Cyclin B1 Availability Is a Rate-limiting Component of the Radiation-induced G2 Delay in HeLa Cells

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ABSTRACT

Irradiation of tumor cells results in a G2 delay, which has been postulated to allow DNA repair and cell survival. The G2 delay after irradiation is marked in HeLa and other cells by delayed expression of cyclin B1. To test whether this depression of cyclin B1 contributes to the G2 delay, we induced cyclin B1 expression in irradiated HeLa cells using a dexamethasone-inducible promoter. Induction of cyclin B1 after radiation abrogated the G2 delay by approximately doubling the rate at which the cells reentered mitosis, whereas dexamethasone itself had no effect. However, overexpression of cyclin B1 did not eliminate the G2 delay in irradiated cells. In unirradiated cells, overexpression of cyclin B1 had no effect on cell cycle progression. Confirmation that reduction of cyclin B1 levels would prolong G2 was provided using antisense oligonucleotides to cyclin B1. These results demonstrate that cyclin B1 levels control the length of the G2 delay following irradiation in HeLa cells but do not exclude additional mechanisms controlling the mitotic delay after irradiation.

INTRODUCTION

It has been known since the first description of the G1 and G2 phases of the cell cycle that radiation with X-rays perturbs the cycling of eukaryotic cells (1). All eukaryotic cells, including yeast, Xenopus oocytes, broad bean roots, and cycling somatic mammalian cells, undergo a division delay after irradiation (2). In primary mammalian cells, delays in the cycle after irradiation are seen in both the G1 and G2 phases and to some degree also in the S phase after high doses of radiation (3). In many transformed cells, however, the division delay is accounted for almost entirely by a delay in the G2 phase of the cell cycle, because transformed cells with mutated or absent p53 fail to arrest in G1 after radiation but still undergo a G2 arrest (4, 5). The mechanisms underlying the G2 delay are only incompletely understood.

In experiments exploring the effects of radiation on the cell cycle, we found previously that both cyclin B1 mRNA and protein levels are reduced substantially in irradiated HeLa cells. This down-regulation by radiation appeared to be a specific effect on cyclin B1, because cyclin A, γ-actin, and rpl32 mRNAs were not affected (6, 7). The decrease in cyclin B1 mRNA levels that is seen during the G2 delay induced by radiation can be accounted for by a significantly decreased stability of the cyclin B1 mRNA in the irradiated cells (7). Although our initial studies were in HeLa cells, we now have data to indicate that the depression of cyclin B1 mRNA can occur in other cell types, including rat embryo cells, Chinese hamster cells, and human glioma and medulloblastoma cells after radiation (8). Datta et al. (9) have shown that cyclin B1 mRNA levels are depressed after irradiation of U937 human myeloid leukemia cells.

In cycling mammalian cells, cyclin B1 mRNA and protein varies markedly in abundance during the cycle, with negligible levels in G1, increased levels in S, and maximal levels in G2-M (10). As the level of cyclin B1 rises, it forms a complex with p34CDC2, which is found at constant levels in cycling mammalian cells (11–13). The association of p34CDC2 with a cyclin is required for activity (14, 15). Because cyclin B1 is required for the transition through G2 to mitosis, the reduced amount of cyclin B1 after irradiation could potentially contribute to the radiation-induced G2 delay. The experiments reported here examine the hypothesis that decreased availability of cyclin B1, secondary to its down-regulation by radiation, is a rate-limiting step in the radiation-induced G2 delay.

The activity of p34CDC2 is essential for the transition from G2 through mitosis (11–13). Exposure to ionizing radiation as well as to other DNA damaging agents results in decreased p34CDC2 kinase activity. Lock and Ross (16), Lock (17), and Lock and Keeling (18) showed that treatment of mammalian cells with X-rays or with etoposide, a topoisomerase II inhibitor that results in DNA breaks, led to drastically reduced p34CDC2 kinase activity (16–18). Subsequently, irradiation has been shown to diminish p34CDC2 kinase activity in CA46, Ramos (Burkitt’s lymphoma), HL 60, and V79 cells (19–26). Decreased p34CDC2 kinase activity could be consistent with decreased availability of cyclin B1, but the mechanisms that account for the diminished p34CDC2 activity and the G2 delay are only beginning to be characterized.

To test the significance of the decrease in cyclin B1 levels in irradiated HeLa cells, we increased the amount of cyclin B1 after irradiation by using a dexamethasone-inducible vector and found that the G2 delay after irradiation was reduced substantially. Conversely, depression of cyclin B1 using antisense oligonucleotides augmented the duration of G2. Together, these results indicate the importance of cyclin B1 levels in regulating the length of G2 after irradiation. They do not, however, exclude a contribution to the G2 delay by other mechanisms.

MATERIALS AND METHODS

Cell Culture, Synchronization, and Irradiation. HeLa cells or HeLa cells transfected with the cyclin B1-inducible vector were grown in DMEM supplemented with 10% FCS, penicillin, and streptomycin. Transfected clones were selected and maintained with hygromycin (200 μg/ml; Calbiochem, La Jolla, CA) but were cultured without hygromycin during the experiments described. HeLa cells were grown, synchronized with sequential thymidine and aphidicolin blocks, and irradiated as described previously (27). Control cells were always mock irradiated.

Isolation of Cyclin B1-Inducible HeLa Cell Clone. pGRE was designed by Mader and White (28) to lead to high levels of mRNA induction driven by five glucocorticoid response elements placed upstream from the adenovirus 2 major late promoter with minimal basal expression. The pGRE/cycB (sense) plasmid was constructed by subcloning the 1.4-kb human cyclin B1 cDNA from pGEM4Z/cyclin (gift of Pines and Hunter (10)) into the BamHI site in the multiple cloning site of the Epstein-Barr plasmid pGRE/S-1. Five μg of the plasmid were transfected into HeLa cells using Lipofectin (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. After 48

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hours, selection was started with hygromycin at 200 μg/ml. Individual colonies were isolated with cloning cylinders and expanded. As described in the text, clone S21 was selected for additional experimentation. Dexamethasone was used at 1 μg/ml in all experiments.

RNA Extraction and Northern Blot Analysis. Total RNA was isolated at indicated times. RNA blots with 5–10 μg of total mRNA in each lane were performed as described previously (27). Hybridization for cyclin B1 mRNA was performed using a radioactive probe made from a 1.3-kb fragment of the human cyclin B1 cDNA cut from the plasmid pGEM4Z, with EcoRI and BamHI (10). The autoradiographs were scanned on an Arcus II scanner (Agfa-Gaevert, Mortsel, Belgium) and digitized using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Immunoblotting for Cyclin B1. For each time point, 100,000 synchronized cells per 6-cm plate were harvested by lysis in 400 μl of sample buffer [10% glycerol, 2% SDS, 10 mM DTT, and 50 mM Tris (pH 6.8)]. Thirty μl of each lysate were loaded per lane and separated by electrophoresis on a 15% SDS polyacrylamide gel prior to overnight transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then probed sequentially with mouse antihuman cyclin B1 monoclonal antibody (Upstate Biotech, Lake Placid, NY) followed by goat antimouse secondary antibody coupled to horseradish peroxidase (Amersham Corp., Arlington Heights, IL). Incubation with the secondary antibody was performed in a solution of 2.5% powdered milk in PBS. Detection was performed by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). Relative levels of cyclin B1 were determined by scanning the blots as described above.

Nocodazole Trapping. For each time point, 100,000 synchronized cells per 6-cm plated were used. Nocodazole (0.04 μg/ml) was added 2–5 hours after release from the aphidicolin block. Mitotic indices were determined after staining cells with propidium iodide using the Zeiss Axioplan microscope with Neofluor lenses (29). For each point, 100 cells were counted and scored visually for mitosis. All counts were repeated three times.

Immunofluorescence for Cyclin B1. Immunofluorescence was performed via a modification of the methods used by Sherwood et al. (30). Briefly, cells were grown on glass coverslips, synchronized using the same procedure described above, and treated as indicated in the figure legends. At the indicated times, they were washed in PBS and fixed by immersion in absolute methanol/acetic acid (1/1, v/v) for 2 min. Air-dried coverslips were incubated at room temperature for 1–2 hours with antihuman cyclin B1 antibody (Pharmingen, San Diego, CA) diluted 1:500 in PBS containing 2% bovine serum albumin. The coverslips were washed in PBS, after which antimouse FITC-labeled goat antibody (Boehringer Mannheim, Indianapolis, IN) was applied for 1 hour. Coverslips prepared in the same way, in which the primary antibody was omitted, showed no immunofluorescence. Photographs were taken using a Zeiss Axioplan microscope with Neofluor lenses. The photographs were scanned and digitized as indicated above.

Oligonucleotide Studies. Pan-modified phosphorothiate-modified oligonucleotides were synthesized by the Core DNA Synthesis Facility of the University of Pennsylvania Cancer Center (Philadelphia, PA). The sequence 5’ CCA TTG GCC TTG GAG AGG 3’ was used as the antisense oligonucleotide, and the complementary sequence was used as the sense control. Oligonucleotides (2 μg/ml), either the antisense or the sense, were applied to synchronized cells via Lipofectin (Life Technologies, Inc.) used according to the manufacturer’s instructions. The oligonucleotides were left on the dishes for the duration of the experiment. Samples for nocodazole trapping and immunoblotting were prepared the same way as in the other experiments.

RESULTS

Inducible Expression of Cyclin B1 in HeLa Cells. To determine the importance that depressed cyclin B1 levels have on the G2 delay after irradiation in HeLa cells, we wished to develop a system in which high levels could be maintained after irradiation. A HeLa cell clone carrying an inducible vector for cyclin B1 was generated to overexpress cyclin B1 at specific times in the cell cycle. Human cyclin B1 cDNA was subcloned into pGRE, a dexamethasone-inducible expression vector. This construct was stably transfected into HeLa cells, and individual hygromycin-resistant clones were isolated. The cyclin B1 mRNA encoded by the vector has additional upstream sequences resulting from the vector, allowing it to be distinguished from the endogenous cyclin B1 mRNA by size. The induced protein should be identical to the endogenous human cyclin B1. Out of 21 selected clones, 8 responded to exposure to dexamethasone with increased amounts of cyclin B1 mRNA coded for by the vector (Fig. 1A). One clone (S21), which was found to express the highest level of cyclin B1 mRNA after induction, was selected for further analysis. Fig. 1B shows the induction of exogenous cyclin B mRNA by dexamethasone in this clone.

Dexamethasone treatment also resulted in increased cyclin B1 protein levels in S21 cells. Both HeLa cells and the vector-bearing S21 cells could be synchronized via a double thymidine/aphidicolin block under identical conditions, because both S21 cells and the parental HeLa cells have a doubling time of 26 h and equivalent distribution through the cell cycle. Dexamethasone was added to S21 cells 2 h after release from the final aphidicolin block, when at least 80% of the cells were in S phase. Cyclin B1 accumulated in G2-M in sequences resulting from the vector, allowing it to be distinguished from the endogenous cyclin B1 mRNA by size. The induced protein should be identical to the endogenous human cyclin B1. Out of 21 selected clones, 8 responded to exposure to dexamethasone with increased amounts of cyclin B1 mRNA coded for by the vector (Fig. 1A). One clone (S21), which was found to express the highest level of cyclin B1 mRNA after induction, was selected for further analysis. Fig. 1B shows the induction of exogenous cyclin B mRNA by dexamethasone in this clone.

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the untreated S21 cells as expected (Fig. 1C). Treatment of S21 cells with dexamethasone resulted in cyclin B1 levels above those seen in untreated cells entering G2-M. Increased expression of cyclin B1 protein could be detected 2 h after addition of dexamethasone in the S21 cells, and levels of cyclin B1 were greater at all subsequent time points measured (Fig. 1, C and D). The timing of cell cycle progression did not vary in the unirradiated S21 cells after induction of cyclin B1 (Fig. 1E). Dexamethasone did not alter cell cycle progression of HeLa cells themselves (data not shown).

To determine whether cyclin B1 expression was being induced in the majority of the S21 cells after dexamethasone treatment, we examined cyclin B1 expression by immunofluorescence. Initially, at 4 h after exposure to dexamethasone, only about 50% of the cells stained strongly, but virtually all showed intense staining after 10 h (Fig. 2). Dexamethasone treatment of control cells had no effect on cyclin B1 staining intensity, but irradiation resulted in decreased intensity of cyclin B1 immunofluorescence as expected (data not shown).

**Induction of Cyclin B1 Protein in Irradiated Cells.** Having established that cyclin B1 could be induced by dexamethasone in synchronized S21 cells, we investigated whether cyclin B1 protein could also be induced by dexamethasone after irradiation in these cells. We had found previously that irradiation results in cyclin B1 mRNA instability (7). Thus, the experiments described here depended on achieving sufficiently high levels of cyclin B1 mRNA to effect higher levels of cyclin B1 in spite of the effects of irradiation on cyclin B1 mRNA stability. The large overexpression of cyclin B1 mRNA driven by the dexamethasone-induced promoter led to increased amounts of cyclin B1 even after irradiation. S21 cells were synchronized, released, and irradiated with 5 Gy. In accord with previously published data, irradiation led to diminished cyclin B1 levels (Fig. 3; Refs. 6 and 27). Increased cyclin B1 protein expression was noted in the irradiated cells 2 h after addition of dexamethasone and reached levels 3- to 5-fold greater than in uninduced cells at later times. The intensity of the cyclin B1 band from the irradiated cells treated with dexamethasone was at least equivalent to or greater than the levels seen in the control cells. Thus, cyclin B1 levels could be induced after irradiation in S21 cells.

**Expression of Cyclin B1 Protein Reduces the G2 Delay following Irradiation.** The effect of induction of cyclin B1 on progression from G2 into mitosis in irradiated cells was examined. To count the number of cells in mitosis, we added nocodazole so that the numbers would reflect the total number of cells that had entered mitosis [as described in O’Connor et al. (19)]. In the unirradiated controls, at least 80% entered mitosis between 12 and 15 h after release from the aphidicolin block. Treatment of unirradiated S21 cells with dexamethasone had no effect on the time of entry into mitosis in spite of the overexpression of cyclin B1 (Fig. 1E).

Radiation of S21 cells in S phase resulted in a delay in the progression into mitosis. After irradiation with 5 Gy, only 30–40% of the irradiated cells had entered mitosis by 22 h. Induction of cyclin B1 with dexamethasone in irradiated S21 cells overcame this delay and resulted in a doubling of the rate of exit from the G2 block. Twenty-two h after release from the aphidicolin block, 75% of the dexamethasone-treated cells had entered mitosis (Fig. 4B). The cyclin B1 protein levels from this experiment are shown in Fig. 4A. Similar experiments were performed using two other clones bearing the inducible vector. Exposure of these cells to dexamethasone after irradiation also resulted in a diminished G2 delay (Fig. 5, A and B). Induction of cyclin B1 by dexamethasone also reduced the G2 delay after 3 Gy (Fig. 6). As a control for the effect of the dexamethasone, the same experiment was performed on the parental HeLa cells. Dexamethasone treatment in this case had no effect on cyclin B1 levels (Fig. 7A) nor on the timing of the G2 delay (Fig. 7B). Dexamethasone treatment also had no effect on the G2 delay in cells bearing the pGRE vector without an insert (Fig. 5C). Thus, overexpression of cyclin B1 resulted in a diminished G2 delay in irradiated HeLa cells.
Antisense Oligonucleotides Diminish Cyclin B1 Expression and Augment the G2 Delay. We wished to confirm that decreasing the expression of cyclin B1 would delay progression into mitosis. Synchronized HeLa cells were treated with a 15-base phosphothiorate-modified oligonucleotide antisense to a portion of the human cyclin B1 mRNA near the methionine start site, which has previously been reported by Minshull et al. (31) to result in destruction of cyclin B1 mRNA in Xenopus egg extracts. A 15-base phosphothiorate-modified oligonucleotide containing the sequence complementary to the antisense oligonucleotide was used as a control. We irradiated synchronized HeLa cells 3 h after release from the aphidicolin block and treated the cells with either antisense or sense oligonucleotides. Immunoblotting showed decreased levels of cyclin B1 in the cells treated with the antisense but not the control oligonucleotides (Fig. 8A). With antisense treatment, progression into mitosis was markedly retarded compared to untreated cells or cells treated with the sense oligonucleotide (Fig. 8B). Again, induction of cyclin B1 by dexamethasone shortened the G2 delay. The experiment shown is after irradiation with 3 Gy, but similar effects were seen in experiments after 5 Gy (data not shown). We have also found that the antisense oligonucleotide to cyclin B1, but not the sense oligonucleotide, results in a G2 block in unirradiated cells (data not shown). Thus, suppression of cyclin B1 expression by antisense oligonucleotides reduced the rate at which cells were able to reenter mitosis after irradiation, further demonstrating a regulatory role for cyclin B1 in the G2 delay.

DISCUSSION

In previous work, we demonstrated that cyclin B1 levels were reduced in HeLa cells after irradiation, leading to the suggestion that cyclin B1 levels could determine the extent of the G2 delay after radiation (6, 27). In this study, we set out to determine whether cyclin B1 expression is the rate-limiting step for this G2 delay. We have now shown that overexpression of cyclin B1 in irradiated HeLa cells resulted in a diminished G2 delay and that decreased expression of cyclin B1 prolonged the G2 delay. These findings demonstrate that the depression of cyclin B1 determines the timing of the G2 delay induced by radiation.

In unirradiated cells, elevation of cyclin B1 levels above normal amounts did not affect the timing of mitosis. Thus, although the levels of cyclin B1 increase markedly in G2 and cyclin B1 has been shown to be essential for the G2-to-mitosis transition, accumulation of cyclin
B1 is not the rate-limiting component for entry into mitosis in unirradiated cells (11, 12).

Induction of cyclin B1 reduced the length of the G2 delay after irradiation, but it did not totally eliminate it. There are several possibilities to explain this result. First, the levels of induction of cyclin B1 might not consistently equal the amounts in normal mitotic cells, and hence, G2 might be prolonged. Even though the immunoblot analysis and immunofluorescence indicated that the average amount of cyclin B1 in the induced cells was equivalent or even higher than that in the mitotic cells, the cell-to-cell variation might still reduce the effective levels for a significant proportion of the cells. A second possibility is that there are redundant pathways of different duration leading to a G2 delay. If this were the case, then the mechanism that leads to the block in G2 for the longest time would be the rate-limiting step. If the longest block were abrogated, the remaining mechanisms would also function to block G2 but for a shorter time. After irradiation of HeLa cells, depression of cyclin B1 appears to be the limiting step in determining the length of the delay, but with recovery of cyclin B1 expression, another block might become evident.

There are a number of candidates for additional, redundant mechanisms that could produce a G2 block. Increased phosphorylation of p34<sup>cdc2</sup> has been shown after irradiation by X-rays and after treatment with other DNA-damaging agents such as etoposide, nitrogen mustard, camptothecin, or UV radiation (17–26). The activity of p34<sup>cdc2</sup> is inhibited by phosphorylation on either threonine 14 or tyrosine 15 during the normal cell cycle. Activation of p34<sup>cdc2</sup> kinase activity through dephosphorylation of threonine 14 and tyrosine 15 is mediated by cdc25C, which is itself activated by phosphorylation (32–36). O'Connor et al. have shown that after treatment with nitrogen mustard, cells undergo a G2 delay and delay phosphorylation of cdc25C (37). Barth et al. (38) have also indicated that phosphorylation of cdc25C is inhibited after irradiation in HeLa cells, making cdc25C a potential target for a redundant mechanism affecting the G2 delay.

The potential involvement of phosphorylation of p34<sup>cdc2</sup> in the radiation-induced G2 delay was confirmed by the experiments of Jin et al. (39), who constructed an inducible system for expressing a p34<sup>cdc2</sup> with mutations at both positions 14 and 15, leading to substitution with amino acids that cannot be phosphorylated and hence cannot be inhibited by phosphorylation. Induction of the mutant p34 after irradiation shortened the G2 delay but did not eliminate it (39). A consideration of the results of Zheng and Ruderman (40) suggests a potential link between the results presented here and those of Jin et al. (39). Zheng and Ruderman (40) defined a domain of cyclin B1 that is required for the dephosphorylation of cdc2 in Xenopus oocyte extracts. Their work suggested that this domain was also required for the activation of cdc25C. Thus, if cyclin B1 is required for the activation of cdc25C, diminished levels of cyclin B1 might be expected to result in increased phosphorylation of cdc2. Whether the expression of cyclin B1 is required for cdc25C activation in mammalian cells is unknown. Hoffmann et al. (32) have postulated that the cyclin B1 cdc2 complex acts as a positive feedback loop to activate cdc25C. However, other kinases may also be able to activate cdc25C (33, 34, 41–43). In particular, the polo-like kinase 1 has been shown at least in Xenopus oocytes to be able to activate cdc25C via phosphorylation (42).

More speculative is the possibility that the targets for radiation effects on G2 could be the kinase(s) that phosphorylate p34<sup>cdc2</sup>, including wee-1, a human homologue of myt-1 (43–48), or mammalian homologues of inhibitors of p34<sup>cdc2</sup> (49, 50). Our results, however, demonstrate that lowered cyclin B1 levels prolonged the G2 delay after radiation and elevation of cyclin B1 levels reduced the delay, thereby establishing the role of cyclin B1 levels in controlling G2 in irradiated cells.
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REFERENCES

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