The G2 Block Induced by DNA Damage: A Caffeine-resistant Component Independent of Cdc25C, MPM-2 Phosphorylation, and H1 Kinase Activity

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ABSTRACT

Treatment of cells with agents that cause DNA damage often results in a delay in G2. There is convincing evidence showing that inhibition of p34cdc2 kinase activation is involved in the DNA damage-induced G2 delay. In this study, we have demonstrated the existence of an additional pathway, independent of the p34cdc2 kinase activation pathway, that leads to a G2 arrest in etoposide-treated cells. Both the X-ray-induced and the etoposide-induced G2 arrest were associated with inhibition of the p34cdc2 H1 kinase activation pathway as judged by p34cdc2 H1 kinase activity and phosphorylation of cdc25C. Caffeine treatment restored these activities after either of the treatments. However, the etoposide-treated cells did not resume cycling, revealing the presence of an alternative pathway leading to a G2 arrest. To explore the possibility that this additional pathway involved phosphorylation of the MPM-2 epitope that is shared by a large family of mitotic phosphoproteins, we monitored the phosphorylation status of the MPM-2 epitope after DNA damage and after treatment with caffeine. Phosphorylation of the MPM-2 epitope was depressed in both X-ray- and etoposide-treated cells, and the depression was reversed by caffeine in both cases. The results indicate that the pathway affecting MPM-2 epitope phosphorylation is involved in the G2 delay caused by DNA damage. However, it is not part of the caffeine-insensitive pathway leading to a G2 block seen in etoposide-treated cells.

INTRODUCTION

DNA damage often leads to a G2 block. This observation raises the question of which biochemical mechanisms leading to M phase induction are inhibited after DNA damage. Mitotic p34cdc2 kinase composed of a complex of p34cdc2 and cyclin B1 is the best characterized crucial M phase inducer. Various studies have examined the potential involvement of this complex in the G2 delay, and it has been shown that p34cdc2 H1 kinase activity is diminished during the G2 delay. (1-9).

Activation of p34cdc2 H1 kinase requires both cyclin B1 binding and the removal of inhibitory phosphorylations on p34cdc2 (10). Alterations in both of these regulatory components may contribute to the diminished p34cdc2 H1 kinase activity seen after irradiation or treatment with DNA-damaging agents. Lowered cyclin B1 levels after irradiation contribute to decreased p34cdc2 activity and the G2 delay in some, but not all cell types (11-18). Cyclin B1 levels fall after irradiation in HeLa cells, transformed rat embryo cells, and some other cell types. Cyclin B1 levels rise when the G2 block is alleviated by caffeine (14). Furthermore, induction of high cyclin B1 levels in irradiated HeLa cells through the use of an inducible expression vector shortens the G2 delay seen after irradiation. Increased cyclin B1 does not affect the normal cell cycle. However, this induction did not eliminate the G2 block, suggesting that the cyclin B1 depression contributes to the length of the G2 delay but is not the only factor contributing to the delay. (18). Thus, in HeLa cells, as well as other cells in which cyclin B1 is depressed after radiation, this decrease is likely to contribute to the length of the G2 delay.

Moreover, p34cdc2 remains phosphorylated during the G2 delay induced either by irradiation or by etoposide. The serine threonine kinase activity of p34cdc2 can be inhibited by phosphorylation of either threonine 14 or of tyrosine 15. During normal cell cycle progression, the inhibitory phosphorylation of p34cdc2 occurs on both of these residues coincidently with the formation of the cyclin B1-p34cdc2 complex (10). Thus, the phosphorylation of p34cdc2 observed after DNA damage could block p34cdc2 activity, potentially contributing to the G2 delay (2-9).

In an attempt to determine whether the increased phosphorylation of p34cdc2 was causal for the G2 block, Jin et al. (19) induced the expression of p34cdc2 that had been mutated at residues 14 and 15 to prevent phosphorylation and hence would be constitutively active. They monitored the cell cycle in irradiated cells after induction of this constitutively active p34cdc2 and found that the G2 delay was shortened but not eliminated, strongly suggesting the involvement of phosphorylation in perpetuating the G2 delay. However, because the delay was not eliminated, phosphorylation of p34cdc2 must be operating in concert with another mechanism to lead to the G2 delay. Blasina et al. (20) found somewhat differing results in a similar system. They also engineered cells with an inducible mutated p34cdc2 but found that the induction was toxic.

The phosphorylation-induced activation of cdc25C, which is responsible for the removal of both of the inhibitory phosphorylations on p34cdc2, has been shown to be decreased after irradiation (21, 22). Phosphorylation of cdc25C occurs at multiple sites and results in activation of cdc25C phosphatase activity (23-27). A reduction in cdc25C phosphatase activity as reflected in decreased phosphorylation would be consistent with the finding of increased phosphorylation of p34cdc2 that has been reported in cells after treatment with DNA-damaging agents. These results are consistent with diminished cdc25C activity being a cause of the G2 delay, but they may merely be a consequence of the G2 arrest. p34cdc2 is capable of performing the activating phosphorylations on cdc25C so that decreased p34cdc2 kinase activity would lead to decreased cdc25C phosphatase activity (25). Recently, Sanchez et al. (28) and Peng et al. (29) have suggested that cdc25C is the target of regulation by the gene chk-l that acts in response to DNA damage. The activity of wee-1, the tyrosine kinase responsible for phosphorylation of tyrosine 15, is not altered by UV radiation in mammalian fibroblasts (30). This contrasts with other data implicating wee-1 as a target of chk-1 in fission yeast (31).

The phosphorylation of the MPM-2\textsuperscript{2} epitope that is shared by a large family of mitotic phosphoproteins is an ill defined biochemical event that is crucial for M-phase induction. Antibodies to these epitopes block initiation of M phase in Xenopus oocytes. These

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3 The abbreviations used are: MPM-2, mitotic phosphoprotein monoclonal-2; TBS, Tris-buffered saline; PP2A, phosphatase-2A.
phosphoepitopes appear slightly before activation of MPF and are not directly the result of p34cdc2 kinase activity. At least some of the cdc25C phosphorylations are MPM-2 antigens (32-36). Thus, MPM-2 epitope formation is distinct from the p34cdc2 kinase pathway but may interact with it proximally via cdc25C. The effect of irradiation on the production of the MPM-2 epitopes has not previously been determined.

In earlier work, we had shown that cyclin B1 levels initially were lowered after treatment of cells with etoposide (37). However, with extended time in the block, cyclin B1 levels rise, yet the cells remain blocked in G2 (37). In this study, we examined the effect of two different DNA-damaging agents, X-rays and etoposide, on both cdc2 kinase activation and MPM-2 epitope phosphorylation. Because caffeine treatment is known to shorten the G2 arrest induced by X-radiation (38, 39), we also examined its effect in damaged cells on cdc2 kinase activation, MPM-2 epitope phosphorylation, and cell cycle progression. These experiments have revealed a G2 block that is independent of cyclin B1-p34cdc2 kinase activity.

**MATERIALS AND METHODS**

**Cell Culture and Synchrony**

HeLa cells were grown in DMEM with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml). For synchrony, 2 × 10⁶ cells were plated in 100-mm dishes, 2 μM thymidine was added in medium and washed off after 12 h, and the cells were left to cycle for 16 h in medium with 2 × 10⁻³ M thymidine and deoxycytidine. Aphidicolin (1 μg/ml) was added to block cells at the G1-S border and then washed out 12 h later. Three h after release from aphidicolin, cells were exposed either to drugs or irradiation, and time points were taken for cell cycle analysis and protein analysis as described previously (18, 40).

**Irradiation and Drug Treatment**

Cells were irradiated with 2.5, 5, and 8 Gy (low, medium, and high doses) 3 h after release using a J. L. Shepherd model 30 Mark 1 ³²³Cs irradiator delivering 12.8 Gy/min with the cells on a rotating platform to ensure uniform irradiation. Ten μM etoposide (diluted from a stock of 5 mM in DMSO; Sigma Chemical Co., St. Louis, MO) was added to cells for 20 or 40 min, or 20 μM etoposide was added for 40 min. The etoposide was removed by washing twice with PBS. Two μM caffeine (Sigma) was added immediately after irradiation or after washout of etoposide or after irradiation.

**Cdc25C Analysis**

**Immunoblots for cdc25C.** Equal amounts of protein were electrophoresed on a 10% SDS-polyacrylamide gel. Protein was transferred overnight with Bio-Rad Transblot to Hybond nitrocellulose membrane. After transfer, membranes were blocked in 5% milk, probed with primary rabbit anti-cdc25C (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) in 2.5% BSA in PBS, and reacted with secondary antirabbit immunoglobulin antibody (1:1500; Santa Cruz Biotechnology, Santa Cruz, CA) in 2.5% BSA in PBS, and branes were blocked in 5% milk, probed with primary rabbit anti-cdc25C or after washout of etoposide or after irradiation. Immunoprecipitation of cyclin B1-specific histone H1 kinase was performed using antihuman cyclin B1 antibody (Upstate Biotechnology, Lake Placid, NY) conjugated to protein A-agarose beads according to the method of Pines and Hunter (43). The specificity of the immunoprecipitation of MPF activity was confirmed using anti-cdk2 antibodies (results not shown). Samples were incubated with histone H1 (Boehringer Mannheim, Indianapolis, IN) with 200 μM γ-labeled P-32 ATP for 20 min. The precipitation of MPF activity was confirmed using anticyclin B1 antibody, and probed with secondary antimouse alkaline phosphatase as described previously (34).

**Flow Cytometry**

After pelleting trypsinized cells, 500 μl of citrate buffer (40 mM citric acid, pH 7.6, with 250 mM sucrose and 5 DMSO) was added, and the samples were stored at −20 C. For analysis, cells were thawed, pelleted, and stained with propidium iodide according to the procedure Vindelov and Christensen (42) as described previously (14). Ten thousand cells were acquired by Becton Dickinson FACScan flow cytometer (San Jose, CA) and analyzed by Cellquest (Version 2.01.2; LCC International, McLean, VA).

**Histone H1 Kinase Assay**

For each sample, 10⁶ cells were washed in ice-cold TBS buffer and scraped into HB buffer (25 mM MOPS (pH 7.2), 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 40 μg/ml aprotinin). The pellet was passed repeatedly through a 26 gauge needle. Immunoprecipitation of cyclin B1-specific histone H1 kinase was performed using antihuman cyclin B1 antibody (Upstate Biotechnology, Lake Placid, NY) conjugated to protein A-agarose beads according to the method of Pines and Hunter (43). The specificity of the immunoprecipitation of MPF activity was confirmed using anti-cdk2 antibodies (results not shown). Samples were incubated with histone H1 (Boehringer Mannheim, Indianapolis, IN) with 200 μM γ-labeled P-32 ATP for 20 min. The reaction was stopped by the addition of 2X sample buffer and boiling for 5 min, and fractionation was performed on 12% SDS-acrylamide gels. Gels were dried prior to autoradiography, and densitometry was performed using NIH Image 1.61 software.

**RESULTS**

**Effect of X-Rays or Etoposide on G2 and cdc25C Phosphorylation.** To study the effect of two different DNA-damaging agents, X-rays and etoposide, on cell cycle progression, we monitored three biochemical markers of the G2 to M-phase transition, cdc25C phosphorylation, p34cdc2 H1 kinase activity, and the formation of MPM-2 epitopes. We synchronized HeLa cells by sequential blocks with thymidine and aphidicolin. In our hands, this method achieves synchrony in G2 for more than 90% of the cells (40). Cells were treated with either X-rays or etoposide at 2–3 h after release from the G1-S block when virtually all of the cells were in S phase. Cell cycle progression was routinely monitored using flow cytometry for DNA content. As can be seen in Fig. 1, under the conditions used, both X-rays and etoposide led to a G2 block. The control population transited through G2-M between 7 and 10 h after release from the aphidicolin block, whereas the majority of the irradiated cells remained in G2 during this time and did not enter G2 until 14 h later. Etoposide treatment resulted in a G2 delay with the cells remaining in G2 throughout the interval of the experiment (see Figs. 1 and 3).

We first investigated the state of cdc25C phosphorylation during the G2 block induced by DNA damage. Activation of maximal cdc25C phosphatase activity is known to require phosphorylation. This phosphorylation, which occurs on at least 4 amino acid residues, results in altered mobility of cdc25C after electrophoresis in polyacrylamide gels (23, 27). To confirm that this mobility shift could be observed directly in lysates examined by Western blotting, we examined the electrophoretic pattern of cdc25C from HeLa cells blocked in mitosis with nocodazole (Fig. 2). Three bands, a doublet of approximately 55 and 57 kDa and a slower band at 60 kDa, were readily apparent. After treatment of the lysate with PP2A, the 60-kDa band disappeared, leaving the unphosphorylated doublet intact. In addition, it has been reported that an inhibitory phosphorylation on serine 216 can occur on cdc25C. However, this phosphorylation did not result in...
with caffeine. It has long been established that caffeine will reverse induced G2 block to the irradiation-induced block, we treated the cells X-Rays or by Etoposide. To compare the nature of the etoposide-place. (Fig. 1), raising the possibility that an additional block might be in results of Barth et al. (21). Phosphorylation of cdc25C was delayed in phosphorylated cdc25C was not seen in the irradiated cells until 10 h activation in HeLa cells is delayed by radiation, consistent with the be noted that this appearance precedes entry into mitosis. In contrast phosphorylated form of cdc25C was seen at 7-8.5 h (Fig. 1). It should shown in Fig. 1. In the untreated cells, the maximal amount of the heLa cells after treatment with X-rays or etoposide in the experiment a mobility shift on one-dimensional gel electrophoresis, and its absence did not alter the slower running bands that we are observing here (29). Thus, the mobility shift of cdc25C was due to phosphorylation, and we could monitor phosphorylation using a gel shift assay. The phosphorylation of cdc25C was examined in synchronized HeLa cells after treatment with X-rays or etoposide in the experiment shown in Fig. 1. In the untreated cells, the maximal amount of the phosphorylated form of cdc25C was seen at 7–8.5 h (Fig. 1). It should be noted that this appearance precedes entry into mitosis. In contrast phosphorylated cdc25C was not seen in the irradiated cells until 10 h (after release from aphidicolin) and persisting at 14 h. Thus, cdc25C activation in HeLa cells is delayed by radiation, consistent with the results of Barth et al. (21). Phosphorylation of cdc25C was delayed in the etoposide-treated cells, but yet the cells remained arrested in G2 (Fig. 1), raising the possibility that an additional block might be in place.

Effect of Caffeine Treatment on the G2 Delay Induced by X-Rays or by Etoposide. To compare the nature of the etoposide-induced G2 block to the irradiation-induced block, we treated the cells with caffeine. It has long been established that caffeine will reverse the radiation-induced G2 block yet has no effect on cell cycle progression in unirradiated cells (Refs. 38, 39, and 44–47 and data not shown). Synchronized irradiated (5 or 8 Gy) HeLa cells or etoposider-treated HeLa cells (treated with either 10 or 20 µM etoposide for 40 min) were also treated with 2 mM caffeine. As seen in Fig. 3, addition of caffeine to the cells irradiated with 5 Gy eliminated the G2 delay and resulted in cell cycle transit with kinetics similar to the control cells. After 8 Gy, caffeine treatment shortened but did not completely eliminate the delay. (Note that the time scale in Fig. 3A, showing the results with 5 Gy, is not the same as that in Fig. 3B, showing the results with 8 Gy.) In contrast, treatment of the etoposide-treated cells with the same dose of caffeine (2 mM) had only a slight effect on the G2 delay (see also DNA histograms in Fig. 4).

cdc25C phosphorylation was monitored after treatment with caffeine. The appearance of the phosphorylated form was delayed in the irradiated cells, corresponding in timing to the G2 delay (Fig. 5). In the caffeine-treated, irradiated cells, the phosphorylated band was detected earlier, correlating with the timing of the abrogation of the G2 delay (Fig. 3). Little phosphorylation of cdc25C could be detected by gel shift soon after treatment with etoposide (Fig. 5). Addition of caffeine also resulted in the appearance of phosphorylated cdc25C in the etoposide-treated cells (10 µM) at 10–12 h, although the cells remained arrested in G2-M. Similarly, the phosphorylated band was found in the cells treated with 20 µM etoposide at 12–30 h, yet the cells remained arrested in G2 from 12–17 h and only 50% of the cells were released from the G2 block at later times (Figs. 3–5).

The activation of cdc25C after exposure of etoposide-treated cells to caffeine led us to ask whether p34cdc2 HI kinase activity was also induced. p34cdc2 HI kinase activity was depressed in the synchronized HeLa cells treated with 10 µM etoposide or 5 Gy of X-rays, as expected (Fig. 6). The activity in control cells was low at this time because the majority of cells were in G1, by this point. Treatment with caffeine led to elevation of p34cdc2 HI kinase activity to equivalent levels in cells treated either with X-rays or with etoposide. Thus, activation of cdc25C coincided with the activation of p34cdc2 Kinase in both X-ray-treated and etoposide-treated cells after the application of caffeine, yet the etoposide-treated cells remained arrested in G2, whereas the G2 block in the X-ray-treated cells was abrogated.

To confirm that the etoposide-treated cells remained arrested in G2 and were not entering mitosis despite phosphorylation of cdc25C and increased H1 kinase activity, we examined the cells for morphological evidence of mitosis after treatment with etoposide and caffeine (Fig. 7). Nocodazole was also added to trap all cells in metaphase, preventing movement into G1. During the time when cdc25C is phosphorylated in cells treated with caffeine and etoposide and flow cytometry indicates that at least 80% of the cells have a DNA content consistent
Fig. 3. G2 delay after DNA damage and treatment with caffeine. Three h after release from the final aphidicolin block, cells were exposed to either 5 or 8 Gy or to 10 or 20 μM etoposide as indicated. Two mM caffeine was added immediately to the indicated groups. Samples were taken at the indicated time points after treatment for cell cycle analysis and protein extraction. The percentage of cells in G2-M after the indicated treatments is shown.

Fig. 4. Failure of caffeine to reverse the etoposide-induced G2 delay. Synchronized cells were treated with caffeine and etoposide as described in the legend to Fig. 3. DNA content was evaluated by flow cytometry, and the histograms are shown here.
with G2-M phase, less than 10% of the cells are in mitosis. Thus, the cells were arrested in G2 and not progressing into mitosis.

Effect of DNA Damage on the Appearance of MPM-2 Epitopes.

To determine whether formation of MPM-2 epitopes might be part of the pathway now blocked in the presence of p34cdc2 activity in etoposide-treated cells, we performed Western blots with anti MPM-2 antibodies on the lysates obtained from the experiments shown above. The formation of MPM-2 epitopes was greatly reduced initially during the G2 delay (Fig. 8) induced by either etoposide or X-rays. This reduction included those bands corresponding to the position of cdc25C. MPM-2 epitopes were seen in the irradiated cells at 15 h as they emerged from the G2 block.

We then asked whether additional treatment with caffeine resulted in the appearance of MPM-2 epitopes. As can be seen in Fig. 8, after treatment with caffeine, the appearance of the majority of the epitopes was restored, even in the etoposide-treated cells that did not resume cell cycle progression. The bands at the position of cdc25C are also seen after exposure of etoposide-treated cells to caffeine, confirming our studies, shown above, examining cdc25C phosphorylation. Thus, DNA damage that results in a cell cycle block also inhibits the appearance of MPM-2 phosphoepitopes, and caffeine reverses this effect in X-ray- or etoposide-treated cells. However, the etoposide-induced G2 block persists in spite of the reappearance of these epitopes.

DISCUSSION

Treatment of mammalian cells with agents that result in DNA damage leads to a prolonged delay in the G2 phase of the cell cycle.

Fig. 8. The appearance of MPM-2 phosphoepitopes is delayed after irradiation or treatment with etoposide. In the experiment shown in Figs. 3 and 4, 3 h after release from aphidicolin, cells were irradiated with 5 Gy or exposed to 10 μM etoposide for 40 min. Cells were harvested at the indicated times. Immunoblots were probed with antibody to MPM-2.
In this paper, we show that the G2 arrest induced by etoposide, a topoisomerase II inhibitor, includes a component independent of p34cdc2 and so can be distinguished from the block induced by X-rays. The G2 arrest induced by X-rays could be reversed by caffeine, but the G2 arrest induced by etoposide could not. It should be noted that caffeine can reverse the irradiation G2 block after much higher doses of radiation than those used here, up to 20 Gy (38, 39). Caffeine treatment of X-irradiated cells restored p34cdc2 H1 kinase activity, cdc25C phosphorylation, and the appearance of MPM-2 epitopes. We had previously shown that cyclin B1 levels, which were depressed by radiation, were restored after treatment of the cells with caffeine.

Unexpectedly, treatment of etoposide-treated cells also restored all of these activities, yet the cells remained arrested in G2. Thus, there appear to be several pathways leading to a G2 arrest. Both X-rays and etoposide result in decreased p34cdc2 H1 kinase activity and decreased phosphorylation of cdc25C and other MPM-2 epitopes. The expression of these activities is influenced by caffeine after DNA damage.

Yao et al. (48) have documented alterations in p34cdc2 activity dependent upon the p53 status of the treated cells after exposure to caffeine, also hypothesizing that caffeine affects pathways affecting p34cdc2 activity. Additional pathways appear to be caffeine insensitive and independent of p34cdc2 H1 kinase activity, phosphorylation of MPM-2 epitopes, and cdc25C phosphorylation. These pathways were revealed in cells treated with etoposide when treatment with caffeine failed to induce cell cycle progression in spite of p34cdc2 H1 kinase activity and phosphorylation of cdc25C and other MPM-2 epitopes.

These data implicate the kinase(s) affecting the formation of MPM-2 epitopes in the caffeine-dependent pathway (34). We found that treatment of cells with either X-rays or etoposide blocked the formation of MPM-2 epitopes during the G2 delay. Treatment of these cells with caffeine restored the formation of MPM-2 epitopes, including phosphorylation of cdc25C, a known MPM-2 epitope (35). These results suggest that MPM-2 kinases may also be a target of regulation after DNA damage.

Etoposide is a topoisomerase II inhibitor that results in double-strand breaks in DNA through the formation of cleavable complexes (49–54). Inhibition of topoisomerase II in the absence of DNA breaks also results in failure of entry into mitosis (55, 56). Etoposide treatment is highly reversible; in the experiments reported here, the etoposide was washed out early in the experiment, so that by the time of G2, topoisomerase II activity should no longer be inhibited. X-irradiation also results in double-strand breaks, although these lesions induced by X-rays, but the exact nature of this difference is not yet clear.

Treatment of X-irradiated cells with caffeine results in a marked shortening of the G2 block. Lock et al. (59) had noted that the effect of caffeine on etoposide-treated cells varied depending upon the dose of caffeine used. At 2 mM caffeine, the G2 block was abolished in this study, whereas at 10 mM, it persisted. In our hands, however, the block induced by etoposide persisted at 2 mM caffeine. This may be due to different conditions of administration of the etoposide and the timing of administration of the caffeine. It should be noted that under the conditions used here, 2 mM caffeine abolished the X-ray induced G2 block, and 2 mM caffeine had no effect on cell cycle progression of synchronized but otherwise untreated HeLa cells. The experiments reported here are consistent with the concept that there are two potential points of action for the G2 block, one affecting p34cdc2 H1 kinase and caffeine responsive, and one independent of p34cdc2 H1 kinase and caffeine independent.


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