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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 39, Issue of September 27, pp. 36832–36838, 2002
Printed in U.S.A.

ATR Enforces the Topoisomerase II-dependent G_2 Checkpoint through Inhibition of Plk1 Kinase*

Received for publication, June 19, 2002, and in revised form, July 19, 2002
Published, JBC Papers in Press, July 29, 2002, DOI 10.1074/jbc.M206109200

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An ATR-dependent G_2 checkpoint responds to inhibition of topoisomerase II and delays entry into mitosis by sustaining nuclear exclusion of cyclin B1-Cdk1 complexes. Here we report that induction of this checkpoint with ICRF-193, a topoisomerase II catalytic inhibitor that does not cause DNA damage, was associated with an ATR-dependent inhibition of polo-like kinase 1 (Plk1) kinase activity and a decrease in cyclin B1 phosphorylation. Expression of constitutively active Plk1 but not wild type Plk1 reversed ICRF-193-induced mitotic delay in HeLa cells, suggesting that Plk1 kinase activity is important for the checkpoint response to ICRF-193. G_2/M synchronized normal human fibroblasts, when treated with ICRF-193, showed a decrease in cyclin B1 phosphorylation and Plk1 kinase activity despite high cyclin B1-Cdk1 kinase activity. G_2 fibroblasts that were treated with caffeine to override the checkpoint response to ICRF-193 displayed a high incidence of chromosomal aberrations. Taken together, these results suggest that ATR-dependent inhibition of Plk1 kinase activity may be one mechanism to regulate cyclin B1 phosphorylation and sustain nuclear exclusion during the G_2 checkpoint response to topoisomerase II inhibition. Moreover, the results demonstrate an important role for the topoisomerase II-dependent G_2 checkpoint in the preservation of human genomic stability.

Cell cycle checkpoints are surveillance systems that ensure orderly and timely replication and segregation of the genome. G_2 checkpoint systems prevent inappropriate mitotic entry when there are abnormalities in DNA due to incomplete DNA replication, insufficient chromatid decatenation, or DNA damage (1–4). The phosphatidylinositol 3-kinase family of protein kinases includes ATM (ataxia telangiectasia-mutated) and ATR (A-T and rad3-related), which are proximal to DNA abnormalities and send signals to regulate the mitosis-promoting cyclin B1-Cdk1 complex during G_2 checkpoint responses.

After DNA damage, one mechanism of G_2 checkpoint control involves regulation of cyclin B1-Cdk1 kinase activity. At the onset of mitosis, cyclin B1-Cdk1 kinase becomes active when the dual specificity phosphatase, Cdc25C, removes inhibitory phosphorylations on Cdk1. Once active, cyclin B1-Cdk1 complexes phosphorylate Cdc25C, which enhances its phosphatase activity. This results in an autocatalytic feedback loop and, therefore, generates a burst of cyclin B1-Cdk1 kinase activity that drives progression into mitosis. The G_2 checkpoint carries out cell cycle arrest in part by inhibiting the activity of cyclin B1-Cdk1 complexes through the effector kinase Chk1. After DNA damage and replication blocks, Chk1 is phosphorylated and activated by ATR and possibly ATM (5–8). Chk1 phosphorylates Cdc25C in a 14-3-3 binding site, which causes 14-3-3 to sequester Cdc25C away from cyclin B1-Cdk1 and prevents activation of cyclin B1-Cdk1 complexes (9, 10). Initiation of the cyclin B1-Cdk1 autoamplification loop requires prior activation of Cdc25C. The kinase that first activates Cdc25C (the mitotic trigger kinase) has not been rigorously identified, although some evidence points to polo-like kinase 1 (Plk1).[1]

Polo-like kinases are a conserved group of protein kinases that are involved at several points in mitotic progression. They are thought to function in diverse processes such as the G_2 to M cycle transition, centrosome assembly and separation, formation of the mitotic spindle, activation of the anaphase-promoting complex, and cytokinesis (11, 12). Human and *Xenopus* polo-like kinases (Plk1 and Plx1, respectively) have been proposed to initiate mitosis by activating Cdc25C and participating in the Cdc25C/cyclin B1-Cdk1 amplification loop (13–16). The regulation of cyclin B1-Cdk1 kinase activity during the G_2 checkpoint has been suggested to occur through inhibition of Plk1 activity, which would presumably result in an inability of Cdc25C phosphatase to activate cyclin B1-Cdk1 complexes. Evidence supporting a role for regulation of Plk1 in the G_2 checkpoint includes the findings that cells experiencing a DNA damage-induced mitotic delay displayed an associated decrease in Plk1 kinase activity, and overexpression of a constitutively active mutant of Plk1 reversed the mitotic delay (17). Moreover, Plk1 activity was decreased in an ATM- and/or ATR-dependent manner after treatment of cells with radiomimetic drugs and UVC, respectively (18).

Another mechanism of G_2 checkpoint control independent of cyclin B1-Cdk1 activity is nuclear exclusion of cyclin B1. At the onset of mitosis, phosphorylation of cyclin B1 causes an inhibition of nuclear export and enhanced nuclear import, resulting in a net accumulation of cyclin B1-Cdk1 complexes in the nucleus (19–23). Interfering with nuclear exclusion of cyclin B1

* This work was supported in part by Public Health Service Grants CA42765 (to W. K. K.), P30-CA16086, and P30-ES10126 and a grant from the Ulster Cancer Foundation (to C. S. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ Supported by NIEHS, National Institutes of Health Environmental Pathology Training Grant T32-ES07017.

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[1] The abbreviations used are: Plk1, polo-like kinase 1; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GFP, green fluorescent protein.

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and down-regulation of Cdk1 kinase activity were required to significantly reverse the DNA damage G_2 checkpoint in HeLa cells (23, 24). In the *Xenopus* system, phosphorylation of Ser-113 (analogous to Ser-147 in human cyclin B1), which resides within the cyclin B1 nuclear export sequence, interfered with the ability of the Crm1 nuclear receptor to bind to cyclin B1 and thereby inhibited nuclear export (25). Plk1 was recently shown to phosphorylate human cyclin B1 at Ser-147 and promote its nuclear accumulation (26). Thus, activation of Plk1 in G_2 appears to drive mitotic progression by promoting the accumulation of cyclin B1 in the nucleus in addition to participating in the Cdc25C-cyclin B1-Cdk1 autocatalytic feedback loop. The G_2 checkpoint may regulate the sub-cellular compartmentalization of cyclin B1-Cdk1 by controlling the phosphorylation status of cyclin B1 through inhibition of Plk1 activity.

The bisdioxopiperazine, ICRF-193, is a topoisomerase II inhibitor that does not cause DNA damage but rather appears to block topoisomerase II at a point in its catalytic cycle after strand passage and religation but before release of the passed DNA and ATP hydrolysis (27). Therefore, ICRF-193 sequesters topoisomerase II in a "closed-clamp" conformation that tethers two DNA strands and blocks additional cycles of strand passage (27). A checkpoint is activated in G_2 when topoisomerase II is inhibited with ICRF-193 (3, 4). The topoisomerase II-dependent G_2 checkpoint response to ICRF-193 was recently reported to be distinct from the DNA damage G_2 checkpoint as it was ATM-independent and did not appear to be enforced through inhibition of cyclin B1-Cdk1 kinase activity. Rather, it was suggested that ATR-dependent signaling acted to sustain the nuclear exclusion of cyclin B1-Cdk1 complexes (4). In this report, we show that inhibition of Plk1 kinase activity occurs in an ATR-dependent manner in cells arrested in G_2 by ICRF-193. The mitotic delay response to ICRF-193 was reversed in cells expressing constitutively active Plk1 alleles, strongly suggesting that Plk1 kinase is an important element of the topoisomerase II-dependent G_2 checkpoint. G_2 cells arrested with ICRF-193 also displayed reduced serine phosphorylation of cyclin B1 *in vivo* and reduced kinase activity on cyclin B1 in cell-free extracts. Taken together these data support a model for the topoisomerase II-dependent G_2 checkpoint whereby ATR signaling inhibits Plk1 activity, which in turn blocks the phosphorylation of cyclin B1 and prevents the accumulation of nuclear cyclin B1-Cdk1 complexes.

EXPERIMENTAL PROCEDURES

Cell Culture, Synchrony, Metaphase Preparation, and Transfections—NHF1-hTERT cells were maintained in minimum Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. For synchrony experiments, NHF1-hTERT cells were grown to confluence arrest and then released into 2 μg/ml aphidicolin for 24 h after which they were rinsed 3 times with phosphate-buffered saline (PBS) (28). Fresh culture media was then added and the cells were returned to the incubator for 8 h. Cells were determined to be in the G_2 phase of the cycle as evidenced by 4N DNA content with very few cells staining for the mitotic marker, phospho-histone H3. G_2 phase cells were harvested or incubated with 0.01% Me_2SO or 2 μM ICRF-193 in the presence of 100 ng/ml Colcemid for an additional 4 h. Cells were prepared for metaphase spreads as previously described (29). The AT diploid fibroblast cell line (GM03395) was maintained in minimum Eagle's medium supplemented with 20% FBS and 2 mM L-glutamine. GM847 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine, and 0.4 mg/ml G418 sulfate (30). For induction of kinase-inactive ATR allele, ATR^{ki}, 5.0×10^5 cells were plated into 100-mm dishes, and 1 μg/ml doxycycline was added for 48 h. HeLa cells were maintained in minimum Eagle's medium containing 10% FBS and 2 mM L-glutamine. For transfections, cells were plated at a density of 8.0×10^5 cells/60-mm dish. After plating, cells were transfected with 0.5 μg of GFP-H2B plasmid together with 2 μg of control vector (pBABE plasmid DNA) or 2 μg of one of the Myc-tagged Plk-1 plasmids (wild type, T210D, or T210D and S137D (TDSD)) (17) using Polyfect (Qiagen) according to the manufacturer's recommendations. Forty-eight hours after transfection, cells were either harvested for Western blot analysis, or ICRF-193-induced G_2 arrest was assessed. Cells were incubated with Me_2SO or 2 μM ICRF-193 for 2 h, and then mitotic index was quantified.

Immunoprecipitation and Western Immunoblot Analysis—For determination of serine phosphorylation of endogenous cyclin B1, this protein was immunoprecipitated from 200 μg of total cell extract using a cyclin B1 monoclonal antibody (Upstate Biotechnology). Immunoprecipitates were then subjected to SDS-PAGE analysis (8% polyacrylamide gel) and immunoblotted with rabbit polyclonal anti-serine antibody (1:1000, Zymed Laboratories Inc.).

Mitotic Index Determination—Mitotic cells were quantified via flow cytometric analysis of cells with 4N DNA that stained positive for the mitotic epitope phospho-histone H3 (31). Briefly, cells were harvested, rinsed with PBS, then resuspended in 1% formaldehyde in PBS and incubated for 20 min at room temperature. Fixed cells were then rinsed once with PBS and stored in 70% ethanol at 4 °C. At the time of staining, cells were rinsed in PBS, pelleted, and resuspended in PBS containing 0.5 μg of anti-phospho-histone H3 antibody (Upstate Biotechnology), 5% FBS, 0.1% sodium azide, and NaCl (IFA solution) and incubated for 2 h at room temperature. Cells were then rinsed twice in IFA, resuspended in fluorescein isothiocyanate-labeled anti-mouse antibody diluted 1:20 (Santa Cruz) in IFA, and incubated for 1 h at room temperature in the dark. After 2 washes in IFA, cells were resuspended in IFA containing propidium iodine (PI) (0.5 μg/ml) and RNase (2.5 μg/ml) and incubated at 37 °C for at least 30 min. For experiments with transfected HeLa cells, cells were fixed with 90% ethanol and 2.5% acetic acid in PBS for 20 min at room temperature. Cells were then rinsed with PBS, and nuclei were stained with DAPI (4′,5′-diamidino-2-phenylindole dihydrochloride). The fraction of mitotic cells in 2000 GFP-positive cells was determined by fluorescence microscopy.

Cyclin B1-Cdk1 Kinase Assay—Cyclin B1-associated Cdk1 kinase activity assays were performed as previously described (4, 32).

Polo-like Kinase Activity Assay—Cells were lysed in buffer containing 50 mM Tris, pH 7.4, 1% Nonidet P-40, 250 mM NaCl, 10 mM NaF, and 5 mM EDTA and incubated on ice for 20 min. Cell lysates were clarified by centrifugation at 45,000 rpm for 20 min. Plk1 was immunoprecipitated using 1 μg of a rabbit anti-Plk1 antibody (a generous gift from Doug Ferris, National Cancer Institute, National Institutes of Health or from Upstate Biotechnology) from 300–500 μg of total protein. Samples were precleared for 30 min with protein G beads previously washed in 10× PBS and 1× kinase buffer. Samples were incubated with anti-Plk1 antibody for 2 h at 4 °C, after which 20 μl of protein G beads was added. After 30 min, samples were washed four times with kinase buffer and once in reaction buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 5 mM NaF, 10 nM orthovanadate). Samples were then incubated with 20 μl of kinase buffer, 5 mg of dephosphorylated α-casein (Sigma), and 1 μCi of [γ-^{32}P]ATP at 34 °C for 50 min. The kinase reaction was stopped by the addition of 2× Laemmli sample buffer, and samples were subjected to SDS-PAGE (12% acrylamide gel) and autoradiography.

In Vitro Cyclin B1 Kinase Assay—Cells were lysed in kinase lysis buffer containing 20 mM Tris, pH 7.5, 10 mM EGTA, 10 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM $NaVO_4$, 10 mM β-glycerophosphate, 20 μg/ml aprotinin, 1 μM okadaic acid, 10 mg/ml leupeptin, 1 μM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride; Sigma) and centrifuged at 14,000 rpm for 20 min. Cell extract (200 μg) was then incubated with 100 μM cold ATP, 15 mM $MgCl_2$, and 2 μCi of [γ-^{33}P]ATP for 45 min at 30 °C. Cyclin B1 was immunoprecipitated from the kinase reaction by adding 1 μg of cyclin B1 monoclonal antibody (Upstate Biotechnology) and lysis buffer and incubating for 2 h at 4 °C. Protein G beads (Sigma) were added, and samples were incubated for an additional 30 min, after which the beads were washed 4 times with kinase lysis buffer. Cyclin B1 immunoprecipitates were then subjected to SDS-PAGE (8% acrylamide gel) and autoradiography.

RESULTS

ICRF-193 Prevents Mitotic Entry and Plk1 Activation in Synchronized Human Fibroblasts—Plk1 activity levels are low in G_1 and S phase but start to rise in G_2 and peak in mitosis (33, 34). Because Plk1 activity is regulated by the DNA damage checkpoint and one function of Plk1 involves regulation of cyclin B1 localization, we determined whether ICRF-193-induced mitotic delay was associated with an inhibition of Plk1 activity. Normal human fibroblasts expressing telomerase (NHF1-hTERT) were synchronized to the G_2 phase of the cell

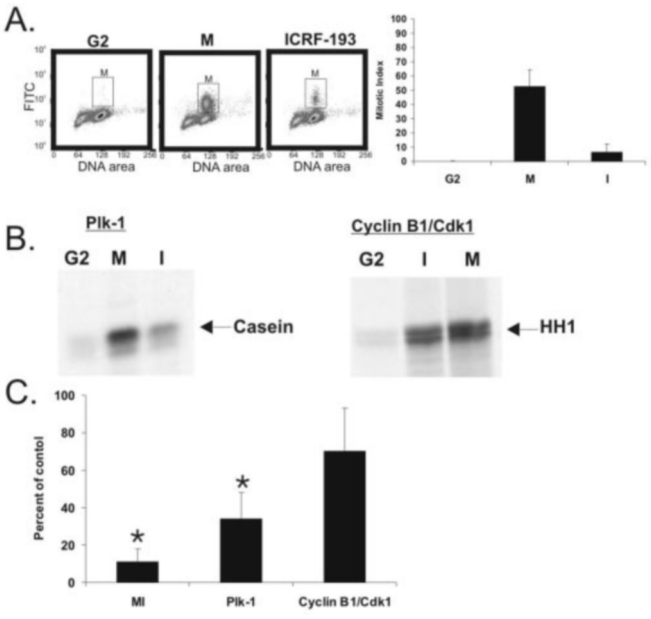

FIG. 1. **ICRF-193 inhibits entry into mitosis, and Plk1 activity in synchronized normal human fibroblasts.** NHF1-hTERT cells were synchronized to the G_2 phase of the cell cycle then harvested directly or further incubated with Me_2SO or 2 μM ICRF-193 in the presence of 100 ng/ml of Colcemid for 4 h. *M* and *I* denote control Me_2SO-treated mitotic and ICRF-193-treated, respectively. *A*, mitotic index was determined via flow cytometric analysis using propidium iodine to stain DNA (*x* axis) and an antibody against phosphorylated histone H3 to stain mitotic cells (*y* axis) as described under "Experimental Procedures." The histogram shows the result from five experiments (mean ± S.D.). *FITC*, fluorescein isothiocyanate. *B*, Plk-1-associated casein kinase activity and cyclin B1-Cdk1-associated histone H1 kinase activity (HH1) were assessed as described under "Experimental Procedures." The autoradiographs are representative of three independent experiments for Plk1 and five independent experiments for cyclin B1-Cdk1. *C*, mitotic, index, Plk1 activity, and cyclin B1-Cdk1 kinase activity observed in ICRF-treated cultures were expressed as percentages of the correspondent values observed in cultures exposed only to the Me_2SO solvent. *, the results observed in the ICRF-193-treated samples compared with the Me_2SO controls were significantly different for mitotic index (analysis of matched pairs, $p = 0.001$) and Plk1 activity (analysis of matched pairs, $p = 0.006$) but not for cyclin B1-Cdk1 activity (analysis of matched pairs, $p = 0.143$).

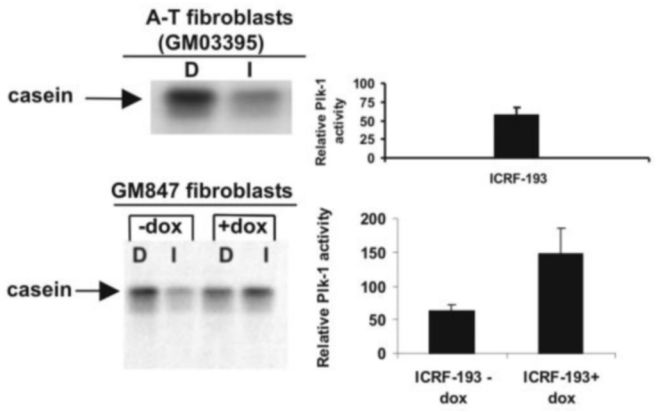

FIG. 2. **ATR regulates Plk1 activity during the topoisomerase II-dependent G_2 checkpoint response.** *Top panel*, AT fibroblasts (GM03395) were incubated with Me_2SO or 2 μM ICRF-193 for 2 h, after which Plk1 kinase activity was determined as described under "Experimental Procedures." The results show the mitotic index in ICRF-193-treated cells expressed as a percentage of the Me_2SO-treated control. *Bottom panel*, non-induced (−*dox*) and induced (+*dox*) GM847 cells were incubated with Me_2SO or 2 μM ICRF-193 for 2 h, after which Plk1 kinase activity was assessed as described under "Experimental Procedures." The autoradiograph shown is representative of all experiments. The values of Plk1 activity determined in ICRF-193-treated cells in 4 independent experiments were expressed as the percentage of the Plk1 activity in Me_2SO-treated cells. Averages (± S.E.) are shown for non-induced and induced GM847 cells.

cycle and then incubated with ICRF-193 or solvent in the presence of Colcemid for an additional 4 h. Under these conditions there were few cells in the G_2 phase population that stained positive for the mitotic marker, phospho-histone H3 (0.1%) (Fig. 1*A*). Me_2SO-treated control cells accumulated in mitosis 8–12 h after release from aphidicolin (53%) (Fig. 1*A*). Treatment of G_2 phase cells with ICRF-193 produced a severe inhibition of entry into mitosis compared with the control cells (89%, $p = 0.001$), as reflected by the low mitotic index (4.5%) (Fig. 1, *A* and *C*). As expected, Plk1 activity was low in G_2-phase cells and significantly increased in control cells that had progressed to mitosis (Fig. 1*B*). Cells treated with ICRF-193 displayed a significant inhibition of Plk1 activity compared with the control Me_2SO-treated mitotic cells (34% of control, $p = 0.006$) (Fig. 1*C*). It is important to note that ICRF-193-treated cells displayed higher Plk1 activity than cells harvested 4 h earlier at the beginning of G_2 (Fig. 1*B*), indicating that Plk1 activation was not completely prevented by the ICRF-193-induced checkpoint.

ICRF-193-induced Inhibition of Plk1 Activity Is Not Associated with a Significant Inhibition in Cyclin B1-Cdk1 Activity—It has been suggested that inhibition of Plk1 activity during G_2 checkpoint responses may control mitotic entry through regulation of cyclin B1-Cdk1 activity (17, 18). Given that we have previously reported a mitotic delay response to topoisomerase II inhibition with ICRF-193 despite high cyclin B1-Cdk1 kinase activity in log phase NHF1-hTERT fibroblasts and other human cell types (4), we investigated whether ICRF-193-induced inhibition of Plk1 activity was associated with an inhibition of cyclin B1 activity in synchronized cells. In synchronized NHF1-hTERT fibroblasts, cyclin B1-Cdk1 activity was low in G_2 phase cells and high in mitotic cells (Fig. 1*B*). Cells undergoing an ICRF-193-induced mitotic delay displayed a substantial increase in cyclin B1-Cdk1 kinase activity when compared with G_2-phase cells harvested 4 h earlier (Fig. 1*B*). Consistent with our previous report in log-phase cells (4), G_2-phase fibroblasts arrested with ICRF-193 displayed only a small inhibition of cyclin B1-Cdk1 kinase activity (28%, $p = 0.14$) compared with the control cells allowed to accumulate in mitosis (Fig. 1*B*). These findings taken together with the observation of strong inhibition of Plk1 kinase activity (66%) (Fig. 1*C*) suggest that inhibition of Plk1 activity and cyclin B1-Cdk1 activation during the topoisomerase II-dependent G_2 checkpoint response are not directly linked.

Plk1 Activity during the ICRF-193-induced Checkpoint Response Is ATR-dependent—It was recently reported that inhibition of Plk1 activity after treatment of cells with DNA-damaging agents occurs in an ATM- and ATR-dependent manner (18). Because ATR was recently shown to be the relevant kinase that enforced the topoisomerase II-dependent G_2 checkpoint (4), we next investigated whether ICRF-193-induced inhibition of Plk1 activity was mediated by ATR. AT cells have been previously shown to display a normal G_2 checkpoint response to ICRF-193 (4). Therefore, as expected, the inhibition of Plk1 activity observed after treatment with ICRF-193 was independent of ATM signaling (Fig. 2, *top panel*). AT cells (GM03395) treated with ICRF-193 displayed an ICRF-193-induced inhibition of Plk1 kinase activity (57% of Me_2SO control) (Fig. 2, *top panel*). The ICRF-193-induced inhibition of Plk1 activity observed in NHF1-hTERT cells was reversed in the presence of 2 mM caffeine, an inhibitor of ATM and ATR (data not shown). Taken together, these observations implied that regulation of Plk1 activity during the topoisomerase II-dependent checkpoint was ATR-dependent. To determine directly

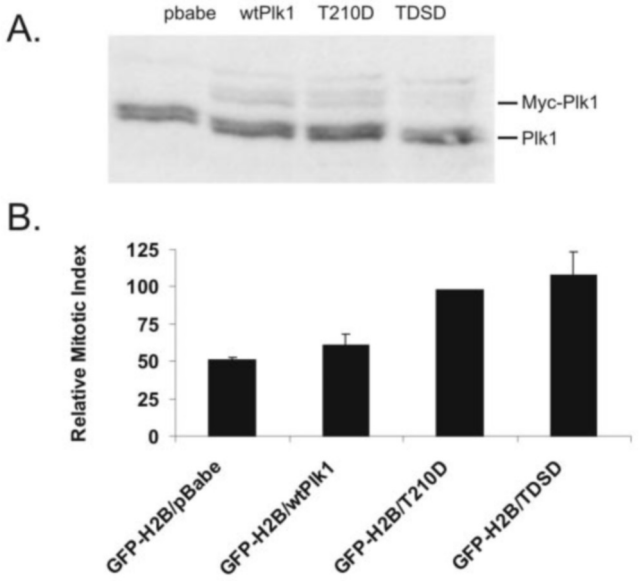

FIG. 3. **Inhibition of Plk1 activity is required for the topoisomerase II-dependent G_2 checkpoint response.** HeLa cells were transfected with GFP-H2B together with pBABE, wtPlk1, T210D, or TDSD plasmids, as described under "Experimental Procedures." 48 h after transfection, cells were incubated with Me_2SO or 2 μM ICRF-193 for 2 h, then harvested for Western immunoblot analysis (*A*) or fixed for mitotic index determination, which was quantified by examining GFP-stained nuclei using a fluorescent microscope. The results in *B* show the mitotic index in ICRF-193-treated cells expressed as a percentage of the mitotic index in the Me_2SO-treated cells (mean ± S.D., $n = 3$).

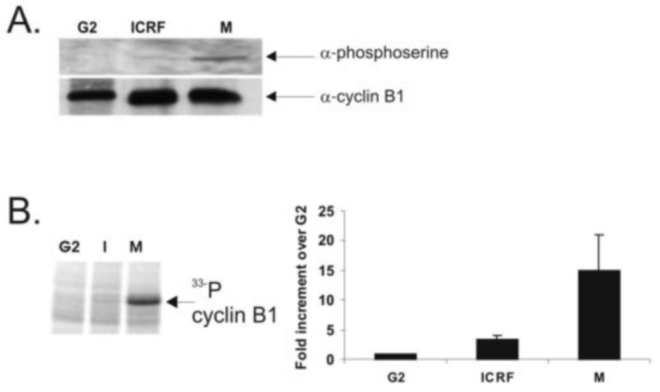

FIG. 4. **The topoisomerase II-dependent G_2 checkpoint inhibits cyclin B1 phosphorylation *in vivo* and *in vitro*.** Synchronized G_2 phase NHF1-hTERT fibroblasts were harvested directly or further incubated with Me_2SO or 2 μM ICRF-193 in the presence of 100 ng/ml of Colcemid for 4 h. At the time of harvest, cells were stored as dry pellets at −80 °C until kinase assays or immunoprecipitation immunoblots were performed. *I* and *M* denote ICRF-193-treated and Me_2SO-treated mitotic phase cells respectively. *A*, cyclin B1 was immunoprecipitated from cell lysates as described under "Experimental Procedures," and immunoblot analysis was performed using antibodies to phosphoserine and cyclin B1. *B*, *in vitro* cyclin B1 phosphorylation was determined as described under "Experimental Procedures." The autoradiograph is representative of four independent experiments. Results show the mean (± S.E., $n = 4$) increment in kinase activity relative to the G_2 phase cells.

whether ATR mediates the ICRF-193-induced inhibition of Plk1 activity, Plk1 kinase assays were performed in GM847 fibroblasts containing an inducible kinase-inactive ATR allele (ATR^{ki}). Treatment of non-induced GM847 fibroblasts with ICRF-193 caused a significant inhibition in Plk-1 kinase (63% of control) (Fig. 2, *bottom panel*). However, cells induced to overexpress ATR^{ki} failed to display the ICRF-193-induced inhibition of Plk1 activity (Fig. 2, *bottom panel*). Like mitotic entry, Plk1 activity was inhibited in an ATR-dependent manner by the topoisomerase II-dependent checkpoint response.

Overexpression of Constitutively Active Plk1 Reverses ICRF-193-induced Checkpoint Function—Plk1 is activated by phosphorylation on serine and threonine residues that lie within a conserved activation loop (17). Mutation of Thr-210 (T210D) or both Thr-210 and Ser-137 to aspartic acid (TDSD) to mimic phosphorylation has been reported to result in constitutive activation of Plk1 (17). Overexpression of these alleles in U20S cells reversed the DNA damage-induced mitotic delay, which implied that Plk1 is a target of the DNA damage checkpoint (17). To demonstrate directly that regulation of Plk1 activity was required for ICRF-193-induced checkpoint function, we tested the ability of human cells overexpressing wild type or constitutively active Plk1 alleles to undergo an ICRF-193-induced mitotic delay. To circumvent the problem of G_1 arrest due to the transfection protocol in NHF1-hTERT fibroblasts, HeLa cells lacking functional p53 were used for these experiments. These HeLa cells had previously been shown to respond to ICRF-193 with a mitotic delay similar to that seen in NHF1-hTERT cells (4). HeLa cells were co-transfected with either Myc-tagged wild type Plk1, Myc-tagged constitutively active Plk1 (T210D or TDSD), or an empty vector plasmid (pBABE) together with a GFP-histone H2B fusion protein (Fig. 3*A*). The transfection efficiency was equivalent between alleles and varied from 12 to 20% between experiments. This is illustrated by the low signal of Myc-tagged Plk1 compared with the endogenous Plk1 observed on the immunoblot from total HeLa cell extracts (Fig. 3*A*). To account for the low transfection efficiency, only cells positive for GFP-H2B expression were examined for ICRF-193-induced mitotic delay. Treatment with ICRF-193 resulted in a 49% inhibition of mitosis in GFP-H2B-positive cells and a 40% inhibition of mitosis in GFP-H2B-positive cells expressing the wild type Plk-1 (Fig. 3*B*). GFP-H2B cells expressing the constitutively active Plk1 mutants (T210D or TDSD) displayed no inhibition of mitosis after treatment with ICRF-193 (98% of control and 105% of control, respectively) (Fig. 3*B*). Thus, inhibition of Plk1 activity is required for the topoisomerase II-dependent checkpoint.

Plk1-dependent Cyclin B1 Phosphorylation Is Inhibited during the Checkpoint Response to ICRF-193—One function of Plk1 is to phosphorylate cyclin B1 and promote its nuclear accumulation (26). Thus, we hypothesized that regulation of Plk1 activity during the checkpoint response to ICRF-193 controls the localization of cyclin B1-Cdk1 complexes by regulating the phosphorylation status of cyclin B1. To test whether treatment with ICRF-193 inhibits serine phosphorylation of cyclin B1 *in vivo*, cyclin B1 was immunoprecipitated from synchronized fibroblasts, then SDS-PAGE and immunoblots were performed using a phosphoserine antibody. As expected, serine phosphorylation on cyclin B1 was not observed in G_2-phase cells but was present in mitotic control cells (Fig. 4*A*). The serine phosphorylation on cyclin B1 was severely diminished in cells that experienced an ICRF-193-induced mitotic delay (Fig. 4*A*). The phosphorylation of cyclin B1 has been shown to be due to the activity of kinases active in mitosis (20). To investigate whether the ICRF-193-induced checkpoint inhibits kinases that phosphorylate cyclin B1 *in vitro*, kinase activity toward endogenous cyclin B1 was assessed in cell-free extracts from NHF1-hTERT fibroblasts that were synchronized with the regimen described above. Kinase activity toward endogenous cyclin B1 in extracts of synchronized mitotic cells was quite high (15× elevated) compared with that detected in extracts from G_2-phase cells that had been harvested 4 h earlier (Fig. 4*B*). Cyclin B1 phosphorylation was slightly increased in extracts from ICRF-193-treated cells when compared with G_2-phase cells (3.3× elevated). However, in extracts from cells that had

Treatment		Breaks	Exchanges	Constrictions	Unifilar
DMSO + Caffeine	Num.	0	0	0	0
	%	0	0	0	0
ICRF-193 + Caffeine	Num.	27	27	103	5
	%	56	60	80	20

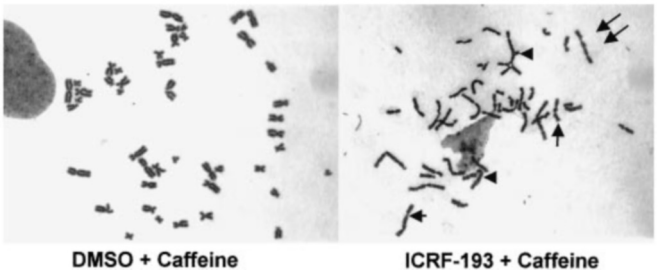

FIG. 5. **Bypass of the topoisomerase II-dependent G_2 checkpoint with caffeine promotes clastogenesis.** Synchronized G_2 phase NHF1-hTERT cells were incubated with Me_2SO (*DMSO*) or 2 μM ICRF-193 in the presence of 2 mM caffeine and 100 ng/ml Colcemid for 4 h. Cells were then harvested, and metaphase spreads were prepared as described under "Experimental Procedures." The *arrows* point to constrictions, and *arrowheads* point to breaks and exchanges. The *double arrow* is an example of a break at a constriction site. *Num.*, the numbers of events in 25 cells; %, the percent of cells with such an event.

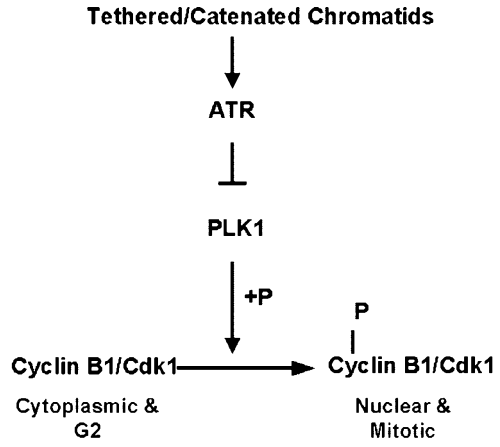

FIG. 6. **Model of topoisomerase II-dependent G_2 checkpoint function.** *P*, phosphorylation.

been treated with ICRC-193, the ability of a kinase or kinases to phosphorylate endogenous cyclin B1 was significantly inhibited (27% of mitotic control) (Fig. 4*B*). Immunodepletion of Plk1 from mitotic cell extracts has been previously shown to abolish phosphorylation of cyclin B1 on Ser-133 and Ser-147 (26). Immunodepletion of Plk1 in extracts from Me_2SO-treated mitotic NHF1-hTERT cells did not completely inhibit the phosphorylation of cyclin B1 in this system (data not shown). This result was not surprising because other kinases such as cyclin B1-Cdk1 are able to phosphorylate the first two serines within the cyclin B1 cytoplasmic retention sequence. In combination, these findings suggest that the topoisomerase II-dependent checkpoint prevents a kinase or kinases from phosphorylating serines on cyclin B1.

Bypass of the ICRF-193-induced Checkpoint with Caffeine Promotes Clastogenesis—ATR is essential for stabilization of the genome during the cell cycle, because disruption of ATR function results in the destabilization of chromosomes and cell death (4, 35). One function of ATR is to enforce the ICRF-193-induced checkpoint (4). Because the consequences of defective topoisomerase II-dependent checkpoint function have not been investigated in normal human cells, the effect of blocking ATR-dependent G_2 checkpoint function in NHF1-hTERT fibroblasts was investigated. NHF1-hTERT cells were synchronized to G_2 as described above. ICRF-193 or Me_2SO were added to the cultures in the presence of 2 mM caffeine, a concentration known to inhibit ATR kinase activity and reverse ICRF-193-induced checkpoint function in normal human fibroblasts (36).[2] The cells were incubated for an additional 4 h in the presence of Colcemid to capture the mitotic cells. No chromosomal aberrations were observed in 25 metaphases from mitotic control cells incubated with caffeine (Fig. 5). However, metaphases from ICRF-193-treated cells that were incubated with caffeine to bypass the checkpoint were abnormal. The chromosomes from ICRF-193-treated cells were much longer and less condensed than those from Me_2SO-treated mitotic cells (Fig. 5). Moreover, 20% of the metaphases from the ICRF-193-treated cells contained chromosomes that were all unifilar, that is, the sister chromatid cores had not separated. These results are consistent with a requirement for topoisomerase II in chromatid condensation (37, 38). The remaining 80% of metaphases contained only bifilar chromosomes. Bifilar chromosomes from cells incubated with ICRF-193 in the presence of caffeine displayed aberrant structural features and frank damage (Fig. 5). All of the metaphases with bifilar chromatids exhibited constrictions or tangles between sister chromatids (Fig. 5). Fifty-six percent of the metaphases contained chromatid breaks and 60% contained exchanges. As shown in Fig. 5, some of the breaks appeared to occur at the site of a constriction.

DISCUSSION

The findings presented here demonstrate that Plk1 kinase activity and cyclin B1 phosphorylation are regulated by a topoisomerase II-dependent G_2 checkpoint. Progression of synchronized human fibroblasts from G_2 to M was associated with activation of Plk1, and this activation was inhibited when cells were treated with ICRF-193 to inhibit topoisomerase II catalytic activity. The regulation of Plk1 activity observed in cells experiencing an ICRF-193-induced G_2 arrest was ATR-dependent because expression of a kinase-inactive ATR allele reversed the ICRF-193-induced inhibition of Plk1 kinase activity. Expression of constitutively active Plk1 mutants reversed the ICRF-193-induced mitotic delay, demonstrating that the inhibition of Plk1 kinase activity observed after treatment with ICRF-193 was not merely due to an inability of the cells to enter mitosis. This finding provides genetic evidence that inhibition of Plk1 activity is required for the topoisomerase II-dependent G_2 checkpoint. Inhibition of mitosis and Plk1 activity in the presence of high cyclin B1-Cdk1 activity occurred in synchronized human fibroblasts that were treated with ICRF-193. Moreover, treatment of G_2 cells with ICRF-193 also resulted in an inhibition of cyclin B1 phosphorylation. These observations support a model for the topoisomerase II-dependent G_2 checkpoint whereby ATR signals to inhibit Plk1 activity, which in turn prevents the phosphorylation of cyclin B1 and, thus, sustains nuclear exclusion of cyclin B1-Cdk1 complexes (Fig. 6).

Nuclear exclusion of cyclin B1-Cdk1 complexes was previously reported to be an important component of topoisomerase II-dependent G_2 checkpoint function (4). Phosphorylation at four serines within cyclin B1 is required for nuclear accumulation of cyclin B1-Cdk1 complexes at the beginning of mitosis (19–22, 25). In support of the hypothesis that one mechanism for checkpoint-enforced nuclear exclusion of cyclin B1-Cdk1 complexes is to prevent the phosphorylation of cyclin B1, serine phosphorylation of cyclin B1 was impaired in checkpoint-arrested cells. Plk1 has recently been shown to phosphorylate cyclin B1 on Ser-147 and target it to the nucleus during prophase in human cells (26). It was suggested that this phos-

[2] P. B. Deming and W. K. Kaufmann, unpublished observations.

phorylation event resulted in inactivation of the nuclear export sequence (26). This is consistent with the finding in the *Xenopus* system that phosphorylation of Ser-113 resulted in the inability of cyclin B1 to bind to the Crm1a nuclear export receptor (25). Plk1 activity was inhibited in ICRF-193-treated cells, and the phosphorylation of cyclin B1 in mitotic cell extracts was diminished when Plk1 was immunodepleted from the reaction, suggesting that the ICRF-193-induced checkpoint regulates cyclin B1 localization at least in part through inhibition of Plk1 activity. Cyclin B1-Cdk1 itself has been reported to phosphorylate *in vitro* the first two serines within the cytoplasmic retention sequence of cyclin B1 (19, 21, 25, 39, 40). Cells arrested in G_2 with ICRF-193 are able to sustain nuclear exclusion of cyclin B1-Cdk1 complexes (4) and significantly inhibit cyclin B1 phosphorylation despite high cyclin B1-Cdk1 activity. Therefore, it does not seem likely that autophosphorylation of cyclin B1 is the rate-limiting event *in vivo* that promotes cyclin B1 nuclear accumulation. Nevertheless, an increased amount of cyclin B1 phosphorylation was observed in ICRF-193-treated cells compared with those in G_2 4 h earlier. It is possible that cyclin B1-Cdk1 autophosphorylation does occur on Ser-126 and/or Ser-128, but that nuclear accumulation requires phosphorylation of all four serines, in particular Ser-147, which inactivates the nuclear export sequence (25, 26).

Despite the finding that the activity of Plk1 kinase was significantly inhibited in G_2-phase cells experiencing a topoisomerase II-dependent checkpoint response, cyclin B1-Cdk1 activity was inhibited only modestly. Therefore, it does not seem probable that the regulation of Plk1 activity mediates the activity of cyclin B1-Cdk1 during the G_2 arrest induced by ICRF-193. Our data do not rule out the possibility that Plk1 initially activates the Cdc25C auto-amplification loop because there was an increase in Plk1 activity during the 4-h treatment with ICRF-193. Activation of the Cdc25C-cyclin B1-Cdk1 loop may require only a minimal amount of Plk-1 activity. Inhibition of Plk1 kinase activity in the presence of substantial cyclin B1-Cdk1 activity during the ICRF-193-induced G_2 arrest may be explained in part by the previous findings that neither Chk1 nor Chk2 is activated in cells treated with ICRF-193 (4). Without Chk1 or Chk2 activation, Cdc25C should be in an unphosphorylated state at Ser-216 (9, 10) and, therefore, capable of interacting with and activating cyclin B1-Cdk1.

Downes *et al.* (3) first proposed that cells express a topoisomerase II-dependent checkpoint that monitors the status of catenated sister chromatids after DNA replication and prevents exit from G_2 until chromatids are sufficiently decatenated (3). We recently reported that unlike the DNA damage G_2 checkpoint, the G_2 checkpoint response to ICRF-193 was independent of ATM signaling and occurred in the absence of Chk1 and Chk2 phosphorylation and in the presence of high levels of cyclin B1-Cdk1 kinase activity (4). This study provided further evidence in support of a topoisomerase II-dependent G_2 checkpoint that monitors the status of chromatid decatenation and is distinct from the DNA damage G_2 checkpoint. However, although ICRF-193 does not directly generate DNA damage, it is not clear if the ICRF-193-induced closed-clamp conformation of topoisomerase II on DNA mimics a DNA lesion or interferes with DNA metabolic machinery. It is also possible that ICRF-193 affects enzymes other than topoisomerase II. Ectopic expression of the G_2-specific human topoisomerase II-α in yeast sensitized cells to killing by ICRF-193 at concentrations that did not affect endogenous topoisomerase II activity (41). This result suggests that the observed effects of ICRF-193 are specifically directed at topoisomerase II and the ICRF-193-induced closed-clamp conformation of human topoisomerase II on DNA interferes with DNA metabolism (41). The signal that is generated by the closed-clamp conformation of topoisomerase II on human chromatin and which activates ATR signaling to inhibit Plk1 and delay mitosis is not known. It seems likely that the signal may be related to the presence of the tethered DNA strands that are induced by ICRF-193 or the catenated chromatids that persist when topoisomerase II function is blocked.

Override of ICRF-193-induced G_2 arrest in normal human fibroblasts using the ATR inhibitor caffeine resulted in clastogenesis. Metaphases from cells that bypassed the topoisomerase II-dependent checkpoint displayed chromatid breaks and exchanges as well as constrictions between daughter chromatids and tangles. ICRF-193 induced similar chromosomal aberrations in a muntjac cell line (42). The ICRF-193-induced chromosomal aberrations are likely due to the inhibition of topoisomerase II catalytic activity producing tethered and catenated chromatids. It is conceivable that these structures interfere with chromatid dis-cohesion producing constrictions and tangles. Torsional strain placed on the chromatids at such constriction sites by condensation forces may result in chromatid breakage, and exchange aberrations result from erroneous repair of juxtaposed breaks. Alternatively the exchange aberrations may be the result of a recombination event targeted at sites of tethering or catenation between non-sister chromatids. Our results suggest that the checkpoint response that arrests cells in G_2 when topoisomerase II catalytic activity is impaired may contribute to genetic stability by delaying mitosis until chromatid catenations have been sufficiently resolved.

The observation that inhibition of topoisomerase II catalytic activity promotes clastogenesis suggests another mechanism of genetic destabilization. The high frequency of nonreciprocal translocations recently observed in prostate cancer cell lines that were engineered to overexpress HMG1(Y) implicated a role for HMG1(Y) in DNA rearrangements (43). It was suggested that because HMG1(Y) colocalizes with topoisomerase II at matrix attachment regions (MARS), the nonreciprocal translocations observed in the cells overexpressing HMG1(Y) may be a result of topoisomerase II processing of the abnormal DNA structures caused by HMG1(Y) overexpression. An alternate explanation for these results is that overexpression of HMG1(Y) interferes with the ability of topoisomerase II to decatenate DNA. Inhibition of topoisomerase II decatenatory activity in cells undergoing chromosome condensation may cause tension at the catenation sites, resulting in breaks, illegitimate DNA repair, and exchange aberrations.

In conclusion, the studies presented here demonstrate a connection between ATR and Plk1 that regulates entry to mitosis. It remains to be defined how ATR regulates Plk1 activity. Because Plk1 does not have any SQ sites, which are preferred ATR phosphorylation sites (44), it is unlikely that ATR regulates Plk1 directly but rather regulates either an activator or inhibitor of Plk1. Increased phosphorylation of Plk1 correlates with an increase in its activity during mitosis (33, 45); therefore, one possibility for regulation of Plk1 during G_2 checkpoint responses is the inhibition of a kinase(s) that directly phosphorylates Plk1. One candidate is the Ste20-like kinase, Slk1 (homologous to xPlkk1), which was recently shown to activate Plk1 during the G_2 phase of the cell cycle (14, 46). Future studies should concentrate on the mechanism by which ATR regulates Plk1 activity and identify the substrate that activates ATR during the topoisomerase II-dependent G_2 checkpoint response.

Acknowledgments—We acknowledge Tim Heffernan and Drs. Dennis Simpson and Alexandra Heinloth for technical advice, Dr. Marila Cordeiro-Stone for critical reading of the manuscript, Drs. Erich Nigg (Max Planck Institute for Biochemistry) and Rene Medema (Jordan Laboratory, University Medical Center Utrecht) for providing the Plk1 plasmids, Dr. Douglas Ferris (National Cancer Institute, National In-

stitutes of Health) for antibody to Plk1, and Dr. Andrew Creighton (Medical Chemistry Laboratory, St. Bartholomew's Hospital Medical College) for providing the ICRF-193.

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