DNA damage triggers multiple checkpoint pathways to arrest cell cycle progression. Polo-like kinase 1 (Plk1) is an important regulator of several events during mitosis. In addition to Plk1 functions in cell cycle, Plk1 is involved in DNA damage checkpoint in G2 phase. Normally, ataxia telangiectasia-mutated kinase (ATM) is a key enzyme involved in G2 phase cell cycle arrest following DNA damage, and inhibition of Plk1 by DNA damage during G2 occurs in an ATM/ATR-dependent manner. However, it is still unclear how Plk1 is regulated in response to DNA damage in mitosis in which Plk1 is already activated. Here, we show that treatment of mitotic cells with doxorubicin and γ-irradiation inhibits Plk1 activity through dephosphorylation of Plk1, and cells were arrested in G2 phase. Treatments of the phosphatase inhibitors and siRNA experiments suggested that PP2A pathway might be involved in regulating mitotic Plk1 activity in mitotic DNA damage. Finally, we propose a novel pathway, which is connected between ATM/ATR/Chk and protein phosphatase-Plk1 in DNA damage response in mitosis.

Mitotic catastrophe of mammalian cells results from a combination of deficient cell cycle checkpoints (DNA damage checkpoint and spindle assembly checkpoint) and cellular damage. The failure to arrest the cell cycle progression before or at mitosis causes aberrant chromosomal segregation and asymmetric division with the consequent generation of aneuploidy, which may lead to oncogenesis (1). Mitotic catastrophe influences by numerous regulatory proteins, in particular, cell cycle-specific kinase (such as cdk1, polo-like kinases, and aurora kinases), cell cycle checkpoint proteins, survivin, p53, caspases, and Bcl-2 family proteins (2–4). Polo-like kinase 1 (Plk1) has important roles during mitosis. To initiate mitosis, Plk1 is required for the phosphorylation of Cdc25C and mitotic cyclin in G2/M boundary (5–7), centrosome maturation (8, 9), and the establishment of a bipolar spindle (6, 9, 10). Activation of the anaphase-promoting complex (APC) by Plk1 initiates anaphase and exit from mitosis (11–13). One of the most intriguing features of Plk1 is the dynamic change of its subcellular localization and kinase activity during mitosis. Plk1 localizes on centrosome during mitosis before anaphase, and moves into the kinetochores/centromere during anaphase, suggesting that Plk1 may regulate chromosome and chromatin separation during this period (14–17). Subsequently, Plk1 moves to the central spindle and midbody (14, 18, 19). In addition to the roles during mitotic progression, several experiments in budding yeast and mammalian cells have shown that Plk1 may be involved in a DNA checkpoint pathway. Cdc5, the polo-like kinase in budding yeast, is controlled by the DNA damage checkpoint. In cdc13-arrested cells, Cdc5p-associated kinase activity is high, and its modification is dependent upon the factors required to activate the DNA damage checkpoint including Mec1p, Mec2/Rad53p, and Rad9p (20, 21). Smits et al. (22) reported that the effects of DNA damage on Plk1 in various human cell lines. DNA damage in G2 phase establishes a block to mitotic entry, and mitotic cells remain arrested at mitosis when exposed to DNA damage agents. Plk1 is inhibited by DNA damage in G2 and mitosis, and its inhibition appears to have a critical role in DNA damage responses. Significantly, expression of constitutively activated mutant of Plk1 can overcome these arrests induced by DNA damage, suggesting that Plk1 is an important target of the DNA damage checkpoint (22). Several reports from mammalian cell studies show that inhibition of Plk1 by DNA damage occurs in an ATM- or ATR-dependent fashion (23), and that Plk1 and Chk2 interact and co-localize to centrosome and midbody (24). These reports on chk2 and Plk1 partly explain how Plk1 is regulated and functions in DNA damage responses and checkpoint pathway. Moreover, Plk1 phosphorylates Chk2 in vitro (24). On the contrary, Plk1 was also identified as new putative target protein of Chk2 through protein data base searching and in vitro kinase assays (25). Although there are many reports on these issues, it is still unclear about the dependence or connection between Plk1 and ATM/Chk, and Plk1 functions in this pathway. Here, we show the inhibition of Plk1 and its effects in mitotic DNA damage. Inactivation of Plk1 is possibly caused by dephosphorylation through the action of protein phosphatase in mitotic DNA damage.

**MATERIALS AND METHODS**

**Cell Culture and Treatments**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal...
Regulation of Polo-like Kinase 1 by DNA Damage in Mitosis

bovine serum. To arrest in mitosis, cells were treated with nocodazole (200 ng/ml) for 16 h, and collected by mitotic shake-off. For DNA damage, doxorubicin (5 μM) was treated for 1 h, or 10–20 Gy of γ-ray was irradiated. Cells were washed twice with 1× PBS buffer thoroughly and incubated for each indicated time in fresh medium. To inhibit phosphatases, cells were pretreated with okadaic acid (Alexis) or tautomycin (Alexis) for 4 h before treatment of doxorubicin.

Determination of G1, G2, or Mitotic Phase—For mitotic spread assay, cells were fixed and stained with Giemsa staining solution as described previously (26). G1 and G2 cells were determined by the intensity of Giemsa signal and the nuclear membrane staining by lamin antibody (anti-lamin A/C antibody from Santa Cruz Biotechnology). Mitotic cells were determined by scattered chromosomes and nuclear membrane breakdown.

Flow Cytometry and Microscopy—For flow cytometry, cells were harvested and fixed in ice cold 80% ethanol. Cells were washed with PBS, stained with propidium iodide (40 μg/ml) and 100 μg/ml RNaseA, and were analyzed by flow cytometry of 10,000 events (Cell Quest, Becton Dickinson). The data were processed with ModFit software for DNA distribution analysis. For microscopic analysis, cells were cultured on glass coverslips, which is coated with fibronectin solution (Sigma). Cells were fixed with 4% paraformaldehyde and ice cold methanol. After a rinse with PBS, cells were blocked in PBS-10% goat serum for 30 min. Cells were stained with mouse monoclonal anti-γ-tubulin antibody (Sigma), and mouse monoclonal anti-MPM-2 antibody (Upstate Biotechnology) for detection of centrosome and mitotic cells, respectively. After overnight treatment, cells were stained with goat anti-mouse IgG–FITC (Santa Cruz Biotechnology). Their fluorescence signals were detected and captured by confocal microscopy (LSM510). The percentage of MPM-2-positive cells was counted using fluorescence microscopy (Axioskop 40, Zeiss).

Western Blotting, Immunoprecipitation, and in Vitro Kinase Assay—Cells were lysed in 0.5% Nonidet P-40, 120 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, and Complete protease inhibitor mixture (Roche Applied Science). For Western blotting, cell lysate (100–300 μg/lane) was electrophoresed through 10% SDS-PAGE (low bis gel, 120:1) to detect endogenous phospho-Plk1 (27). Proteins were transferred to polyvinylidene difluoride membrane (Millipore) and blocked in TBS with 5% skim milk. After incubation with mouse monoclonal anti-Plk1 antibody (Zymed Laboratories Inc.), and rabbit polyclonal anti-cyclin B1 and anti-phospho-histone H3(ser10) antibodies (Santa Cruz Biotechnology), blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Signals were visualized using ECL TM system (Amersham Biosciences). For immunoprecipitation of Plk1, cell lysate (500 μg) was incubated with mouse monoclonal anti-Plk1 antibody (Zymed Laboratories Inc.) and protein/A/G-agarose bead. Beads were washed thoroughly and used for in vitro kinase assay.

PP2A Phosphatase Assay by Immunoprecipitation—PP2A phosphatase assay was performed by Malachite Green phosphate detection system (Upstate Biotechnology). In Brief, Endogenous PP2A was immunoprecipitated by anti-PP2A antibody (Upstate Biotechnology) from cell lysates. Beads were washed with TBS, followed by wash with 50 mm Tris-HCl, pH 7.0, 100 μM CaCl2. Synthetic phosphopeptide (KRPITRR) was incubated with the immunoprecipitate at 30 °C, and degradation of phosphopeptide was detected by Malachite Green phosphate detection solution at 650 nm.

PP2A siRNA Design, Preparation, and Transfection—SiRNA duplexes were synthesized by Bioneer (Daejeon, Korea). All siRNA duplexes have the sense strand sequence of 5′-N19-dTdT-3′, where N19 is the target sequence in PP2A-D (NM_002715) and PP2A-E (NM_004156) and dTdT is 2–3 nt 3′ overhang. Antisense strand has the sequence complementary to N19 and dTdT 3′ overhang sequence. All six siRNAs were designed by Turbo si-Designer™ (Bioneer) and their N19 target sequences were as follows: PP2A-D-1446, 5′-CACA-TGGACCAAAAGATGT-3′; PP2A-D-1523, 5′-CAGTTCAT-TGCATGCTGAA-3′; PP2A-D-2212, 5′-CATTTGGTGTGT-GCATT-3′; PP2A-E-1297, 5′-CACATCATGGACCAAA-TGT-3′; PP2A-E-1550, 5′-CACAAGCTACTTCCCATA-3′; PP2A-E-1720, 5′-GTCATGACAGTGTGTGCAT-3′. After validation of silencing efficiency, we choose two constructs, PP2A-D-1446 and PP2A-E-1720 for further study. Twenty-four hours before transfection, HeLa cells were plated in 6-well dishes at a density of 2.5 × 105 cells/well. Transfection was performed using Lipofectamine 2000™ (Invitrogen) as recommended by the manufacturer. In brief, cells were washed once with Opti-MEM (Invitrogen), and 500 μl of Opti-MEM was added to each well. For each transfection, 3.5 μl of Lipofectamine was mixed with 250 μl of Opti-MEM and incubated for 5 min at room temperature. Then, siRNA (final concentration of 100 nM) was added to 250 μl of Opti-MEM and the mixture was added to the Lipofectamine mixture and incubated for an additional 20 min at room temperature to allow complex formation. The solutions were added to the cells in the 6-well plate to the final volume of 1 ml. Cells were incubated at 37 °C in the presence of the transfection solution for 6 h. The Opti-MEM medium containing the complexes was then replaced with 2 ml of standard growth media and cultured at 37 °C for 18 h. Twenty-four hours after transfection, total RNA was isolated from transfected cells using the RNeasy mini kit (Qiagen) and subjected to Northern blot analysis. For Western blot analysis, transfected cells were treated with nocodazole (200 ng/ml) at 36 h after transfection. After 16 h, doxorubicin (5 μM) was added to the culture and incubated for 1 h. Cells were trypsinized and washed twice with 2 ml of 1× PBS. Cells were cultured for 5 h before harvested.

Northern Blot Analysis—Northern blot analysis was performed with 8 μg of total RNA as previously described (28). The filters were first probed with PP2A cDNAs and rehybridized with β-actin cDNA as a control to normalize for sample variation. The probes labeled were prepared with 670 bp of the PP2A-α cDNA 3′-UTR region (NM_002715, nucleotide 1341–2010), 600 bp of PP2A-β cDNA 3′-UTR region (NM_004156, nucleotide 1171–1770), and 788 bp of the β-actin cDNA (NM_001101, nucleotide 433–1220) by using a random primer labeling kit (Stratagene) and [α-32P]dCTP (Amersham Biosciences) (29).
Regulation of Polo-like Kinase 1 by DNA Damage in Mitosis

RESULTS

Dephosphorylation of Plk1 and Inhibition of Plk1 Activity by Treatment with DNA Damage Agents—Previously, we reported that Plk1 is phosphorylated on threonine 210 during the mitotic phase of the cell cycle, and that this phosphorylation is correlated with Plk1 activation (27). As shown in lane 2 of Fig. 1, endogenous Plk1 in mitotic nocodazole-treated HeLa cells is highly phosphorylated and activated, whereas Plk1 in G2 cells is not phosphorylated and inactive (Fig. 1, lane 1). As demonstrated in our previous report (27), the phosphorylated form of Plk1 (the upper band on the gel in Fig. 1, indicated by phospho-Plk) is correlated with active Plk1; therefore, we used this modification of Plk1 as an assay for activated Plk1 in the present study.

Because DNA damage can occur during mitosis as well as interphase, we investigated how cells overcome DNA damage in the mitotic phase when Plk1 is fully activated. For this study, HeLa cells were synchronized at prometaphase by nocodazole treatment for 16 h, followed by doxorubicin treatment for 1 h to induce DNA damage. The cells were then transferred to fresh medium and incubated. As expected, Plk1 was highly phosphorylated and activated following nocodazole treatment (Fig. 2, lane 3). Five hours after removing the nocodazole, endogenous Plk1 remained in the phosphorylated form, but its kinase activity had slightly decreased (Fig. 2, lane 4). Just after doxorubicin treatment, Plk1 was phosphorylated and activated, similar to Plk1 without doxorubicin treatment (Fig. 2, lane 1); however, when doxorubicin-treated mitotic cells were incubated in fresh medium for more than 5 h to induce continuous mitosis, endogenous Plk1 was dephosphorylated, and its kinase activity decreased (Fig. 2, lane 2).

These data showed that doxorubicin treatment inhibits the kinase activity of Plk1 through dephosphorylation. To confirm the inactivation of Plk1 by DNA damage over time, mitotic HeLa cells were treated with or without doxorubicin for 1 h, washed, and incubated. After incubation, cells were harvested and endogenous Plk1 was analyzed. As expected, after 5 h most of the untreated cells were still in the mitotic phase (Fig. 3A, lane 1). After 7 h of incubation in fresh medium, more than 40% of the cells had reentered mitosis, and cells at the G1 phase could be observed (Fig. 3A, lane 6). The G1 population increased after 7 h of incubation, whereas the cells in G2/M phase decreased. These data indicate that cells with damaged DNA remain in mitosis and do not enter the next round of cell division (Fig. 3, A, lanes 8–12 and C). The association of DNA with histones results in densely packed chromatin, which restricts the binding of proteins involved in gene transcription to DNA. Histone H3, the core protein in nucleosomes, is phosphorylated at the end of prophase at two major mitosis-specific sites: Ser-10 and Ser-28 (30). Our data demonstrated that the phosphorylation of the histones in DNA-damaged cells disappeared rapidly, meaning that the cells had returned to interphase (Fig. 3B, lanes 5–8). As the cells entered G1, the histone phosphorylation signal weakened (lane 4 in α-p-His). These data indicated that cells with damaged DNA remain in interphase, and that the Plk1 activity is diminished. Analysis by the ModFit program revealed that the population of doxorubicin-treated cells with 4N DNA (G2/M phase) was unchanged after the 7-h incubation, whereas the cells with undamaged DNA entered G1.

To determine the status of the DNA-damaged mitotic HeLa cells after incubation, we used several approaches. First, cells were immunostained with anti-MPM2 antibody, and then the MPM2-positive mitotic cells were counted. Mitotic cells treated with nocodazole were not arrested in mitosis after nocodazole removal, and the number of MPM2-positive cells
FIGURE 3. DNA-damaged mitotic cells are accumulated in the 4N DNA stage. HeLa cells were synchronized in prometaphase by nocodazole treatment. After 16 h, cells were treated with or without doxorubicin for 1 h as previously described. Cells were transferred into fresh medium and harvested after incubation for indicated times (1, 3, 5, and 7 h). A, DNA contents of cells harvested after incubation during indicated times. After ethanol-fixation, cells were treated with propidium iodide and analyzed by FACScan™. noco, cells synchronized in mitosis; noco+ doxo, cells treated with nocodazole following doxorubicin treatment. B, Plk1 protein was detected by anti-Plk1 immunoblot (α-Plk1). The Plk1 immunoprecipitate was used in the kinase assay (casein). Endogenous cyclin B1 and the phosphorylated histone were detected by anti-cyclin B1 and anti-p-histone (Ser-10) antibodies, respectively (α-Cyclin B1 and α-p-His). Lanes 1 and 5, 1-h incubation; lanes 2 and 6, 3 h; lanes 3 and 7, 5 h; lanes 4 and 8, 7 h. C and D, quantification of cell population in A. Total numbers of G2/M cells were analyzed by ModFit™ (C). Mitotic cell populations were detected and counted by immunostaining with anti-MPM2 antibody (D). E, centrosome and nucleus in normal cells and in cells treated with nocodazole and doxorubicin. Nuclei and centrosomes were detected with propidium iodide and tubulin antibody, respectively. Panel a, cells treated with nocodazole for 16 h; panel b, cells incubated for 7 h after treatment of nocodazole; panel c, cells incubated for 7 h after serial treatment of nocodazole and doxorubicin. F, quantification of cell population in E. Cell phenotypes in the cell cycle stage are indicated by confocal images. In mito, all kinds of mitotic cells are counted. G, mitotic spread assay in normal cells and in cells treated with nocodazole and doxorubicin. Giemsa-stained types were classified, counted, and quantified as percentage. H, immunostaining with anti-lamin antibody for detection of nuclear membrane. Cells were treated with drugs and incubated under the same conditions as E.
DNA damage during mitosis may inhibit cell cycle progression and not in prometaphase anymore after the 7-h incubation. The mitotic cells with DNA damage were in G2 or early mitosis, and these cells exhibited intact nuclear envelopes (Fig. 3, upper panels). Immediately following 16 h of nocodazole treatment, the cells were round and mostly unattached to the culture dish, the centrosomes were apparently separated, and their nuclear boundaries were not detectable by lamin antibody (Fig. 3, E, panel a and H, upper panels). After 7 h, several cells were at the G1 stage and were well attached with intact nuclear envelopes (Fig. 3H, middle panels). At this stage, it was difficult to locate the centrosomes in each cell (Fig. 3E, panel b). Cells damaged by doxorubicin, however, were not round or well attached like the undamaged cells after the 7-h incubation, but their centrosomes were detectable and slightly separated. Moreover, these cells exhibited intact nuclear envelopes (Fig. 3E, panel c and H, lower panels). These phenotypes suggest that most of the mitotic cells with DNA damage were in G2 or early mitosis, and not in prometaphase anymore after the 7-h incubation. DNA damage during mitosis may inhibit cell cycle progression as a result of Plk1 inactivation and return cells to early mitosis. The percentage of cells with DNA damage that contained prometaphasic figures decreased significantly after incubation in fresh medium (Fig. 3G, black bar in panel b), whereas the percentage of cells containing G2/prophasic figures increased (Fig. 3G, gradient bar in panel b). The features of G2 cells are that DNA content is 4N, the Giemsa signal is increased, and the nuclear membrane is still remained (Fig. 3, A–H). The cells without DNA damage showed an increase in G1 figures (weak Giemsa signal and intact nuclear membrane) in fresh medium (Fig. 3, G, gray bar in panel a and H, middle panels).

Similar to doxorubicin, when mitotic cells were irradiated with 10 Gy and 20 Gy of γ-rays, dephosphorylation of Plk1 was induced, and the cells were arrested at the 4N DNA stage, indicating that the phenomena described above are not doxorubicin-specific effects (Fig. 4). Cells given 20 Gy of γ-rays remained at the 4n stage after a 7-h incubation, whereas G1 cells were detected 5 h after irradiation with 10 Gy (Fig. 4A). In mitotic-irradiated cells, Plk1 was dephosphorylated in a manner similar to Plk1 in doxorubicin-treated cells (Fig. 4B).

PP2A Is Involved in Dephosphorylation of Plk1 in Mitotic Cells with DNA Damage—During normal cell division, the level of Plk1 increases from G2 to mitosis, and the protein is modified by an upstream kinase (27). At the end of mitosis and cytokinesis, Plk1 is degraded by an anaphase-promoting complex, indicating that Plk1 inactivation by dephosphorylation may play a major role in Plk1 down-regulation. We showed that inactivation of Plk1 by dephosphorylation occurs in response to DNA damage during mitosis, suggesting that protein phosphatase(s) might be involved in mitotic Plk1 inactivation after DNA damage.

The serine/threonine protein phosphatase family contains four distinct groups: protein phosphatase-1 (PP1), protein phosphatase-2A (PP2A), protein phosphatase-2B (PP2B, also called calcineurin), and protein phosphatase-2C (PP2C) (31). Among the numerous family members, PP1 and PP2A are referred to as the two principal enzymes because they are ubiquitous and have broad specificity (31). By using two specific phosphatase inhibitors, okadaic acid and tautomycin, we sought to determine the phosphatase(s) that might be involved in regulating Plk1 in mitotic DNA-damaged cells. Okadaic acid has a 100-fold greater specificity for PP2A than for PP1. In
Regulation of Polo-like Kinase 1 by DNA Damage in Mitosis

contrast, tautomycin specificity for PP1 is 10-fold greater than for PP2A.

The specificity of these inhibitors allowed the activities of the individual phosphatases to be analyzed. Treatment of HeLa cells with various concentrations of okadaic acid for 4 h before DNA damage did not affect PP1 activity (Fig. 5B, white bars), but PP2A activity decreased in proportion to the amount of okadaic acid used (Fig. 5B, black bars). Moreover, treatment with 10 μM tautomycin for 4 h before DNA damage did not affect PP2A activity, whereas PP1 activity was inhibited (Fig. 5D, white bars). Western blot analyses with anti-PP1/-PP2A specific antibodies did not reveal any significant changes because of treatment with 100 nM okadaic acid, 10 μM tautomycin, or doxorubicin (Fig. 5, A and C). These data indicate that 100 nM okadaic acid inhibited PP2A activity in mitotic HeLa cells, and that 10 μM tautomycin inhibited PP1 activity. Notably, the effective concentrations of these phosphatase inhibitors in HeLa cells are in agreement with the results of a previous report (32). HeLa cells were then synchronized at prometaphase by nocodazole treatment for 14 h, and endogenous phosphatase activity was blocked by treatment with okadaic acid or tautomycin at their effective concentrations for 4 h.

After DNA damage, the cells were transferred to fresh medium, incubated for 7 h, and endogenous Plk1 was analyzed in cell extracts by Western blot analysis. Mitotic Plk1 was dephosphorylated by DNA damage (Fig. 6, A and B, lane 3). Interestingly, when 100 nM okadaic acid was added, dephosphorylation of mitotic Plk1 was not detected (Fig. 6A, lane 7). Because this concentration of okadaic acid specifically blocks PP2A (Fig. 5B), we postulated that PP2A might dephosphorylate mitotic Plk1. Inhibition of PP1 by tautomycin treatment had no effect on Plk1 dephosphorylation after DNA damage (Fig. 6B, lane 6), even though its activity decreased (Fig. 5D). These data indicate that PP2A, but not PP1, is involved in the inhibition of mitotic Plk1 following DNA damage.

The effect of okadaic acid on mitotic Plk1 is also shown in Fig. 7. HeLa cells were arrested at mitosis by nocodazole treatment (Fig. 7B, panel 1), and mitosis-specific phosphorylation of endogenous proteins was detected with MPM2 antibody (Fig. 7A, lane 1 in panel c). As expected, Plk1 was highly phosphorylated and activated (Fig. 7A, lane 1 in panels a and b). After DNA damage, the mitotic cell population, indicated by MPM2-positive cells, decreased (Fig. 7A, lane 3 in panel c), and Plk1 was dephosphorylated and inactive (Fig. 7A, lane 3 in panels a and b); however, PP2A inhibition by okadaic acid before DNA damage prevented the inhibition of Plk1 and increased the size of the mitotic cell population (Fig. 7, A and C, lane 4).

These data suggest that Plk1 is regulated by PP2A after DNA damage during mitosis. Plk1 interacted with endogenous PP2A in mitotic cells with and without damaged DNA (Fig. 8A). An in vitro phosphatase assay indicated that phosphoorylated Plk1 may be a substrate of PP2A, given that the upper band of Plk1 was diminished (Fig. 8B). To investigate the effect of PP2A on Plk1 inhibition, endogenous PP2A expression was silenced by siRNA. Mixed siRNA constructs for both the α and β isoforms of PP2A...
efficiently blocked PP2A siRNA expression (Fig. 9A); however, the PP2A protein level did not significantly decrease from the siRNA treatment, and PP2A signal was detected after a 4-min exposure during Western blotting (see “Materials and Methods”) (Fig. 9B, middle lower panel, 4 min, lanes 2 and 4). Although a large amount of PP2A protein remained, dephosphorylation of mitotic Plk1 after DNA damage was diminished by the siRNA treatment (Fig. 9B, upper panel, lane 4). In addition, phosphorylation of Plk1 after DNA damage was steady following okadaic acid treatment (Fig. 9B, lane 5). These data provide direct evidence for the involvement of PP2A in the regulation of mitotic Plk1 after DNA damage.

DISCUSSION

To date, most studies of DNA damage checkpoints have investigated the S and G2 phases; however, DNA damage responses in mammalian cells are not restricted to interphase and may occur during mitosis. When DNA damage occurs, cell division should not proceed until the damage is repaired. Thus, when DNA damage is detected during mitosis, mitotic progression is blocked by DNA damage checkpoint activation. Plk1 is a key protein in both the initiation and termination of mitosis, and may routinely regulate mitosis, arresting the cell cycle at several points during its progression by DNA damage.

In this report, we focused on two issues: how Plk1 is regulated following DNA damage during mitosis and at which stage cells are arrested by DNA damage. First, we examined whether Plk1 is directly regulated by the DNA damage specific kinase(s). Based on this, two questions remain regarding the inhibition of Plk1 upon mitotic DNA damage. Is Plk1 phosphorylated and inactivated, or is Plk1 dephosphorylated and inactivated?

Two classes of kinases play key roles in the response to DNA damage: phosphatidylinositol 3-kinase-related kinases and checkpoint (Chk) kinases (33). Recently, several reports showed that Plk1 is a target of the DNA damage checkpoint (22, 23), and that inactivation of Plk1 by DNA damage occurs in an ATM-or ATR-dependent manner (23). Despite these reports, it is still unclear whether ATM/ATR directly inhibits Plk1 activity. These reports suggested that inhibition of Plk1 by ATM/ATR probably requires other checkpoint kinases such as Chk1/Chk2. Seo et al. (25) included Plk1 as an in vitro target of Chk2, meaning that Plk1 is a possible downstream substrate of Chk2. If true, this may explain how Plk1 is regulated in an ATM-dependent manner upon DNA damage; however, the relationship between Chk1/Chk2 and Plk1 at DNA damage checkpoints appears complex. Tsvet-
kov et al. (24) reported that Plk1 and Chk2 interact and co-localize to the centrosomes and midbody, which are target organelles of Plk1. They also showed that Plk1 phosphor-ylates recombinant Chk2 in vivo, suggesting that Plk1 might be an upstream regulator of Chk2, and not a target of Chk2. In vivo verification of these results is required.

Previously, we reported that the phosphorylation of Plk1 increases during mitosis, and we identified the phosphorylation site of Plk1 in mitotic HeLa cells (27). As expected, Plk1 was highly phosphorylated at prometaphase regardless of whether the cells had been treated with DNA-damaging agents. Interestingly, phosphorylated Plk1 decreased in several hours after treatment with doxorubicin (Fig. 2, lane 2 and Fig. 3 B).

Based on FACS analysis, immunoassay with anti-MPM2, anti-phosphohistone, or anti-lamin antibodies, and mitotic spread assay, cells arrested at prometaphase began to divide after several hours in fresh medium, but mitotic cells treated with doxorubicin could not progress continuously through the cell cycle. Under these conditions, we observed that Plk1 was dephosphorylated and inactive (Fig. 3, A and B). These results indicate that inhibition of mitotic Plk1 by DNA-damaging agents is probably caused by its dephosphorylation.

So far, several groups have reported that phosphatase inhibitors such as okadaic acid inhibit the initiation of mitosis and induce a mitotic arrest, suggesting that protein phosphatase 1 and/or 2A might be involved in DNA damage responses (34–36).

In our experiments, when doxorubicin-treated mitotic cells were pretreated with okadaic acid, but not with tautomycin, the dephosphorylation and inactivation of Plk1 was suppressed (Fig. 6 A), suggesting that protein phosphatases are targets of the DNA damage checkpoint via the ATM/ATR pathway. Although the inhibition or activation of abundant and largely unspecialized proteins such as protein phosphatases might make it difficult to demonstrate specific effects, we have shown here that PP2A is likely involved in the response to DNA damage by applying its specific inhibitor or PP2A-specific siRNA to cells. Our results show that Plk1 does not seem to be regulated by the DNA damage checkpoint kinases Chk1/Chk2 directly, but its inactivation is caused by dephosphorylation, which requires protein phosphates(s) such as PP2A. Although PP2A participates in various cellular events, its activity is necessary during times of stress, such as in the G2 arrest that follows a viral infection or DNA damage by ionizing radiation (37, 38). A recent report indicated that Chk2 associates with and phosphor-ylates the B′ regulatory subunit of PP2A, and that PP2A activity is regulated by this phosphorylation (39). Although

FIGURE 8. PP2A interacts with and dephosphorylates phospho-Plk1 in mitotic DNA damage. A, endogenous PP2A in mitotic cells treated with or without doxorubicin was immunoprecipitated, and bound Plk1 was detected (upper panel). Middle and lower panels were indicated the amount of proteins in cell extract. Arrowhead indicated immunoglobulin heavy chain. B, purified PP2A from bovine kidney dephosphorylates mitotic phospho-Plk1. Phospho-Plk1 was purified in mitotic cell extract by immunoprecipitation.

FIGURE 9. PP2A depletion prevents Plk1 dephosphorylation by mitotic DNA damage. A, HeLa cells were transfected with RNA oligos against PP2Aα and PP2Aβ (see “Materials and Methods”). Twenty-four hours after transfection, cells were harvested. 8 µg of total RNAs were used for Northern analysis. For probes, PP2A cDNA and β-actin cDNA fragments were isotope-labeled and hybridized. The expression of endogenous PP2As was effectively blocked. B, HeLa cells were transfected with mixed RNA oligos against PP2Aα and PP2Aβ. Thirty-six hours after transference, cells were treated with 100 ng/ml nocodazole for 16 h, and then treated with doxorubicin for 1 h. After releasing for 5 h, cells were harvested, and 150 µg of cell lysates were directly resolved by SDS/PAGE, and subjected to Western blot (exposures for 20 s and 4 min in α-PP2A immunoblot) using the antibodies indicated on the left.
Regulation of Polo-like Kinase 1 by DNA Damage in Mitosis

FIGURE 10. Functions of Plk1 and PP2A in mitotic DNA damage checkpoint. DNA damage in prometaphase activates ATM/ATR pathway. The downstream kinases in this pathway, Chk kinases probably activate protein phosphatase 2A. Mitotic Plk1 is dephosphorylated by PP2A, and is inactivated in this condition. Because of inactivation of Plk1, mitosis is blocked and cannot progress forward in the cell cycle, or even revert from the middle of mitosis to the early stage of mitosis and G2. The dotted line is not validated in this report.

The phosphorylation of Plk1 is mostly restored following okadaic acid treatment (Fig. 7, lane 4), PP2A-controlled dephosphorylation probably contributes to Plk1 inactivation, but may not be the only way for Plk1 regulation, because the PP2A knockdown, the other evidence for Plk1 regulation, was not sufficient for showing the restoring of Plk1 activation.

We also considered the state of the DNA-damaged cells. G2 and early mitotic cell populations accumulated after DNA damage (Figs. 3 and 7C), and remained in prometaphase following nocodazole treatment. After a 7-h incubation, mitotic cells damaged by doxorubicin seemed to revert to a G2-like state (Fig. 3, A–H). A possible explanation is that if DNA damage occurs in cells prior to spindle checkpoint activation, cells would be accumulated in the G2 phase. It is a very interesting concept that cell division can be reversed if DNA damage in prometaphase activates ATM/ATR pathway. The downstream kinases in this pathway, Chk kinases probably activate protein phosphatase 2A. Mitotic Plk1 is dephosphorylated by PP2A, and is inactivated downstream kinases in this pathway, Chk kinases probably activate protein phosphatase 2A. Mitotic Plk1 is dephosphorylated by PP2A, and is inactivated in this condition. Because of inactivation of Plk1, mitosis is blocked and cannot progress forward in the cell cycle, or even revert from the middle of mitosis to the early stage of mitosis and G2. The dotted line is not validated in this report.

Moreover, the population of cells in G2 increases and is probably involved in Plk1 inhibition directly. As a result of Plk1 inactivation by dephosphorylation, cells cannot complete mitosis. Consequently, the population of cells in G2 increases after DNA damage in mitosis. These data suggest that prometaphasic cells might revert and remain in G2 as a result of Plk1 inactivation by DNA damage.

Acknowledgments—We thank Prof. Raymond L. Erikson in Harvard University for critical reading. We thank Prof. Dae-Sik Lim in KAIST, Prof. Yoon-Sun Seong, Chong-Heon Lee, and Chung-Hun Oh in Dankook University, and Prof. Eun-Yi Moon in Sejong University for much help and technical discussion.

REFERENCES
1. Castedo, M., Perfettini, J. L., Roumier, T., Andreau, K., Medema, R., and Kroemer, G. (2004) Oncogene 23, 2825–2837
2. Bunz, F., Dutriaux, A., Lengauer, C., Walldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998) Science 282, 1497–1501
3. Chen, Z., Xiao, Z., Chen, J., Ng S. C., Sowin, T., Sham, H., Rosenberg, S., Fesik, S., and Zhang, H. (2003) Mol Cancer Ther 2, 543–548
4. Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999) Nature 401, 616–620
5. Glover, D. M., Ohkura, H., and Tavares, A. (1996) J Cell Biol 135, 1681–1684
6. Hamanaka, R., Smith, M. R., O’Connor, P. M., Maloid, S., Mihalic, K., Spivak, J. L., Longo, D. L., and Ferris, D. K. (1995) J Biol Chem 270, 21086–21091
7. Lane, H. A., and Nigg, E. A. (1996) J Cell Biol 135, 1701–1713
8. Ohkura, H., Hagan, I. M., and Glover, D. M. (1995) Genes Dev 9, 1059–1073
9. Qian, Y. W., Erikson, E., Li, C., and Maller, J. J. (1998) Mol Cell Biol 18, 4262–4271
10. Tavares, A. G., Glover, D. M., and Sunkel, C. E. (1996) EMBO J 15, 4873–4883
11. Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Haging, A., Pines, J., and Peters, J. M. (2003) EMBO J 22, 6598–6609
12. Kotani, S., Tugendreich, S., Fuji, M., Jorgensen, P. M., Watanabe, N., Hoog, C., Hieto, P., and Todokoro, K. (1998) Mol Cell 1, 371–380
13. Brassac, T., Castro, A., Lorca, T., Le Peuch, C., Doree, M., Labbe, J. C., and Galas, S. (2000) Oncogene 19, 3782–3790
14. Arnaud, L., Pines, J., and Nigg, E. A. (1998) Chromosoma 107, 424–429
15. Jang, Y. J., Ji, J. H., Ahn, J. H., Hoe, K. L., Won, M., Im, D. S., Chae, S. K., Song, S., and Yoo, H. S. (2004) Biochem. Biophys. Res. Commun. 325, 257–264
16. Casenghi, M., Meraldi, P., Weinhardt, U., Duncan, P. I., Korner, R., and Nigg, E. A. (2003) Dev Cell 5, 113–125
17. Seong, Y. S., Kamijo, K., Lee, J. S., Fernandez, E., Kuriyama, R., Miki, T., and Lee, K. S. (2002) J Biol Chem 277, 32282–32293
18. Zhou, T., Aumaiz, J. P., Liu, X., Yu-Lee, L. Y., and Erikson, R. L. (2003) Dev Cell 5, 127–138
19. Wianny, F., Tavares, A., Evans, M. J., Glover, D. M., and Zernicka-Goetz, M. (1998) Chromosoma 107, 430–439
20. Cheng, L., Hanke, L., and Hardy, C. F. (1998) Mol Cell Biol 18, 7360–7370
21. Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Eldredge, S. J. (1999) Science 286, 1166–1171
22. Smits, V. A., Klompmarker, R., Arnaud, L., Rijksen, G., Nigg, E. A., and Medema, R. H. (2000) Nat Cell Biol 2, 672–676
23. van Vught, M. A., Smits, V. A., Klompmarker, R., and Medema, R. H. (2001) J Biol Chem 276, 41656–41660
24. Tsvetkov, L., Xu, X., Li, J., and Stern, D. F. (2003) J Biol Chem 278, 8468–8475
25. Seo, G. J., Kim, S. E., Lee, Y. M., Lee, J. W., Lee, J. R., Hahn, M. I., and Kim, S. T. (2003) Biochem. Biophys. Res. Commun. 304, 339–343
26. Chow, J. P., Siu, W. Y., Fung, T. K., Chan, W. M., Lau, A., Arozoo, T., Ng, C. P., Yamashita, K., and Poon, R. Y. (2003) Mol Cell Biol 14, 3989–4002
27. Jang, Y. J., Ma, S., Terada, Y., and Erikson, R. L. (2002) J Biol Chem 277, 44115–44120
28. Choi, Y. C., Gu, W., Hecht, N. B., Feinberg, A. P., and Chae, C. B. (1996) DNA Cell Biol 15, 495–504
29. Feinberg, A. P., and Vogelstein, B. (1984) Anal Biochem 137, 266–267
30. Shibata, K., Inagaki, M., and Ajiro, K. (1990) Eur J Biochem 192, 87–93
Regulation of Polo-like Kinase 1 by DNA Damage in Mitosis

31. Wera, S., and Hemmings, B. A. (1995) Biochem. J. 311, 17–29
32. Favre, B., Turowski, P., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 13856–13863
33. Zhou, B. B., and Elledge, S. J. (2000) Nature 408, 433–439
34. Ghosh, S., Schroeter, D., and Pawelz, N. (1996) Exp. Cell Res. 227, 165–169
35. Roberge, M., Tudan, C., Hung, S. M., Harder, K. W., Jirik, F. R., and Ander-
   son, H. (1994) Cancer Res. 54, 6115–6121
36. Kawabe, T. (2004) Mol Cancer Ther. 3, 513–519
37. Douglas, P., Moorhead, G. B., Ye, R., and Lees-Miller, S. P. (2001) J. Biol.
   Chem. 276, 18992–18998
38. Zhao, R. Y., and Elder, R. T. (2005) Cell Res. 15, 143–149
39. Dozier, C., Bonyadi, M., Baricault, L., Tonasso, L., and Darbon, J. M. (2004)
   Biol. Cell 96, 509–517