Histone H1 and H3 Dephosphorylation Are Differentially Regulated by Radiation-induced Signal Transduction Pathways

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ABSTRACT

We recently demonstrated that linker histone H1, which is thought to have a fundamental role in higher-order chromatin structure, becomes transiently dephosphorylated after ionizing radiation (IR) in a mutated ataxia telangiectasia (ATM) dependent manner. To establish whether H1 dephosphorylation was a component of a damage-response pathway that included dephosphorylation of other histones, we asked whether H3 was dephosphorylated in response to IR in a manner similar to H1. H1 and H3 are maximally phosphorylated in metaphase and both are dephosphorylated after IR. However, the duration of IR-induced H3 dephosphorylation is significantly longer than that of IR-induced H1 dephosphorylation. Moreover, H1 dephosphorylation is ATM-dependent, whereas H3 dephosphorylation is ATM-independent. These observations suggest that the damage-sensing pathways regulating H3 and H1 dephosphorylation diverge upstream of ATM.

INTRODUCTION

Cellular responses to DNA damage include activation of repair pathways, control of cell cycle checkpoints and apoptosis (1–3). Many of these processes are regulated through signal transduction pathways that are thought to be triggered by DNA damage. The ultimate targets for DNA-damage sensing pathway(s) are unknown, but because many of the acute responses to IR are DNA templated, one potential target of importance, therefore, is chromatin. Several levels of chromatin organization are required to package eukaryotic genomes within nuclei. The fundamental unit of chromatin organization is the nucleosome, in which 146 bp of DNA are wrapped in one and three-quarter turn around an octamer assembled from two molecules each of the core histones H2A, H2B, H3, and H4 (4). A fifth type of histone, either H1 or a related linker histone, binds to the outer surface of the nucleosome and portions of the “linker” DNA that joins adjacent nucleosomes. The unfolding of the thin nucleosomal filament gives rise to higher-order chromatin structures such as the 30-nm chromatin fiber (4). Linker histones are required for chromatin folding in vitro (5) and have been shown to play an important role in regulating the folding and activity of chromatin in vivo (6, 7).

All four core histones possess a COOH-terminal globular domain rich in arginyl residues comprising 60–70% of the molecule that is involved in octamer assembly and a flexible NH2-terminal domain containing sites of posttranslational modification (acetylation, phosphorylation, methylation). Similarly, linker histones contain a central globular domain that is flanked by flexible N- and COOH-terminal domains containing known sites of phosphorylation. Posttranslational modifications of the flexible “tails” of linker and core histones are thought to contribute to the regulation of higher-order chromatin folding through direct (electrostatic) effects (8) as well as by altering the accessibility of regulatory DNA sequences to trans-acting factors (9, 10).

In mammalian cells, H1 and H3 are phosphorylated in a cell cycle-dependent manner (10–12). Cell cycle-regulated phosphorylation of H3 appears to be largely but not exclusively restricted to mitosis, when many H3 molecules are phosphorylated at Ser 10 (12) and Ser 28 (13). Although phosphorylation of a subset of H3 molecules at Ser 10 correlates with mitogen-stimulated gene activation in quiescent cells (14), a large body of evidence suggests that mitotic phosphorylation of H3 is associated with chromatin condensation. Agents that induce premature chromosome condensation also induce H3 phosphorylation (15, 16), and analyses using an antibody specific for Ser 10 phosphorylated H3 have demonstrated that H3 phosphorylation correlates with (12) and is causally linked to mitotic chromatin condensation in a variety of eukaryotes (17).

In contrast, H1 is progressively phosphorylated during the cell cycle. One phosphate is added to a significant fraction of H1 molecules in late G1, an additional 2–3 phosphates are added to many molecules during S and G2, and essentially all molecules become hyperphosphorylated, bearing as many as six phosphates, in M. H1 is then quantitatively dephosphorylated when cells enter telophase (18, 19). Although the coincidence of H1 hyperphosphorylation with mitosis has long suggested a role for H1 phosphorylation in chromatin condensation, the molecular consequences of H1 phosphorylation are not known (see “Discussion”).

Despite growing evidence that histone modifications play an important role in transcriptional regulation (9, 20), little attention has focused on modification of histones in response to DNA damage. A small subset of H2AX has been shown to be phosphorylated in response to IR (21), and recently we demonstrated that H1 is transiently dephosphorylated after IR in an ATM-dependent manner through inhibition of cyclin-dependent kinases as well as via activation of nuclear protein phosphatase (22). To establish whether changes occur in H3 phosphorylation that are mediated by the same damage response pathway that results in H1 dephosphorylation, we monitored H3 phosphorylation after IR treatment of cells. Here we show that IR induces prolonged H3 dephosphorylation in an ATM-independent manner by inducing dephosphorylation in G2-M cells as well as by arresting cells in G2 before mitotic phosphorylation.

MATERIALS AND METHODS

Cell Culture. CHO cells were grown as monolayers in DMEM medium (Life Technologies, Inc.) containing 10% FBS (Life Technologies, Inc.), penicillin, and streptomycin in 5% CO2 in a 37°C incubator. Jurkat cells (a human T-cell lymphoma cell line) were grown in RPMI 1640 medium (Life Technologies, Inc.) with penicillin and streptomycin and 10% FBS. FT/pEBS7 and FT/pEBST-YZ5 cells were both derived from the AT22JET-T line, an immortalized fibroblast line (23) containing a homozygous frameshift mutation at codon 762 of the ATM gene (24). AT22JE-T cells were transfected with the mammalian expression vector pEBS7 (25) containing either the hygromycin resistance marker to yield FT/pEBS7-YZ5 cells. FT/pEBS7 and FT/pEBST-YZ5 were generously provided by Y. Shiloh (Tel Aviv University, Tel Aviv, Israel) and grown in DMEM with 15% FBS and 100 μg/ml
RESULTS

IR Dephosphorylates H3 with Different Kinetics than H1. Asynchronously growing Jurkat and CHO cells were irradiated with 5 or 10 Gy, respectively. Histones were extracted at various times after irradiation and analyzed by Western analysis (Fig. 1) using phospho-specific H1 and H3 antibodies (27, 28). Decreased phosphorylation of H1 was detectable within 30 min in both Jurkat and CHO cells and recovered to basal levels within 2–4 h. The levels of phosphorylated H3 also decreased in both cell types within 1 h. In contrast to the transient (2–3 h) H1 dephosphorylation induced by IR, H3 dephosphorylation was much more prolonged, lasting 12–16 h. The total levels of H1 and H3 were not altered by IR treatment, even when doses as high as 50 Gy were used. Thus, IR decreased the phosphorylation of H1 and H3 in vivo without affecting the levels of the H1 and H3 proteins themselves.

Because H1 and H3 are both maximally phosphorylated in mitosis (29), FACS analysis of Jurkat and CHO cells after 5 and 10 Gy, respectively, was performed to determine whether dephosphorylation of these histones correlated with G2–M arrest. IR-induced G2 arrest was found to be maximal at 24 h post-IR for both cell lines (see Fig. 3A). Thus, H1 dephosphorylation did not correlate with G2 arrest because normal levels of phosphorylated H1 were reacquired before the point at which most cells were arrested in G2.

IR-induced H3 Dephosphorylation Is Associated with G2 Arrest. To directly test whether IR-induced dephosphorylation of H1 and H3 were cell cycle-dependent, CHO cells were arrested at the G1–S boundary by isoleucine starvation, followed by mimosine treatment (30). Cells were then released into complete medium and irradiated at 4.5 or 9 h after release when 80% of cells were in mid-S phase and 90% of cells were in G2–M, respectively, by FACS. One h after irradiation, histones were extracted and analyzed by immunoblotting using antibodies specific for phosphorylated H1 and phosphorylated H3 (Fig. 2). H1 phosphorylation was detected in both S and G2–M cells, and IR induced H1 dephosphorylation in both phases. In contrast, phosphorylated H3 was detected only in G2–M cells, and IR caused significant dephosphorylation of H3 in these cells. It is important to note that this finding demonstrates that the apparent dephosphorylation of H3 after IR treatment of asynchronous cells (Fig. 1) is not attributable exclusively to an absence of H3 phosphorylation attributable to IR-induced G2 arrest before the point at which H3 phosphorylation occurs. Thus, IR induced H1 dephosphorylation in S and G2–M, whereas IR induced H3 dephosphorylation only in G2–M.

IR-induced H3 dephosphorylation could be dependent or independent of cell cycle progression. Damage-responsive signaling pathways could potentially inactivate relevant kinases or activate relevant phosphatases independent of triggering cell cycle arrest in S or G2.

Fig. 1. Radiation decreases the levels of phosphorylated H1 and phosphorylated H3 in Jurkat and CHO cells with different kinetics. Asynchronously growing Jurkat and CHO cells were exposed to 5 and 10 Gy, respectively. Cultures were then returned to the incubator for the indicated times after which histones were extracted. Immunoblots were performed with antibodies to phosphorylated H1 and phosphorylated H3. Total H1 levels were monitored using an antibody to H1. Total H3 and other core histone levels were monitored by staining blots with Coomassie Blue.

Fig. 2. IR induces H1 dephosphorylation in S and G2–M, whereas IR-induced H3 dephosphorylation is limited to G2–M. CHO cells were synchronized at the G1–S border after 45 h in isoleucine-free medium, followed by a 14-h exposure to 200 μM mimosine. Cells were released into complete medium and then irradiated with 10 Gy at 4.5 or 9 h after release. Histones were extracted 1 h post-IR. Immunoblots were performed with antibodies to phosphorylated H1 and phosphorylated H3. Equivalent sample loading and transfer was confirmed by staining blots with Ponceau S and monitoring total levels of H1 using an antibody to H1 (data not shown).
Fig. 3. Caffeine abrogates the IR-induced G2 arrest as well as IR-induced H3 dephosphorylation. Asynchronously growing Jurkat cells were treated with 2 mM caffeine for 30 min before irradiation with 5 Gy. A, FACS analysis was performed at the times indicated on control cultures as well as cultures treated with caffeine, 5 Gy (IR), and caffeine followed by IR. B, histones were extracted at various times postirradiation and Western analysis was performed using antibody to phosphorylated H3. Equivalent sample loading and transfer was confirmed by staining blots with Ponceau S and monitoring total levels of H1 using an antibody to H1 (data not shown).
As expected, FACS analysis revealed that IR-induced H3 dephosphorylation is ATM-independent, H3 dephosphorylation in response to a wide range of doses of IR was monitored (Fig. 5B). As expected, IR-induced dephosphorylation of H1 at 60 min after irradiation was much less prominent in the vector-empty cell line than in the reconstituted cell line (Fig. 5B). In contrast, IR-induced dephosphorylation of H3 was equivalent in both cell lines (and other AT fibroblast lines; data not shown) over the dose range examined, supporting the notion that ATM does not mediate IR-induced H3 dephosphorylation.

Because ATM has been shown to be upstream of p53 (34), we asked whether IR-induced H1 and H3 dephosphorylation was p53 dependent. To address this question, we used RKO cells in which the p35-p21 pathway was antagonized by the E6 oncoprotein (26). Cells stably transfected with vector alone or with E6 vector were subjected to irradiation (Fig. 6). IR induced H3 dephosphorylation to a similar extent in both vector only and in two different E6-expressing cell lines, with near complete loss of phosphorylated H3 at 2 and 4 h post-IR. Similarly, IR-induced dephosphorylation of H1 was not influenced by the presence of the E6 oncoprotein (data not shown). Additionally, because IR induced dephosphorylation of both H1 and H3 in CHO cells (Fig. 1), which are known to bear a mutation in the p53 DNA-binding domain, and failed to arrest in G1 in response to a

Alternatively, checkpoint activation could be necessary for kinase inactivation and or phosphatase activation leading to H3 dephosphorylation, i.e., the IR-induced G2 arrest point occurs before the point of H3 phosphorylation in the cell cycle. Because phosphorylated H1 and phosphorylated H3 are not readily detected in noncycling populations of mammalian cells, it is not possible to determine directly whether IR induces dephosphorylation of these histones independent of inhibition of cell cycle progression. Therefore, to distinguish between these alternatives, we examined the effect of caffeine on IR-induced H3 dephosphorylation (Fig. 3). Caffeine is known to abrogate the IR-induced G2 arrest (31–33). As expected, FACS analysis revealed that irradiation (5 Gy) of asynchronous Jurkat cells led to a pronounced arrest in G2 phase and that this arrest was abrogated by pretreatment with caffeine (Fig. 3A). In the presence of caffeine, IR did not induce H3 dephosphorylation (Fig. 3B). Thus, caffeine abrogates both the IR-induced G2 arrest and IR-induced H3 dephosphorylation.

If IR-induced H3 dephosphorylation is dependent on an IR-induced G2 arrest that occurs before H3 phosphorylation in the cell cycle, then cells arrested in mitosis with Colcemid should not dephosphorylate H3 in response to IR. To test this prediction, Jurkat cells were arrested in late mitosis with Colcemid and subjected to 5 Gy irradiation (Fig. 4). Histones were extracted at various times after irradiation and subjected to immunoblotting with antiserum to phosphorylated H3. IR-induced dephosphorylation of H3 was not detected in Colcemid-arrested cells. Thus, IR-induced inhibition of kinases and or activation of nuclear phosphatases in Colcemid-arrested cells is not sufficient to cause net H3 dephosphorylation. The failure of IR to cause H3 dephosphorylation in Colcemid-arrested cells is consistent with the model that a G2 arrest is necessary for IR-induced H3 dephosphorylation.

H1 but not H3 Dephosphorylation Is ATM-dependent. Neither H1 nor H3 IR-induced Dephosphorylation is Dependent on the p53-p21 Axis. Because ATM has been shown to influence IR-induced H1 dephosphorylation (22), we asked whether ATM also influenced IR-induced H3 dephosphorylation. The effect of IR on H3 dephosphorylation in an AT fibroblast cell line transfected with either empty vector or with a vector containing full-length wild-type ATM was compared at both 5 and 20 Gy. Previous studies have established that the reconstituted cell line behaves like wild-type cells in terms of radiation sensitivity and the S phase checkpoint (25). After 5 Gy, significant IR-induced dephosphorylation of H3 was evident at 4–12 h post-IR (Fig. 5A). After 10 Gy (data not shown) and 20 Gy, phosphorylated H3 was not detectable at 4–12 h post-IR. The similar extent and duration of IR-induced H3 dephosphorylation in the AT and reconstituted cells indicates that IR-induced H3 dephosphorylation in these cell lines does not require ATM. Similar results were obtained with control and AT lymphoblasts (data not shown). To further establish that H3 dephosphorylation is ATM-independent, H3 dephosphorylation in response to a wide range of doses of IR was monitored (Fig. 5B). As expected, IR-induced dephosphorylation of H1 at 60 min after irradiation was much less prominent in the vector-empty cell line than in the reconstituted cell line (Fig. 5B). In contrast, IR-induced dephosphorylation of H3 was equivalent in both cell lines (and other AT fibroblast lines; data not shown) over the dose range examined, supporting the notion that ATM does not mediate IR-induced H3 dephosphorylation.

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Mitotic chromosome condensation can occur by the dephosphorylation of H1 and H3 can also promote chromosome decondensation. However, our data demonstrate that dephosphorylation of H1 and H3 in response to IR are p53-independent.

**DISCUSSION**

Chromatin is not an inert structure. Cycles of chromatin condensation and decondensation that occur during the cell cycle are presumably driven by the presence or absence of unique polypeptides or by the modification of existing chromosomal proteins. Sites of posttranslational modification in histones potentially represent targets exploited by damage-sensing pathways to effect changes in chromatin structure that accompany the activation of checkpoints and repair processes. Histone phosphorylation has been observed in a number of different organisms ranging from protozoa to mammals (36) and, thus, is likely to be an important event in histone metabolism. The striking correlation of extensive phosphorylation of H1 and H3 with mitotic chromatin condensation, e.g., has led to the long-standing hypothesis that these modifications are required for chromosome condensation.

However, an increasing body of evidence suggests that phosphorylation of H1 and H3 can also promote chromosome decondensation. Mitotic chromosome condensation can occur in vivo without linker histone (6) and in extracts lacking H1 in vitro (37, 38). Because mitotic hyperphosphorylation of H1 can be dissociated from chromosome condensation, the phosphorylation and/or participation of other proteins such as condensins are better correlated with this process (39–41). Antibodies specific for phosphorylated H1 (27) and phosphorylated forms of other linker histones (42) have been used to show enrichment of phosphorylated H1 in extended, and in some cases, transcriptionally active chromatin in normal (27, 43, 44) and transformed (45–47) cells. The phosphorylation status of H1 has also been implicated in the control of DNA replication in several systems (46, 48, 49) and more recently in transcriptional regulation (50). Thus, although hyperphosphorylation of H1 accompanies chromosome condensation, it has become clear that H1 phosphorylation alone does not cause this condensation and that linker histone phosphorylation can also accompany chromatin decondensation. Similarly, phosphorylation of H3 accompanies chromosome condensation in many eukaryotes, and it has been shown directly that phosphorylation of H3 at Ser 10 is required for proper chromosome condensation and segregation in Tetrahymena (17). Yet, H3 phosphorylation also occurs during transcriptional responses to mitogenic (9, 14, 51) and hormonal stimuli (52) and decondensation of sea urchin sperm chromatin during fertilization (53, 54).

Our working model is that H1 and H3 phosphorylation contribute to localized chromatin decondensation, allowing trans-acting nonhistone proteins to gain access to repressed chromatin templates, as originally proposed by Roth and Allis (54). This model suggests that transient phosphorylation of these histones, either by weakening their interaction with DNA or by destabilizing supranucleosomal structures, functions as a first-step mechanism to enhance the accessibility and subsequent binding of trans-acting factors to target DNA sequences. The final outcome of this process (transcriptional activation, replication, repair, and chromatin condensation), however, depends on the nature of the trans-acting factors themselves. In contrast, the model proposes that dephosphorylation of both H1 and H3 is involved in the generation or stabilization of condensed or quiescent chromatin that is less accessible to trans-acting factors. What then is the significance of IR-induced dephosphorylation of H1 and H3?

IR causes both single- and double-stranded breaks that activate DNA repair and lead to inhibition of DNA synthesis. How are these seemingly opposing responses to DNA damage, the former potentially requiring unfolding of chromatin and the latter presumably associated with more compact chromatin facilitated by the IR-induced dephosphorylation of H1? One possible explanation is that in the absence of damage, appropriate cell cycle, stage-specific phosphorylation of H1 and H3 may facilitate access of factors required for normal progression while impeding access or function of positive regulators of repair. IR-induced dephosphorylation of H1, possibly in combination with other chromatin-modifying activities, could serve to "reset" the receptivity of chromatin to signals leading to inhibition of DNA synthesis and activation of repair. Dephosphorylation of H1 and H3 are not likely to be the only damage-induced posttranslational modifications of histones. It is likely that multiple modifications (phosphorylation, acetylation, methylation, and ubiquitination) as proposed by Strahl (55) acting in a combinatorial or sequential fashion specify unique downstream functions. On the other hand, IR-induced dephosphorylation of H1 could be a consequence of inhibition of DNA synthesis and activation of repair if the relevant trans-acting factors involved in both of these processes signaled directly or through pathways to the phosphatases responsible for H1 dephosphorylation.

Although the phosphorylation of both H1 and H3 may serve similar functions, the IR-induced signaling pathways controlling dephosphorylation are clearly different. Not only are the kinetics of dephosphorylation significantly different, but also the points in the cell cycle when the two histones are dephosphorylated by IR are also different. IR-induced dephosphorylation of H1 requires ATM and occurs in S phase as well as in G2-M, whereas IR-induced dephosphorylation of H3 does not require ATM and can only occur in G2-M. Interestingly, both the AT and reconstituted AT cells have an intact G2 checkpoint (data not shown), further supporting a connection between activation of the G2 checkpoint and dephosphorylation of H3. These observations suggest that the damage-sensing pathways regulating H1 and H3 dephosphorylation diverge upstream of ATM. Although damage-sensing signaling pathways and their targets are not yet fully defined, the present work clearly demonstrates that IR leads to dephosphorylation of H1 and H3 through different pathways. Understanding these pathways and the functional significance of damage-induced modifications of histones and other chromatin proteins can potentially identify novel targets for antineoplastic therapy.

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