Inhibition of Polo-like Kinase-1 by DNA Damage Occurs in an ATM- or ATR-dependent Fashion*

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Polo-like kinases play multiple roles in different phases of mitosis. We have recently shown that the mammalian polo-like kinase, Plk1, is inhibited in response to DNA damage and that this inhibition may lead to cell cycle arrests at multiple points in mitosis. Here we have investigated the role of the checkpoint kinases ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related) in DNA damage-induced inhibition of Plk1. We show that inhibition of Plk1 kinase activity is efficiently blocked by the radio-sensitizing agent caffeine. Using ATM−/− cells we show that under certain circumstances, inhibition of Plk1 by DNA-damaging agents critically depends on ATM. In addition, we show that UV radiation also causes inhibition of Plk1, and we present evidence that this inhibition is mediated by ATR. Taken together, our data demonstrate that ATM and ATR can regulate Plk1 kinase activity in response to a variety of DNA-damaging agents.

To monitor genomic integrity, cells are equipped with a variety of checkpoint mechanisms (1). One such checkpoint is the G2 DNA damage checkpoint, preventing mitotic entry when DNA is damaged (2). This checkpoint is highly conserved from Schizosaccharomyces pombe to mammalian cells and requires the function of a family of checkpoint kinases that share homology with phosphatidylinositol 3-kinase. In yeast, this family of checkpoint kinases is comprised of scTel1, scMec1, spTel1, and spRad3 (2). The mammalian checkpoint kinases that belong to this family are ATM, the gene mutated in ataxia telangiectasia, and ATR (ATM- and Rad3-related). The ATM kinase plays a very important role in the linkage of DNA damage detecting and the induction of a subsequent cell cycle arrest. This is illustrated by the fact that ataxia telangiectasia cells were described to be defective for the G1, S, and G2-M checkpoints (3). Also, ataxia telangiectasia patients show extreme sensitivity to radiation and increased predisposition to tumor formation. In the DNA damage checkpoint, activation of ATM has been shown to result in the activation of other checkpoint kinases, such as Chk1 and Chk2/hCds1 (4, 5). At least for Chk2, this was shown to occur through direct phosphorylation by ATM in vivo (6). In addition, ATM was also shown to be responsible for damage-induced phosphorylation of p53, MDM-2, BRCA1, and NBS1 (7–11). Thus, ATM appears to sit at the top of the checkpoint signaling cascades that trigger cell cycle arrests in response to DNA damage.

The cell cycle arrest that is provoked when the G2 DNA damage pathway is activated is achieved at least in part by inhibition of the cyclin B-cdc2 complex (12, 13). Activation of this complex is critically required to enter mitosis, and this activation is brought about via dephosphorylation of cdc2, which is mediated by the Cdc25c phosphatase (14, 15). This dual specificity phosphatase is the target of the G2 DNA damage checkpoint, and dephosphorylation of Tyr15 on cdc2 is efficiently impaired when DNA is damaged (16). Inactivation of Cdc25C was suggested to be achieved by phosphorylation of Cdc25C by the serine/threonine kinases Chk1 and Chk2 (5, 17). Phosphorylation of Cdc25C by Chk1 or Chk2 subsequently inhibits cyclin B-cdc2 activation (18, 19). In contrast to the inhibitory phosphorylation by Chk1 and Chk2, Cdc25C also requires phosphorylation to become activated. Activating phosphorylation is thought to require both cyclin B-cdc2 complexes and the action of Polo-like kinases (Plks) (20, 21). Because depletion of Plk1, the Xenopus homologue of Polo-like kinases, from oocyte extracts, results in a block of Cdc25C activation, an essential role for Plks in the initiation of the activation-loop of Cdc25C has been suggested (22).

Recently, Plks were also shown to be regulated in response to DNA damage. Cdc5, the Saccharomyces cerevisiae homologue of Plk, was shown to be post-translationally modified in response to DNA damage (23). Furthermore, Cdc5 appeared to act downstream of Rad53, the S. cerevisiae homologue of Chk2, because a loss-of-function mutant of Cdc5 could revert a Rad53 defect, and overexpression of Cdc5 resulted in a DNA damage checkpoint override (24). Recent work from our laboratory has demonstrated that the mammalian Polo-like kinase, Plk1, is also a target of the G2-M DNA damage checkpoint (25).

Here we have investigated the role of the ATM and ATR checkpoint kinases in the inhibition of Plk1 by DNA damage. We show that inhibition of Plk1 occurs in an ATM-dependent fashion but that under certain conditions ATR can also signal inhibition of Plk1. Moreover, the data presented here demonstrate that Plk1 is also inhibited by UV irradiation, in addition to the DNA-damaging agents described previously.

EXPERIMENTAL PROCEDURES

Cell Culture—The human osteosarcoma cell line U2OS and the ATM−/− (ATRBR1 and GM05849) and ATM+/+ (GM00498) fibroblasts were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. ATM−/− (GM05849) and ATM+/+ (GM00498) fibroblasts were purchased from the Coriell Institute (Camden, NJ). Media for all cell lines were supplemented with 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. To inflict DNA damage with adriamycin, the cells were incubated with different concentrations of adriamycin in Dulbecco's modified Eagle's medium for 1 h and subsequently extensively washed. For bleomycin...
treatment, the cells were washed twice with PBS1 containing 1 mM CaCl₂ and permeabilized with 4 µg/ml of L-α-lysophosphatidylcholine (LPC) in PBS-CaCl₂ for 2 min. Subsequently, the cells were treated with indicated concentrations of bleomycin for 30 min. LPC alone was taken along as a control. After treatment, the cells were extensively washed.

Antibodies and Reagents—Mouse monoclonal anti-MPM-2 and rabbit polyclonal anti-plk-1 were from Upstate Biotechnology, Inc. The mouse monoclonal anti-cyclin B1 and protein A/Gagarose were from Santa Cruz. Protein A-Sepharose was from Amersham Pharmacia Biotech. Fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody was from Becton Dickinson. Histone H1 was from Roche Molecular Biochemicals. Adriamycin, bleomycin, LPC, wortmannin, caffeine, nocodazole, propidium iodide, and dephosphorylated α-casein were from Sigma.

Immunoprecipitations and in Vitro Kinase Reactions—For Plk1 kinase assays, the cells were lysed in E1A lysis buffer (ELB, 150 mM NaCl, 50 mM Hepes, pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 50 kallikrein-inactivating units of aprotinin, 10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 0.5 mM sodium orthovanadate, 20 mM β-glycerophosphate) for 1 h at 4 °C. Total cell lysate was incubated with protein A-Sepharose beads and anti-Plk1 for overnight immunoprecipitation. Protein A-Sepharose beads were extensively washed, and subsequently kinase reactions were performed in kinase buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM NaF) in the presence of 0.5 mg/ml dephosphorylated α-casein, 10 µM ATP, and 4 µCi of [γ-32P]ATP. For cyclin B-associated kinase reactions, the cells were lysed in lysis buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 400 mM NaCl, 0.5% Nonidet P-40, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 50 kallikrein-inactivating units of aprotinin, 10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 0.5 mM sodium orthovanadate, 20 mM β-glycerophosphate). 5 µg of total cell lysate, in the presence of protein A/G-Sepharose and anti-cyclin B, was used for overnight immunoprecipitations. Protein A/G-Sepharose beads plus were extensively washed, and subsequently kinase reactions were performed in kinase buffer (50 mM Hepes, pH 7.5, 5 mM MgCl₂, 2.5 mM MnCl₂, 1 mM dithiothreitol) in the presence of 10 µg of histone H1, 50 µM ATP, and 2.5 µCi of [γ-32P]ATP.

MPM-2 Staining—The cells were fixed in 70% ethanol for 2 h at 4 °C. Ethanol was washed away with PBS, and subsequently the cells were incubated with anti-MPM-2 antibody for 1 h at 4 °C. Then the cells were washed with PBS and subsequently incubated with fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody for 30 min at 4 °C, protected from light. Next, the cells were washed with PBS and then were incubated with RNase (0.25 mg/ml) and propidium iodide for 15 min at 37 °C. DNA content and MPM-2 positivity were analyzed using flow cytometry using Cell Quest software (Becton Dickinson).

RESULTS

To investigate the role of ATM in Plk1 regulation, we examined the influence of the radio-sensitizing agent caffeine on DNA damage-induced inhibition of Plk1. Work from a number of laboratories has shown that the radio-sensitizing effect of caffeine is due to specific inhibition of the ATM/ATR checkpoint kinases (26–29). Because we had obtained data that indicated that the damage-induced inhibition of Plk1 was reverted by the addition of caffeine (25), we decided to investigate the involvement of ATM/ATR and the effects of caffeine in more detail. As shown in Fig. 1, the activity of Plk1 is high in U2OS cells trapped in mitosis with the microtubuli-distabilizing drug nocodazole, but this activity is severely inhibited by the DNA-damaging agent adriamycin, consistent with our previous findings. The addition of caffeine completely prevented DNA damage-induced Plk1 inhibition (Fig. 1a), suggesting a role for ATM and/or ATR in the effects of DNA damage on Plk1. To extend these findings, we investigated the effects of adriamycin and caffeine on mitotic entry in the presence of DNA-damaging agents. To this end, we analyzed the appearance of the MPM-2 phospho-epitope. The monoclonal anti-MPM-2 antibody recognizes mitosis-specific phospho-epitopes and thus can be used to specifically detect mitotic cells (30). Cells trapped in mitosis with nocodazole showed high MPM-2 positivity, whereas adriamycin-treated cells showed a loss of MPM-2 positivity (Fig. 1b), consistent with a block in mitotic entry. However, caffeine was able to fully rescue this effect of DNA damage, because a full restoration of the MPM-2 positivity was observed in the presence of DNA-damaging agents. These data demonstrate that caffeine can drive a very efficient checkpoint escape in these cells and suggest that inhibition of Plk1 by DNA damage depends on the ATM and/or ATR kinases.

To distinguish between ATM and ATR as an upstream regulator of Plk1 activity, the activity of Plk1 was examined in fibroblast cell lines lacking functional ATM, derived from different AT patients. Indeed, we observed a clear difference in inhibition of Plk1 kinase activity in response to the DNA-damaging agent adriamycin in both ATM−/− cell lines compared with their normal counterparts (Fig. 2a). Whereas Plk1 activity was inhibited very efficiently in the ATM−/− cells using 0.25 µM adriamycin, no significant inhibition was seen at this concentration of adriamycin in the ATM+/− cells. This firmly supports a role for ATM in the DNA damage-induced inhibition of Plk1 activity. To further strengthen these conclusions, the effects of another radiomimetic agent, bleomycin, were examined (Fig. 2b). Bleomycin was added in combination with LPC to facilitate the uptake of bleomycin (31). Similar to the effects

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1 The abbreviations used are: PBS, phosphate-buffered saline; LPC, L-α-lysophosphatidylcholine.
investigated whether the observed inhibition of Plk1 correlated with a similar inhibition pattern of cyclin B/cdc2 kinase activity (Fig. 2c). Whereas asynchronous cells showed low cyclin B-associated kinase activity, these levels were strongly elevated when cells were blocked in mitosis using nocodazole. As expected, DNA damage resulted in a loss of cyclin B/cdc2 kinase activity. This effect, however, was clearly rescued by caffeine, similar to the effect of caffeine on Plk1 kinase activity. Moreover, inhibition of cyclin B/cdc2 kinase activity by adriamycin showed a similar dependence on ATM, as demonstrated by a lack of inhibition in ATM−/− cells (Fig. 2d). A similar difference in checkpoint response in ATM−/− versus ATM+/+ cells was also seen at the level of mitotic entry in these cells (Fig. 2f). In ATM−/− cells, we observed a 97% inhibition in mitotic entry in the presence of adriamycin, whereas this effect was fully overcome by the addition of caffeine. In contrast, in ATM+/+ cells, the addition of adriamycin could only reduce mitotic entry by 13%.

We subsequently tested the effect of higher doses of adriamycin with respect to Plk1 inhibition in ATM−/− cells to explore a possible difference in dose response. Interestingly, higher doses of adriamycin did result in inhibition of Plk1 activity in ATM−/− cells (Fig. 3a), without affecting its expression levels (data not shown). Thus, high levels of DNA damage can result in ATM-independent inhibition of Plk1, indicating that other checkpoint kinases could also be involved. We noted, however, that Plk1 inhibition at high doses of adriamycin in ATM−/− cells was fully rescued by the addition of caffeine (Fig. 3b), suggesting that the ATM-related kinase, ATR, was respon-
were determined using Western blotting. The protein levels in the immunoprecipitation kinase activity was measured. Plk1 protein levels in the immunoprecipitation were determined by Western blotting. ATPM−/− (GM05849) and ATM−/− cells were treated with 25 J m−2 UV or 0.5 μM adriamycin for 1 h or left untreated. Subsequently, the cells were blocked in mitosis using nocodazole (250 ng ml−1) for 16 h. The cells were lysed, and Plk1 kinase activity was determined by immunoprecipitation kinase assay, using dephosphorylated casein as a substrate. After kinase assay, Plk1 protein levels in the immunoprecipitation were determined by Western blotting.

In contrast, the ATM homologue ATR was shown to be activated in response to ionizing radiation only when functional ATM is present (6, 32). Activation of the checkpoint kinase Chk2 is observed in response to ionizing radiation for instance. Activation upon ionizing radiation is dependent on ATM, whereas UV only activates ATR, and subsequently gives rise to partial inhibition. Alternatively, it could reflect a difference in the ability of ATM and ATR to inhibit Plk1. To pinpoint the UV-mediated inhibition of Plk1 to ATR, we examined the effects of caffeine on Plk1 activity after UV treatment of ATM−/− cells (Fig. 4b). Whereas UV-treated ATM−/− cells showed a clear inhibition in Plk1 kinase activity, the addition of caffeine completely rescued this effect, confirming the role of ATR in regulating Plk1 activity. We also examined the effects of wortmannin on ATM/ATR-mediated Plk1 inhibition. Wortmannin has been shown to potently inhibit ATM function but is far less efficient in inhibiting ATR function, when added to cells in culture (37). Wortmannin indeed clearly rescues the inhibitory effects of adriamycin on Plk1 activity in ATM−/− cells; however, no clear effects on UV-induced inhibition of Plk1 activity were observed after UV treatment in A-T cells (Fig. 4b), consistent with a role for ATM in regulating Plk1 activity in response to radiomimetic drugs. When we next assayed the block to mitotic entry, the observed pattern of inhibition by UV irradiation was similar to that of Plk1 activity (Fig. 4c). Mitotic entry was blocked by UV radiation in ATM−/− and ATM−/− cells, as determined by the reduction in MPM-2 positivity. Thus, inhibition of Plk1 correlates well with the block in mitotic entry seen in these cells. In this respect, it is important to note that Plk1 is also inhibited by adriamycin and UV radiation in cells that are blocked in mitosis (Fig. 4d). Therefore, the effects of adriamycin and UV radiation on Plk1 activity are also seen in cells that have passed the G2 DNA damage checkpoint,
demonstrating that inhibition of Plk1 is not an indirect consequence of a DNA damage-induced G2 arrest. Also, this indicates that DNA damage checkpoints are also active at later stages of mitosis, consistent with what we described previously (25).

DISCUSSION

The results shown here indicate that the inhibition of Plk1 kinase activity, which is seen when the DNA damage checkpoint is activated, requires functional ATM kinase: 1) because the inhibition can be rescued by the addition of caffeine, shown to interfere with the function of ATM and 2) because we were able to show that Plk1 inhibition by the radiomimetic agents adriamycin and bleomycin was impaired in cells lacking functional ATM. Only at very high doses of adriamycin did we observe a partial inhibition of Plk1 activity in ATM−/− cells. We speculate that this is due to activation of ATR under these circumstances, because the inhibition could be rescued by caffeine. Indeed, using UV irradiation to specifically activate ATR, and not ATM, we found that Plk1 was also inhibited. UV-induced inhibition of Plk1 occurred in ATM−/− and ATM+/+ cells, clearly demonstrating furthermore that UV irradiation can inhibit Plk1 in an ATM-independent fashion. Furthermore, the UV-mediated inhibition of Plk1 activity in ATM−/− cells was efficiently rescued by caffeine, a potent inhibitor of ATM/ATR. When UV-treated cells were incubated with wortmannin, a rescue of Plk1 activity was not observed. This is in good agreement with the inefficient inhibitory effects of wortmannin on ATR in vivo that was shown by others (37). Taken together, our results show that Plk1 can be regulated in an ATM- or ATR-dependent way in response to DNA damage and that the type of damage determines which one of these checkpoint kinases will be utilized. Because Plk1 was shown to function at different stages of mitosis, it would be very interesting to study at which stages of mitosis ATM and ATR can exert their checkpoint function and whether ATM or ATR can directly inhibit Plk1 activity. BRCA-1, p53, and Chk2, are phosphorylated on residues in so-called SQ/TQ cluster domains by ATM (6–8, 38). Because Plk1 does not contain such a SQ/TQ cluster domain, we suggest that inhibition of Plk1 by ATM and ATR probably requires yet other checkpoint kinases, such as Chk1 or Chk2.

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