Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress

Irene M. Ward and Junjie Chen

Guggenheim 1342
Division of Oncology Research
Mayo Clinic
200 First Street, S.W.
Rochester, Minnesota 55905
USA

1Corresponding author: Junjie Chen, Ph. D.
E-mail: Chen.junjie@mayo.edu
Phone: 507-538-1545
Fax: 507-284-3906

Running title: H2AX phosphorylation in response to replication arrest
**Summary**

H2AX, a member of the histone H2A family, is rapidly phosphorylated in response to ionizing radiation. This phosphorylation, at an evolutionarily conserved C-terminal phosphatidylinositol-3 OH kinase related kinase (PI3KK) motif, is thought to be critical for recognition and repair of DNA double strand breaks. Here we report that inhibition of DNA replication by hydroxyurea or ultraviolet irradiation also induces phosphorylation and foci formation of H2AX. These phospho-H2AX foci colocalize with PCNA, BRCA1 and 53BP1 at the arrested replication fork in S-phase cells. This response is ATR-dependent but does not require ATM or Hus1. Our findings suggest that, in addition to its role in the recognition and repair of double strand breaks, H2AX also participates in the surveillance of DNA replication.
Introduction

Histone H2AX has been implicated in the maintenance of genomic stability in response to DNA double strand breaks (DSBs). It is phosphorylated at an evolutionary conserved (PI3KK) motif in the carboxyl terminus within seconds after exposure to ionizing radiation (IR) (1). Immunofluorescence studies have revealed that phosphorylated H2AX (γ-H2AX) forms nuclear foci at the sites of DSBs. These foci appear within 1 minute after exposure of cells to IR. Their numbers increase in the first 10-30 min after irradiation before they gradually decline correlating with the predicted value of slowly re-joining DSBs (2). γ-H2AX foci are also found at sites of V(D)J recombination-induced DSBs in developing thymocytes (3), and at sites of recombinational DSBs during meiosis (4). In addition, phosphorylation of H2AX is also induced by initiation of DNA fragmentation during apoptosis (5). Thus, H2AX is phosphorylated in response to DSBs.

Several proteins involved in the DNA damage response (e.g. 53BP1, Rad50, NBS1, MRE11, BRCA1) have been shown to subsequently co-localize with γ-H2AX at the sites of strand breaks in response to IR (6-8). Prevention of H2AX phosphorylation by treatment with the PI3KK inhibitor wortmannin eliminated the foci formation of other repair proteins suggesting a role of γ-H2AX in recruitment of repair factors to sites of DNA double strand breaks (6). Furthermore, mutations in the C-terminal PI3KK motif of Saccharomyces cerevisiae histone H2A1 resulted in hypersensitivity to agents that lead to DNA double strand breaks (9). Taken together, these results strongly suggest that H2AX is required for the recognition and repair of DNA double strand breaks.
The roles of H2AX in the cellular responses to replication block and/or other types of DNA damage have not yet been studied. Surveillance of DNA replication and progression through the cell cycle is regulated by checkpoints that ensure the temporal coordination of critical cell cycle events. The S-phase checkpoint prevents the initiation of mitosis until DNA replication is completed. This checkpoint was originally described in the budding yeast *Saccharomyces cerevisiae* by the isolation of loss of function mutants that initiate mitosis despite an HU-induced replication block (10). One of the proteins that control HU-mediated replication arrest in *S. cerevisiae* is the PI3K-related kinase MEC1, the homologue of mammalian ATR. ATR null mice die early in embryogenesis and ATR-/- blastocytes show a phenotype resembling mitotic catastrophe (11,12). Recently, it has been shown that ATR forms foci at the sites of stalled replication forks in response to replication arrest (13). These foci overlap with foci formed by the product of the breast cancer susceptibility gene BRCA1 (13). Furthermore, BRCA1 is phosphorylated by ATR following exposure to HU or UV (13,14). Taken together, these data suggest that ATR and BRCA1 are involved in the replication checkpoint controls.

Here we show that replicational stress due to HU or UV treatment also induces phosphorylation and foci formation of H2AX. These γ-H2AX foci colocalize with proliferating cell nuclear antigen (PCNA) and BRCA1 at the arrested replication fork in S-phase cells. Cells lacking wild-type ATM (ataxia-telangiectasia-mutated) showed no difference in HU-induced γ-H2AX foci formation, nor was the response impaired in Hus1 (hydroxyurea sensitive)-deficient cells. In contrast, overexpression of kinase-inactive ATR (ataxia-telangiectasia-Rad3 related) inhibited the phosphorylation and foci formation of
H2AX upon treatment with HU or UV, suggesting that H2AX is phosphorylated by ATR in response to replication blocks.
Experimental Procedures

Cell culture and genotoxic agents

Human cell lines were grown in RPMI1640 supplemented with 10% fetal bovine serum (FBS). Hus1-/-p21-/- or Hus1+p21-/- mouse fibroblasts were grown in DMEM plus 10% FBS. For cell cycle experiments, MCF-7 cells were synchronized by serum starvation for 24 h and release into RPMI plus 20% serum (15).

Irradiation was performed using a $^{137}$Cs source, UV light was delivered in a single pulse using a Stratalinker and HU was added to final volume of 2mM. If not indicated otherwise, the cells were harvested 1 h after drug application or exposure to IR and UV.

Antibodies

Rabbit polyclonal anti-$\gamma$-H2AX, mouse monoclonal anti-BRCA1 and mouse monoclonal anti-53BP1 antibodies were raised as described (7). The anti-PCNA specific antibody was purchased from Santa Cruz Biotechnology, Inc.

Immunostaining

Cells grown on coverslips were fixed for 5 minutes in a 1:1 methanol:acetone solution prior incubation with the primary antibodies for 20 minutes at 37°C. FITC-conjugated goat anti-mouse and/or Rhodamine-conjugated goat anti-rabbit serum (Jackson Immunoresearch) were used as secondary antibodies. All antibodies were diluted in 5% goat serum. Cells were counterstained with Hoechst dye for 30 sec, mounted and viewed with a Nikon ECLIPSE E800 fluorescence microscope using a 60x objective. Images were processed using Adobe Photoshop and Canvas software.
**Western blotting**

Cells were lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH8], 0.5% Nonidet P-40) and the insoluble fraction was pelleted for 5 min in a microcentrifuge. Histones were extracted from the pellets with 0.1 M HCl, boiled in SDS gel sample buffer and loaded on a 15% or 17% SDS polyacrylamide gel. The separated proteins were transferred to a PVDF membrane (Immobilon-P, Millipore). The membrane was blocked with 5% nonfat milk for 30 min prior incubation with 50ng/ml anti-γ-H2AX antibodies for 1 h. The blots were washed in TBST, incubated with HRP-conjugated Protein A (Amersham) and visualized by chemiluminescence using the Supersignal kit purchased from Pierce.
Results

H2AX is phosphorylated at the sites of stalled replication in response to HU and UV treatment

To investigate the phosphorylation and foci formation of H2AX we raised polyclonal anti-phospho-H2AX antibodies against a C-terminal peptide containing phosphorylated Ser-139. Results obtained by immunofluorescence studies and Western blotting demonstrated that these antibodies specifically recognize phosphorylated H2AX and do not cross-react with the unphosphorylated form (7). As described earlier for various cell lines (2,7,8), exposure of HBL100 cells to IR resulted in the rapid and dose-dependent formation of γ-H2AX foci (Figure 1A, and data not shown). In addition, extensive γ-H2AX staining was observed in a subpopulation of cells treated with 2 mM hydroxyurea or low dose (10 J/m²) UV radiation (Figure 1A). Western blotting analyses confirmed that H2AX is phosphorylated in response to HU or UV treatment (Figure 1B).

HU blocks DNA replication by inhibiting ribonucleotide reductase, which results in decreased intracellular deoxynucleoside triphosphates that are required for DNA synthesis. Similarly, exposure of S-phase cells to UV results in replication arrest since most DNA polymerases are unable to replicate templates containing UV-induced DNA lesions (16). We therefore asked whether the observed γ-H2AX staining is restricted to the arrested DNA replication forks. Coimmunostaining with PCNA revealed that H2AX is phosphorylated at the replication fork upon treatment with HU (Figure 1C). A similar response was observed after exposure to 10 J/m² UV (Figure 1C), while a high dose of
UV radiation (100 J/m²), which introduces multiple types of DNA damage, induced γ-H2AX foci formation both in S-phase and non-S-phase cells (data not shown).

BRCA1 has been reported to form nuclear foci at the sites of stalled replication forks (15). Coimmunostaining analyses revealed that γ-H2AX foci greatly overlap with BRCA1 foci in response to HU treatment (Figure 1D). We have demonstrated previously that p53 binding protein-1 (53BP1) also forms nuclear foci in response to replication arrest (7). As shown in Figure 1D, lower panel, these 53BP1 foci colocalize with γ-H2AX following treatment with HU. Thus, γ-H2AX co-localizes with PCNA, BRCA1 and 53BP1 at the arrested replication forks.

To further confirm that the phosphorylation of H2AX upon exposure to HU or low dose UV is related to DNA replication, we synchronized MCF7 cells by a cycle of serum starvation and release into high serum. Only a few cells showed γ-H2AX staining when exposed to HU or UV 12 hours after release into high serum when the majority of cells are found in the G1 phase of the cell cycle (Figure 2, left panel). In contrast, most cells showed HU- or UV- induced phosphorylation of H2AX when analyzed during early or late S-phase (24 h and 36 h after serum release, respectively) correlating with the pattern of PCNA staining (Figure 2). While phosphorylation of H2AX in response to HU or UV treatment proofed to be cell cycle dependent, exposure to IR induced H2AX phosphorylation and foci formation both in S-phase and G1-phase cells (Figure 2). Together these results suggest that H2AX is phosphorylated at the replication fork in response to replication block.
H2AX phosphorylation in response to replication arrest requires ATR but is independent of ATM and Hus1

Although the cellular responses to DNA damage and replication blocks are similar, there is growing evidence suggesting that ATR plays a central role in controlling downstream responses to replicational stress, while ATM primarily controls the cellular response to IR-induced DSBs (17). We therefore investigated to what extent these kinases are involved in HU- and UV-induced H2AX phosphorylation. Wortmannin, which inhibits PI3KK family members, greatly reduced the HU-induced phosphorylation of H2AX (Figure 3A). To explore which PI3KK is required for H2AX phosphorylation we used ATM-deficient cells and cells overexpressing kinase dead ATR. ATM-deficient cells (FT169) and cells reconstituted with wild-type ATM (YZ5) showed similar H2AX phosphorylation in response to HU or UV treatment (Figure 3B), suggesting that ATM is not required for H2AX phosphorylation following exposure to HU or UV. In contrast, HU- or UV-induced H2AX phosphorylation was greatly reduced in GM847kd fibroblasts, which overexpress a kinase-inactive mutant of ATR (18), when compared with that in parental wild-type ATR expressing cells (Figure 3C). Notably, γ-H2AX foci formation upon introduction of DSBs by exposure to 1 Gy of IR was only slightly reduced in the presence of overexpressed kinase-inactive ATR (Figure 3C, lower panel). Western blotting analyses confirmed that ATR is required for H2AX phosphorylation in response to HU and UV but not in response to IR (Figure 3D).

Besides ATR and BRCA1, Hus1 is another protein implicated in the early response to replicational stress (19,20). Hus1-deficient mouse fibroblasts are highly sensitive to HU and UV but only show slightly increased sensitivity to IR (20). To test
whether Hus1 is required for HU-induced H2AX phosphorylation, we compared Hus1-/-p21-/- mouse fibroblasts with Hus1+ p21-/- cells (20). Inactivation of p21 is necessary to allow for the growth of Hus1-deficient cells, which fail to proliferate otherwise (20). As shown in Figure 3E, HU-induced phosphorylation of H2AX was not reduced in the absence of Hus1 suggesting that Hus1 either acts downstream of γ-H2AX or is involved in a parallel pathway in response to replication stress.

Discussion

The results presented here demonstrate that H2AX phosphorylation, an event believed to be DSBs specific, also occurs in response to replication arrest. Phosphorylated H2AX forms nuclear foci at the sites of stalled replication forks in response to HU-mediated replication arrest or exposure of S-phase cells to UV radiation. This implies that DSB and replication block pathways are intimately connected. In both pathways, H2AX is phosphorylated at the sites of DNA lesions (arrested replication forks or DSBs) and colocalizes with 53BP1 and BRCA1 at these DNA damage sites regardless of the nature of these DNA lesions. Chromatin modification appears to be a general theme in these two DNA damage signaling pathways. While it is possible (although not likely) that DNA damage "sensor" proteins open every chromatin/nucleosome to find DNA lesions, our study provides a more general and simplified model for DNA damage detection: certain DNA lesions, like DSBs or replication block, lead to changes in local chromatin structure. These chromatin changes facilitate the phosphorylation of H2AX and probably other chromatin components, and also recruit BRCA1 and 53BP1 to DNA lesions. Thus, we propose that chromatin changes are the initial signals of DNA damage.
While growing evidence suggests that H2AX has an evolutionary conserved function in the recognition and/or repair of DSBs (2,6-9), the kinase(s) involved in the phosphorylation of H2AX following DSBs is (are) still not elusive. Our data suggest that following replication block, phosphorylation of H2AX is ATR-dependent. This finding supports that ATR is a critical player in the replication block pathway, and that chromatin changes or modifications play a role in the replication block pathway. Hus1 probably acts in conjunction with ATR in the DNA replication checkpoints (20). While H2AX phosphorylation requires ATR, this phosphorylation event is independent of Hus1. Thus, the phosphorylated H2AX may function upstream of Hus1 in the transduction of DNA damage/replication block signals, or in a parallel repair pathway. The kinetics of H2AX phosphorylation in Hus1-deficient cells following UV radiation are similar to that in wild-type cells (unpublished observations), arguing that phospho-H2AX may participate in a pathway distinct from that of Hus1.
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Figure Legends

Figure 1. H2AX is phosphorylated in response to replication arrest. (A) HBL100 cells were immunostained with anti-γ-H2AX specific antibodies before and after treatment with HU (2 mM), UV (10 J/m²) or IR (1 Gy). (B) Extracts from HBL100 cells before an after treatment with HU, UV or IR were either separated by SDS PAGE or stained with Coomassie blue. (C) MCF-7 cells were coimmunostained with anti-γ-H2AX and anti-PCNA antibodies before and after treatment with HU (2 mM) or UV (10 J/m²). (D) HU-treated MCF-7 cells were coimmunostained with anti-γ-H2AX and anti-BRCA1 or anti-53BP1 antibodies.

Figure 2. H2AX colocalizes with PCNA at the arrested replication fork in S-phase cells. MCF-7 cells were synchronized by a cycle of serum starvation and subsequent release into 20% serum. 12 h (G1-phase), 24 h (early S-phase) and 36 h (late S/G2-phase) after release the cells were coimmunostained with anti-γ-H2AX and anti-PCNA antibodies.

Figure 3. H2AX phosphorylation in response to replication stress depends on ATR. (A) MCF-7 cells were coimmunostained with anti-γ-H2AX and anti-PCNA antibodies before and after a 1 h treatment with HU (2mM) or a combination of HU and wortmannin (200µM). (B) ATM-deficient FT169 fibroblasts and isogenic ATM wild-type –reconstituted YZ5 cells were immunostained with anti-γ-H2AX antibodies before and after exposure to HU (2 mM) or UV (10 J/m²). (C) GM847 fibroblasts (ATR wt) or GM847 fibroblasts overexpressing Flag-tagged kinase-dead ATR construct (ATR kd)
were coimmunostained with anti-Flag and anti-γ-H2AX antibodies before or 1 h after exposure to HU (2 mM), UV (10 J/m²) or IR (1 Gy). (D) GM847 cells (ATR wt) or GM847 cells overexpressing kinase-dead ATR (ATR kd) were treated with various genotoxic agents as indicated. Extracted histones were separated by SDS PAGE and either immunoblotted with anti-γ-H2AX or stained with Coomassie blue. Note that a 10 times higher IR dose was used for Western blotting than that used for immunostaining. (E) Mouse Hus1+p21-/- and Hus1-/-p21-/- fibroblasts were immunostained with anti-γ-H2AX antibodies before and after treatment with 2 mM HU.
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Fig. 1, Ward & Chen
Untreated

HU / 1 h

UV / 1 h

IR / 1 h

G₁  early S  late S/G₂

PCNA  γ-H2AX  PCNA  γ-H2AX  PCNA  γ-H2AX

Fig. 2, Ward & Chen
Fig. 3A+B, Ward & Chen
C

|         | ATR wt | ATR kd |
|---------|--------|--------|
| Flag    | γ-H2AX | Flag   | γ-H2AX |
| Untreated | ![Image](untreated.png) | ![Image](untreated.png) |
| HU      | ![Image](huh.png) | ![Image](huh.png) |
| UV      | ![Image](uv.png) | ![Image](uv.png) |
| IR      | ![Image](ir.png) | ![Image](ir.png) |

D

|         | ATR wt | ATR kd |
|---------|--------|--------|
| Untreated | ![Image](untreated.png) | ![Image](untreated.png) |
| 2 mM HU | ![Image](huh.png) | ![Image](huh.png) |
| 20 J/m² UV | ![Image](uv.png) | ![Image](uv.png) |
| 10 Gy IR | ![Image](ir.png) | ![Image](ir.png) |

E

|         | Hus1+ | Hus1-/|
|---------|-------|-------|
| Untreated | ![Image](untreated.png) | ![Image](untreated.png) |
| HU / 1 hr | ![Image](hu.png) | ![Image](hu.png) |

Fig. 3C-E, Ward & Chen
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