

Differential Contribution of Inhibitory Phosphorylation of CDC2 and CDK2 for Unperturbed Cell Cycle Control and DNA Integrity Checkpoints*

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Inhibition of cyclin-dependent kinases (CDKs) by Thr¹⁴/Tyr¹⁵ phosphorylation is critical for normal cell cycle progression and is a converging event for several cell cycle checkpoints. In this study, we compared the relative contribution of inhibitory phosphorylation for cyclin A/B1-CDC2 and cyclin A/E-CDK2 complexes. We found that inhibitory phosphorylation plays a major role in the regulation of CDC2 but only a minor role for CDK2 during the unperturbed cell cycle of HeLa cells. The relative importance of inhibitory phosphorylation of CDC2 and CDK2 may reflect their distinct cellular functions. Despite this, expression of nonphosphorylation mutants of both CDC2 and CDK2 triggered unscheduled histone H3 phosphorylation early in the cell cycle and was cytotoxic. DNA damage by a radiomimetic drug or replication block by hydroxyurea stimulated a buildup of cyclin B1 but was accompanied by an increase of inhibitory phosphorylation of CDC2. After DNA damage and replication block, all cyclin-CDK pairs that control S phase and mitosis were to different degrees inhibited by phosphorylation. Ectopic expression of nonphosphorylated CDC2 stimulated DNA replication, histone H3 phosphorylation, and cell division even after DNA damage. Similarly, a nonphosphorylation mutant of CDK2, but not CDK4, disrupted the G₂ DNA damage checkpoint. Finally, CDC25A, CDC25B, a dominant-negative CHK1, but not CDC25C or a dominant-negative WEE1, stimulated histone H3 phosphorylation after DNA damage. These data suggest differential contributions for the various regulators of Thr¹⁴/Tyr¹⁵ phosphorylation in normal cell cycle and during the DNA damage checkpoint.

Cyclins and cyclin-dependent kinases (CDKs)¹ are key regulators of the eukaryotic cell cycle. In mammalian cells, differ-

ent cyclin-CDK complexes are involved in regulating different cell cycle transitions: cyclin D-CDK4/6 for G₁ progression, cyclin E-CDK2 for the G₁-S transition, cyclin A-CDK2 for S phase progression, and cyclin A/B-CDC2 (CDK1) for entry into M phase (reviewed in Refs. 1 and 2).

The kinase activity of CDK is tightly regulated by an intricate system of phosphorylation and protein-protein interactions. By definition, the activation of CDKs is dependent on a cyclin subunit. Activation of CDKs requires binding to a cyclin partner and phosphorylation of a conserved threonine residue (Thr¹⁶¹ in CDC2). Inhibition of some CDKs is carried out by phosphorylation of residues close to the catalytic site (Thr¹⁴ and Tyr¹⁵ in CDC2), and binding to protein inhibitors of the p21^{CIP1/WAF1} and p16^{INK4A} families.

The regulation of the archetype CDK, CDC2, remains the best understood. CDC2 exists as an unphosphorylated and inactive monomer during G₁. During S phase, cyclin B1 accumulates and forms an inactive complex with Thr¹⁴-, Tyr¹⁵-, and Thr¹⁶¹-phosphorylated CDC2. This inactive cyclin B1-CDC2 complex continues to accumulate during G₂ until it is activated by dephosphorylation of Thr¹⁴ and Tyr¹⁵ during mitosis. CDC2 is inactivated when cyclin B1 is degraded by APC/C as the cell exit mitosis. The phosphorylation of CDC2 returns to the ground state in G₁ when Thr¹⁶¹ is dephosphorylated.

For mammalian CDC2, Thr¹⁴ and Tyr¹⁵ are phosphorylated by WEE1 and MYT1, and dephosphorylated by members of the CDC25 family. WEE1 is a dual specificity protein kinase that phosphorylates Tyr¹⁵ but not Thr¹⁴ (3). The activity of human WEE1 is suppressed during mitosis, when it is transiently hyperphosphorylated and degraded (4–6). Although the precise mechanism of how WEE1 is regulated is not completely understood, data from yeast and other organisms suggest that WEE1 is negatively regulated by phosphorylation of its N-terminal noncatalytic region, possibly by cyclin B1-CDC2 in a feedback loop. MYT1 is an endoplasmic reticulum- and Golgi complex-bound protein kinase that can phosphorylate both the Thr¹⁴ and Tyr¹⁵ residues of CDC2, but has a stronger preference for Thr¹⁴ (7, 8). MYT1 activity decreases during M phase, coinciding with the phosphorylation of its C-terminal domain. The C-terminal domain of MYT1 also has a separate inhibitory function for CDC2, as it can bind cyclin B1-CDC2 and prevent its entry into the nucleus (9, 10). Thr¹⁴ and Tyr¹⁵ are dephosphorylated by the dual specificity phosphatase CDC25. Mammalian cells contain three isoforms of CDC25 (A, B, and C), which are believed to function at discrete periods during the cell cycle (reviewed in Ref. 11). CDC25C is the major phosphatase that activates CDC2 for entry into mitosis, CDC25B may have roles in both G₂ and S phase, and CDC25A functions in G₁. Recently, CDC25A has also been implicated for the control of mitosis (12). Active cyclin B1-CDC2 phosphorylates the N-

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¹ The abbreviations used are: CDK, cyclin-dependent kinase; ADR, adriamycin; CMP, camptothecin; Dox, doxycycline; HU, hydroxyurea; GST, glutathione S-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; GFP, green fluorescent protein; BrdUrd, bromodeoxyuridine; TRITC, tetramethylrhodamine isothiocyanate.

terminal region of CDC25C and increases its activity (13), and phosphorylates CDC25A and stabilizes the phosphatase (12). These autocatalytic mechanisms lead to a rapid and complete activation of all the cyclin B1-CDC2 as cells enter mitosis.

For cyclin B-CDC2, the involvement of Thr¹⁴/Tyr¹⁵-dependent regulation appears to be well conserved through evolution. The notable exception is budding yeast, in which phosphorylation of Cdc28p (the CDC2 homolog) is dispensable for normal cell cycle progression (14, 15). The involvement of inhibitory phosphorylation in the regulation of other cyclin-CDK pairs during normal cell cycle is not as well defined. Cyclin A-CDC2 is activated and destroyed slightly ahead of cyclin B1-CDC2. Cyclin A-CDC2 is not regulated by inhibitory phosphorylation in *Xenopus* embryonic cell cycle (16), but is inhibited by phosphorylation in *Drosophila* (17). Vertebrate CDK2 is phosphorylated on Thr¹⁴ and Tyr¹⁵ during S phase and G₂, and can be activated by CDC25 (18–22). Whereas inhibitory phosphorylation is crucial for the regulation of CDC2 *in vivo*, the case for CDK2 is not as clear. It has been shown that inhibitory phosphorylation of CDK2 plays no essential role in *Drosophila* development (23). For vertebrate cells, ectopic expression of a nonphosphorylatable CDK2 in HeLa cells induces premature chromosome condensation (24), suggesting that inhibitory phosphorylation of CDK2 may play a role in normal cell cycle control. Similarly, G₁-S transition is blocked by microinjection of antibodies against CDC25A (25, 26) and is accelerated by ectopic expression of CDC25A (22, 27). However, whether exogenous CDC25A reduces inhibitory phosphorylation of CDK2 is a contentious issue (22, 27).

Proper control of cell cycle progression requires several checkpoint controls, which among other things, regulate the activities of CDKs to ensure that each stage of the cell cycle is completed before the next stage is initiated. Deregulation of checkpoints may allow cell cycle progression to become insensitive to genotoxic stress and give rise to genomic instability. The G₂ DNA damage checkpoint exerts its effects mainly through the inhibitory phosphorylation of CDC2 (reviewed in Ref. 28). Upon DNA damage, ATM/ATR phosphorylates and activates CHK1/CHK2, which in turn phosphorylates CDC25C on Ser²¹⁶. This either inactivates the phosphatase activity of CDC25C directly, or indirectly through the creation of a 14-3-3 binding site (29, 30). This tips the balance of Thr¹⁴/Tyr¹⁵ phosphorylation toward CDC2 inactivation. Destruction of CDC25A (12) and activation of WEE1 by CHK1 and 14-3-3 (31) may also contribute to Thr¹⁴/Tyr¹⁵ phosphorylation of CDC2 after DNA damage. In agreement with an essential role of inhibitory phosphorylation of CDC2 in the G₂ DNA damage checkpoint, expression of a nonphosphorylatable CDC2 impairs the irradiation-induced G₂ delay and CDC2 inactivation (24, 32).

It is believed that the checkpoint that responds to a block in replication is executed through similar mechanisms as the G₂ DNA damage checkpoints (reviewed in Ref. 33). In agreement with this, CDC2 is phosphorylated on Thr¹⁴/Tyr¹⁵ during replication block (34). Moreover, expression of a nonphosphorylatable CDC2 renders cells highly sensitive to killing by replication block (32).

The G₁ DNA damage checkpoint inhibits cyclin-CDK2 through mechanisms comprised of p53 and the CDK inhibitor p21^{CIP1/WAF1} (reviewed in Ref. 35). A parallel pathway that monitors DNA damage during G₁ and S phase involves inhibitory phosphorylation of CDK2. Several DNA damage-induced protein kinases, including ATM, ATR, CHK1, and CHK2, phosphorylate and promote the destruction of CDC25A (36, 37). This prevents the dephosphorylation of Thr¹⁴/Tyr¹⁵ in CDK2 and impairs both G₁-S transition and S phase progression. CDK2 participates in the phosphorylation of pRb and releases

the transcription factor E2F (reviewed in Ref. 38). E2F controls the expression of a number of genes required for S phase. CDK2 also phosphorylates components of the prereplication complex. This serves two purposes: one involves the loading of CDC45 and activation of the origin by phosphorylation of MCM proteins; the other involves prevention of re-replication by phosphorylation of CDC6 (39–41). Inhibitory phosphorylation of CDK4 also plays a role in the G₁ DNA damage checkpoint. CDK4 is phosphorylated on Tyr¹⁷ (the Tyr¹⁵ equivalent site) after UV-induced G₁ checkpoint, and ectopic expression of a nonphosphorylatable CDK4 abolished the checkpoint (42).

Recent data have placed some uncertainties on the functions of inhibitory phosphorylation of CDC2 in the DNA damage and replication checkpoints. Inhibitory phosphorylation of CDC2 in response to replication block is abolished in mammalian cells without ATR (34). However, the replication checkpoint remains intact in ATR knockout cells, suggesting that incomplete DNA replication can prevent M phase entry independently of Thr¹⁴/Tyr¹⁵ phosphorylation of CDC2 (34).

In this study, we compared the relative contribution of inhibitory phosphorylation for cyclin A/B1-CDC2 and cyclin A/E-CDK2 complexes during unperturbed cell cycle, DNA damage, and replication block. We found that inhibitory phosphorylation plays a critical role in the regulation of CDC2, but only a minor role for CDK2 during unperturbed cell cycle. However, expression of nonphosphorylation mutants of both CDC2 and CDK2 is cytotoxic. We further investigated the relative contribution of inhibitory phosphorylation of CDC2, CDK2, CDK4, as well as regulators of Thr¹⁴/Tyr¹⁵ (CDC25 family and WEE1) in the DNA damage checkpoints.

EXPERIMENTAL PROCEDURES

Materials—All reagents were obtained from Sigma unless stated otherwise.

DNA Constructs—All clones in this study were from human origin. CDC2 cDNA was amplified by PCR with the primers 5'-GAGAATTC-ATGGAAGATTATACCAAAA-3' and 5'-TCGAATTCCTACATCTTCTT-ATCTG-3' (CDC2 reverse). The PCR fragment was cut with *EcoRI* and ligated into pUHD-P1 (43) to produce FLAG-CDC2 in pUHD-P1. CDC2(T14A/Y15F) mutation was introduced by PCR with the primers 5'-GAGAATTCATGGAAGATTATACCAAAAATAGAGAAAATTGG-AGAAGGTGCCTTTGGAG-3' and CDC2 reverse, cleaved with *EcoRI*, and ligated into pUHD-P1/PUR (pUHD-P1 with a puromycin-resistant gene introduced into the *BamHI* site) to produce FLAG-CDC2(AF) in pUHD-P1/PUR. FLAG-CDK2 in pUHD-P1 was created as described (44). The CDK2(T14A/Y15F) mutation was introduced with a PCR method (45) using the primer 5'-GGAGAGGGCGCGTTCGGAGTTGTG-3' and its antisense, and put into pUHD-P1/PUR. CDK4-HA construct was a gift from Motoaki Ohtsubo (Hiroshima University, Japan). The *BamHI*-*BamHI* fragment of CDK4-HA was subcloned into pUHD-P1 to obtain FLAG-CDK4-HA in pUHD-P1. The Y17F mutation was introduced by PCR using the primer 5'-GACCATGGCTACCTCTCGATATGAGCCAGTGGCTGAAATTGGTGTCTGGTGCCTTTGGGA-3' and a pUHD-P1 reverse primer. The PCR fragment was cut with *NcoI*-*BamHI*, and ligated into pUHD-P1 to produce FLAG-CDK4(Y17F)-HA in pUHD-P1. Constructs for GST-CDC25B (46) and β -galactosidase (47) were obtained for sources as described previously. GST-CDC25A in pGEX-KG, 3HA-CDC25A in pCAGGS, and 3HA-CDC25B in pCAGGS were gifts from Katsumi Yamashita (Kanazawa University, Japan). CDC25A was amplified by PCR with a pCAGGS reverse primer and 5'-ACCATGGCTGGGGCCAGCCCCAA-3'; the PCR product was cleaved with *NcoI*-*HindIII* and ligated into pUHD-P2 (44) to create HA-CDC25A(N Δ 294) in pUHD-P2. The CDC25C cDNA was amplified by PCR with the primers 5'-CACCATGGGGGCAAAAAGGCCCCG-3' and 5'-AGGATCCTCATGGGCTCATGTCTT-3'; the PCR product was cleaved with *NcoI*-*BamHI*, and ligated into pUHD-P1 to obtain FLAG-CDC25C(N Δ 274) or into pUHD-P2 to obtain HA-CDC25C(N Δ 274). Mammalian expression constructs for WEE1 and WEE1(K328R) were gifts from Nobumoto Watanabe (RIKEN, Wako-shi, Japan). Construction of FLAG-CHK1(KR) will be described in detail elsewhere.² The

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histone H2B-GFP construct was a gift from Tim Hunt (Cancer Research, United Kingdom).

Cell Culture and Synchronization—HtTA1 cells are HeLa cells (human cervical carcinoma) expressing the tTA tetracycline repressor chimera (44). H1299 (non-small cell lung carcinoma) and Swiss 3T3 mouse fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). Chinese hamster ovary cells were obtained from Clontech (Palo Alto, CA). E1A-immortalized wild type mouse embryonic fibroblasts and p53^{-/-} mouse embryonic fibroblasts were gifts from Dr. Richard Woo (Hong Kong University of Science and Technology, Hong Kong). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum (for HtTA1) or fetal bovine serum (for H1299) in a humidified incubator at 37 °C in 5% CO₂. Cells were transfected with a calcium phosphate precipitation method (48). Cell-free extracts (49) and cell cycle synchronization by double thymidine block (50) were as described previously. For mitotic block synchronization, cells were first blocked with thymidine for 24 h before being released into medium containing nocodazole for 9 h. The cells were then washed and seeded into fresh medium without nocodazole. Unless stated otherwise, cells were treated with the following reagents at the indicated final concentration: adriamycin (ADR) (0.02 µg/ml), camptothecin (CMP) (700 nM), Dox (1 µg/ml), G418 (100 µg/ml), hydroxyurea (HU) (1.5 mM), nocodazole (0.1 µg/ml), puromycin (1 µg/ml), and thymidine (2 mM). Stable inducible cell lines were produced by co-transfection of FLAG-tagged CDKs in pUHD-P1/PUR and pLINX (a gift from Tony Hunter, The Salk Institute, La Jolla, CA) into H1299. Two days after transfection, the cells were then selected in medium supplemented with puromycin, G418, and Dox. After about 3 weeks of selection, individual colonies were isolated and expanded. The expression of FLAG-tagged proteins was analyzed after growing cells in medium in the presence or absence of Dox for 24 h. The established cell lines were propagated in the presence of Dox but without other antibiotic selection.

Cell Growth Analysis—Cell growth was measured by seeding ~10⁴ cells/60-mm plate and recording an image from the same randomly selected areas (within 2-mm diameter circles fixed at the bottom of the plate) every 24 h using a light microscope and a cool CCD camera. The number of attached cells was counted and the population doubling time was estimated by plotting the log of cell number against time. For clonogenic survival assays, 500 cells were seeded per 60-mm plate either in the presence or absence of Dox. Dox was supplemented into the medium at the indicated time and fresh medium (with or without Dox) was replenished every 3 days. After 2 weeks, colonies were fixed with methanol:acetic acid (2:1, v/v) and visualized by staining with 2% (w/v) crystal violet in 20% methanol. For β-galactosidase staining, cells were fixed in 0.05% (v/v) glutaraldehyde in PBS at 25 °C for 10 min. The cells were then washed four times with PBS, and stained with a solution containing 20 mM K₃Fe(CN)₆, 20 mM K₃Fe(CN)₆·3H₂O, 2 mM MgCl₂, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in PBS at 37 °C for 16 h.

Flow Cytometry—Flow cytometry analysis after propidium iodide staining were performed as described (51). For double analysis of cell cycle and histone H3 phosphorylation, cells were harvested by trypsinization, fixed in ice-cold 80% EtOH, and washed twice with PBST (PBS supplemented with 0.5% (v/v) Tween 20 and 0.05% (w/v) bovine serum albumin). The cell pellet was resuspended in the residue buffer and incubated with 1 µg of primary antibody at 25 °C for 30 min. The cells were washed twice with PBST, resuspended in the residue buffer, and incubated with 2.5 µl of fluorescein isothiocyanate-conjugated anti-mouse IgG or fluorescein isothiocyanate-conjugated anti-rabbit IgG antibodies (DAKO, Glostrup, Denmark) at 25 °C for 30 min. After washing once in PBST, the cells were processed for propidium iodide staining and two-dimensional flow cytometry analysis. In some experiments, transfected cells were distinguished from non-transfected cells by co-transfection of a membrane-targeted GFP reporter (52) (a gift from Wai Jiang and Tony Hunter, The Salk Institute). Cells were harvested by propidium iodide staining and flow cytometry as usual, and the DNA content of GFP-positive and GFP-negative cells was analyzed.

BrdUrd Incorporation—Cells were incubated in medium supplemented with 10 µM BrdUrd for 2 h. The cells were then harvested and fixed with 80% ethanol. After washing twice with PBS, the cells were treated with 2 M HCl at 25 °C for 20 min. The cells were washed twice with PBS followed by once with PBST. The cell pellet was resuspended in residue buffer and incubated with 2 µl of anti-BrdUrd antibody (BD Biosciences) at 25 °C for 30 min. The cells were then washed twice with PBST and incubated with 5 µl of fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibodies (DAKO) at 25 °C for 30 min. The cells

were washed with PBST, and processed for propidium iodide staining and two-dimensional flow cytometry analysis.

Phosphatase Treatment and Histone H1 Kinase Assays—Expression of recombinant GST-CDC25A and GST-CDC25B proteins in bacteria and purification with GSH-agarose chromatography were as described (49). Immunoprecipitates were incubated with 1 µg of GST fusion proteins in 10 µl of phosphatase buffer (10 mM HEPES, pH 7.2, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol) at 25 °C for 30 min. After washing in kinase buffer (80 mM Na β-glycerophosphate, 20 mM EGTA, 15 mM MgOAc, 1 mM dithiothreitol), the histone H1 kinase activity was assayed as described previously (46). Phosphorylation was quantified with a PhosphorImager (Amersham Biosciences).

Antibodies and Immunological Methods—Immunoblotting and immunoprecipitation were performed as described (49). Indirect immunofluorescence microscopy were performed as described previously (53), except that the cells were centrifuged at 2,000 rpm for 5 min onto the coverslips just before fixing. Rat monoclonal antibody YL1/2 against tubulin, monoclonal antibodies E23 against cyclin A, A17 against CDC2, and AN4.3 against CDK2 were gifts from Tim Hunt and Julian Gannon (Cancer Research, UK). Polyclonal antibodies against cyclin E were gifts from Motoaki Ohtsubo (Hiroshima University, Japan). Rabbit polyclonal antibodies against CDC2 (54), CDK2 (54), cyclin A (43), cyclin B1 (50), FLAG tag (44), monoclonal antibody 12CA5 against HA tag (44), and monoclonal antibody M2 against FLAG tag (55) were obtained from sources as described previously. Monoclonal antibodies GNS1 against cyclin B1 (sc-245), HE12 against cyclin E (sc-247), F6 against CDC25A (sc-7389), polyclonal antibodies against CDC25C (sc-327), WEE1 (sc-325), and phospho-histone H3(Ser¹⁰) (sc-8656R) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Inhibitory Phosphorylation Plays a Major Role in CDC2 Regulation but Not CDK2 Regulation during Unperturbed Cell Cycle—To study the relative contribution of inhibitory phosphorylation in the control of CDC2 and CDK2, HeLa cells were synchronously released into the cell cycle from mitosis, and cell extracts were prepared at different time points (Fig. 1). The quality of the synchronization was indicated by immunoblotting for various cell cycle regulators (Fig. 1A). As expected, histone H3 was highly phosphorylated during mitosis, but decreased after the cells were released into G₁. Histone H3 phosphorylation peaked again later (*t* = 18 h), indicating that the cells progressed through at least one cell cycle synchronously. Cyclin E, cyclin A, and cyclin B1 were orderly synthesized and destroyed. On the other hand, the levels of their CDK partners remained relatively constant throughout the cell cycle.

To see whether cyclin A/B1-CDC2 and cyclin A/E-CDK2 complexes were inhibited by phosphorylation, they were immunoprecipitated from the synchronized lysates, treated with either GST-CDC25 or GST *in vitro*, and the kinase activities were then assayed (Fig. 1B). As expected, the kinase activities of CDC2 were high during mitosis, decreased as cells entered G₁, and resurged again in the next cell cycle. Treatment with recombinant CDC25 did not affect the activities of CDC2 isolated from mitosis. On the other hand, dephosphorylation by CDC25 robustly activated CDC2 isolated from interphase. These data indicate that CDC2 was largely inhibited by Thr¹⁴/Tyr¹⁵ phosphorylation outside mitosis. In agreement with this, immunoblotting analysis showed that cyclin A and cyclin B1 accumulated before the phosphorylation of histone H3 during the second mitosis (Fig. 1A). Similar analysis for CDK2 revealed that its activities fluctuated during the cell cycle as expected. However, the kinase activities were only slightly increased by CDC25 throughout the cell cycle. These results indicate that in contrast to CDC2, inhibitory phosphorylation plays a relatively minor role in the regulation of CDK2 during normal cell cycle.

An independent synchronization procedure from another position of the cell cycle was used to confirm the relative roles of inhibitory phosphorylation of CDC2 and CDK2. Cells were

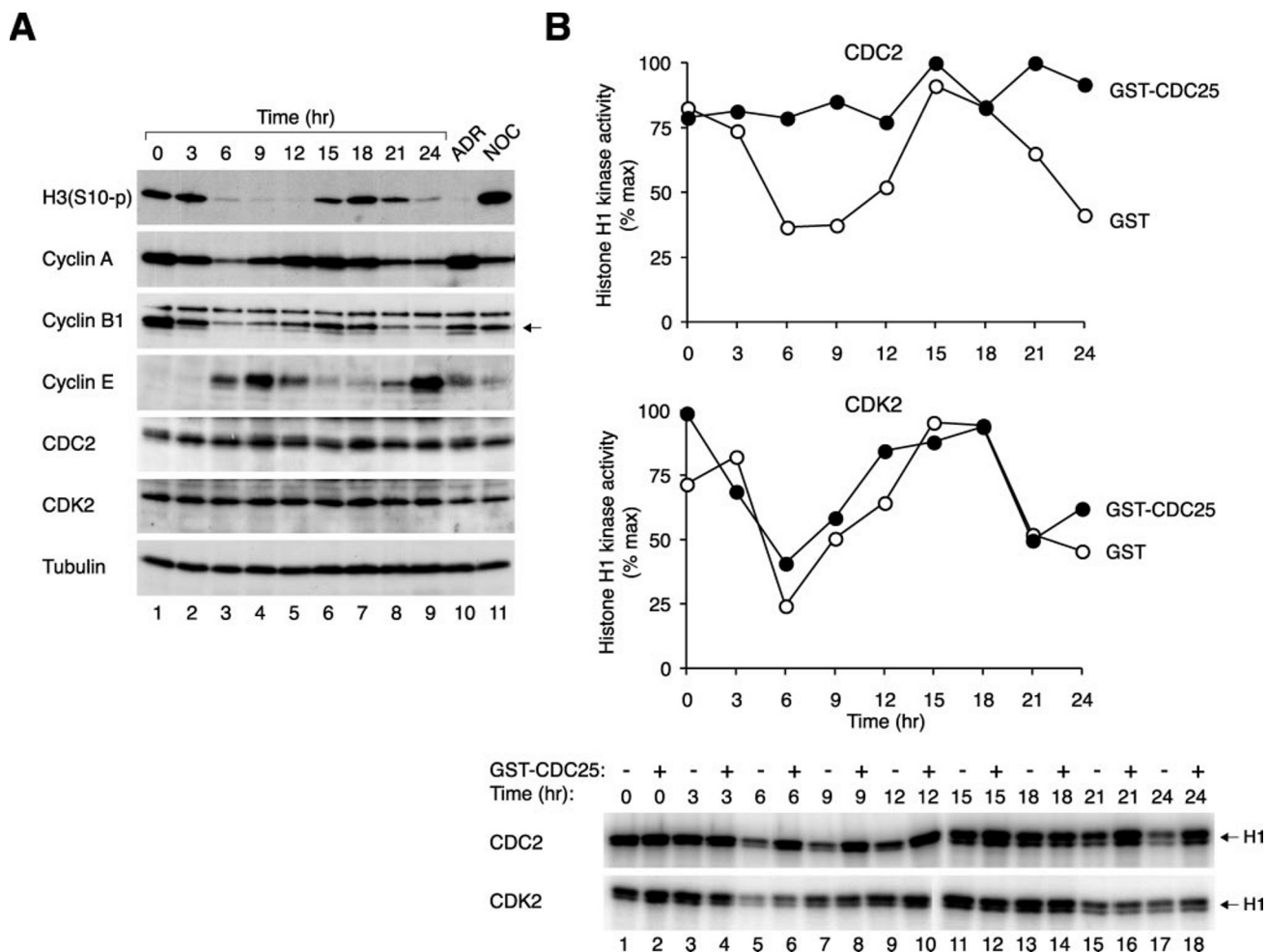


FIG. 1. Inhibitory phosphorylation plays a major role on CDC2 activity but a relatively minor role on CDK2 activity during normal cell cycle. *A*, cell cycle synchronization by mitotic block release. HtTA1 cells were synchronously released into the cell cycle from mitotic block as described under "Experimental Procedures." Cell-free extracts were prepared from samples taken at 3-h intervals, and were subjected to immunoblotting with antibodies against Ser¹⁰-phosphorylated histone H3, cyclin A, cyclin B1, cyclin E, CDC2, and CDK2 as indicated. HtTA1 cells treated with ADR (lane 10) and nocodazole (lane 11) for 16 h were included as controls. Immunoblotting for tubulin indicates similar sample loadings. *B*, activation of CDC2 and CDK2 by recombinant CDC25 in synchronized cell cycles. Cell-free extracts from different time points were prepared as described in panel *A*. CDC2 or CDK2 were immunoprecipitated and were treated with either GST-CDC25 (a mixture of GST-CDC25A and GST-CDC25B) (filled circles) or GST control (open circles). Histone H1 kinase activities were assayed as described under "Experimental Procedures" (bottom panel), and the phosphorylated bands were quantified with a PhosphorImager.

released from early S phase following a double thymidine block method (Fig. 2). Immunoblotting analysis indicated that the cells completed about one cell cycle synchronously after they were released from the block. Histone H3 phosphorylation was at a low level during S phase, accumulated to a maximum during mitosis before returning to basal level. Cyclin E was destroyed before cyclin A and cyclin B1, and peaked again in the next cycle. We found that CDC2 was inhibited by phosphorylation from the initial S phase block up to mitosis (Fig. 2B). Unlike synchronization from mitosis described above, CDK2 was also inhibited by Thr¹⁴/Tyr¹⁵ phosphorylation during the initial S phase block. This was likely to be the effect of the thymidine-imposed replication checkpoint, as CDK2 was no longer inhibited by phosphorylation during the next S phase.

Taken together, these data show that inhibitory phosphorylation plays a major role in the regulation of CDC2. In contrast, only a minor portion of CDK2 is regulated by inhibitory phosphorylation during unperturbed cell cycle, but inhibitory phosphorylation of CDK2 becomes important during the replication checkpoint.

Inactivation of CDC2 by Thr¹⁴/Tyr¹⁵ Phosphorylation during Checkpoint Activation Is Accompanied by a Buildup of Cyclin B1—It is well established that one of the major mechanisms of

the G₂ DNA damage checkpoint and replication checkpoint is by inhibitory phosphorylation of CDC2. However, we found that CDC2 was not inactivated after DNA damage or replication block in a variety of cell lines. We used the ribonucleotide reductase inhibitor HU to activate the replication checkpoint, and the topoisomerase II inhibitor ADR to activate the G₂ DNA damage checkpoint. The checkpoints induced by HU and ADR effectively blocked HeLa cells in early S phase and late S phase/G₂, respectively (Fig. 3A).

The levels of CDC2 and CDK2 were unaltered after HU or ADR treatments (Fig. 3B), but CDC2 was shifted to a slower mobility form representing Thr¹⁴/Tyr¹⁵ phosphorylation (56). Not all the CDC2 was phosphorylated upon ADR or HU treatments. This is well established because only the cyclin-bound form of CDC2 can be phosphorylated, and CDC2 is in excess of its cyclin partner. Analysis of the CDC2 after immunoprecipitation of cyclin B1 confirmed that all the cyclin B1-associated CDC2 were in the slower mobility form (Fig. 3C). Moreover, the mobility of the cyclin B1-associated CDC2 was restored to the basal state after dephosphorylation with GST-CDC25.

Up to half of the CDC2 were phosphorylated after ADR or HU treatment (Fig. 3B). These data are inconsistent with the relative levels of cyclins and CDC2 in the cell. We have directly

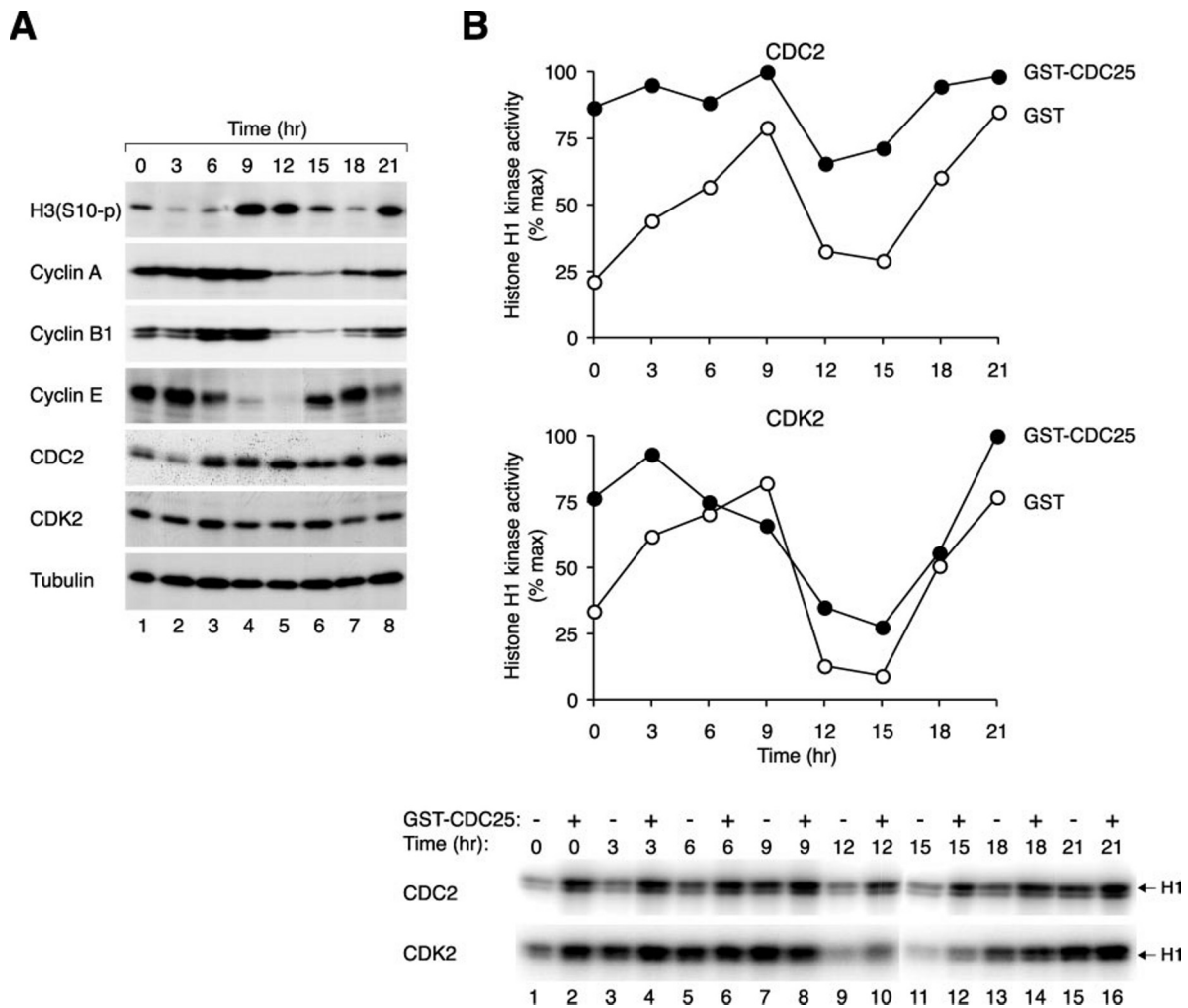


FIG. 2. The relative role of inhibitory phosphorylation of CDC2 and CDK2 during double thymidine synchronization. *A*, cell cycle synchronization by double thymidine block. HtTA1 cells were synchronously released into the cell cycle after double thymidine block as described under "Experimental Procedures." Cell-free extracts were prepared from samples taken at 3-h intervals, and were subjected to immunoblotting with antibodies against Ser¹⁰-phosphorylated histone H3, cyclin A, cyclin B1, cyclin E, CDC2, and CDK2 as indicated. Immunoblotting for tubulin indicates similar sample loadings. *B*, activation of CDC2 and CDK2 by recombinant CDC25. Cell-free extracts from different time points were prepared as described in *panel A*. CDC2 or CDK2 were immunoprecipitated and were treated with either GST-CDC25 (filled circles) or GST control (open circles). The histone H1 kinase activities were assayed as described under "Experimental Procedures" (*bottom panel*), and the phosphorylated bands were quantified with a PhosphorImager.

quantified the amount of cyclins and CDKs in HeLa cells, and found that the levels of cyclin A and cyclin B1 are ~30 times less than CDC2 during normal G₂ (50). One possibility is that during ADR- or HU-induced checkpoints, the cyclin partners of CDC2 accumulated to a level much higher than in normal G₂. In support of this hypothesis, we found that there was a significant accumulation of cyclin B1 following ADR treatments in a variety of mammalian cell lines (Fig. 3, *D* and *F*). Similarly, cyclin B1 accumulated in mammalian cells after HU treatment (Fig. 3*E*). We used serial dilution of the samples to estimate the increase in cyclin B1 after checkpoint activation (Fig. 3*F*). After normalizing the kinase activities of CDC2 with the levels of cyclin B1, we found that CDC2 was inactivated after ADR or HU treatments (Fig. 3*G*).

Taken together, these data show that although CDC2 is phosphorylated after DNA damage and replication block, it is not necessarily inactivated when equal amounts of cell extracts are assayed. This is in part because of the enrichment of cyclin B1 as cells are blocked in S phase and G₂ by the checkpoints.

Cyclin-CDK Complexes That Control S Phase to Mitosis Are Regulated to a Different Degree by Inhibitory Phosphorylation—There remain several unanswered questions concerning

inhibitory phosphorylation of CDKs. In particular, it is not clear whether cyclin A-, cyclin B1-, and cyclin E-bound CDKs are equally regulated by inhibitory phosphorylation in growing cells. It is also not clear whether all these cyclin-CDK pairs are concurrently phosphorylated on Thr¹⁴/Tyr¹⁵ after DNA damage and replication block.

The kinase activities of different cyclin-CDK complexes were assayed after immunoprecipitation. The kinase activities associated with cyclin B1 immunoprecipitates from growing cells were activated by either CDC25A or CDC25B (Fig. 4*A*). A significantly larger portion of cyclin B1-CDC2 was inhibited by Thr¹⁴/Tyr¹⁵ phosphorylation after ADR or HU treatments. Cyclin A-CDC2/CDK2 (Fig. 4*B*) and cyclin E-CDK2 (Fig. 4*C*) complexes from growing cells were also inhibited by phosphorylation. In contrast to cyclin B-CDC2, however, CDC25A/CDC25B only slightly activated cyclin A- and cyclin E-associated kinases after ADR or HU treatments. These data indicate that the various complexes that control S phase to mitosis: cyclin E-CDK2, cyclin A-CDC2/CDK2, and cyclin B1-CDC2, are inhibited to different degrees by Thr¹⁴/Tyr¹⁵ phosphorylation after DNA damage and replication block.

Ectopic Expression of Nonphosphorylatable CDC2 or CDK2 Is Cytotoxic in H1299—Ectopic expression of nonphosphorylat-

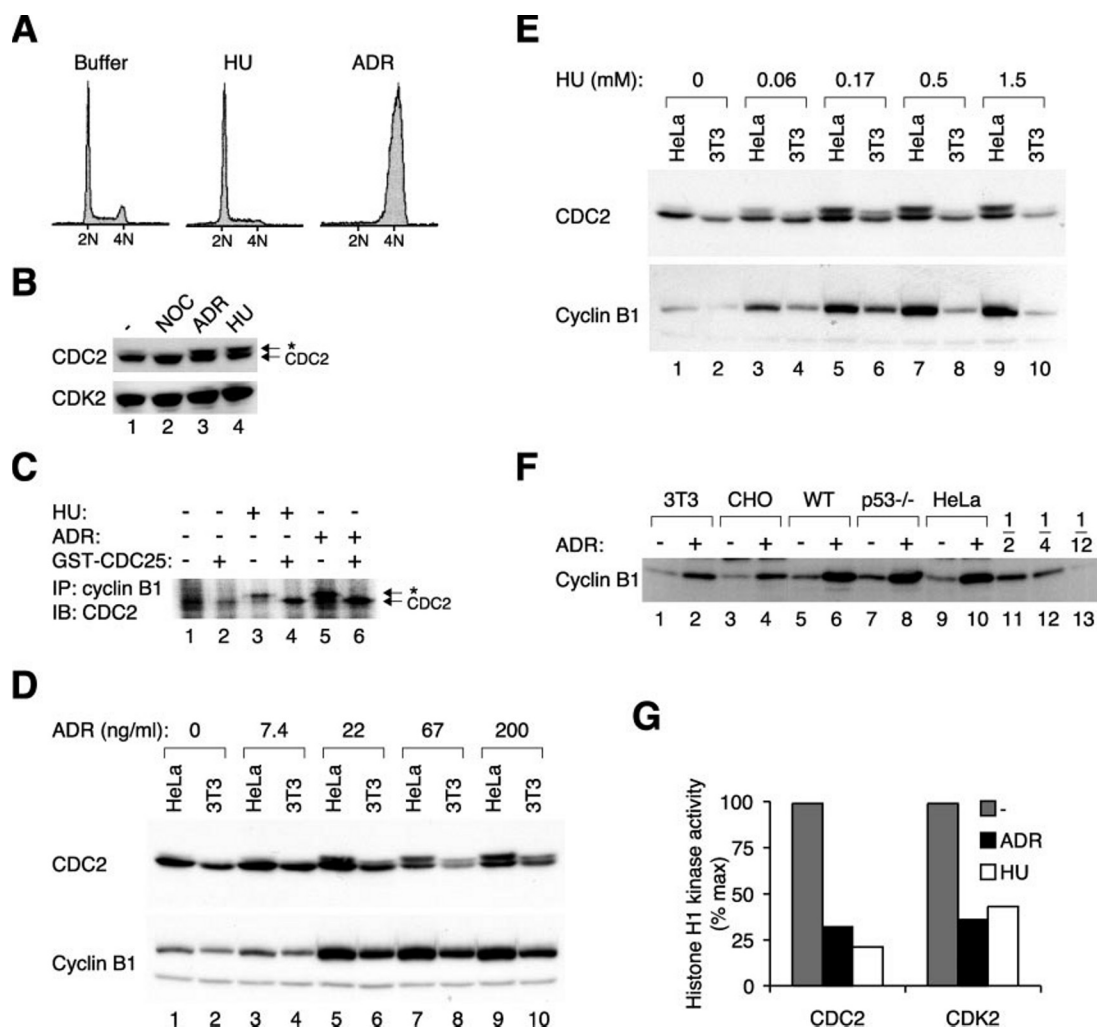


FIG. 3. Inactivation of CDC2 and CDK2 during DNA damage checkpoint and replication checkpoint is accompanied by a buildup of cyclin B1. *A*, cell cycle arrest induced by HU and ADR. HtTA1 cells were treated with buffer, HU, or ADR for 16 h before harvesting for flow cytometry analysis. The positions of 2 N and 4 N DNA content are indicated. *B*, gel mobility shifts of CDC2 during checkpoint activation. HtTA1 cells were treated with buffer, nocodazole, ADR, or HU as indicated. Cell extracts were prepared after 16 h and were subjected to immunoblotting (IB) for CDC2 and CDK2. The asterisk indicates the position of the Thr¹⁴/Tyr¹⁵-phosphorylated forms of CDC2. *C*, cyclin B1-bound CDC2 is completely phosphorylated during checkpoint activation. HtTA1 cells were treated with buffer, HU, or ADR for 16 h as indicated. Cell extracts were prepared and were subjected to immunoprecipitation with antiserum against cyclin B1. The immunoprecipitates (IP) were treated with either GST control or GST-CDC25. CDC2 was detected by immunoblotting. The asterisk indicates the position of the Thr¹⁴/Tyr¹⁵-phosphorylated forms of CDC2. *D*, accumulation of cyclin B1 during the DNA damage checkpoint. HeLa and Swiss 3T3 cells were treated with the indicated concentrations of ADR for 24 h. Cell extracts were prepared and were subjected to immunoblotting for cyclin B1 and CDC2. *E*, accumulation of cyclin B1 during the DNA replication checkpoint. HeLa and Swiss 3T3 cells were treated with the indicated concentrations of HU for 24 h. Cell extracts were prepared and were subjected to immunoblotting for cyclin B1 and CDC2. *F*, cyclin B1 accumulates after DNA damage in a variety of cell lines. Swiss 3T3, Chinese hamster ovary (CHO), wild type mouse embryonic fibroblast, p53^{-/-} mouse embryonic fibroblast, and HtTA1 cells were treated with either buffer or ADR for 16 h. Cell extracts were prepared and subjected to immunoblotting for cyclin B1. Serial dilutions of the sample in lane 10 were loaded (lanes 11–13) to estimate the increase of cyclin B1 after ADR treatment. *G*, checkpoints inactivate both CDC2 and CDK2. HtTA1 cells were treated with buffer, ADR, or HU for 16 h. Cell extracts were prepared, subjected to immunoprecipitation with antiserum against CDC2 or CDK2, and the histone H1 kinase activities were assayed. For CDC2, the kinase activity was normalized with the level of cyclin B1.

able mutants of CDC2 and CDK2 induces premature chromosome condensation and cytotoxicity (18, 24, 32). Given that CDC2 (and to a lesser extent CDK2) could be activated by CDC25 at multiple points of the cell cycle (Figs. 1 and 2), we wished to understand at what stages of the cell cycle do CDC2 and CDK2 mutants induce mitotic events.

Because the cytotoxicity triggered by CDC2 and CDK2 mutants has only been demonstrated in HeLa cells (18, 24, 32), we first confirmed that the effects of CDC2 and CDK2 mutants are not only limited for HeLa cells. The Thr¹⁴ and Tyr¹⁵ residues of CDC2 and CDK2 were mutated to nonphosphorylatable alanine and phenylalanine to create CDC2(AF) and CDK2(AF), respectively. The two mutants were tagged with a FLAG epitope to distinguish them from the endogenous proteins. Non-small cell lung carcinoma H1299 cells were transfected

with CDC2(AF) or CDK2(AF) expression constructs, and stable transfectants were established as described under "Experimental Procedures." Individual clones were isolated and inducible expression of CDC2(AF) or CDK2(AF) was confirmed by immunoblotting. The expression of CDC2(AF) (Fig. 5A) and CDK2(AF) (Fig. 5B) was suppressed in the presence of Dox. CDC2(AF) and CDK2(AF) were only transiently expressed after the removal of Dox (Fig. 5, C and D). Consistent with their expression, the kinase activities of CDC2(AF) and CDK2(AF) increased transiently after removal of Dox (Fig. 5E). These results raised the possibility that expression of CDC2(AF) and CDK2(AF) were toxic, and the cells could only tolerate a transient expression of these proteins. In agreement with this, induction of CDC2(AF) or CDK2(AF) severely compromised cell proliferation (Fig. 5F). As a control, Dox had no effect on pro-

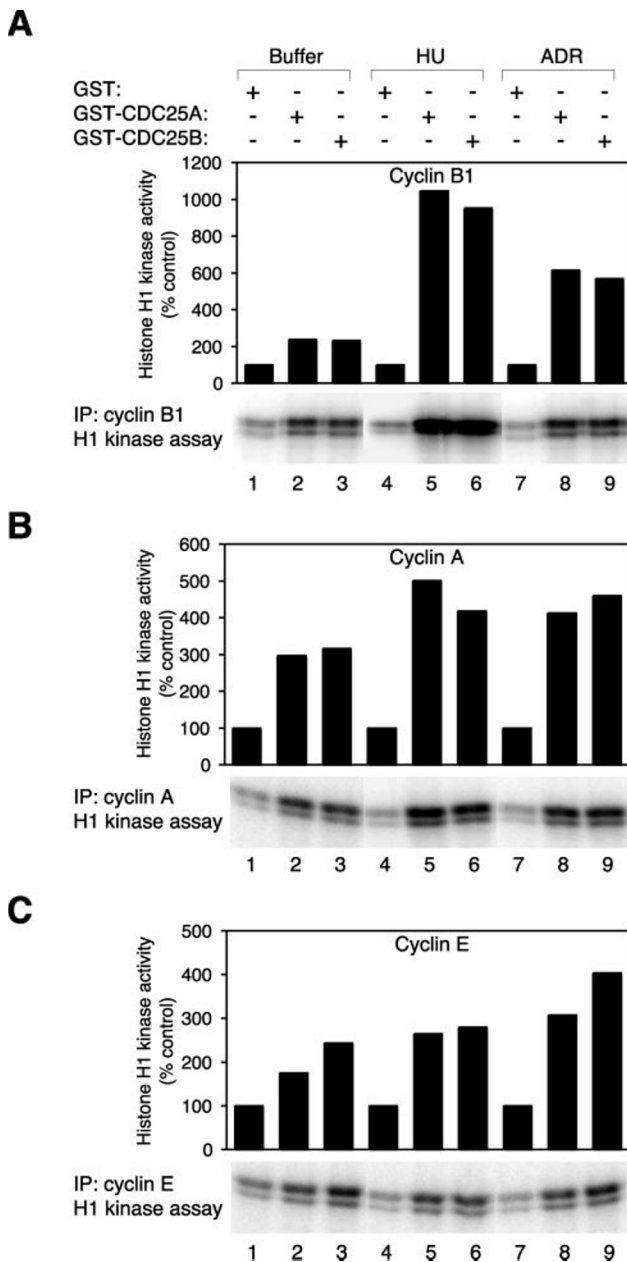


FIG. 4. The relative contribution of inhibitory phosphorylation of cyclin-CDK complexes during asynchronous cell growth and checkpoint arrest. HtTA1 cells were treated with buffer, HU, or ADR for 16 h. Cell extracts were prepared and were subjected to immunoprecipitation (IP) with antiserum against cyclin B1 (A), cyclin A (B), or cyclin E (C). The immunoprecipitates were treated with GST, GST-CDC25A, or GST-CDC25B as indicated before the histone H1 kinase activities were assayed. Phosphorylation was quantified with a PhosphorImager and normalized to the GST control.

liferation of the parental H1299 cells.³ Clonogenic survival analysis indicated that CDC2(AF) and CDK2(AF) triggered a long term inhibition of cell proliferation (Fig. 5G). Expression of CDC2(AF) or CDK2(AF) for 48 h was sufficient to inhibit colony formation even when Dox was added back for the rest of the experiment. Taken together, these data show that ectopic expression of CDC2(AF) or CDK2(AF) is detrimental in H1299, and cells were not able to recover even when these mutants are subsequently suppressed after 48 h.

Nonphosphorylatable CDC2 and CDK2 Induce Unscheduled

Histone H3 Phosphorylation during Unperturbed Cell Cycle and Checkpoint Activation—CDC2(AF) and CDK2(AF) stable cell lines rapidly became very heterogeneous even when Dox was frequently replenished, and the expression of the recombinant proteins were diminished in a portion of the cells. To overexpress the mutant CDC2/CDK2 so that they could overwhelm the endogenous proteins, cells were transiently transfected with FLAG-tagged CDC2(AF) and CDK2(AF). As controls, FLAG-tagged wild type CDC2 and CDK2 were expressed to the same levels as the mutants (Fig. 6A). Interestingly, whereas the kinase activities associated with CDC2(AF) were significantly higher than wild type CDC2, the kinase activities of CDK2(AF) were not much higher than CDK2 (Fig. 6B). The percentage of cells that expressed a GFP-tagged histone H2B reporter was rapidly diminished when co-expressed with CDC2(AF), but not when co-expressed with wild type CDC2 (Fig. 6C). This indicates that like the inducible cell lines, transient expression of CDC2(AF) was anti-proliferative. We worked with cells at 24 h after transfection, when the cytotoxicity caused by CDC2(AF) was less pronounced. The efficiency of transient transfection in HeLa cells was typically above 50% (see Fig. 6C).

Expression of CDC2(AF) induced mainly a G₁ delay (Fig. 6D). Whereas the non-transfected cells were predominantly trapped in mitosis (4 N DNA content) by nocodazole, a large portion of the CDC2(AF)-transfected cells was arrested with a G₁ DNA content. In contrast, CDK2(AF) caused only a slight delay in both G₁ and G₂/M (Fig. 6E). To see whether CDC2(AF) or CDK2(AF) affected mitotic entry, we analyzed the phosphorylation of histone H3 at Ser¹⁰ (an event that normally occurs as cells enter prophase). Unexpectedly, expression of both wild type CDC2/CDK2 and the nonphosphorylatable mutants induced a slight increase in histone H3 phosphorylation (Fig. 6A). This increase was specific because transfection of control vectors of the same backbone did not affect histone H3 phosphorylation. Because CDC2 and CDK2 were already in excess of their cyclin partners, we are not sure why their overexpression affected histone H3 phosphorylation.

To see whether preventing the inhibitory phosphorylation of CDC2 could suppress the DNA damage checkpoint and replication checkpoint, cells were transfected with CDC2(AF) before ADR or thymidine were applied (Fig. 7A). Nocodazole was included to trap cells in the mitotic state after checkpoint bypass. Expression of CDC2(AF) increased histone H3 phosphorylation after DNA damage or replication block. In contrast, histone H3 phosphorylation was not rescued by wild type CDC2 or control vector. Similarly, expression of CDK2(AF), but not wild type CDK2 or control vector, increased histone H3 phosphorylation after ADR or thymidine treatment (Fig. 7B).

To demonstrate that histone H3 phosphorylation was not induced by all CDKs, a nonphosphorylatable mutant of CDK4 was constructed. The Tyr¹⁷ residue of CDK4 was mutated to a phenylalanine to create CDK4(F) (CDK4 contains a Tyr¹⁵ equivalent residue but not a Thr¹⁴ residue). In contrast to CDC2(AF) and CDK2(AF), neither wild type CDK4 nor CDK4(F) increased histone H3 phosphorylation after DNA damage or replication block (Fig. 7C).

One limitation for the above experiments is that not all of the cells were transfected. We next analyzed histone H3 phosphorylation in individual transfected cells by indirect immunofluorescent microscopy (Fig. 7D). Cells were transfected with FLAG-tagged CDK-expressing constructs before being treated with ADR. Nocodazole was subsequently added to trap cells in the mitotic state after checkpoint bypass. Transfected cells were distinguished from non-transfected cells by immunostaining with an antibody against the FLAG epitope. The de-

³ J. P. H. Chow, W. Y. Siu, H. T. B. Ho, K. H. T. Ma, C. C. Ho, and R. Y. C. Poon, unpublished results.

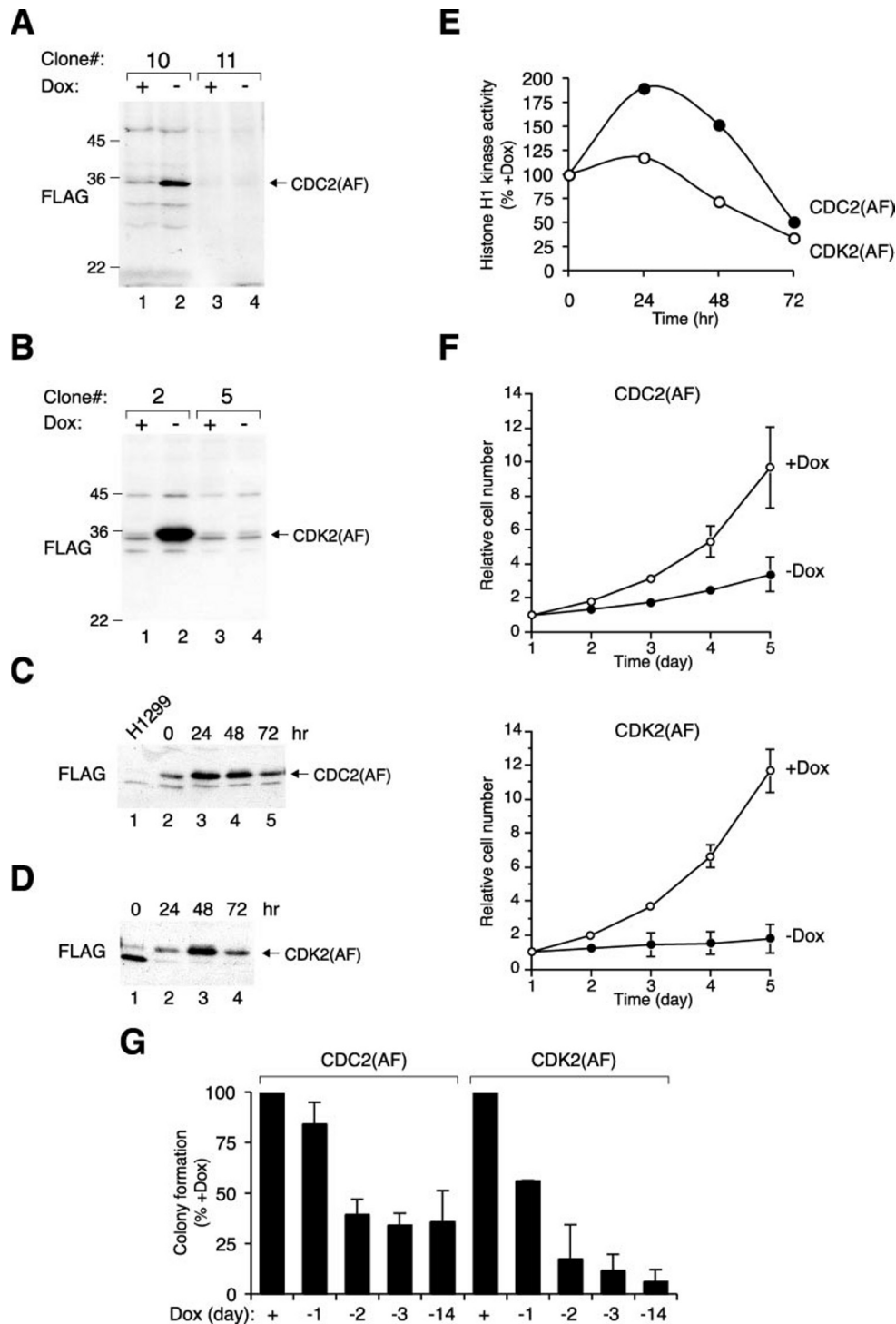


FIG. 5. Expression of CDC2(AF) or CDK2(AF) is cytotoxic in H1299. *A*, inducible expression of CDC2(AF). Stable H1299 cell lines that inducibly expressed FLAG-CDC2(AF) were created as described under "Experimental Procedures." Cells were grown in the presence or absence of Dox for 24 h as indicated. Cell extracts were prepared and were subjected to immunoblotting for FLAG tag. One clone that expressed FLAG-CDC2(AF) and another that did not are shown. The positions of the molecular size standards (in kDa) are shown on the left. *B*, inducible expression of CDK2(AF). Stable H1299 cell lines that inducibly expressed FLAG-CDK2(AF) were created as described under "Experimental Procedures." Samples were prepared and analyzed as in panel *A*. *C*, FLAG-CDC2(AF) expression is only transient. Cells that inducibly expressed FLAG-CDC2(AF) were grown in the absence of Dox and were harvested at the indicated time points. Cell extracts were prepared and were subjected to immunoblotting for FLAG tag. Cell extracts from the parental H1299 cells were loaded in lane 1. *D*, FLAG-CDK2(AF) expression is only transient. Cells that inducibly expressed FLAG-CDK2(AF) were grown in the absence of Dox and were harvested at the indicated time points. Cell extracts were prepared and were subjected to immunoblotting for FLAG tag. *E*, the kinase activities of nonphosphorylatable CDC2/CDK2 decrease over time. Cells that inducibly expressed FLAG-CDC2(AF) or FLAG-CDK2(AF) were grown in the absence of Dox and were harvested at the indicated time points. Cell extracts were prepared and the histone H1 kinase activities associated with the FLAG-immunoprecipitates were assayed. *F*, CDC2(AF) and CDK2(AF) inhibit cell growth. Cells that inducibly expressed FLAG-CDC2(AF) or FLAG-CDK2(AF) were grown in the presence or absence of Dox. Cell number was counted as described under "Experimental Procedures." The mean \pm S.D. from three independent experiments are shown. *G*, transient expression of CDC2(AF) and CDK2(AF) inhibit clonogenic formation. Clonogenic survival assays were performed as described under "Experimental Procedures." Cells that inducibly expressed FLAG-CDC2(AF) or FLAG-CDK2(AF) were grown in the absence of Dox for the indicated number of days before Dox was added back to the medium. Cells were fixed on day 14. Control cells were grown in the presence of Dox throughout the experiment. The mean \pm S.D. from three independent experiments are shown.

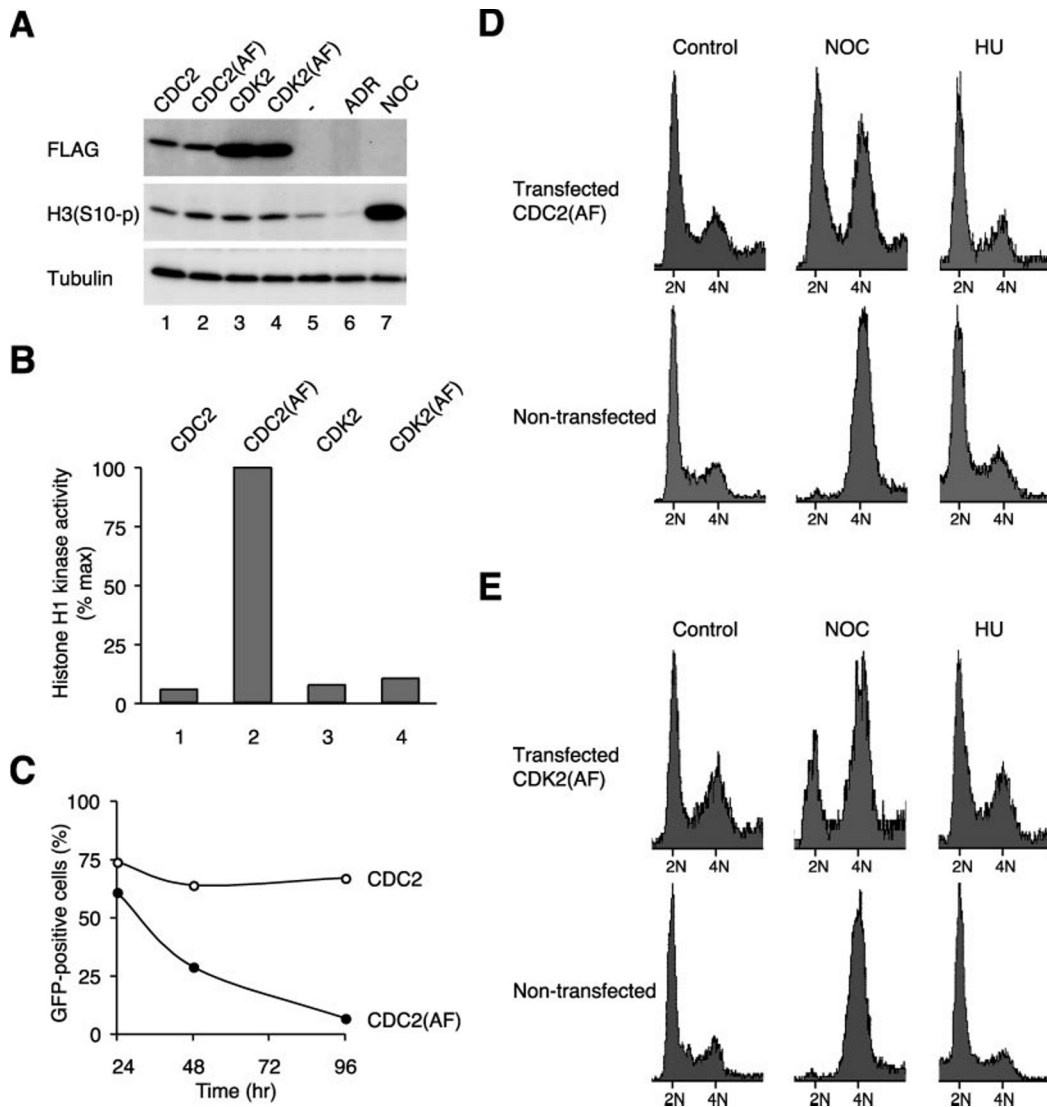


FIG. 6. Transient overexpression of CDC2(AF) and CDK2(AF) causes a G₁ delay. *A*, HtTA1 cells were transfected with constructs expressing FLAG-tagged CDC2, CDC2(AF), CDK2, and CDK2(AF) as indicated. Cell extracts were prepared at 24 h after transfection and subjected to immunoblotting for FLAG tag, Ser¹⁰-phosphorylated histone H3, and tubulin. Extracts were derived from growing cells, ADR-treated, and nocodazole (NOC)-treated cells were loaded as controls. *B*, kinase activities of transiently transfected CDC2(AF) and CDK2(AF). Extracts from HtTA1 cells transiently transfected with FLAG-tagged CDC2, CDC2(AF), CDK2, and CDK2(AF) were subjected to immunoprecipitation with antiserum against FLAG tag. The histone H1 kinase activities associated with the immunoprecipitates were assayed. *C*, transient expression of CDC2(AF) is cytotoxic. FLAG-tagged CDC2 or CDC2(AF) was co-transfected with a GFP-tagged histone H2B marker into HtTA1 cells. The percentage of GFP-containing cells was counted under fluorescence microscopy at different time points. *D*, expression of CDC2(AF) caused a G₁ delay. FLAG-tagged CDC2(AF) was co-transfected with a membrane-targeted GFP reporter into HtTA1 cells. After 12 h, nocodazole, HU, or buffer was added, and cells were incubated for another 16 h before being harvested for flow cytometry analysis. Cell cycle distribution was analyzed for GFP-positive (transfected) and GFP-negative cells (non-transfected cells). The positions of 2 N and 4 N DNA content are indicated. *E*, this experiment was performed as in *panel D* except that CDK2(AF) was used instead of CDC2(AF).

crease of phosphorylated histone H3 signal in the non-transfected cells served as internal controls for each transfection. In agreement with the above immunoblotting data, CDC2(AF) or CDK2(AF) increased the histone H3 phosphorylation after DNA damage. In contrast, wild type CDC2 or CDK2 did not affect histone H3 phosphorylation. Taken together, these data indicate that interference of the inhibitory phosphorylation of CDC2, CDK2, but not CDK4, impairs the G₂ DNA damage checkpoint and the replication checkpoint.

To see whether the histone H3 phosphorylation induced by CDC2(AF) and CDK2(AF) was specific for a certain period during the cell cycle, we next analyzed histone H3 phosphorylation and DNA content simultaneously with flow cytometry (Fig. 8). In normal cells or cells transfected with control vectors, histone H3 was phosphorylated exclusively in cells with 4 N DNA content. Phosphorylated histone H3 was detected in

2–4% of asynchronously growing cells and was reduced to less than 1% after ADR treatment. Unexpectedly, CDC2(AF) induced histone H3 phosphorylation not only in G₂/M, but also in cells with 2 N DNA content. Moreover, CDC2(AF) increased histone H3 phosphorylation after ADR-induced damage mainly in cells with 2 N but not 4 N DNA content. These data suggested that CDC2(AF) triggered unscheduled histone H3 phosphorylation early in the cell cycle. Unlike CDC2(AF), CDK2(AF) only lead to a slight increase in histone H3 phosphorylation in S phase. After DNA damage, however, most of the histone H3 phosphorylation signals were found in S phase cells. As controls, wild type CDC2 or CDK2 did not elevate histone H3 phosphorylation. Taken together, these data suggest that ectopic expression of CDC2(AF) and CDK2(AF) bypasses ADR-induced DNA damage by causing unscheduled mitotic events before G₂.

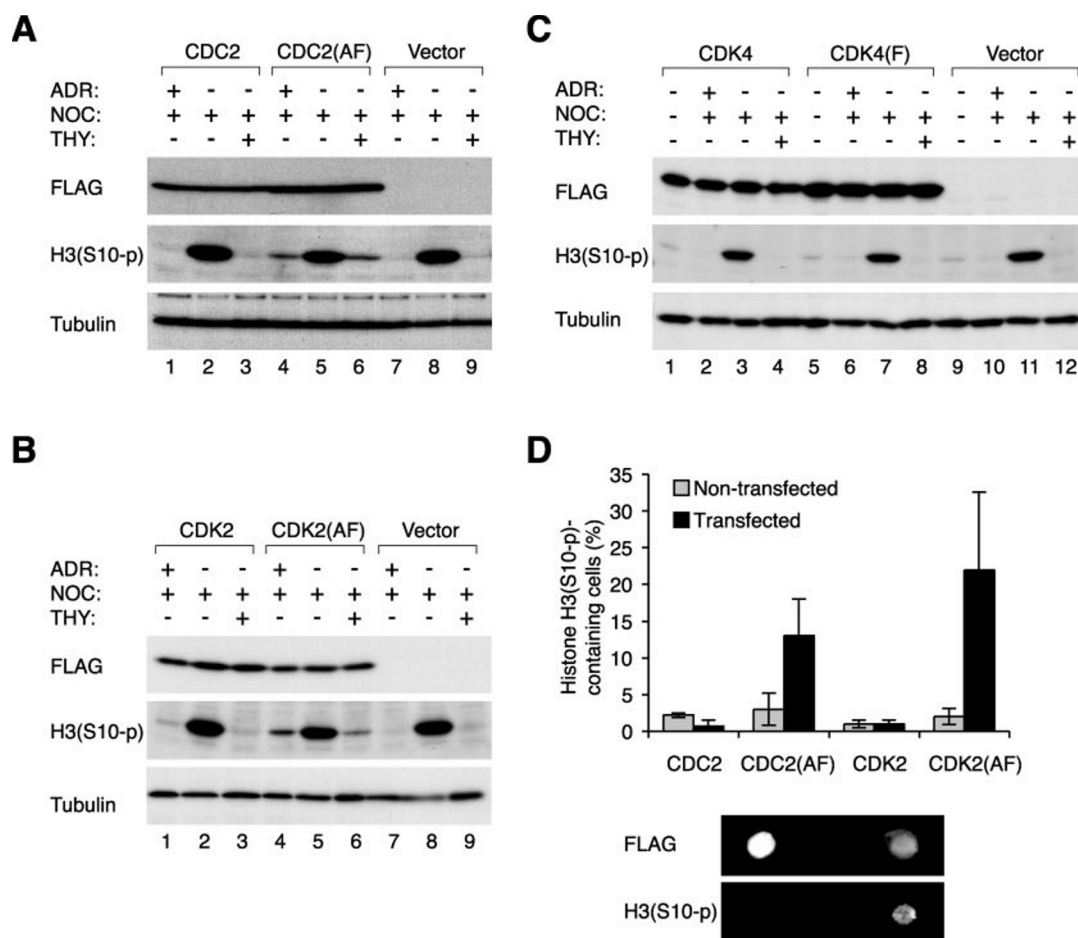


FIG. 7. Nonphosphorylatable CDC2 and CDK2 increase histone H3 phosphorylation after DNA damage and replication block. *A*, nonphosphorylatable CDC2 increases histone H3 phosphorylation after DNA damage and replication block. HtTA1 cells were transfected with constructs expressing FLAG-tagged CDC2, CDC2(AF), or control vector as indicated. Buffer, ADR, or thymidine were added and the cells were incubated for 3 h. Nocodazole (NOC) was then added for another 16 h to trap the cells that bypassed the ADR- or thymidine-induced checkpoints. Cell extracts were prepared and were subjected to immunoblotting for Ser¹⁰-phosphorylated histone H3. Expression of CDC2 and CDC2(AF) was confirmed by immunoblotting for FLAG tag. Immunoblotting for tubulin indicates similar sample loadings. *B*, nonphosphorylatable CDK2 increases histone H3 phosphorylation after DNA damage and replication block. This experiment was performed as in *panel A* except that CDK2 and CDK2(AF) were used instead of CDC2 and CDC2(AF), respectively. *C*, nonphosphorylatable CDK4 does not affect histone H3 phosphorylation after DNA damage and replication block. HtTA1 cells were transfected with constructs expressing FLAG-tagged CDK4, CDK4(F), or the control vector as indicated. Cells were treated as in *panel A*. Cell extracts were prepared and were subjected to immunoblotting for FLAG tag and Ser¹⁰-phosphorylated histone H3. *D*, CDC2(AF) and CDK2(AF) increase histone H3 phosphorylation after ADR treatment. HtTA1 cells were transfected with FLAG-tagged CDC2, CDC2(AF), CDK2, or CDK2(AF). Cells were treated with ADR for 3 h before nocodazole was added for another 16 h. Cells were processed for double immunostaining with antibodies against FLAG tag and Ser¹⁰-phosphorylated histone H3 (detected by fluorescein isothiocyanate-conjugated and TRITC-conjugated secondary antibodies, respectively). The percentage of transfected and non-transfected cells (300 each were counted) that are also positive for Ser¹⁰-phosphorylated histone H3 is plotted. The mean \pm S.D. of counting by three individuals are shown. Examples of transfected cells that contain (*right*) or without (*left*) Ser¹⁰-phosphorylated histone H3 signals are shown.

Suppression of the G₂ DNA Damage Checkpoint by Specific Regulators of Thr¹⁴/Tyr¹⁵—We next investigated whether the G₂ DNA damage checkpoint can be disrupted by the various regulators of Thr¹⁴/Tyr¹⁵. Ectopic expression of CDC25A (but not control vector) increased histone H3 phosphorylation after DNA damage (Fig. 9A). Although the expression of CDC25A was lower in the presence of ADR, it was sufficient to increase histone H3 phosphorylation. A similar increase in histone H3 phosphorylation was obtained with a more stable N-terminal truncated mutant of CDC25A (N Δ 294). Similar to CDC25A, CDC25B increased histone H3 phosphorylation after DNA damage (Fig. 9B). However, expression of full-length CDC25C did not increase histone H3 phosphorylation.³ Because it is possible that CDC25C was inactivated by phosphorylation of the N-terminal regulatory domain, we expressed a CDC25C mutant lacking the regulatory domain (N Δ 274). Expression of CDC25C(N Δ 274) with two different epitope tags did not increase histone H3 phosphorylation after DNA damage or replication block (Fig. 9C).

We expressed a kinase-inactive mutant of WEE1(KR) to disrupt the function of WEE1. Neither the overexpression of wild type WEE1 nor WEE1(KR) affected histone H3 phosphorylation after DNA damage (Fig. 9D). In contrast, deregulation of the checkpoint kinase CHK1 (an upstream regulator for both CDC25 and WEE1) disrupts the DNA damage checkpoint. Overexpression of a kinase-inactive mutant of CHK1(KR) increased histone H3 phosphorylation following ADR treatment (Fig. 9D). These data suggest that different regulators of Thr¹⁴/Tyr¹⁵ may have different roles in the control of the DNA damage checkpoint.

Suppression of Inhibitory Phosphorylation of CDC2 Promotes Cell Division after DNA Damage—Although the previous experiments show that CDC2(AF) and CDK2(AF) disrupted the G₂ DNA damage checkpoint, they were performed in the presence of nocodazole to prevent cells to progress beyond mitosis. One question is whether suppression of the inhibitory phosphorylation of CDC2/CDK2 allows cells to complete mitosis and cytokinesis in the presence of DNA damage. To examine cyto-

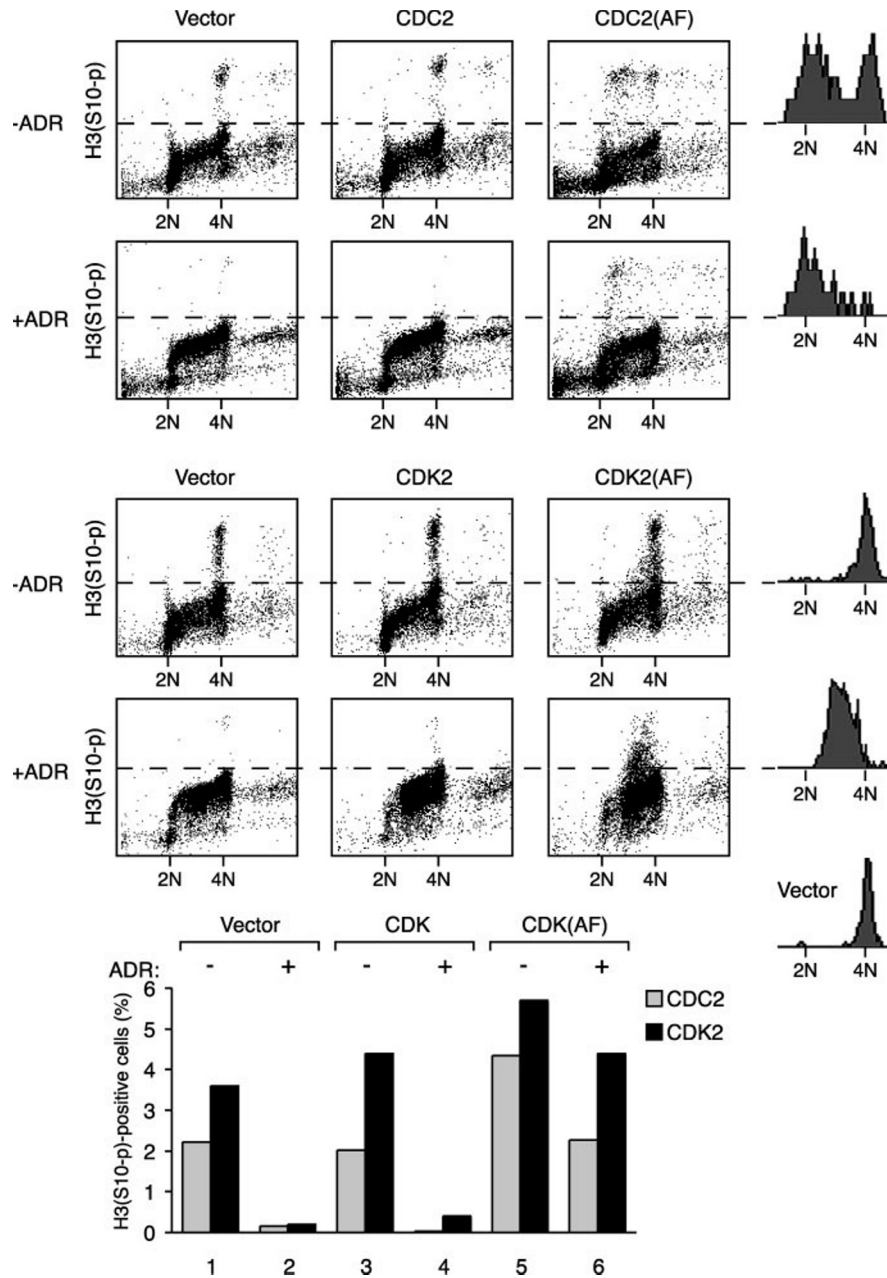


FIG. 8. Nonphosphorylatable CDC2 and CDK2 induce unscheduled histone H3 phosphorylation at different parts of the cell cycle. HtTA1 cells were transfected with constructs expressing FLAG-tagged CDC2, CDC2(AF), CDK2, CDK2(AF), or control vector. Cells were treated either with ADR or buffer for 3 h, before nocodazole was added for another 16 h. Cells were analyzed for Ser¹⁰-phosphorylated histone H3 (y axis) and DNA content (x axis) by flow cytometry as described under "Experimental Procedures." The dotted lines indicate the cut-off level for background versus positive histone H3 phosphorylation signals. The percentage of cells containing phosphorylated histone H3 is indicated in the bottom panel. The right-hand panels show the DNA content profiles of cells containing phosphorylated histone H3 (for CDC2(AF)- and CDK2(AF)-transfected cells only). The bottom right panel shows control vector-transfected cells with phosphorylated histone H3.

kinesis, cells were co-transfected with CDC2(AF) and a β -galactosidase reporter, treated with ADR, and plated at low density in the absence of nocodazole. The percentage of transfected cells that underwent cell division over a period of 24 h was scored (Fig. 10A). Over 80% of cells transfected with control vector or wild type CDC2 divided within 24 h, but the number of cell divisions was about halved in the presence of ADR. In contrast, cell division in CDC2(AF)-transfected cells was not inhibited by ADR. These data indicate that suppression of the inhibitory phosphorylation of CDC2 allows cells to complete mitosis in the presence of DNA damage.

Suppression of Inhibitory Phosphorylation of CDC2 but Not

CDK2 Abolishes the Intra-S Phase DNA Damage Checkpoint— Both CDC2(AF) and CDK2(AF) impair the G₂ DNA damage checkpoint (18, 24, 32). It is not yet established whether CDC2(AF) and CDK2(AF) are equally effective in disruption of DNA damage checkpoints specific for S phase. CMP is a topoisomerase I inhibitor that induces DNA damage only during S phase. Treatment with CMP decreased DNA synthesis as measured by BrdUrd incorporation (Fig. 10B). Expression of CDC2(AF), but not wild type CDC2, restored BrdUrd incorporation of CMP-treated cells to the level of untreated controls. Interestingly, although CDK2 is involved in S phase control, CDK2(AF) did not restore BrdUrd incorporation in CMP-

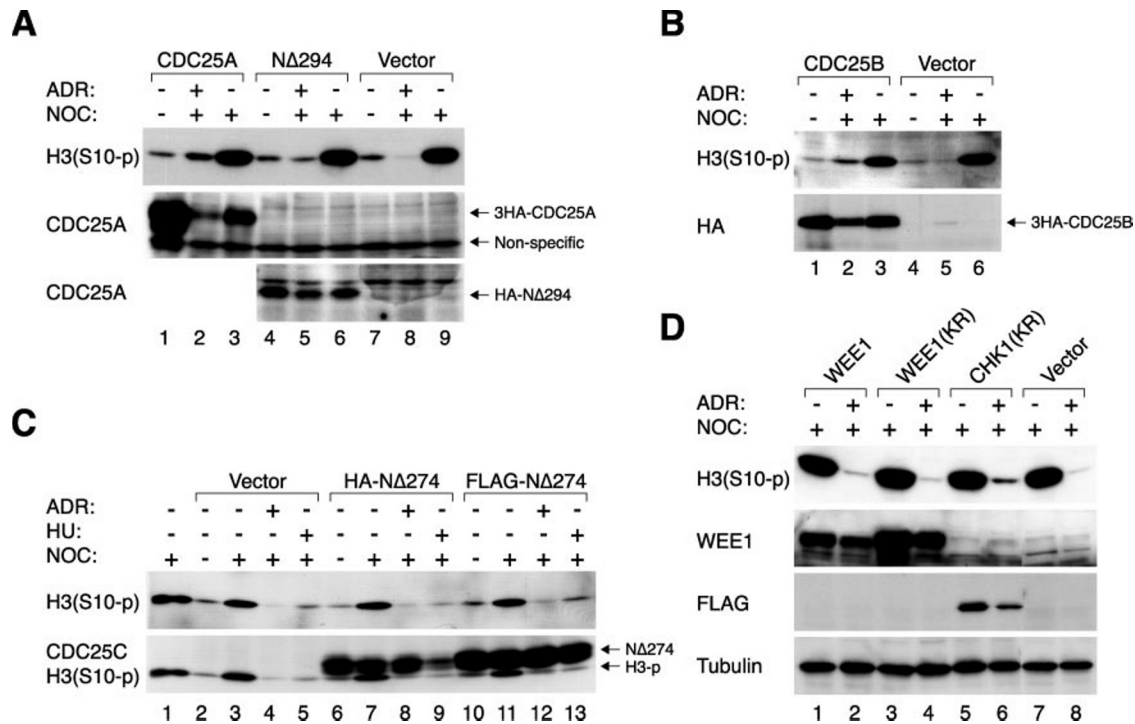


FIG. 9. CDC25A and CDC25B stimulate histone H3 phosphorylation after DNA damage. *A*, bypass of DNA damage checkpoint by CDC25A. HtTA1 cells were transfected with 3HA-CDC25A, HA-CDC25A(Δ), or control vector. Each transfection was divided into three portions, and incubated with buffer or ADR for 3 h before nocodazole (NOC) was added for another 16 h. Cell extracts were prepared and were subjected to immunoblotting for Ser¹⁰-phosphorylated histone H3 or CDC25A. A nonspecific band recognized by the anti-CDC25A antibodies serves to indicate constant sample loadings. *B*, bypass of DNA damage checkpoint by CDC25B. Experiments were performed as in *panel A* except that 3HA-CDC25B was transfected. Immunoblotting was performed with antibodies against Ser¹⁰-phosphorylated histone H3 and HA tag. *C*, CDC25C does not increase histone H3 phosphorylation after DNA damage. HtTA1 cells were transfected with control vector or plasmids expressing HA-tagged or FLAG-tagged CDC25C(Δ). Each transfection was divided into four portions, and incubated with buffer, ADR, or HU for 3 h before nocodazole was added for another 16 h. Cell extracts were prepared and were subjected to immunoblotting for Ser¹⁰-phosphorylated histone H3 and HA tag. *D*, dominant-negative WEE1 does not impair the G₂ DNA damage checkpoint. HtTA1 cells were transfected with plasmids expressing WEE1, WEE1(KR), FLAG-CHK1(KR), or control vector. Each transfection was divided into two portions, and incubated with buffer or ADR for 3 h before nocodazole was added for another 16 h. Cell extracts were prepared and subjected to immunoblotting for Ser¹⁰-phosphorylated histone H3, WEE1, or FLAG tag. Immunoblotting for tubulin indicates similar sample loadings.

treated cells. These data show that preventing the inhibitory phosphorylation of CDC2 also abolishes the intra-S phase DNA damage checkpoint.

DISCUSSION

Our data indicate that during normal cell cycle, inhibitory phosphorylation plays a critical role in controlling the timing of CDC2 activation (Figs. 1 and 2). Treatment with recombinant CDC25 affected the timing but not the magnitude of CDC2 activation, indicating that CDC2 was already fully dephosphorylated during G₂/M. On the other hand, inhibitory phosphorylation plays a relatively minor role in the regulation of CDK2 during normal cell cycle (Fig. 1). A caveat is that we cannot exclude the possibility that CDK2 was inhibited by both phosphorylation and CDK inhibitors at the same time. In this scenario, CDC25 treatment would dephosphorylate CDK2 but not relieve the inhibition by the CDK inhibitors. In agreement with the relatively minor role of inhibitory phosphorylation for CDK2, Lents *et al.* (57) found that the time course of activation of a nuclear targeting CDK2 by the extracellular signal-regulated kinase pathway is the same as the respective CDK2(AF) mutant.

The relative importance of inhibitory phosphorylation of CDC2 and CDK2 may reflect their distinct cellular functions. Although human CDC2 and CDK2 are highly related (65% identity in amino acid sequence) and can bind to a common cyclin partner (cyclin A), the main populations of CDC2 and CDK2 are activated at different times during the cell cycle. It is thought that abrupt activation and inactivation of cyclin B1-

CDC2 are required for proper control of mitosis. Mitotic events need to be highly synchronized because processes like chromosome condensation and sister chromatid separation are not easily reversible. Inhibitory phosphorylation of CDC2 serves as an elegant mechanism to convert a progressive synthesis of cyclin B1 into an abrupt activation of cyclin B1-CDC2. Rapid inactivation of CDC2 is carried out by the removal of cyclin B1 by ubiquitin-mediated proteolysis. CDK2 is involved in the control of G₁-S transition and S phase progression. Among other things, cyclin E-CDK2 is involved in the assembly of a prereplication complex and cyclin A-CDK2 is involved in preventing re-replication after the firing of the origins (reviewed in Ref. 58). In comparison to mitosis, S phase is carried out over a longer period of time and with less synchrony. It is conceivable that different pieces of DNA can be replicated at different times, as long as each piece is replicated once and there is a mechanism to prevent re-replication. This nature of the S phase control may explain the relative insignificant role of inhibitory phosphorylation for CDK2.

Although inhibitory phosphorylation of CDK2 appears to be less pronounced than for CDC2 during unperturbed cell cycles, expression of either CDC2(AF) or CDK2(AF) was cytotoxic (Fig. 5). The exact reasons of why CDK2(AF) is anti-proliferative are unclear. Jin *et al.* (24) demonstrated that expression of CDK2(AF) in HeLa cells induced premature chromosome condensation. One possibility is that because CDK2(AF) can bind to both cyclin E and cyclin A, overexpression of CDK2(AF) may replace some of the cyclin A-CDC2 complexes with cyclin

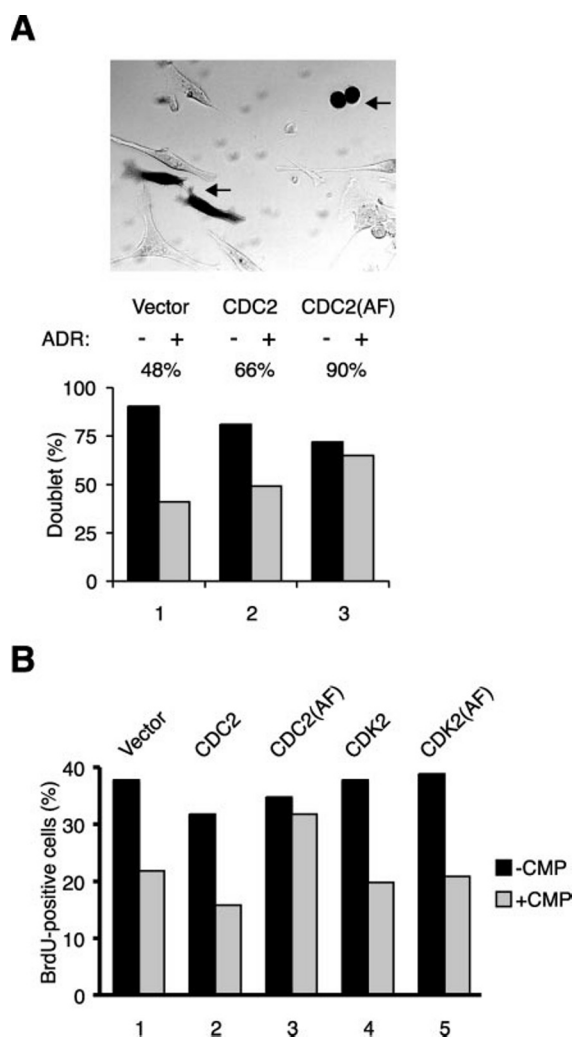


FIG. 10. Nonphosphorylatable CDC2 promotes DNA synthesis and cell division after DNA damage. A, CDC2(AF) increases cell division after ADR-induced DNA damage. HtTA1 cells were co-transfected with a β -galactosidase reporter and constructs expressing CDC2, CDC2(AF), or control vector. The transfected cells were then seeded at a low density, treated with buffer or ADR for 24 h, and fixed for β -galactosidase staining as described under "Experimental Procedures." The number of transfected cells that are single or closely placed doubles is counted under light microscopy (300 units were counted). An example of two pairs of doublets is shown in the top panel (arrows). B, CDC2(AF) increases BrdUrd incorporation after CMP-induced DNA damage. HtTA1 cells were transfected with constructs expressing CDC2, CDC2(AF), CDK2, CDK2(AF), or control vector. After 12 h, the cells were either mock-treated or treated with CMP for a further 6 h. The cells were then pulsed with BrdUrd and analyzed for BrdUrd incorporation as described under "Experimental Procedures."

A-CDK2(AF). The formation of cyclin A-CDK2(AF) complexes may also explain the increase in histone H3 phosphorylation during S phase (Fig. 8).

Similar to CDK2(AF), expression of CDC2(AF) induced histone H3 phosphorylation early in the cell cycle (Fig. 8). The unscheduled phosphorylation of histone H3 (and possibly other G_2 substrates) during S phase or earlier in the cell cycle may underlie the cytotoxicity of CDC2(AF) and CDK2(AF). Both cyclin A and cyclin B1 are already synthesized during S phase (Figs. 1 and 2), hence they could be activated by either expression of CDC2(AF) *in vivo* or by dephosphorylation with CDC25 *in vitro*. However, it is not clear why CDC2 from G_1 cells was activated by CDC25 *in vitro* (Figs. 1 and 2), and why CDC2(AF) increased histone H3 phosphorylation in cells with 2N DNA content (Fig. 8). It is unlikely that CDC2 was activated by the G_1 cyclins, because neither the endogenous nor transfected

CDC2 could bind cyclin E.³ One possibility is that cyclin B1 were not completely destroyed and persisted during G_1 . The presence of residue cyclin B1 during G_1 could be the actual case *in vivo* or because of an artifact of the synchronization procedure. Irrespective of the mechanism, the fact that CDC2 from G_1 could be activated by CDC25 but could not be activated during mitosis (Fig. 1) implies that the cyclin B1-CDC2 in G_1 was Thr¹⁴/Tyr¹⁵-phosphorylated. A provocative hypothesis is that the cyclin B1-CDC2 complexes that are not destroyed after mitosis are then inhibited by phosphorylation. In this connection, inhibitory phosphorylation is not the only mechanism that inhibits the activity of cyclin B1-CDC2 during S phase. Recently, it was shown that cyclin B1-CDC2 can also stimulate S phase if it is relocated from the cytoplasm into the nucleus and is moderately activated (59). The roles of subcellular localization of cyclin-CDK are not examined here as total cell lysates were used for our experiments.

Thr¹⁴/Tyr¹⁵ of CDK2 is dephosphorylated by CDC25A and CDC25B. In contrast to CDK2(AF), ectopic expression of CDC25A and CDC25B is not anti-proliferative. CDC25A and CDC25B are overexpressed in some human cancer cell lines (60), and expression of CDC25A and CDC25B cooperates with an activated RAS mutant and loss of retinoblastoma protein to transform primary rodent cells (61). Similarly, CDC25A and CDC25B are overexpressed in a variety of primary cancers (reviewed in Ref. 62). It is not clear why expression of CDK2(AF) is toxic but CDC25A or CDC25B are not. One possibility is that CDK2 is not completely dephosphorylated even when CDC25A or CDC25B is overexpressed. Under *in vitro* or overexpressed conditions, there was no significant difference between CDC25A and CDC25B in the activation of CDC2/CDK2 (Fig. 4). However, it is likely that the two CDC25 isoforms have distinct roles in the cell.

DNA damage or replication block inhibited mitotic entry and decreased histone H3 phosphorylation (Figs. 6 and 7). However, no reduction of the kinase activities associated with cyclin B1-CDC2 was observed when it is assayed *in vitro*. We attributed these conflicting results to an accumulation of cyclin B1 during the block in S phase and G_2 (Fig. 3), so that the pool of cyclin B1-CDC2 immunoprecipitated after DNA damage or replication block was actually more than that from growing cells. Accumulation of cyclin B1 was because of both an activation of transcription and a repression of proteolysis. Transcription from the human cyclin B1 promoter is induced during S phase and repressed in G_1 (63–67). APC/C-dependent degradation of cyclin B1 is inhibited during S phase and G_2 cell cycle block. The accumulation of cyclins during cell cycle block may also in part explain the previously published results showing that the activities of CDK2 are only transiently inhibited after HU treatment (68, 69) or irradiation (36, 70).

We found that CDC2(AF) or CDK2(AF) increased histone H3 phosphorylation after DNA damage (Fig. 7). One interpretation is that DNA damage normally inhibits mitotic entry by causing inhibitory phosphorylation of CDC2 and CDK2, and overexpression of the nonphosphorylatable mutants acts in a dominant-negative manner and impairs the checkpoint. This is also in agreement with previously published works (24, 32) that show that CDC2(AF) increases cyclin B1-CDC2 kinase activity and promotes progression through mitosis after irradiation. Intriguingly, CDC2(AF) and CDK2(AF) increased histone H3 mainly in G_1 and S phase, respectively (Fig. 8). As nocodazole was included in the experiment, it is unlikely that cells that bypassed the G_2 DNA damage checkpoint went through mitosis and entered the next cell cycle. One interpretation is that CDC2(AF) and CDK2(AF) caused mitotic events in other phases of the cell cycle after ADR-induced DNA damage. The

ability to induce unscheduled mitotic entry may be one of the explanations of the cytotoxicity associated with CDC2(AF) and CDK2(AF).

Interestingly, not all potential regulators of Thr¹⁴/Tyr¹⁵ have the same effect on the G₂ DNA damage checkpoint. We found that like CDC2(AF) and CDK2(AF), CDC25A and CDC25B also increased histone H3 phosphorylation after DNA damage (Fig. 9). In contrast, histone H3 phosphorylation was not increased by CDC25C or dominant-negative WEE1. This may be because of the fact that not all regulators of Thr¹⁴/Tyr¹⁵ are of equal importance. For example, mice with disrupted CDC25C display no obvious abnormalities and embryonic fibroblasts from these mice have normal DNA damage responses (62). Dominant-negative MYT1 was not examined here because it has been reported that kinase-dead MYT1 still retain the ability to inhibit the cell cycle by preventing the entry of cyclin B1-CDC2 into the nucleus (9, 10).

CDK may be inhibited by phosphorylation in more situations than once thought. We recently found that apart from its role in the G₂ DNA damage checkpoint, inhibitory phosphorylation of CDC2 is also involved in the DNA damage response during mitotic block. ADR treatment during nocodazole block triggers Thr¹⁴/Tyr¹⁵ phosphorylation and inactivation of CDC2 (71). This is accompanied with the destruction of CDC25A and a loss of histone H3 phosphorylation. Similarly to the G₂ DNA damage checkpoint, the decrease of histone H3 phosphorylation during the mitotic DNA damage response can be reversed by overexpression of CDC2(AF) or CDC25A.

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