ATM Phosphorylates Histone H2AX in Response to DNA Double-strand Breaks*

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A very early step in the response of mammalian cells to DNA double-strand breaks is the phosphorylation of histone H2AX at serine 139 at the sites of DNA damage. Although the phosphatidylinositol 3-kinases, DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated), and ATR (ATM and Rad3-related), have all been implicated in H2AX phosphorylation, the specific kinase involved has not yet been identified. To definitively identify the specific kinase(s) that phosphorylates H2AX in vivo, we have utilized DNA-PKcs−/− and Atm−/− cell lines and mouse embryonic fibroblasts. We find that H2AX phosphorylation and nuclear focus formation are normal in DNA-PKcs−/− cells and severely compromised in Atm−/− cells. We also find that ATM can phosphorylate H2AX in vitro and that ectopic expression of ATM in Atm−/− fibroblasts restores H2AX phosphorylation in vivo. The minimal H2AX phosphorylation in Atm−/− fibroblasts can be abolished by low concentrations of wortmannin suggesting that DNA-PK, rather than ATR, is responsible for low levels of H2AX phosphorylation in the absence of ATM. Our results clearly establish ATM as the major kinase involved in the phosphorylation of H2AX and suggest that ATM is one of the earliest kinases to be activated in the cellular response to double-strand breaks.

DNA double-strand breaks (DSBs)1 are probably the most dangerous of the many different types of DNA damage that occur within the cell. DSBs are generated by exogenous agents such as ionizing radiation (IR) or by endogenously generated reactive oxygen species and occur as intermediates during meiotic and V(D)J recombination (1). A very early step in the cellular response to DSBs is the phosphorylation of a histone H2A variant, H2AX, at the sites of DNA damage (2). H2AX is rapidly phosphorylated (within seconds) at serine 139 when DSBs are introduced into mammalian cells (3) resulting in discrete γ-H2AX (phosphorylated-H2AX) foci at the DNA damage sites (4). In experiments involving the use of “laser scissors” to introduce breaks into living cells, γ-H2AX foci localized specifically with the laser path through the cell nuclei clearly demonstrating that H2AX phosphorylation is specific to the sites of DNA damage (4, 5). H2AX phosphorylation also appears to be a general cellular response to processes involving DSB intermediates including V(D)J recombination in lymphoid cells (6) and meiotic recombination in mice (7). Phosphorylation of yeast H2A at serine 129 (homologous to serine 139 of mammalian H2AX) causes chromatin decondensation and is required for efficient DNA double-strand break repair (8). In mammals, phosphorylation of H2AX appears to play a critical role in the recruitment of repair or damage-signaling factors to the sites of DNA damage (5, 9).

As H2AX phosphorylation plays a very early and important role in the cellular response to DNA double-strand breaks, it is important to specifically identify the kinase(s) involved in this event. Members of the PI 3-kinase family, including DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated), and ATR (ATM and Rad3-related), are involved in the responses of mammalian cells to DSBs (10). γ-H2AX focus formation is inhibited by the PI 3-kinase inhibitor wortmannin, and H2AX phosphorylation is reduced in the DNA-PK-deficient human cell line M059J (5). This led to the conclusion that DNA-PK and at least one other kinase, possibly ATM and/or ATR, can phosphorylate H2AX upon DNA damage (2, 5, 10, 11).

To unambiguously define the roles of ATM and DNA-PK in H2AX phosphorylation, we utilized cells derived from knockout mice for ATM or DNA-PKcs (the catalytic subunit of DNA-PK). We observed normal H2AX phosphorylation and γ-H2AX focus formation in irradiated fibroblasts derived from wild type or DNA-PKcs−/− mice. In contrast, H2AX phosphorylation and γ-H2AX focus formation were strikingly reduced to near background levels in fibroblasts from Atm−/− mice. Ectopic expression of ATM in Atm−/− cells restored H2AX phosphorylation. Moreover, we show that immunoprecipitated ATM can phosphorylate recombinant H2AX in vitro. These results indicate that ATM, not DNA-PK, is the major kinase responsible for modifying H2AX upon irradiation. The minimal H2AX phosphorylation in Atm−/− cells could be abolished by low concentrations of wortmannin suggesting that DNA-PK, rather than ATR, is responsible for low levels of γ-H2AX formation in the absence of ATM.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Induction of DNA Damage**—Spontaneously immortalized mouse fibroblasts, derived from wild type, DNA-PKcs−/− (12), or Atm−/− mice (13), were maintained in a humidified atmosphere with 5% CO₂ in α-minimum Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Mouse embryonic fibroblasts (MEFs) were isolated from 13.5-day-old embryos and maintained in α-minimum Eagle’s medium supplemented with 15% fetal calf serum. Cells were grown to about 70% confluence and irradiated with x-rays (300-kV, 12-ma, 0.5-mm Cu) at the rate of 5.5 gray/min to achieve a cumulative dose of 10 gray for all experiments.

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§The abbreviations used are: DSB, double-strand break; IR, ionizing radiation; PI, phosphatidylinositol; MEF, mouse embryonic fibroblasts.
RESULTS

Histone H2AX Is Phosphorylated Specifically in Response to DNA Double-strand Breaks—To examine H2AX phosphorylation in mouse cells, a rabbit polyclonal antibody (anti-γ-H2AX) was generated against a synthetic phosphorylated polypeptide consisting of the last nine amino acids of H2AX with phospho-Ser-139 as described before (3). SDS extracts from Western blotting were prepared from mock-irradiated or irradiated cells as described previously (14). The antibodies used for Western blotting are anti-γ-H2AX, anti-H2A (H-124; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-ATM monoclonal antibody MAT3–4G10/8 (15).

Transient Transfection of Atm−/− Cells—Transient transfection of exponentially growing Atm−/− spontaneously immortalized fibroblasts with the ATM DNA expression vector pMAT1 (16) was carried out using Superfect transfection reagent (Qiagen Inc., Valencia, CA) as per the manufacturer’s protocols. Immediately after transfection, cells were induced for ATM expression with 5 μM CdCl₂ for 16 h and then mock-irradiated or irradiated as described above.

ATM Kinase Assay—ATM immunoprecipitations were carried out as described (17). Approximately 1 × 10⁷ spontaneously immortalized mouse fibroblasts were grown to 70% confluence, mock-irradiated or irradiated, harvested after 30 min, and lysed in fresh cold lysis buffer containing protease and phosphatase inhibitors. The lysate was cleared by centrifugation, and the supernatant was incubated with 10 μg of anti-ATM monoclonal antibody MAT3–4G10/8 (15) for 2 h at 4 °C followed by incubation with protein A/G-Sepharose beads for an additional 2 h. The beads were washed repeatedly with lysis buffer, once with high salt buffer, and twice with kinase buffer. The beads were then incubated in a kinase mix (20 μl of kinase buffer, 500 ng of recombinant H2AX (purified from bacteria), 2 μl of 100 μM ATP, and 10 μCi of γ⁰³²P[ATP] at 30 °C for 10 min. After SDS-polyacrylamide gel electrophoresis, the reaction products were visualized by autoradiography. Anti-ATM monoclonal antibody MAT3 (15).

Immunofluorescence—Spontaneously immortalized fibroblasts were grown on chamber slides to about 70% confluence and then mock-irradiated or irradiated and incubated for 30 min. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized for 10 min in 0.2% Triton X-100, and blocked in 10% normal goat serum for 1 h at room temperature. The slides were incubated with anti-γ-H2AX antibody for 1 h, washed in phosphate-buffered saline, and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. Cells were washed in phosphate-buffered saline and mounted using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratory, Burlingame, CA). Fluorescence images were captured using an Olympus BH2 epifluorescent microscope equipped with a CCD camera and Cytovision software (Applied Imaging, Santa Clara, CA). To allow direct comparisons, all the cells were irradiated and processed simultaneously, and all the images were obtained using the same parameters (brightness, contrast, etc.).

Fig. 1. Inhibition of H2AX phosphorylation by wortmannin. A, anti-γ-H2AX antibody (directed against H2AX phosphorylated at serine 139) was used to immunoblot synthetic peptides comprised of the last nine amino acids of H2AX with (134–142) or without (134–142) phosphorylation at serine 139. B, spontaneously immortalized wild type mouse fibroblasts were mock-irradiated or irradiated with x-rays or UV rays as indicated and harvested after 30 min. Cells were mock-treated or treated for 1 h with the DSB-inducing agents neocarzinostatin (NCS), bleomycin (BLM), or etoposide or with the DNA-alkylating agent methyl methanesulfonate (MMS) as indicated and then harvested. SDS extracts were analyzed for H2AX phosphorylation by Western blotting with anti-γ-H2AX antibody. The blots were stripped and re-probed with anti-H2A antibody as a normalizing control. HU, hydroxyurea. C, wild type fibroblasts were mock-irradiated (C) or irradiated with x-rays after a 30-min incubation with increasing concentrations of wortmannin (0–100 μM). Cells were harvested 30 min after irradiation, and SDS extracts were analyzed by Western blotting with anti-γ-H2AX or anti-H2A antibodies.

irradiated samples when the immunizing polypeptide (phosphorylated at serine 139) was used as competitor in Western blotting (data not shown). Significant phosphorylation of H2AX was also observed after treatment of cells with the DSB-inducing agents neocarzinostatin, bleomycin, and etoposide. In contrast, there was no increase in γ-H2AX formation when these cells were irradiated with UV rays or treated with the DNA-alkylating agent methyl methanesulfonate confirming that...
H2AX is phosphorylated at serine 139 specifically in response to DNA double-strand breaks. Low levels of H2AX phosphorylation were also observed in cells treated with the DNA replication inhibitor hydroxyurea. This is probably because cells treated with hydroxyurea accumulate DSBs because of replication fork collapse (18, 19).

Ionizing Radiation-induced H2AX Phosphorylation Can Be Inhibited by Low Concentrations of Wortmannin—As the PI3-kinases, DNA-PK, ATM, and ATR, have all been implicated in H2AX phosphorylation (2, 5, 10, 11), we wanted to determine which of these three kinases played a major role in the process. The fungal PI 3-kinase inhibitor wortmannin inhibits the kinase activities of ATM and DNA-PK in intact cells with half-maximal inhibition at concentrations of about 5 μM (20). The kinase activity of ATR is significantly more resistant to this drug with half-maximal inhibition at concentrations higher...
than 100 μM. Spontaneously immortalized wild type mouse fibroblasts were treated with increasing concentrations of wortmannin for 30 min, irradiated with x-rays, harvested after 30 min, and analyzed by Western blotting. We found that H2AX phosphorylation was inhibited by low concentrations of wortmannin (1–10 μM) indicating that ATM and/or DNA-PK, but not ATR, is involved in this process (Fig. 1c).

H2AX Phosphorylation Is Abrogated in Atm−/−, but Not in DNA-PKcs−/−, Cells—Spontaneously immortalized fibroblasts from wild type, DNA-PKcs−/−, or Atm−/− mice were mock-irradiated or irradiated, harvested at time points ranging from 5 min to 8 h, and assayed for H2AX phosphorylation by Western blotting. H2AX phosphorylation in both wild type and DNA-PKcs−/− cells occurred very rapidly (within 5 min) and lasted for about 2 h, with maximum levels of phosphorylation observed at 30 min (Fig. 2a). In striking contrast, we observed minimal H2AX phosphorylation in irradiated Atm−/− cells. Although we observed robust H2AX phosphorylation in DNA-PKcs−/− cells at 30 min post-irradiation, γ-H2AX formation in Atm−/− cells was reproducibly reduced to about 5% of that in wild type cells (Fig. 2b) indicating that ATM is the major kinase responsible for H2AX phosphorylation upon DNA damage.

Atm−/− fibroblasts were treated with increasing concentrations of wortmannin for 30 min, irradiated with x-rays, harvested after 30 min, and analyzed by Western blotting. We found that the minimal H2AX phosphorylation in Atm−/− cells was completely abolished by low concentrations of wortmannin (1–10 μM) (Fig. 2c). As ATR is inhibited by high concentrations of wortmannin (>100 μM) (20), our results suggest that DNA-PK, rather than ATR, is responsible for low levels of γ-H2AX formation in the absence of ATM.

Lack of H2AX Phosphorylation in Atm−/− MEFs—It is possible that other mutations in the Atm−/− cell line used could also be responsible for the lack of H2AX phosphorylation in these cells. We, therefore, examined H2AX phosphorylation in a panel of isogenic, early passage (p2 or p3) ATM+/+ or −/− MEFs. Normal H2AX phosphorylation was observed in irradiated Atm+/+ MEFs (Fig. 3a, upper panel). In contrast, very low levels of γ-H2AX formation was observed in two independent Atm−/− MEFs confirming that ATP is required for H2AX phosphorylation in response to IR. No significant difference in H2AX phosphorylation was observed between irradiated DNA-PKcs+/− and −/− MEFs (Fig. 3a, lower panel).

Ectopic Expression of ATM Restores H2AX Phosphorylation in Atm−/− Cells—To confirm that ATM is required in vivo for
H2AX phosphorylation, the ATM cDNA expression vector pMAT1 (16) was transiently transfected into Atm−/− spontaneously immortalized fibroblasts. The ectopic expression of ATM in the transfected cells resulted in restoration of H2AX phosphorylation upon irradiation (Fig. 3b, compare lanes 2 and 4). On the other hand, cells transfected with the vector alone showed no increase in γ-H2AX formation (Fig. 3b, compare lanes 2 and 6). The correlation between ATM expression and H2AX phosphorylation establishes that ATM is required in vivo for γ-H2AX formation in response to ionizing radiation.

**ATM Can Phosphorylate Recombinant H2AX in Vitro**—To determine whether ATM can directly phosphorylate H2AX in vitro, ATM was immunoprecipitated from spontaneously immortalized wild type fibroblasts using an anti-ATM monoclonal antibody raised against a peptide representing positions 1967–1988 of murine ATM (15). The immunoprecipitated ATM efficiently phosphorylated recombinant H2AX in vitro (Fig. 3c, lane 1). Furthermore, irradiation of cells resulted in a significant increase in H2AX phosphorylation (Fig. 3c, lane 2). Essentially no ATM protein or kinase activity was detected when immunoprecipitation was performed with normal mouse IgG or from Atm−/− fibroblasts (Fig. 3c, lanes 3–6). The in vitro phosphorylation of H2AX by ATM suggests that ATM could directly phosphorylate H2AX within the cell in response to DNA damage.

**Poor γ-H2AX Focus Formation in Atm−/− Cells—H2AX phosphorylation in response to DNA damage results in the formation of discrete γ-H2AX foci at the sites of DNA double-strand breaks (4). To determine the status of γ-H2AX focus formation in wild type, DNA-PKcs−/−, and Atm−/− spontaneously immortalized fibroblasts, these cells were irradiated and allowed to recover for 30 min before fixation and immunostaining with anti-γ-H2AX antibody. We observed robust γ-H2AX focus formation upon irradiation of both wild type and DNA-PKcs−/− cells (Fig. 4). In striking contrast, focus formation was very poor in Atm−/− cells confirming that ATM is required for γ-H2AX focus formation at the sites of DSBs.**

**DISCUSSION**

Histone H2AX is rapidly phosphorylated at serine 139 in response to DNA double-strand breaks (3). The PI 3-kinases, DNA-PK, ATM, and ATR, have all been implicated in this process (2, 5, 10, 11). Although the substrate specificities of these kinases are overlapping in vitro, they have clearly distinct functions in vivo (10). For example, whereas ATM phosphorylates p53, Chk2, and Nbs1 leading to cell cycle arrest, DNA-PKcs−/− cells (Fig. 4). In striking contrast, focus formation was very poor in Atm−/− cells confirming that ATM is required for γ-H2AX focus formation at the sites of DSBs. This is therefore important to definitely delineate the roles of these kinases in the phosphorylation of H2AX.

We demonstrate that ATM can phosphorylate H2AX in vitro and that H2AX phosphorylation and γ-H2AX focus formation are severely compromised in Atm−/− cells. Ectopic expression of ATM corrects this defect. In contrast, these functions are normal in DNA-PKcs−/− cells. Interestingly, DNA-PK, but not ATR, may be responsible for the minimal levels of H2AX phosphorylation in Atm−/− cells as this can be abolished by low concentrations of wortmannin. We also find that immunoprecipitated ATM can directly interact with recombimant H2AX in vitro, and experiments will be performed to examine complex formation between H2AX and ATM in vivo.

Our results establish that ATM is the major kinase responsible for histone H2AX phosphorylation in response to DNA double-strand breaks in murine fibroblasts. The reduced

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2 Sandeep Burma and David J. Chen, unpublished results.
Phosphorylation of H2AX by ATM
