on a SMART Superdex 200 PC column. Aliquots of the column fractions were subjected to gel electrophoresis and silver staining. The peak fraction of the complex is depicted. (B) The Cdc2-AF/Δcyclin B complex is stable in egg extracts. A complex in which the Cdc2 subunit was radiolabeled with ^{35}S (lane a) was added to interphase egg extracts containing no nuclei (lane c) or 1000 nuclei per μl of extract and 100 μg/ml aphidicolin (lane d). After a 30-min incubation, nickel-IDA beads were added. The complexes were isolated, subjected to SDS gel electrophoresis, and either immunoblotted with anti-cyclin B antibodies (top panel) or exposed directly to a phosphorimaging screen for detection of the ^{35}S-labeled Cdc2-AF protein. As a control (lane b), we added IDA agarose beads lacking nickel ions to an aliquot of the extract used in lane c. (C) The purified Cdc2-AF/Δcyclin B complex was added to interphase egg extracts at a final concentration of 4 nM (panel a), 6 nM (panel b), or 8 nM (panel c). The interphase extracts contained: 200 nuclei per μl of extract (closed squares); 1000 nuclei per μl (closed triangles, in panel b only); or 1000 nuclei per μl and 100 μg/ml aphidicolin (closed circles). At the indicated times, aliquots were removed, and nuclear envelope breakdown (NEB) was monitored by microscopy.

cles). As a control, we showed that even a twofold higher concentration of Cdc2-AFA mutant complex (20 nM) could not induce mitosis in cycloheximide-containing extracts (closed squares), demonstrating both that the mutant Cdc2 was indeed inactive and that its bound Δcyclin B partner could not activate the normal, endogenous Cdc2 protein in the extracts. These experiments suggest that a titratable inhibitor, which acts upon the Cdc2/cyclin B complex, interferes with mitotic initiation when incompletely replicated DNA is present. Further experiments have indicated that this inhibitory activity remains in the particulate fraction after isolation of the soluble Cdc2-AF/cyclin B complex (our unpublished observation), suggesting that the inhibitor might be tightly associated with the nucleus.

DISCUSSION

In these studies, we have examined the regulation of the Cdc2/cyclin B complex in *Xenopus* egg extracts, which cannot enter mitosis in the presence of the replication inhibitor aphidicolin (Dasso and Newport, 1990). This drug triggers the replication checkpoint, a regulatory system of unknown nature that prevents mitosis until the replication of the genome has reached completion (Hartwell and Weinert, 1989; Dasso and Newport, 1990). To study the mechanisms of this checkpoint-induced arrest of the cell cycle, we established an experimental system in which *Xenopus* extracts containing a recombinant Cdc2/cyclin B complex cannot undergo mitosis in the presence of aphidicolin. The cyclin subunit of the recombinant

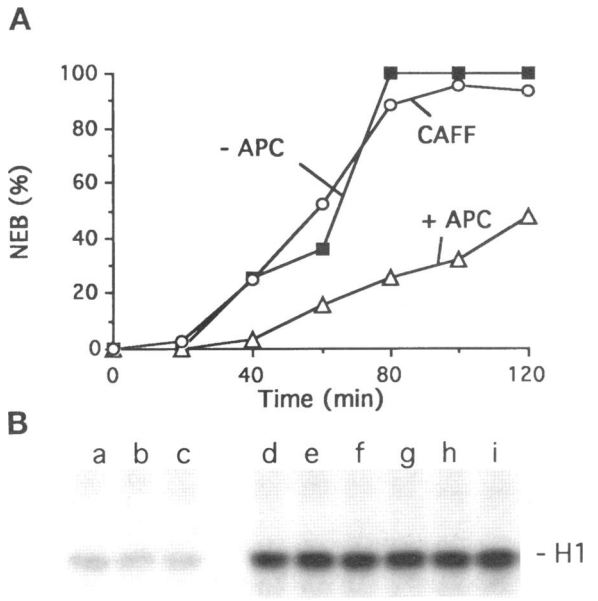

Figure 9. Characterization of the Cdc2-AF/Δcyclin B complex in aphidicolin-containing extracts. (A) The Cdc2-AF/Δcyclin B complex (6 nM) was added to interphase extracts containing: 1000 nuclei per μl (closed squares); 1000 nuclei per μl and 100 μg/ml aphidicolin (open triangles); or 1000 nuclei per μl, 100 μg/ml aphidicolin, and 5 mM caffeine (open circles). At the indicated times, NEB in the extracts was determined. (B) H1 kinase activities of the Cdc2-AF/Δcyclin B complexes after re-isolation from interphase extracts. The complex was isolated from the extracts with nickel-IDA after 15 min (lanes d, f, and h) or 30 min (lanes e, g, and i) of incubation. The interphase extracts contained: no nuclei (lanes a, d, and e); 1000 nuclei per μl and 100 μg/ml aphidicolin (lanes b, f, and g); or 1000 nuclei per μl, 100 μg/ml aphidicolin, and 3 μM okadaic acid (lanes c, h, and i). As a control, we demonstrated that there was no H1 kinase activity associated with nickel-IDA beads that had been added to extracts that lacked the recombinant Cdc2/cyclin complex (lanes a, b, and c).

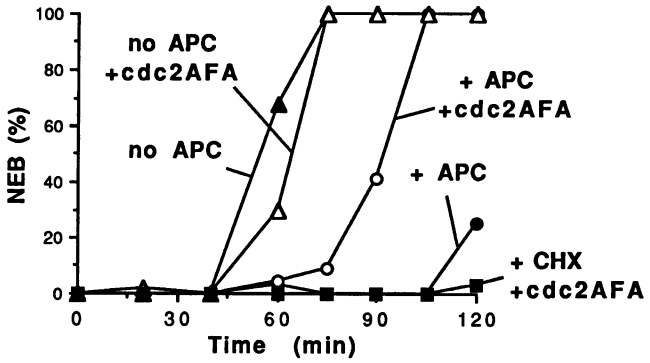

Figure 10. A complex between a triple-mutant Cdc2 protein (T14AY15FT161A) and Δcyclin B overcomes the aphidicolin-induced blocks to mitosis. Except as indicated, the interphase extracts lacked cycloheximide but contained 1000 nuclei per μl in the absence (closed triangles, open triangles, closed squares) or presence (open circles, closed circles) of 100 μg/ml aphidicolin. The Cdc2-T14AY15FT161A/Δcyclin B complex was added to a final concentration of either 10 nM (open triangles, open circles) or 20 nM (closed squares). The incubation denoted by the closed squares contained 100 μg/ml cycloheximide. NEB was quantitated at the indicated times. T14AY15FT161A is abbreviated as AFA.

Cdc2/cyclin B complex contains a six-histidine tag, so it was possible to re-isolate the complex from aphidicolin-treated extracts and biochemically characterize its constituents under conditions in which the extracts had remained stably arrested in interphase. To aid in these experiments, we were able to make use of a variety of Cdc2 mutants that contain amino acid substitutions at key phosphorylation sites. Thus, we could program the *Xenopus* extracts to undergo mitotic-checkpoint regulation with known amounts of a Cdc2/cyclin B complex of predetermined composition.

In cycling *Xenopus* extracts, newly synthesized cyclin B normally binds to Cdc2 and triggers the phosphorylation of Cdc2 on T161, Y15, and T14 (Solomon *et al.*, 1990). Later, one or more Cdc25 proteins generate active MPF by dephosphorylating Y15 and T14 (Galaktionov and Beach, 1991; Kumagai and Dunphy, 1991; Sebastian *et al.*, 1993). It seemed plausible that one of the enzymes regulating phosphorylation at Y15, T14, or T161 might be modulated directly by unreplicated DNA in checkpoint-arrested extracts. In the case of phosphorylation at Y15, it is well established that the experimental manipulation of the concentrations of the Cdc25 and Wee1 proteins can alter the length of interphase in both fission yeast and *Xenopus* extracts (Russell and Nurse, 1986, 1987; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Mueller *et al.*, 1995). Moreover, there is a report that aphidicolin induces an increase of total Cdc2-specific tyrosine kinase activity in *Xenopus* extracts (Smythe and Newport, 1992). However, in our studies, we could not detect an aphidicolin-dependent stimulation of Cdc2-specific tyrosine kinase activity. One explanation for the discrepancy might be that the GST-sea urchin cyclin B that was utilized to form the tyrosine kinase substrate in earlier studies can override the replication checkpoint in a dominant manner (Kornbluth *et al.*, 1992; Smythe and Newport, 1992). Finally, in other studies, we observed that aphidicolin did not increase the activity of a recently cloned *Xenopus* Wee1-like kinase (Mueller *et al.*, 1995). However, this observation does not eliminate the possibility that *Xenopus* extracts might contain additional Wee1-like kinases that could be regulated differently. The studies in this paper show that total Cdc2-specific tyrosine kinase activity is not modulated by the replication checkpoint.

We proceeded to analyze other enzymes that control the phosphorylation of Cdc2. First, we corroborated earlier results that indicated that the activity of the Cdc25 protein, which dephosphorylates Y15 and most probably T14, is not suppressed further below its normally low interphase levels by the presence of unrep-

licated DNA (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). Second, we could not detect an increase in T14-specific kinase activity in aphidicolin-containing extracts. Finally, the checkpoint did not appear to affect T161 phosphorylation or dephosphorylation of Cdc2, which is consistent with the fact that the Cdc2/cyclin B complex that accumulates in aphidicolin-treated extracts is fully phosphorylated on T161 (see Figure 9).

These paradoxical results were clarified when we observed that a Cdc2/cyclin B complex containing the Cdc2-AF mutant (which cannot be phosphorylated on Y15 or T14) displays an obvious reduction in its ability to induce mitosis in aphidicolin-containing extracts. This observation indicates that in the *Xenopus* system, another inhibitory mechanism that does not involve phosphorylation of Cdc2 on Y15 or T14 directs the suppression of MPF in the presence of unreplicated DNA. Obviously, it will be important to elucidate the molecular identity of this inhibitory factor and the mechanism by which unreplicated DNA controls its action.

In the yeast systems, the role of tyrosine phosphorylation in the replication checkpoint has been analyzed in a variety of genetic experiments. In the budding yeast *S. cerevisiae*, a strain harboring a mutant Cdc2/CDC28 protein that cannot be phosphorylated on either T18 or Y19 (the equivalents of T14 and Y15 in fission yeast and frogs) displays no defects in the replication checkpoint, and appears to grow normally in all other respects (Amon *et al.*, 1992; Sorger and Murray, 1992; Booher *et al.*, 1993; Stueland *et al.*, 1993). Moreover, Stueland *et al.* were able to demonstrate that the Cdc2/cyclin B complex in budding yeast arrested at the replication checkpoint was significantly smaller during gel filtration than the complex from control cells. These observations do not support the notion that the replication checkpoint in budding yeast operates exclusively through the inhibitory tyrosine and/or threonine phosphorylation of the Cdc2/CDC28 protein.

However, studies in fission yeast have suggested a different conclusion. Specifically, a fission yeast strain containing mutations in both the *wee1* and *mik1* genes (whose products collaborate in the tyrosine phosphorylation of Cdc2) cannot arrest its cell cycle in the presence of the replication inhibitor hydroxyurea (Lundgren *et al.*, 1991). Intriguingly, single mutants in either *mik1* or *wee1* display an apparently normal replication checkpoint, even though *wee1* mutants have obvious defects in G2 size control (Enoch and Nurse, 1990; Lundgren *et al.*, 1991; Rowley *et al.*, 1992; Barbet and Carr, 1993). Finally, a fission yeast strain containing a mutant Cdc2 that cannot be phosphorylated on Y15 does not undergo a mitotic delay in the presence of hydroxyurea (Gould and Nurse, 1989; Enoch *et al.*, 1991).

Our observations indicate that the replication checkpoint in *Xenopus* extracts shares common features with both the budding and fission yeast systems. On the one hand, we found that *Xenopus* extracts containing the Cdc2-AF mutant still undergo a pronounced mitotic delay in the presence of aphidicolin, which fits well with the observations in the budding yeast system. Conversely, the *Xenopus* extracts can tolerate only limited amounts of the mutant Cdc2-AF complex; at concentrations well above the threshold for mitotic initiation in control extracts, this mutant complex induces mitosis in the presence of aphidicolin, albeit with delayed kinetics (see also Norbury *et al.*, 1991). As a result, the extracts enter mitosis inappropriately, as is the case with fission yeast harboring the Y15F mutant of the Cdc2 protein.

A plausible interpretation of these findings is that a limiting inhibitor impedes the MPF-dependent induction of mitosis in the presence of unreplicated DNA. In parallel, one or more Wee1-like kinases, which are highly active in interphase egg extracts, phosphorylate Cdc2 on Y15 and T14, resulting in the formation of a stably inactivated Cdc2/cyclin B complex. In this scenario, the Cdc2-inhibitory tyrosine and threonine kinases would not be regulated directly by the replication checkpoint. However, these kinases would play an ancillary role in helping the checkpoint function more efficiently by stably turning off the Cdc2/cyclin B complex as its concentration increases throughout interphase due to ongoing cyclin synthesis. In *Xenopus* extracts, this back-up system appears to be important because high concentrations of the Cdc2-AF protein can override the replication checkpoint. It is possible that a similar situation may exist in fission yeast. In fission yeast, the replication checkpoint does not operate effectively when tyrosine phosphorylation of Cdc2 cannot occur, but conversely there is no evidence that Cdc2-specific tyrosine kinase activity is modulated by the presence of unreplicated DNA (Enoch and Nurse, 1990; Lundgren *et al.*, 1991; Rowley *et al.*, 1992). Finally, one might surmise that in budding yeast, the back-up system afforded by Cdc2-specific tyrosine phosphorylation is not essential.

Throughout this paper, we have addressed the problem of how unreplicated DNA leads to the initial suppression of the Cdc2/cyclin B complex. A related issue is how the inactive Cdc2/cyclin B complex that accumulates in interphase extracts containing unreplicated DNA eventually undergoes activation when replication is complete. In principle, the mechanism which impedes mitotic induction by the Cdc2-AF protein could be reversed at the end of S-phase. This event could lead to the formation of a small amount of active Cdc2, which has been hypothesized to be a trigger for the activation of Cdc25 and inactivation of Wee1 at mitosis (Solomon *et al.*, 1990; Clarke *et al.*, 1993; Izumi and Maller, 1993; Dunphy, 1994; Lee *et al.*,

1994). It is well established that the activation of Cdc25 and the inactivation of Wee1 at mitosis results from the extensive phosphorylation of their regulatory domains (reviewed in Dunphy, 1994). In the case of Wee1 and Cdc25, multiple kinases most probably contribute to this regulatory process, including Cdc2 and another unidentified enzyme referred to as kinase X (Tang *et al.*, 1993; Kuang *et al.*, 1994; Mueller *et al.*, 1995). It is not known whether kinase X is regulated by Cdc2 or controlled independently. In the latter case, it is conceivable that the completion of DNA replication could trigger the activation of kinase X (or the inactivation of a competing PP2A-like phosphatase). In this scheme, although Cdc25 and Wee1 would not direct the imposition of the checkpoint-dependent arrest of interphase, these enzymes could play a role in receiving a signal for the completion of S-phase.

In conclusion, we have demonstrated that the checkpoint-induced arrest of the *Xenopus* cell cycle in the presence of unreplicated DNA does not operate principally through the inhibitory tyrosine and threonine phosphorylation of Cdc2 but instead involves a titratable inhibitor of the Cdc2/cyclin B complex. The identification of this inhibitor and the elucidation of its regulation will greatly expand our understanding of the replication checkpoint.

ACKNOWLEDGMENTS

We thank our colleagues for comments on the manuscript. We are grateful to D. Morgan (University of California, San Francisco) for providing a baculovirus vector for histidine tagging. M. Solomon (Yale University) kindly provided the Cdc2 mutant plasmids. This work was supported by grants from the National Institutes of Health, Lucille P. Markey Charitable Trust, National Science Foundation, and Gustavus and Louise Pfeiffer Foundation. W.G.D. is an investigator of the Howard Hughes Medical Institute.

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