Histone H1 Dephosphorylation Is Mediated through a Radiation-induced Signal Transduction Pathway Dependent on ATM*

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†The abbreviations used are: IR, ionizing radiation; CDK, cyclin-dependent kinase; AT, ataxia telangiectasia; PI, phosphatidylinositol; Gy, gray; TdR, thymidine; Mes, 4-morpholineethanesulfonic acid.

Ionizing radiation is known to activate multiple signal transduction pathways, but the targets of these pathways are poorly understood. Phosphorylation of histone H1 is thought to have a role in chromatin condensation/decondensation, and we asked whether ionizing radiation (IR) would alter H1 phosphorylation. Our data demonstrate that low doses of IR result in a dramatic, but transient, dephosphorylation of H1 isoforms. The in vivo IR-induced dephosphorylation of H1 is completely blocked by wortmannin and is abrogated in ataxia telangiectasia cells. Furthermore, we measured radiation-induced inhibition of cyclin dependent kinase activity and activation of histone H1 phosphatase activity. Both activities were affected by radiation-induced signals in an ATM-dependent manner. Thus, the rapid IR-induced dephosphorylation of H1 involves a pathway including ATM and a wortmannin-sensitive step leading to both inhibition of cyclin-dependent kinase activities as well as activation of H1 phosphatase(s).

Exposure of mammalian cells to ionizing radiation (IR) induces complex cellular responses, including activation of cell cycle checkpoints. Checkpoints function by anticipating whether conditions are appropriate for successful entry into and completion of the DNA replication (S) or chromosome segregation (M) phases of the cell cycle (1). Passage through either one of these cell cycle phases in the presence of strand breaks or other lesions could lead to cell death or genetic instability by chromosomal loss and/or rearrangement. Checkpoints are controlled through signal transduction pathways involving protein kinases and phosphatases. The pathway leading from double-stranded breaks to both G1/S arrest and G2 arrest have been partially delineated (1), although the molecular details of the S phase arrest remain unknown (2). In the case of the G1 arrest, the prototype cyclin-dependent kinase (CDK) inhibitor, p21, is transcriptionally activated by p53 through the gene product ATM (mutated in ataxia telangiectasia), an autosomal recessive disease characterized by cerebellar and thymic degeneration, radiation sensitivity, and predisposition to cancer (3). AT cells have increased levels of spontaneous and induced chromosomal fragility, are hypersensitive to ionizing radiation, and fail to activate checkpoints following IR (4–6). ATM has recently been cloned and has a C-terminal domain with PI 3-kinase homology whose activity is important in a number of cell cycle checkpoints (7). p21 in turn inhibits multiple cyclin-dependent kinases including CDK2, and CDK4, which prevents the phosphorylation of pRB, resulting in a G1 arrest (8, 9). The maintenance of CDC2 in the phosphorylated (inhibited) state is thought to be the key regulator of the G2 arrest (10). Recently it has been demonstrated that CDC25, the dual specificity protein phosphatase that controls entry into mitosis by dephosphorylating CDC2, is phosphorylated at serine 216 by human CHK1, which itself is activated in response to DNA damage (10). The 216 CHK1 serine modification has been shown to abrogate binding to 14-3-3 proteins, which would otherwise inactivate CDC25 through sequestration (10). More recently it has been demonstrated that Chk2 negatively regulates CDC25 in an ATM-dependent manner (11). Abrupt changes in the phosphorylation status of target proteins likely requires the opposing activation/inactivation of a protein kinase and the corresponding protein phosphatase, respectively. Thus, although CDC2 is preeminent among the cell cycle regulators, the question arises whether the damage signaling pathways that inactivate CDC2 and other CDKs also activate phosphatases, leading to dual regulation of downstream targets such as histone H1.

Histone H1 is a potentially important target of damage-sensing pathways, not only because its phosphorylation status has been linked to chromatin condensation/decondensation, but also because it has been implicated in the control of DNA replication in several systems (12–14). Covalent modifications of histones potentially alter chromatin structure so as to allow repair or permit checkpoint proteins to gain access to damaged DNA. Here we show that IR acutely, but transiently, decreases levels of phosphorylated H1 in vivo in an ATM-dependent manner. The rapid IR-induced dephosphorylation of H1 results from dual regulation of cyclin-dependent kinase activity (inhibition) as well as H1 phosphatase activity (activation). Our data suggest the phosphorylation status of H1 is tightly regulated in response to DNA damage and, therefore, may be an important target of damage sensing pathways.

MATERIALS AND METHODS

Cell Culture—RKO cells, derived from a human colon cancer, were grown as monolayers in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.) penicillin, and streptomycin in 5% CO2 at 37 °C incubator. Jurkat cells (a human T cell lymphoma cell line) were grown in RPMI 1640 medium (Life Technologies, Inc.) containing penicillin and streptomycin and 10% fetal bovine serum. FT/pEBS7 and FT/pEBS7-YZ5 cells were both derived from the AT22IJE-T line, an immortalized fibroblast line (15) containing a homozygous frameshift mutation at codon 762 of the ATM gene (16). AT22IJE-T cells were transfected with the mammalian expression vector pEBS7 (17) containing either the hygromycin resistance marker to yield FT/pEBS7 cells or with full-length ATM open reading frame to yield FT/pEBS7-YZ5 cells. FT/pEBS7 and FT/pEBS7-YZ5 were generously provided by Y. Shiloh (Tel Aviv University) and

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RESULTS

Radiation Reduces Phosphorylation Levels of H1—Asynchronously growing Jurkat and RKO cells were exposed to various doses of radiation and analyzed at 45 min post-radiation by Western blotting using a phospho-specific H1 antibody (20). Phosphorylated H1 levels were decreased at doses as low as 1.25 Gy in both cell lines and ramped down as IR dose was increased from 1.25 to 2.5 to 5.0 Gy (Fig. 1). At higher doses, i.e. > 5 Gy, phosphorylated H1 levels were dramatically decreased to nearly undetectable levels. As a control for the amount of H1 fraction in total histone, the blot was re-probed with an anti-H1 antibody (Fig. 1A). No significant decrease in total H1 occurred, even at a dose of 50 Gy. Thus, IR decreased phosphorylation of H1 without decreasing total H1 levels. In addition, the effect of IR on phosphory-H3 levels was examined (Fig. 1A). In contrast to the H1 dephosphorylation, no significant changes in phosphorylation of H3 were noted following doses as high as 50 Gy. To determine the kinetics of H1 dephosphorylation, asynchronous Jurkat and RKO cells were radiated with 5 Gy, and samples were harvested at various times post-radiation (Fig. 1B). Maximal dephosphorylation occurred at 30–60 min post-radiation. Within 2 h, levels of phosphorylated H1 returned to basal levels. Again, as in Fig. 1A, there was no decrease in total H1 in either RKO or Jurkat cells due to IR. The dephosphorylation of H1 was transient and fully reversible.

Reactive Oxygen Intermediates Cause Dephosphorylation of H1—IR is associated with the formation of reactive oxygen intermediates (ROI) and recent evidence indicates that radiation induces several transcription factors through ROI (21, 22). Although cycloheximide did not abrogate IR-induced dephosphorylation of H1 (data not shown), we asked whether ROI were capable of inducing dephosphorylation of H1. Jurkat cells were treated with 5 μM hydrogen peroxide for 30 min at 37°C. Histones were extracted and measured by DNA synthesis activity.

Histone Preparation and Western Analyses—Cells were seeded at the same number per dish and at various times post-radiation washed in phosphate-buffered saline buffer. Following centrifugation, cells were resuspended in extraction buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 1.5 mM phenylmethylsulfonyl fluoride) and sulfuric acid was added to a final concentration of 0.4 M. Cells were incubated on ice for 30 min and then centrifuged for 10 min, and the acid-insoluble pellet discarded. Histones were precipitated with 20% trichloroacetic acid and washed with acidified (0.1% HCl) acetone and subsequently with acetone alone. The histones were then resuspended in Laemmli buffer. Fifty micrograms of protein lysate as determined by a Bradford assay (Bio-Rad) was loaded into each lane. Immunoblots were done with an anti-phospho-H1 or anti-phospho-H3 antibody generously supplied by C. D. Allis (University of Virginia). Immunoblots for total H1 were performed with an anti-H1 antibody (Upstate Biotechnology, Lake Placid, NY). Reactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Pharmacia Biotech). Histone H1 Kinase and Phosphatase Assays—Cells were collected, washed with cold phosphate-buffered saline, and resuspended in a final lysis buffer (1% Nonidet P-40, 0.15 mM NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 100 units/ml aprotinin). The suspensions were kept on ice for 30 min, and the lysate was collected by centrifugation at 15,000 × g for 20 min at 4°C. Protein concentrations were determined using a Bradford assay. Extracts were diluted to 1 mg/ml with lysis buffer. Immunoprecipitations with anti-CDC2 and anti-CDK2 were performed by incubating 500 μg of extract and 4 μg of antibody for 2 h at 4°C. The immune complexes were then incubated at 4°C with 15 μl of a 50% suspension of protein A (previously washed in lysis buffer). The complexes were then incubated at 4°C with 15 μl of the kinase assay buffer containing 25 μM ATP, 2.5 μM of [γ-32P]ATP, and histone H1 at 1.0 mg/ml. Reactions were terminated with the addition of 8 μl of boiling 6× electrophoresis sample buffer and run on a 12.5% SDS-polyacrylamide gel. The gel was fixed and stained with Coomassie Blue (in 45% methanol, 10% acetic acid), destained (40% methanol, 10% acetic acid), dried, and exposed to x-ray film. For quantification, the histone H1 bands were excised, and [32P]incorporation was determined by liquid scintillation counting. Amount of CDC2 and CDK2 proteins was determined by resuspending the immunoprecipitates-protein A beads in 2× SDS sample buffer and incubating in a boiling water bath for 5 min. The resulting supernatant was resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted.

Assays of protein phosphatase activities were performed using [32P]-labeled histone H1 prepared by using catalytic subunit of cAMP-dependent protein kinase (in 0.5 mM Mes, pH 7.1, 100 mM MgCl₂, 0.2 mM ATP, 5 mg/ml histone H1), purified with a Sepharose G-25 column. Nuclei were prepared by digitonin as described by Psachal (19). Phosphatase activity was measured as the release of trichloroacetic acid-soluble phosphate from [32P]-histone H1. To distinguish between PP1 and PP2A activity, the assays were carried out in the presence and absence of 1 mM okadaic acid and/or 10 nM tautomycin.

Chemicals—Tautomycin and okadaic acid were obtained from Calbiochem. Calyculin A was obtained from Sigma. Histone H1 was obtained from Roche Molecular Biochemicals (Meylan, France).
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Histone H1 is a target of a radiation-induced signaling pathway. The rapid dephosphorylation of H1 in response to IR is complete within 30 min post-irradiation. Histones were extracted 45 min post-irradiation and Western analysis was performed using anti-H1 and anti-phospho-H1 antibodies. The effect of IR on the dephosphorylation of H1 is completely abrogated in AT cells (26). To determine whether this also occurred in Jurkat cells and in reconstituted AT cells, we performed H1 kinase assays. IR led to a time-dependent inhibition of both CDK2 and CDC2 kinase activity in Jurkat and normal cells, but the response was blunted in AT cells (Fig. 6).

We investigated the possibility that IR activated a phosphatase by testing cell permeable serine-threonine phosphatase inhibitors. Calyculin A increased phospho-H1 levels at concentrations between 2 and 20 nM (Fig. 7) and at these concentrations also blocked the radiation-induced dephosphorylation of H1. Similar to calyculin A, okadaic acid and tautomycin also prevented IR-stimulated dephosphorylation of H1. These data show that a type 1 or 2A phosphatase activity is necessary for IR-induced dephosphorylation of H1, and essentially eliminate calcineurin (phosphatase 2B) and Mg2+-dependent (phosphatase 2C) as H1 phosphatases responsive to IR.

We performed phosphatase assays on nuclear extracts prepared from irradiated and nonirradiated Jurkat, AT, and reconstituted AT cells. At various times following 5-Gy irradiation, nuclei were prepared and H1 phosphatase activity determined using 32P-labeled H1 as substrate. IR increased phosphatase activity in both Jurkat and reconstituted AT cells (Fig. 8). However, H1 phosphatase activity was only minimally increased in the AT cells. Furthermore, H1 phosphatase activity was maximal 30 min post-irradiation and declined to baseline at 120 min post-irradiation. These kinetics are consistent with the in vivo dephosphorylation as shown in Fig. 2. Both okadaic acid and tautomycin were added to the nuclear extracts prior to the addition of phosphorylated H1 to inhibit type-1 and type-2 phosphatases. Okadaic acid fully inhibited the radiation-induced activation of phosphatase. Tautomycin, on the other hand, failed to block the radiation-induced phosphatase activation. These data suggest that PP2A is activated by irradiation in vitro but do not establish which phosphatase(s) is activated by irradiation in vivo.

DISCUSSION

Although the effects of radiation on histone turnover and phosphorylation were investigated many years ago (27), this...
report presents the first documentation that radiation acutely and transiently causes net dephosphorylation of H1 through an ATM-dependent pathway. The fact that the phosphorylation status of H1 returns to normal within 2h following IR argues that acute dephosphorylation of H1 is not an early readout of cell death, but rather an important response to DNA damage. Little is known about the signaling pathway that is activated by IR that ultimately leads to altered phospho-H1 levels. For many IR-induced DNA damage sensing pathways, including induction of p53, it is believed that DNA double-stranded breaks trigger the response (28). But, we have observed rapid H1 dephosphorylation in Chinese hamster ovary cells, which do not have an intact p53 G1 arrest pathway (29). Thus, it is likely that the pathway leading from ATM to H1 is p53 independent. Evidence exists to suggest that the signal that ultimately leads to dephosphorylation of H1 originates in the nucleus. Not only is ATM thought to be a sensor of DNA damage (3), but also it

**Fig. 4.** Effect of IR on DNA replication in Jurkat, RKO, and AT cells. Cells growing in 12 well dishes were prelabeled for 48h with 10 nCi/ml of [14C]TdR for 48h. Cultures were irradiated and at 35 min post-irradiation were pulse-labeled with 2.5 μCi of [3H]TdR/ml of culture medium for 20min and were fixed with citric acid. The quantity of trichloroacetic acid-insoluble radioactivity was then quantified at the end of the experiments as described previously (21). A, RKO cells (●); B, Jurkat (●) and Jurkat cells pretreated for 30 min with 30 μM wortmannin (●); and C, AT fibroblasts transfected with either empty vector FT/pEBS7 (●) or recombinant wild-type ATM FT/pEBS7-YZ5 (●).

**Fig. 5.** The IR-induced decrease in phospho-H1 is influenced by the ATM protein. AT fibroblasts transfected with either empty vector FT/pEBS7 or recombinant wild type ATM FT/pEBS7-YZ5 were irradiated with doses between 0 and 50 Gy. Cultures were returned to the incubator for 45min, after which histones were extracted. Western analysis was performed with anti-phospho-H1 and anti-H1 antibodies.

**Fig. 6.** Radiation inhibits CDC2 and CDK2 activity in Jurkat and normal fibroblasts but not AT cells. In vitro H1 kinase activity was determined on immunoprecipitates with antibodies to CDC2 and CDK2 at various times following 5 Gy. All experiments were performed at least three times, and a representative experiment is shown. Equal loading of H1, CDC2, and CDK2 are shown as controls. Percentage inhibition of kinase activity was determined by excising H1 bands from the gel and measuring 32P incorporation by liquid scintillation counting Jurkat cells (A), FT/pEBS7 cells (B), and ATM FT/pEBS7-YZ5 cells (C).
has recently been demonstrated that laser treatment limited to the nuclei of mitotic Hela cells results in dephosphorylation of H1 (30). Dephosphorylation of H1 does not require protein synthesis, since cycloheximide does not block the IR-induced response (data not shown).

A simple model (Fig. 9) is that IR induces DNA damage, which is sensed by the ATM protein. The ATM protein then transduces the DNA damage signal to the involved kinase(s) and phosphatase(s). Although ATM has homology to the lipid PI 3-kinase, it and other members of the PI3-like kinase family have protein kinase activity (31–33). Indeed, ATM has been shown to enhance the phosphorylation of, or to phosphorylate, several proteins, including the nuclear tyrosine kinase ABL, p53, and CHK1. Wortmannin inhibits IR-induced dephosphorylation of H1 potently in wild type cells. The effect of wortmannin is greater than the ATM lesion in AT cells (compare Figs. 4 and 5). Therefore, the possibility exists that multiple PI3-like kinases, including ATR or DNA-PK, may be involved in the pathway leading to H1 dephosphorylation.

It is noteworthy that the kinetics of phosphatase activation closely parallels the in vivo radiation-induced dephosphorylation of H1. On the other hand, neither CDC2 nor CDK2 activity returns to basal levels within 2 h of the radiation insult. One possibility is that CDC2 and CDK2 activities remain decreased at 2 h post-radiation, since both of these kinases are known to participate in both the G1 and G2 arrest, both of which are in early stages 2 h post 5 Gy. Indeed, in Chinese hamster ovary cells in which the p53 G1 arrest pathway is nonfunctional (29), D'Anna et al. (34) observed both inhibition and recovery of cyclin A CDK and cyclin E CDK activity within 2 h following radiation. Thus, while overall CDC2 and CDK2 activities are not regulated in parallel, it is possible that H1 kinase and H1 phosphatase activities are regulated in tandem.

A major question not resolved by the present data is whether the damage signal transduced by ATM acts on a single component to control both the kinases and phosphatases, as outlined in the model shown in Fig. 9. Alternatively, phosphatase activity could be regulated by a kinase cascade or phosphatase activation could lead to kinase inactivation. Although both H1 kinase activity is inhibited and H1 phosphatase activity activated by radiation, the data presented do not allow us to distinguish between these possibilities. The rapid and extensive changes in phosphorylated H1 levels following irradiation are, however, likely related to dual regulation by both kinase inhibition and phosphatase activation.

The functional significance of H1 phosphorylation is controversial. H1 becomes phosphorylated starting in late G1, with maximal phosphorylation apparent in metaphase (35–37). It has been proposed that the function of phosphorylated H1 is to enhance mitotic chromosome condensation (35). Therefore, it could be argued that the decreased phospho-H1 in response to...
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IR is an early readout of a G2/M arrest. However, the phosphorylation of H1 is restored 2 h post-IR when G2/M arrest has not yet reached its maximum which occurs at 24 h post 5 Gy (data not shown). Thus, the IR-induced dephosphorylation of H1 can not be easily explained by the G2 arrest. What then is the consequence of H1 dephosphorylation in S phase? One possibility is that H1 dephosphorylation facilitates DNA repair. If dephosphorylation of H1 causes a decondensation of chromatin structure, then repair factors could potentially have better access to damaged sites.

On the other hand, phosphorylation of H1 in S phase may be responsible for opening chromatin structure (13, 37, 38) and thereby facilitating replication. Likewise dephosphorylation of H1 in S phase would be associated with inhibition of replication. This model is supported by our data showing that AT cells have both an impaired ability to dephosphorylate H1 as well as lower DNA synthesis in response to DNA damage in AT cells. Additionally, a temperature-sensitive mouse cell line with low phospho-H1 levels has been noted to have impaired S phase progression (14). And, it has been demonstrated that H1 extracted from S phase cells is more efficient in terms of supporting DNA synthesis in SV40 mini-chromosomes than H1 extracted from G0 or M cells (13).

In summary, our data establish that ATM is part of a radiation-induced signaling pathway resulting in phosphorylation activation and kinase inactivation, with net dephosphorylation of H1. It will be important to determine whether inhibiting this pathway downstream of ATM reduces cell survival rates after a radiation challenge. If so, then the development of novel targets for radiation and possibly chemotheraphy sensizers may be facilitated.

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