Polo-Like Kinase 1 Inactivation Following Mitotic DNA Damaging Treatments Is Independent of Ataxia Telangiectasia Mutated Kinase

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Abstract

Polo-like kinase 1 (Plk1) is an important regulator of several events during mitosis. Recent reports show that Plk1 is involved in both G_2 and mitotic DNA damage checkpoints. Ataxia telangiectasia mutated kinase (ATM) is an important enzyme involved in G_2 phase cell cycle arrest following interphase DNA damage, and inhibition of Plk1 by DNA damage during G_2 occurs in an ATM-/ATM-Rad3–related kinase (ATR)–dependent fashion. However, it is unclear how Plk1 is regulated in response to M phase DNA damage. We found that treatment of mitotic cells with DNA damaging agents inhibits Plk1 activity primarily through dephosphorylation of Plk1, which occurred in both p53 wild-type and mutant cells. Inhibition of Plk1 is not prevented by caffeine pretreatment that inhibits ATM activity and also occurs in ATM mutant cell lines. Furthermore, ATM mutant cell lines, unlike wild-type cells, fail to arrest after mitotic DNA damaging treatments. The phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, reduces Plk1 dephosphorylation following mitotic DNA damaging treatments, suggesting that the PI3K pathway may be involved in regulating Plk1 activity. Earlier studies showed that inhibition of Plk1 by G_2 DNA damage occurs in an ATM-dependent fashion. Our results extend the previous studies by showing that ATM is not required for dephosphorylation and inhibition of Plk1 activity following mitotic DNA damage, and also suggest that Plk1 is not a principal regulator or mediator of the mitotic DNA damage response.
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Introduction

The polo-related kinases are a highly conserved family of serine/threonine kinases, and family members have been identified from yeasts to mammals (1). *Drosophila* polo was first identified as a mutation that caused abnormal mitotic spindle formation and subsequently was characterized as a critical mitotic protein kinase (2, 3). Single polo-related kinase genes are present in yeasts, and mutations in these genes result in severe mitotic abnormalities. In mammals, three polo-related kinases have been identified, Polo-like kinase 1 (Plk1; refs. 4-8), serum-inducible kinase (Snk, Plk2; refs. 9), and fibroblast growth factor–inducible kinase (Fnk/Prk, Plk3; refs. 10, 11). These closely related mammalian enzymes seem to have distinct, although possibly overlapping, roles in cellular metabolism as both Plk1 and Plk3 can complement mutations in cdc5, the polo homolog in *Saccharomyces cerevisiae* (10, 12). Both Plk1 and Plk3 are activated during mitosis in mammalian cells (10, 13, 14). Plk1 is the most thoroughly studied of the mammalian Plks and clearly represents the closest mammalian polo homolog. Plk1 mRNA (15), protein expression, and the kinase activity (13, 16) are tightly regulated during the cell cycle. Plk1 protein is first detectable during S phase and its expression peaks during late G_2. Plk1 is phosphorylated and activated at the G_2-M phase transition (13, 16, 17) and is rapidly degraded during late mitosis through the ubiquitin-proteasome pathway (18). Several putative Plk1 substrates have been identified, including CHO1, a kinesin-like motor protein (16); α, β, and γ tubulins (19); GRASP65, a golgi membrane protein (20, 21); APC (anaphase promoting complex; refs. 22, 23); and proteasome subunits (24).

In addition to regulating normal cell cycle progression, Plks also seem to respond to DNA damage and are integrated into checkpoint control pathways. In our initial report on the cell cycle regulation of Plk1, we showed that checkpoint activation by S-phase DNA damage prevented mitotic entry and activation of Plk1 and Cdc2. This finding first suggested that Plk1 kinase activation, like Cdc2 activation, is integrated into the G_2 checkpoint response to DNA damage (13). Our observations were confirmed by a more recently published article (25), showing that DNA damage prevented activation of both Plk1 and cyclin B–associated kinase activity and cells were arrested in mitosis. These data provided an important extension to the existing models for control of DNA damage checkpoints in mammalian cells by showing that responses to DNA damage are

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not restricted to interphase but also occur during mitosis. Plk1 represents a particularly attractive target of the DNA damage checkpoint in mitosis as it is thought to perform multiple functions from prophase onward to cytokinesis.

The mechanisms behind the DNA damage–induced G_2 checkpoint are relatively well known. Two phosphoinositide kinase–related kinases, ataxia telangiectasia mutated kinase (ATM) and ATM-Rad3–related kinase (ATR), are crucial in detecting the most lethal types of DNA damage (26, 27). ATM primarily controls the response to ionizing radiation (IR), whereas ATR responds to other types of damage (26). ATM/ATR activate two downstream checkpoint kinases Chk1 and Chk2. The substrates of ATM/ATR and Chk1/Chk2, such as Cdc25c, p53, and BRCA1, are required for cell cycle regulation and DNA repair. p53 is phosphorylated and activated in response to DNA damage (28), thereby inducing transcription of its downstream targets, p21, Gadd45, and 14-3-3σ (29, 30). The regulation of these proteins in response to DNA damage converges to inhibit Cdc2/cyclin B, causing a delay in mitotic entry and thereby allowing DNA repair. However, little is known about the checkpoint(s) triggered by mitotic DNA damage. Mikhailov et al. (31) reported that M phase DNA damage delays the metaphase/anaphase transition via the spindle-assembly checkpoint, but how Plk1 is regulated in response to such damage is unclear.

Recently, mitotic DNA damage was reported to inhibit the kinase activity of Plk1 and block mitotic exit. But no biochemical mechanism for Plk1 inhibition was revealed (25). However, expression of a Plk1 T210D mutant that mimics the effect of an activating phosphorylation could override the G_2 arrest implying that dephosphorylation of Plk1 was responsible for the inhibition. A subsequent report showed that when DNA damage was introduced in asynchronous cells, the inhibition of Plk1 activity was caffeine sensitive and ATM/ATR dependent, and the cells arrested in G_2 phase (32). However, whether the same pathways are required to arrest cells after mitotic DNA damage is not clear. The purpose of this study is to investigate the response of Plk1 to mitotic DNA damage and also examine signal transduction pathways that could be involved in relaying the damage signal. Here we show that mitotic DNA damage causes down-regulation of Plk1 activity primarily through dephosphorylation of Plk1 protein. However, the length of time required for Plk1 to be dephosphorylated, hours rather than minutes, suggests that Plk1 is not an early mediator of the mitotic checkpoint. We also test a variety of inhibitors and mutant cell lines to elucidate details of the pathway linking mitotic DNA damage to Plk1 dephosphorylation. Our data suggest that Plk1 dephosphorylation caused by mitotic DNA damage is independent of ATM but may involve phosphatidylinositol 3-kinase (PI3K) and possibly other members of the extended PI3K family.

Results

Inhibition of Plk1 Activity by Mitotic DNA Damage Is Correlated With Dephosphorylation of Plk1

We previously showed that Plk1 is hyperphosphorylated and activated in mitotic cells, and that phosphorylated Plk1 has a slower mobility in SDS gels (13). Smits et al. (25) reported that mitotic DNA damage inhibited Plk1 activity but did not address how the inhibition occurred. There are three possibilities leading to the reduction of Plk1 activity during M phase: dephosphorylation of the activation site(s), phosphorylation of the inhibitory site(s), and degradation of Plk1 protein. To investigate these possibilities, we used a gel system that allows separation of hyper- and hypophosphorylated Plk1. To reduce the confounding variables that mitotic exit might introduce, we maintained the cells in culture medium containing nocodazole, a microtubule depolymerizing agent that arrests cells in prometaphase (25, 33). When mitotic CA46 cells were treated with the DNA strand-breaking drug adriamycin, Plk1 was dephosphorylated over a time course of 8 hours as shown by immunoblotting of lysate samples (Fig. 1A, *panel a*). The adriamycin-induced dephosphorylation of Plk1 correlated precisely with the reduced phosphorylation of casein (*panel b*) by immunoprecipitated Plk1 (*panel c*) during *in vitro* kinase reactions. Cyclin B protein was stable during the entire time course, indicating that the cells remained arrested in prometaphase (*panel d*). To more thoroughly address that issue, we did both mitotic index analysis (not shown) and flow cytometry analysis (FACS; Fig. 1B). The vast majority of the cells remained arrested in prometaphase (as expected since they were maintained in nocodazole) and no significant cell death occurred during the time course (Fig. 1B). These results indicate that in CA46 cells mitotic DNA damage induces a slow dephosphorylation of Plk1, resulting in eventual inhibition of Plk1 kinase activity. Significant dephosphorylation and inhibition of Plk1 required more than 2 hours, which leads us to suggest that Plk1 may not be an important early mediator of the mitotic DNA damage checkpoint.

Oxidative stress is another threat to cells which can cause DNA damage through increasing the cellular load of reactive oxygen species (34). To study the effect of reactive oxygen-induced DNA damage on Plk1 during mitosis, we treated nocodazole-blocked CA46 cells with various concentrations of hydrogen peroxide (H_2O_2) and harvested the cells 6 hours after the treatment. We examined Plk1 phosphorylation status by immunoblotting lysate samples following 7.5% SDS-PAGE (Fig. 1C, *panel a*). Plk1 was partially dephosphorylated at 0.2 mmol/L, and totally dephosphorylated at concentrations $\geq$0.6 mmol/L (*panel a*). Plk1 kinase activity (*panels b* and *c*) was also reduced in a dose-dependent manner paralleling its dephosphorylation (*panel a*).

We next determined the time required between H_2O_2 treatment and Plk1 dephosphorylation. As shown in Fig. 1D (*panel a*), Plk1 was partially dephosphorylated 2 hours after treatment and was completely dephosphorylated by 6 hours. Once again, we found that the reduction of Plk1 immune-complex kinase activity (*panels b* and *c*) correlated closely with its dephosphorylation (*panel a*). In both of the hydrogen peroxide experiments, cyclin B protein amounts were relatively constant (*panel d*), indicating that the cells did not exit mitosis. This was also confirmed by mitotic index analysis (not shown). Irradiation of CA46 cells caused a similar dephosphorylation of Plk1 and reduction of its *in vitro* kinase activity (data not shown), demonstrating that the results are not specific to adriamycin or hydrogen peroxide. Taken together,

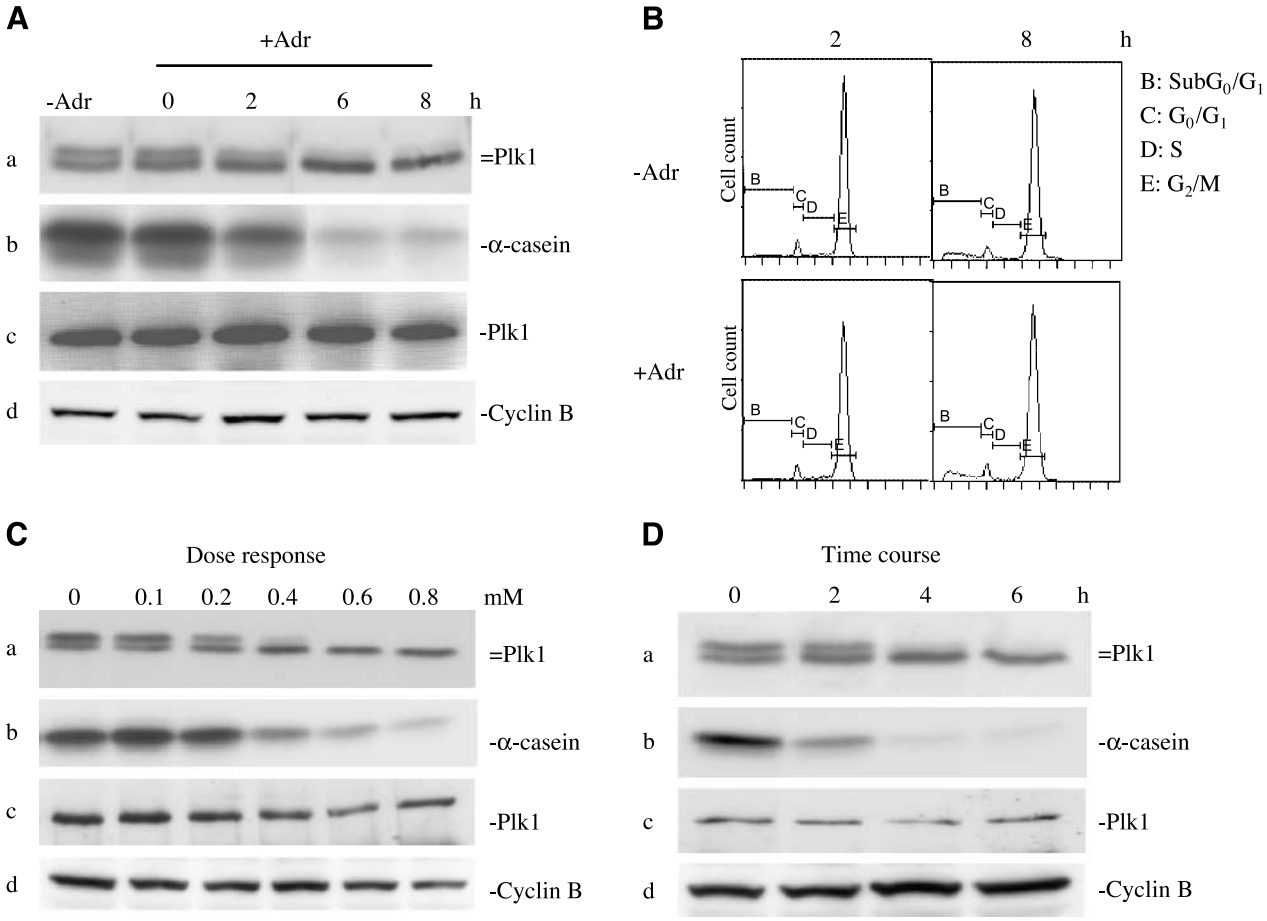

FIGURE 1. DNA damaging treatments cause dephosphorylation of Plk1 in mitotically arrested cells. **A.** Mitotic CA46 cells were treated with adriamycin and collected at indicated times. Plk1 lysate immunoblot after 7.5% 120:1 acrylamide:bis SDS-PAGE (*panel a*). Phospho-casein autoradiography from Plk1 immune-complex kinase assay (*panel b*). Plk1 protein present in the immune-complexes was detected by immunoblot following 12% SDS-PAGE (*panel c*). Cyclin B protein levels were assessed by immunoblotting (*panel d*). **B.** Cell cycle FACS analysis. **C.** Mitotic CA46 cells were treated with H_2O_2 at the indicated doses, then harvested after 6 hours. *Panel a*, Plk1 lysate immunoblot after 7.5% 120:1 acrylamide:bis SDS-PAGE. *Panel b*, Plk1 immune-complex kinase assay. Autoradiography is shown. *Panel c*, immune-complex immunoblot after 12% SDS-PAGE. *Panel d*, immunoblot of cyclin B. **D.** Mitotic CA46 cells were treated with 0.4 mmol/L H_2O_2 and collected at the indicated times. *Panel a*, Plk1 lysate immunoblot after 7.5% 120:1 acrylamide:bis SDS-PAGE. *Panel b*, Plk1 immune-complex kinase assay. Autoradiography is shown. *Panel c*, immune-complex immunoblot following 12% SDS-PAGE. *Panel d*, cyclin B immunoblot. These experiments were performed three times with similar results.

the results in Fig. 1 indicate that Plk1 dephosphorylation occurs following different types of DNA damaging treatments and that Plk1 immune-complex kinase activity is also concurrently reduced. Both the dephosphorylation of Plk1 and inhibition of its *in vitro* kinase activity require hours to occur and, therefore, Plk1 may not be a major target of the mitotic DNA damage checkpoint response.

DNA-Damaging Agents Block Mitotic Exit

We next wished to confirm that the mitotic cells treated with adriamycin were actually arrested by the treatment and to determine the fate of Plk1 in such cells after release from nocodazole. Nocodazole-arrested CA46 cells were mock treated, or treated with adriamycin for 1 hour and then released into fresh medium. Cells were harvested at various times for immunoblot (Fig. 2A) and cell cycle analysis (Fig. 2B). Approximately 85% of the cells were arrested in prometaphase by the nocodazole treatment as determined by mitotic index analysis (not shown) and FACS. After release from nocodazole, 60% of mock-treated cells proceeded through mitosis and entered G_1 within 9 hours. During that time, Plk1 protein was gradually reduced such that by 9 hours, very little Plk1 protein was detected (Fig. 2A, *panel a*). In contrast, treatment of cells with adriamycin impaired their ability to exit mitosis following nocodazole release while Plk1 dephoshorylation remained similar to that seen in cells maintained in nocodazole (compare Fig. 2A, *panel a* to Fig. 1A, *panel a*). There was a reduction in the amount of Plk1 protein most likely due to mitotic exit of a significant percentage of the cells, but dephosphorylation was the most pronounced effect (compare the 5 and 9 hours + and − adriamycin time points).

We found that approximately 85% of nocodazole-arrested cells had condensed chromosomes as assessed by DAPI staining and fluorescence microscopy (not shown). By 9 hours after adriamycin treatment and release from nocodazole, only 8% of the cells retained condensed chromosomes, although about 65% remained in G_2-M as determined by FACS analysis.

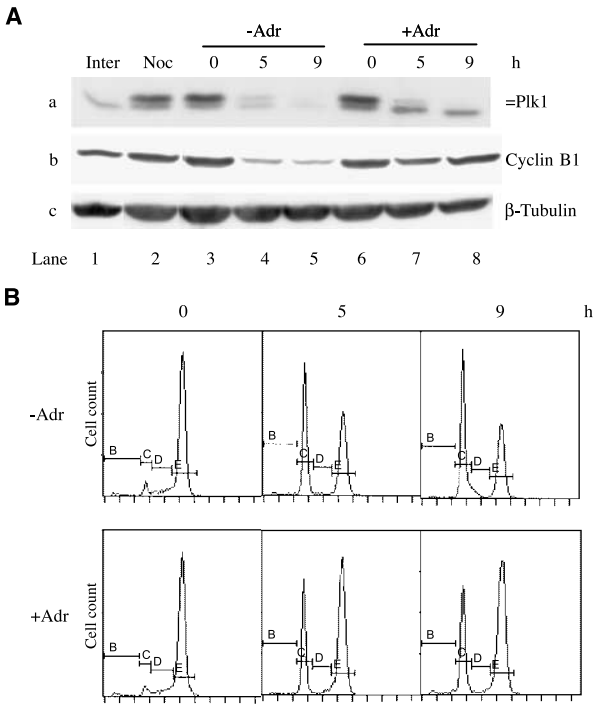

FIGURE 2. Dephosphorylation of Plk1 is correlated with mitotic arrest. Mitotic CA46 cells were either untreated or treated with 0.5 μmol/L of adriamycin for 1 hour. Cells were washed and re-plated without nocodazole and harvested at the indicated times for immunoblot and cell cycle FACS analyses. **A.** Plk1 lysate immunoblot (*panel a*). Cyclin B1 lysate immunoblot (*panel b*) and reprobing with β-tubulin antibody (*panel c*) to demonstrate that equal protein was loaded in each lane. **B.** FACS analysis. These experiments were conducted three times with similar results.

To further clarify that those cells with decondensed chromosomes had not progressed into G_1, cyclin B1 levels were analyzed (Fig. 2A, *panel b*). The amount of Cyclin B1 protein was significantly reduced in the cells released from nocodazole alone compared with cells treated with adriamycin (compare the 5 and 9 hours + and − adriamycin time points). Thus, the adriamycin-induced dephosphorylation of Plk1 correlates with an inability to proceed through mitosis and a return to a G_2-like state with decondensed chromosomes in about 50% of the cells. The changes seen in Plk1 and cyclin B were not due to differences in protein loading as β-tubulin protein levels were constant (*panel c*).

Plk1 Dephosphorylation Caused by Mitotic DNA Damage Is p53 Independent

p53 plays an important role in protecting mammals from neoplasia by inducing cell cycle arrest, DNA repair, and apoptosis in response to a variety of stresses. The p53-dependent arrest of cells in the G_1 phase is an important component of the cellular response to stress. Recent evidence further shows that p53 is involved in controlling the G_2-M transition after DNA damage or whenever DNA synthesis is interrupted (35). For this reason, we compared the effects of mitotic DNA damage on Plk1 dephosphorylation in two B lymphoma cell lines: WMN B lymphoma cells that contain wild-type p53 and CA46 B lymphoma cells that express inactive p53 and display little ability to arrest at G_1 after irradiation (36). Plk1 dephosphorylation was found to occur in both cell lines (Fig. 3A), indicating that p53 status is irrelevant in the down-regulation of Plk1.

Adriamycin-Induced Mitotic Plk1 Dephosphorylation Does Not Require the ATM Kinase

The ATM kinase is known to be an important regulator of the G_2 checkpoint and has also been reported to be involved in

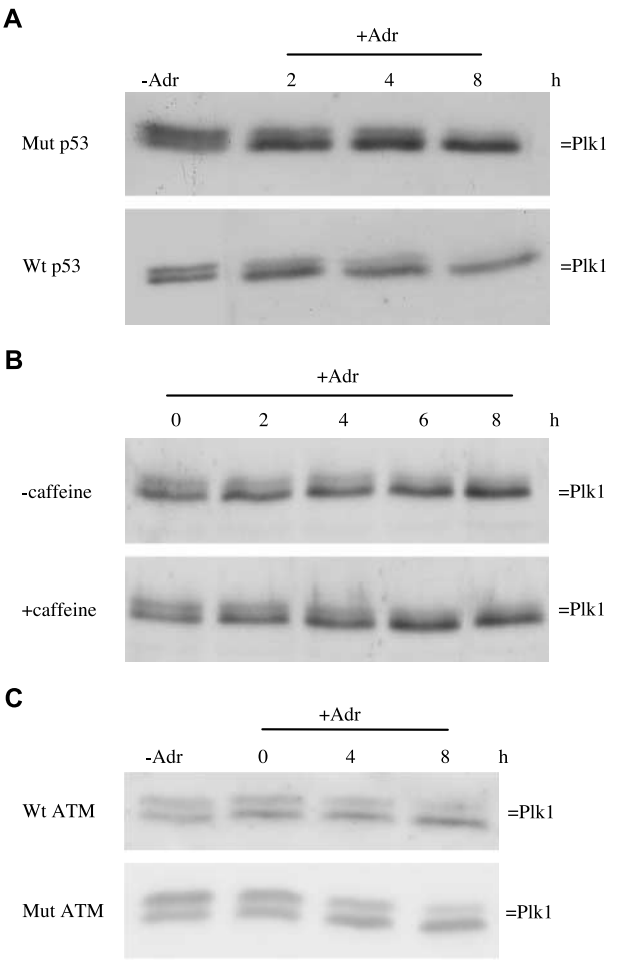

FIGURE 3. Dephosphorylation of Plk1 following mitotic DNA damage is independent of p53 and ATM. **A.** Dephosphorylation of Plk1 is p53 independent. Cells were synchronized with nocodazole and treated with 0.5 μmol/L adriamycin for 1 hour, replated with fresh media containing nocodazole, and harvested at the indicated times for Plk1 lysate immunoblot analysis. *Top panel*, CA46 cells (mutant p53). *Bottom panel*, WMN cells (wild-type p53). **B.** Caffeine does not affect DNA damage–induced Plk1 dephosphorylation. Mitotic CA46 cells were mock treated or pretreated with 2.5 mmol/L caffeine for 1 hour, after which 0.5 μmol/L adriamycin was added to medium for 1 hour. Cells were washed and cultured in fresh media containing nocodazole without (*top panel*) or with caffeine (*bottom panel*) and harvested at the indicated times for lysate Plk1 immunoblot. **C.** Mitotic dephosphorylation of Plk1 is independent of ATM. Nocodazole-arrested ATM mutant GM08436A (*bottom panel*), and a normal control cell line, GM09622 (*top panel*), were treated with 0.5 μmol/L adriamycin for 1 hour, washed, and re-plated with media containing nocodazole. Cells were harvested for lysate Plk1 immunoblot analysis at the indicated times. Experiments were conducted at least three times for **A** and **B** with similar results. Experiments were conducted three times in two ATM mutant cell lines and two normal control cell lines with similar results.

the down-regulation of Plk1 activity after DNA damage (25). We, therefore, tested whether caffeine, a frequently used inhibitor of the ATM kinase, could prevent the adriamycin-induced dephosphorylation of Plk1 in M phase. We found that caffeine had no effect on Plk1 dephosphorylation after mitotic DNA damage (Fig. 3B).

To confirm that the ATM kinase is not essential for down-regulation of Plk1 after mitotic adriamycin treatment, we tested responses in four lymphoblast cell lines harboring ATM mutations. Adriamycin treatment caused Plk1 dephosphorylation in all ATM mutant cells (Fig. 3C, *top panel* and data not shown) as well as in two control normal lymphoblast cell lines (Fig. 3C, *bottom panel* and data not shown). Plk1 *in vitro* kinase activity was also inhibited and correlated with dephosphorylation (data not shown). The dephosphorylation and down-regulation of Plk1 activity was slower in both the ATM mutant and wild-type lymphoblasts compared with other cell lines, such as CA46 (Fig. 1). However, because there were no pronounced differences in the Plk1 dephosphorylation and inhibition between the mutant and control lymphoblasts, our results suggest that ATM is not required for Plk1 dephosphorylation and the inhibition after mitotic DNA damage.

We next tested whether ATM mutant cells had an intact mitotic checkpoint by performing a nocodazole release following adriamycin treatment (Fig. 4). In ATM mutant cells maintained in nocodazole and treated for 1 hour with 0.5 μmol/L adriamycin (Fig. 4A, *top panel*), Plk1 was partially dephosphorylated within 4 hours and completely dephosphorylated by 20 hours. As expected, the majority of the cells remained in prometaphase as determined by FACS analysis (Fig. 4B, *top panel*) and mitotic index analysis (not shown). In accord with those findings, cyclin B levels were relatively constant during the 20-hour time course. In contrast, when cultures blocked with nocodazole were released without adriamycin treatment, the majority of the cells exited mitosis and entered G_1 within 4 hours, by which time Plk1 was completely dephosphorylated because of mitotic exit and cyclin B protein was greatly reduced (Fig. 4B, *middle panel*). The results in cells treated with 0.5 μmol/L adriamycin and released from a nocodazole block were similar to mock adriamycin-treated cultures. Within 4 hours, most cells exited mitosis and entered G_1 and only dephosphorylated Plk1 was detected. The same experiment was done in a second ATM mutant cell line with virtually identical results. Thus, the ATM mutant cells failed to arrest in mitosis in response to adriamycin treatments that were sufficient to arrest wild-type cells.

We next tested whether higher doses of adriamycin might be able to induce an ATM-independent mitotic arrest. When ATM mutant cells were exposed to 100 μmol/L adriamycin for 1 hour and then released from the nocodazole arrest, the results were strikingly different from what we had observed at lower dosages (Fig. 4C and D). Plk1 was dephosphorylated within 4 hours as seen in the previous experiments, but the majority of the cells were unable to complete cell division. Interestingly, both cyclin B and Plk1 protein levels were significantly reduced at 8 and 24 hours even though most cells remained arrested in mitosis. Microscopic observation of these cells suggests that many of them are arrested after anaphase but before cytokinesis. Therefore, ATM mutant cells subjected to high doses of adriamycin are able to arrest in late mitosis by an ATM-independent mechanism. However, Plk1 is dephosphorylated in both ATM wild-type and mutant cells regardless of whether or not the cells undergo arrest following adriamycin treatment. In addition, we saw no difference in Plk1 dephosphorylation after treatment with 0.5 or 100 μmol/L adriamycin when the cells were maintained in nocodazole (data not shown).

PI3K May Be Involved in Mitotic DNA Damage-Induced Plk1 Dephosphorylation

We tested a variety of specific signal transduction inhibitors to identify required components of the M phase pathway leading to the inhibition of Plk1, including olomoucine (cdc2 kinase inhibitor), PD98059 (p42,44 mitogen-activated protein kinase inhibitor), SB212090 (p38 mitogen-activated protein kinase inhibitor), caffeine (ATM/ATR inhibitor), UCN01 (Chk1 kinase inhibitor), and okadaic acid (serine/threonine phosphatase inhibitor). We found that these inhibitors had no effect on Plk1 dephosphorylation after M phase adriamycin treatment, suggesting that these pathways are not necessary for Plk1 down-regulation following mitotic DNA damage (data not shown).

There are five PI3K family members in mammalian cells, including PI3K, FRAP (FKBP12 and rapamycin-binding protein kinase), DNA-PK (DNA-dependent protein kinase), ATM, and ATR (37). It is known that ATM, ATR, and DNA-PK are important for DNA damage responses (38). Recently, PI3K was shown to be involved in the DNA damage response during both G_1 and G_2-M phases (39, 40). We examined the potential role of PI3K in the mitotic DNA damage response by using the PI3K inhibitor LY294002. Akt is a downstream target of PI3K, and, thus, Akt activity can be used as an indicator of PI3K activation. We first wanted to confirm that mitotic DNA damaging treatments activate Akt and that LY294002 could inhibit this activation. Mitotic CA46 cells that were mock treated or pretreated with LY294002 were exposed to 0.4 mmol/L H_2O_2 for the indicated times. Akt was activated after 5 minutes of H_2O_2 treatment and LY294002 inhibited this activation (Fig. 5A, *top panel*), while total Akt protein remained constant (Fig. 5A, *bottom panel*).

We next tested whether PI3K was involved in Plk1 regulation in response to H_2O_2 treatment. Nocodazole-blocked CA46 cells were pretreated with LY294002, exposed to H_2O_2 for 1 hour, and the cells were analyzed at various times after treatment. We found LY294002 delayed, but did not completely prevent, the damage-induced dephosphorylation of Plk1 (Fig. 5B). Plk1 dephosphorylation was also inhibited by LY294002 after adriamycin treatment of mitotic cells (Fig. 5C). Similar results were obtained using wortmannin (Fig. 5D), a microbial metabolite that is a specific inhibitor of PI3K (41, 42) and that is unrelated to LY294004. To confirm that LY294002 was not affecting Plk1 phosphorylation status through inhibiting the ATM kinase, we analyzed the ATM mutant cell line GM08436A. Mitotic Plk1 dephosphorylation after either adriamycin or H_2O_2 treatment was inhibited to a similar extent

by LY294002 in both wild-type and mutant ATM cell lines (Fig. 5E and F). However, because LY294002 and wortmannin can also inhibit DNA-PK (43), the results do not exclude the possibility that DNA-PK may be involved in this response. Both inhibitors of PI3K protein delay but do not prevent Plk1 dephosphorylation, suggesting that other pathways unrelated to PI3K may also play a role in regulation of Plk1 dephosphorylation.

Discussion

In the present study, we examined the effects of DNA damage introduced during mitosis on the phosphorylation and activity of Plk1 and on cell cycle arrest. We confirmed earlier reports (25) that Plk1 is down-regulated in response to mitotic DNA damage, and showed that the reduction of Plk1 activity correlates with Plk1 dephosphorylation. One activating phosphorylation site for Plk1 has, in fact, been mapped

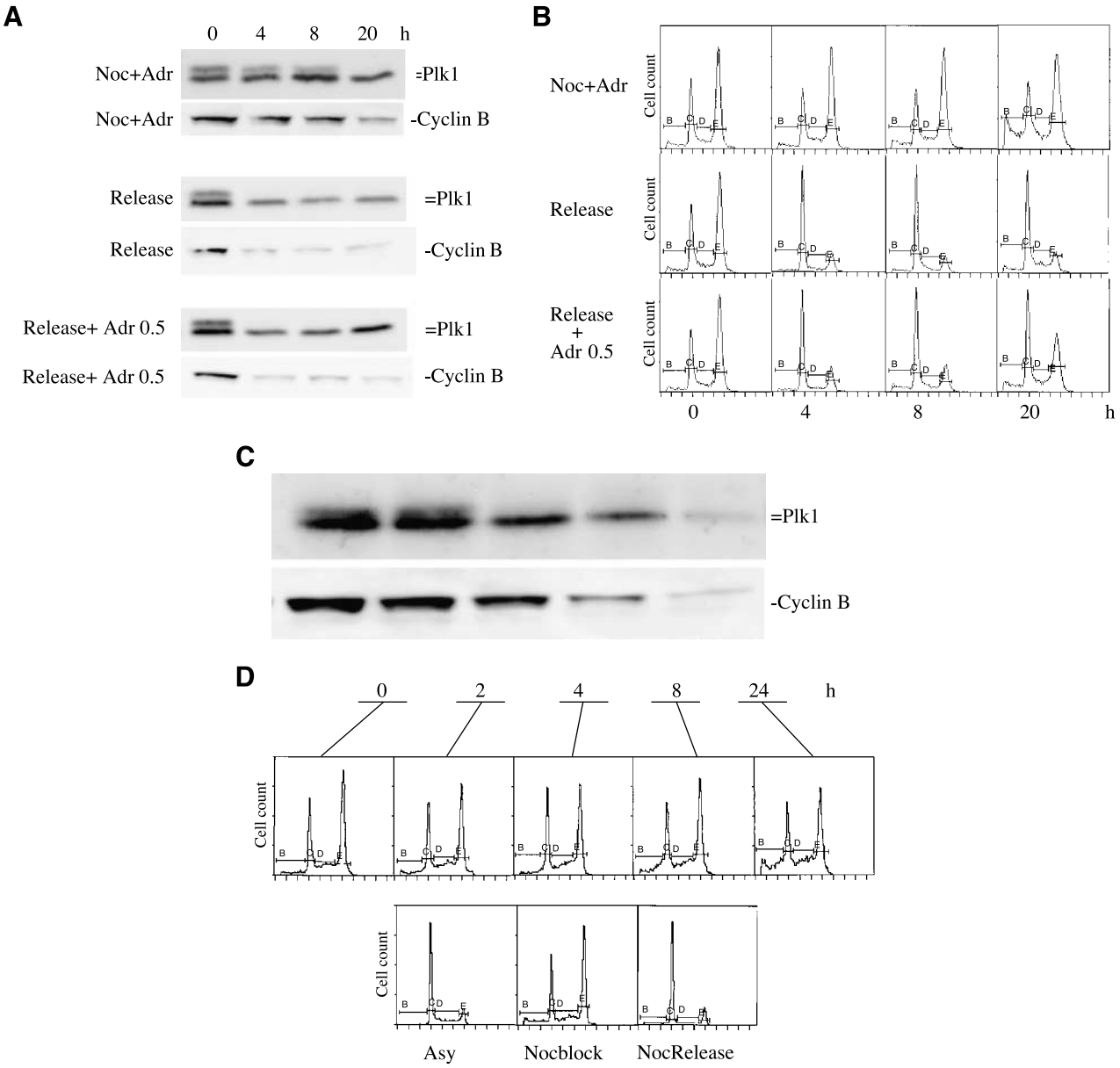

FIGURE 4. G_2 and M phase checkpoints are differentially sensitive to DNA damaging agents in ATM mutant cells. Nocodazole-blocked GM08436A cells were treated with 0.5 or 100 μmol/L adriamycin for 1 hour, washed three times using cold PBS with or without nocodazole, and re-plated in media with or without nocodazole (0 hour). Cells were harvested at various times. **A.** Plk1 lysate immunoblot. Cells were treated with 0.5 μmol/L adriamycin, washed, and re-plated in media with nocodazole (*top panel*). Cells were released from nocodazole without adriamycin treatment (*middle panel*), released from nocodazole with 0.5 μmol/L adriamycin treatment (*bottom panel*). Cyclin B was detected by immunoblot in each experiment. **B.** Corresponding cell cycle FACS analysis. **C.** Nocodazole-blocked GM08436A cells were treated with 100 μmol/L adriamycin for 1 hour. Cells were released from nocodazole after treatment with 100 μmol/L adriamycin and analyzed by lysate immunoblot at the indicated times. **D.** Corresponding cell cycle FACS analysis (*top panel*). *Bottom panel* shows FACS analysis for untreated control. *Asy*, asynchronous GM08436A cells. *Nocblock*, cells remained in nocodazole. *NocRelease*, cells released from nocodazole 2 hours. Three independent experiments were performed with similar results.

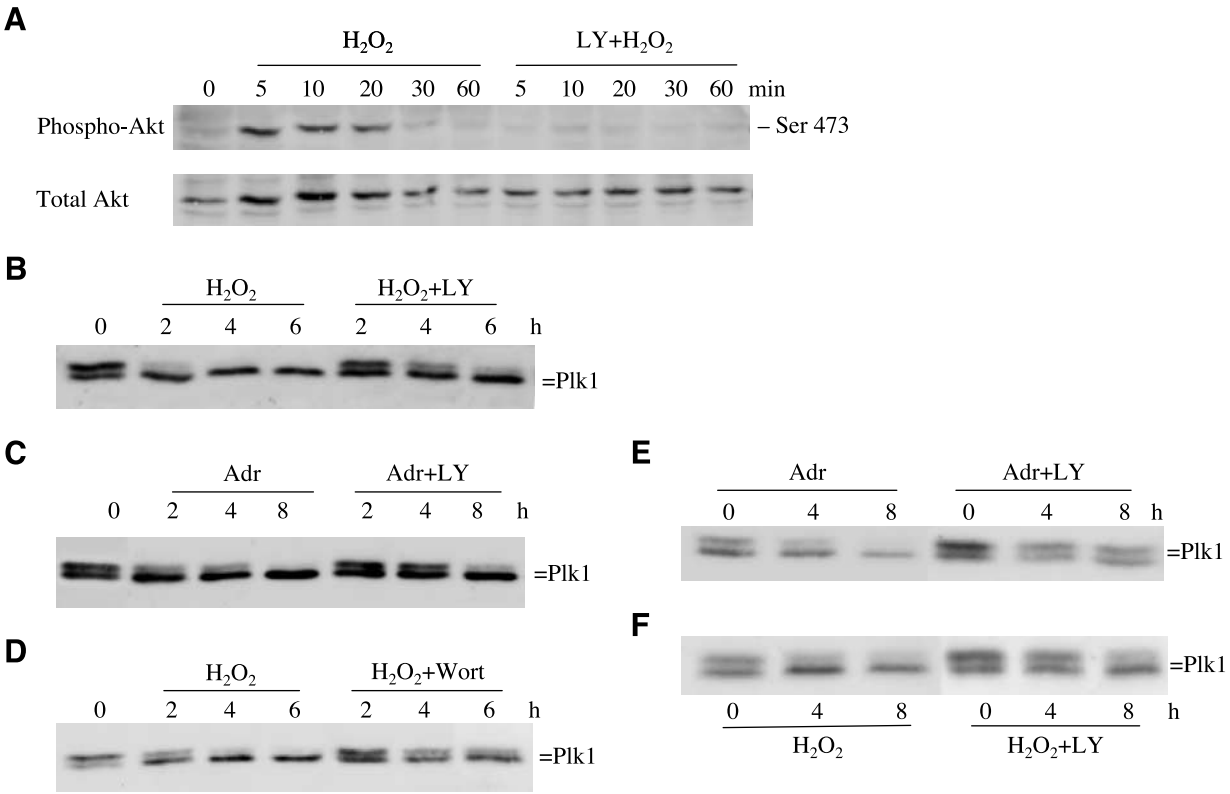

FIGURE 5. PI3K inhibitors LY294002 and wortmannin inhibit Plk1 dephosphorylation following mitotic DNA damage. **A.** Mitotic CA46 cells were mock treated or pretreated with 50 μmol/L LY294002 for 1 hour, after which 0.4 mmol/L H_2O_2 was added and cells were collected at the indicated times for immunoblot analysis of phospho-Akt (*top panel*) and total Akt immunoblot (*bottom panel*). **B**–**D.** Mitotic cells were mock treated or pretreated with 50 μmol/L LY294002 or 20 μmol/L wortmannin for 1 hour, after which adriamycin or H_2O_2 was added for 1 hour. Cells were washed and cultured in fresh media containing nocodazole with or without inhibitors and harvested at the indicated times for lysate Plk1 immunoblot analysis. **B.** 0.4 mmol/L H_2O_2 treatment in Jurkat cells pretreated with LY294002. **C.** 0.5 μmol/L adriamycin treatment in CA46 cells pretreated with LY294002. **D.** 0.4 mmol/L H_2O_2 treatment in Jurkat cells pretreated with wortmannin. **E** and **F.** Nocodazole-blocked GM08436A cells were mock treated or pretreated with 50 μmol/L LY294002 for 1 hour, after which 0.5 μmol/L adriamycin or 0.4 mmol/L H_2O_2 was added to medium for 1 hour. Cells were washed and cultured in fresh media containing nocodazole with or without LY294002 and lysates harvested at the indicated times for Plk1 immunoblot. **E.** 0.5 μmol/L adriamycin treatment. **F.** 0.4 mmol/L H_2O_2 treatment. Experiments were performed three times with similar results.

to T210 (44); however, it is not clear whether phosphorylation at this site is responsible for the shift in electrophoretic mobility we detect when Plk1 is activated. The equivalent site in *Xenopus* Plx1 has been mapped to T201 and three additional phosphorylation sites have been identified only one of which, S340, seemed to be responsible for the shift in Plx1 mobility (45).

In agreement with the earlier reports, we also found that activation of the mitotic DNA damage pathway in response to low-dose (0.5 μmol/L) adriamycin, requires functional ATM. A study by van Vugt et al. (32) examined the G_2 DNA damage checkpoint and reported that inhibition of Plk1 activity by DNA damage occurs in an ATM-/ATR-dependent fashion. Our findings differ significantly, however, in that we found no requirement for ATM in Plk1 dephosphorylation following mitotic DNA damage. In addition, a preliminary examination in ATR inactive cells indicates that ATR is also not required for Plk1 dephosphorylation following mitotic DNA damage (data not shown).

Interestingly, we found no correlation between Plk1 down-regulation and induction of the mitotic DNA damage checkpoint. Plk1 dephosphorylation after mitotic DNA damage requires at least 1 to 2 hours or longer, and that it occurs similarly in cells containing wild-type or mutant ATM. However, only the ATM wild-type cells have an intact mitotic checkpoint in response to the damage introduced by 0.5 μmol/L adriamycin. Although ATM mutant cells failed to arrest following 0.5 μmol/L adriamycin treatment, we found that 100 μmol/L adriamycin did induce a mitotic arrest following release from a nocodazole block. In contrast, we detected essentially no difference concerning Plk1 dephosphorylation after these two treatments although the outcomes in terms of cell cycle arrest were strikingly different. These results suggest that high levels of DNA damage can activate a mitotic checkpoint that does not require ATM, and that Plk1 is unlikely to be involved in either the ATM-dependent or -independent pathways because in both cases Plk1 down-regulation is quite slow. Aside from the question of ATM's potential involvement in Plk1 dephosphorylation, which our data do not support, it seems that the slow time course of Plk1 down-regulation following mitotic DNA damage also argues against the idea that Plk1 is a principal target of this checkpoint response. Mitosis is a comparatively rapid process, being completed within a few hours in most mammalian cell types. Recent reports have

shown that Plk3 is activated within minutes after DNA damaging treatments or in response to microtubule poisons (46, 47). We, therefore, suggest that Plk3 is more likely than Plk1 to be an important mitotic checkpoint target.

While this article was in preparation, Mikhailov et al. (31) reported that cells blocked in metaphase by chromosome damage contain one or more Mad2-positive kinetochores, and the block is rapidly overridden when the cells are microinjected with dominant-negative Mad2. Their results suggest that the damage leads to defects in kinetochore attachment and function that maintains an intrinsic Mad2-based spindle-assembly checkpoint that is ATM independent. Their findings are consistent with our studies using high doses of adriamycin (100 μmol/L) in which we found that mitotic progression was delayed in an ATM-independent fashion. In contrast, we found that lower doses of adriamycin (0.5 μmol/L) caused a mitotic delay that did require functional ATM. After both high and low doses of adriamycin, however, Plk1 was slowly dephosphorylated in cells maintained in nocodazole.

Chow et al. (33) used a similar treatment (0.4 μmol/L adriamycin) to cause DNA damage in nocodazole arrested cells. Consistent with the results of Chow et al. (33), we found that mitotic DNA damage induced chromosome decondensation in nocodazole-blocked cells and the treated cells returned to a G_2-like stage with 4N DNA.

We tested a wide variety of kinase and phosphatase inhibitors targeting well-known signal transduction pathways for their ability to interfere with mitotic DNA damage–induced Plk1 dephosphorylation and only found significant effects with the PI3K inhibitors. Unlike ATM/ATR and DNA-PK, class I PI3K is a phosphoinositide kinase that phosphorylates phosphoinositide to produce ptdIns(3,4,5)P3, which subsequently activates several downstream targets, including Akt/PKB. Shtivelman et al. (48) examined Akt kinase activity throughout the cell cycle in epithelial cells and found that the activity is highest in G_2-M. Previous reports indicated that PI3K signaling may both positively and negatively affect mitotic progression. Alvarez et al. (49) showed that the PI3K/Akt pathway must be repressed in G_2 so that Forkhead transcription factors can be activated to induce transcription of cyclin B and Plk1. Furthermore, the expression of constitutively activated PI3K or Akt causes cells to arrest in telophase with high levels of cyclin B protein. Their conclusion is that Forkhead transcription factors regulate both the synthesis and degradation of important mitotic proteins, including cyclin B and Plk1. In striking contrast, Roberts et al. (50) reported that the PI3K/Akt pathway is activated during late S phase and maintained throughout G_2 and mitosis, and that inhibition of the pathway with LY294002 blocked both cdc2 activation and mitotic entry. Similar results about the PI3K/Akt pathway were found by Shtivelman et al. (48) who reported that Akt activity is elevated during G_2-M in epithelial cells, and that PI3K inhibition prevents cdc2/cyclin B activation and results in apoptosis at the G_2-M transition. These effects of PI3K inhibition were relieved by expression of constitutively activated Akt.

Recent studies have examined the potential involvement of PI3K/Akt pathway in response to DNA damage during G_2-M. Kandel et al. (51) reported that constitutively activated Akt can overcome a G_2-M cell cycle checkpoint induced by DNA damage probably by preventing inactivation of cdc2. While this manuscript was in preparation, Shtivelman (52) reported that activated Akt/PKB decreases the G_2 delay following DNA damage and maintains high levels of Plk1 protein and activity though Akt/PKB regulation of the checkpoint protein CHFR.

In the present study, we specifically focused on the responses of Plk1 to DNA damage induced during mitosis rather than during interphase, thus, likely accounting for our rather different results about the relationship between Plk1 and the PI3K pathway. In contrast with the effects of PI3K inhibition following interphase DNA damage described by the previous studies, we found that inhibitors of PI3K significantly reduced DNA damage–induced mitotic Plk1 dephosphorylation. In addition, we noted that PI3K inhibition also resulted in enhanced phosphorylation and activation of Plk1 in nocodazole-arrested cells not treated with DNA damaging agents (not shown). The apparent discrepancies in the literature about the activity of PI3K and Akt during G_2-M and our findings that PI3K inhibitors enhance Plk1 activity following mitotic DNA damage suggest that additional work is required before these issues can be fully resolved.

Materials and Methods

Chemicals

Nocodazole, paclitaxel, adriamycin (Doxorubicin HCl), hydrogen peroxide (H_2O_2), wortmannin, and caffeine were purchased from Sigma Chemical Co. (St. Louis, MO) SB202190, PD98059, and LY294002 were purchased from Cell Signaling Technology (Beverly, MA). Okadaic acid was purchased from Life Technologies (Rockville, MD). UCN-01 was a gift from DCTDC, NCI. Olomoucine was purchased from Promega (Madison, WI).

Cell Culture and Drug Treatment

The human B lymphoma cell lines, CA46 (mutant p53) and WMN (wild-type p53), and the human T lymphoma cell line Jurkat, were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. The human ATM mutant lymphoblast cell lines, GM06315 and GM13996, were gifts from Nikki K. Holbrook, NIA. ATM mutant cell lines, GM08436A and GM01526E, and normal control lymphoblast cell lines, GM 09622 and GM 10959A, were purchased from Coriell Cell Repository, NJ. All ATM mutant and related cell lines were maintained in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum. Cells were synchronized in mitosis with nocodazole (100 ng/mL) or paclitaxel at 10 nmol/L for 16 hours. Adriamycin (0.5 or 100 μmol/L) or H_2O_2 (at indicated concentrations) were added to synchronized cells for 1 hour, treatment conditions previously shown to cause DNA damage and previously used to study Plk1 responses to DNA damage (25, 46, 53-55). Cells were washed in PBS/nocodazole, and then cultured in fresh medium with or without nocodazole. Cells were collected at the indicated times after release from drug treatments. Cells were lysed in NP40 lysis buffer (50 mmol/L Tris, pH 7.4; 250 mmol/L NaCl; 1% NP40; 5 mmol/L EDTA; 10 mmol/L NaF; supplemented with 1 μg/mL

aprotinin, leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L DTT). The cell lysates were centrifuged at 100,000 × *g* (1 hour at 4°C), the supernatants were recovered and analyzed.

Gel Electrophoresis and Immunoblot Analysis

A special 7.5% (120:1) acrylamide:bis resolving gel was used to separate the mitotic Plk1 doublet in lysate samples as reported previously (13). Samples were then transferred to Immobilon (Millipore, Bedford, MA) membranes and immunoblotted with Plk1 polyclonal antibody, 8845 or 8847, as described (13). Standard SDS-PAGE and transfer to Immobilon was carried out to examine other proteins and following *in vitro* kinase reactions to detect phosphorylated casein and for immnoblot analysis of the Plk1 protein immnoprecipitated for the reactions. Immunoblot analyses of proteins other than Plk1 were done using the following antibodies: Cyclin B1 antibody (GNS1; Santa Cruz Biotechnology, Santa Cruz, CA), Cyclin B antibody (Oncogen Research Products, San Diego, CA), β-tubulin antibody (Sigma), phospho-Akt (Ser473) antibody, and Akt Antibody (Cell Signaling Technology). All immunoblots of lysate samples were re-probed with the β-tubulin antibody to ensure equal loading of protein in each lane. In all lysate blots shown, equal amounts of protein were loaded in each lane.

Kinase Reactions

To test Plk1 activity in cell lysates, Plk1 protein was immunoprecipitated with 3 μL of Plk1 polyclonal antibody and protein G-Sepharose (Sigma) from equal amounts of total protein lysate. The immunoprecipitates were washed extensively in the lysis buffer, and once in the kinase buffer (20 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 10 mmol/L $MgCl_2$; 1 mmol/L EGTA; 5 mmol/L NaF; 0.1 mmol/L orthovanadate; 0.5 mmol/L DTT; ref. 19). The kinase reactions were done with 5 μg dephosphorylated α-casein, 10 μCi [γ-^{32}P]ATP (Amersham Biosciences, Piscataway, NJ, 10 mCi/mL) in 20 μL kinase buffer at 37°C for 1 hour. The reactions were quenched with 3× SDS sample buffer, boiled for 5 minutes, and loaded onto a 12% polyacrylamide gel (acrylamide:bis, 37.5:1). After SDS-PAGE, the gels were transferred to Immobilon membranes. Phosphorylation of casein was detected by autoradiography and Plk1 protein was detected by immunoblotting of the same membrane. On these 12% gels, hyperphosphorylated and hypophosphorylated Plk1 migrate as a single band and the immunoblotting is only done to confirm that similar amounts of Plk1 protein were present in the *in vitro* kinase reactions. The phosphorylation status of Plk1 is accessed by immunoblotting lysate samples run on the 7.5% 120:1 acrylamide:bis gels.

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