Sensing of Ionizing Radiation-induced DNA Damage by ATM through Interaction with Histone Deacetylase*

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The ATM gene is mutated in individuals with ataxia telangiectasia, a human genetic disease characterized by extreme sensitivity to radiation. The ATM protein acts as a sensor of radiation-induced cellular damage and contributes to cell cycle regulation, signal transduction, and DNA repair; however, the mechanisms underlying these functions of ATM remain largely unknown. Binding and immunoprecipitation assays have now shown that ATM interacts with the histone deacetylase HDAC1 both in vitro and in vivo, and that the extent of this association is increased after exposure of MRC5CV1 human fibroblasts to ionizing radiation. Histone deacetylase activity was also detected in immunoprecipitates prepared from these cells with antibodies to ATM, and this activity was blocked by the histone deacetylase inhibitor trichostatin A. These results suggest a previously unanticipated role for ATM in the modification of chromatin components in response to ionizing radiation.

The human genetic disease ataxia telangiectasia (AT), which is characterized by extreme sensitivity to radiation, is caused by mutations in the ATM gene (1, 2). The protein encoded by this gene acts as a sensor of radiation-induced cellular damage and plays important roles in cell cycle regulation, signal transduction, and DNA repair (2–6). However, the mechanisms by which ATM performs these various functions remain largely uncharacterized. Exposure of cells to ionizing radiation results in the arrest of cell cycle progression, induction of the transcription of specific genes, modification of nucleosomal structure, and activation of the DNA repair machinery (3, 6). Histone acetylation and deacetylation are thought to play important roles in the modification of chromatin structure and in monitoring chromosomal integrity during the cell cycle and transcriptional regulation (7–9). Various non-histone proteins that participate in regulation of the cell cycle and transcription are associated with histone acetylation or deacetylation activities (10–14). Certain transcriptional coactivators, including pCAF, BRCA2, and ATM-like proteins, possess intrinsic acetyl transferase activities (15–18). Conversely, transcriptional repressors have been shown to associate with histone deacetylases (19–24). Recent studies have shown that the product of the retinoblastoma gene (Rb) represses transcription of the E2F gene by recruiting the mammalian deacetylase proteins HDAC1 and HDAC2, to which it binds through an LXXCX motif in its pocket domain (24–27).

Sequence analysis has revealed that the NH2 terminus of ATM contains an LXXCX motif (amino acids 115–119) (Fig. 1a). We therefore investigated whether ATM also interacts with HDAC1. We have now shown that the two proteins indeed interact both in vitro and in vivo and that the extent of the association in vivo is increased by exposure of the cells to ionizing radiation.

EXPERIMENTAL PROCEDURES

Cell Culture and Irradiation—Human normal (MRC5CV1) and AT (AT5BIVA, AT4BIVA, and AT3BIVA) fibroblasts were maintained at 37 °C under an atmosphere of 5% CO2 in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. Exponentially growing cells were exposed to 20 Gy of γ-radiation (J. L. Shepherd Mark I Radiator) with a 137Cs source emitting at a fixed dose rate of 3.83 Gy min−1 and were harvested at various intervals thereafter.

GST-ATM Constructs—An expression vector encoding a glutathione S-transferase (GST) fusion protein containing residues 1–300 of ATM (GST-ATM(1–300)) was generated by inserting the corresponding polynucleotide chain reaction-generated BamHI-EcoRI fragment of human ATM cDNA (2) into pGEX-4T-1 (28) incorporating the pcDNA3 vector (Invitrogen) was subjected to in vitro transcription and translation in the presence of [35S]methionine with a TNN radiol (J. L. Shepherd Mark I Radiator) with a 137Cs source emitting at a fixed dose rate of 3.83 Gy min−1 and were harvested at various intervals thereafter.

In Vitro Translation and GST Precipitation Assays—The 1.4-kb full-length human HDAC1 cDNA, a gift from Dr. Schreiber (28), incorporated into the pcDNA3 vector (Invitrogen) was subjected to in vitro transcription and translation in the presence of [35S]methionine with a TNT T7-coupled transcription and translation kit (Promega). Beads coated with GST fusion proteins (10 mg) were incubated for 1 h at 4°C with in vitro translated 35S-labeled HDAC1 (10 µg) or nuclear extracts (1 mg of protein) in a final volume of 400 µl containing TNN buffer (40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% (v/v) Nonidet P-40, and protease inhibitors) (5). After extensive washing of the beads, bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis and either autoradiography or immunoblot analysis with antibodies to HDAC1 (Santa Cruz Biotechnology).

Immunoprecipitation and Immunoblot Analysis—Nuclear extracts were prepared as described (5), and the concentration of protein was determined with the Bradford reagent (Bio-Rad). The extracts (1 mg of protein) were subjected to immunoprecipitation for 2 h at 4°C with antibodies to HDAC1 in a final volume of 20 µl of TNN buffer. After the addition of protein A/G-agarose (Santa Cruz Biotechnology), the reaction mixtures were incubated for an additional 2 h. The immunoprecipitates were washed extensively and subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were then transferred to a nitrocellulose membrane (Schleicher & Schuell) and subjected to immunoblot analysis as described (5).

Histone Deacetylase Assay—[3H]Acetyllysine-labeled histones were isolated from HeLa cells as described (29, 30). Nuclear extracts (1 mg of...
RESULTS AND DISCUSSION
To investigate whether ATM interacts with HDAC1, we performed in vitro protein-protein binding assays. In vitro translated $^{35}$S-labeled HDAC1 was incubated with beads coated with a bacterially produced GST fusion protein containing residues 1–300 of ATM. Beads coated with GST or with a GST fusion protein containing the NF-kB inhibitor protein I$x$B$\alpha$ were used as controls. The $^{35}$S-labeled HDAC1 bound to the beads coated with GST-ATM(1–300) but not to those coated with GST or GST-I$x$B$\alpha$ (Fig. 1b). To assess further the specificity of this interaction, we generated the mutant protein GST-ATM(C117F), in which Cys$^{117}$ of the LXXE motif of ATM was replaced by phenylalanine with the use of site-directed mutagenesis (24). The extent of the interaction of GST-ATM(C117F) with HDAC1 was greatly reduced relative to that observed with GST-ATM(1–300). The electrophoretic mobilities of the wild-type and mutant GST-ATM proteins differed slightly, possibly because of a conformational change induced by the mutation. Together, these data suggest that the LXXE motif of ATM is required for binding of the protein to HDAC1.

We next determined whether the interaction between ATM and HDAC1 contributes to the cellular response to ionizing radiation-induced DNA damage. Normal human fibroblasts (MRC5CV1) were exposed to 20 Gy of $\gamma$-radiation, and after various intervals, nuclear extracts were prepared and subjected to the in vitro binding assay with beads coated with GST-ATM(1–300). Immunoblot analysis with antibodies to HDAC1 of proteins that bound to the beads revealed that HDAC1 present in the nuclear extracts bound to GST-ATM(1–300) (Fig. 2c). The amount of HDAC1 bound to GST-ATM(1–300) was maximal 30 min after irradiation and then gradually decreased to basal levels over the next ~3 h. HDAC1 in nuclear extracts did not bind to beads coated with GST alone (data not shown). Immunoprecipitates prepared from the nuclear extracts with antibodies to HDAC1 also contained ATM (Fig. 2b). The amount of ATM present in the immunoprecipitates was maximal between 30 and 60 min after irradiation and had returned to basal levels by 3 h. The amount of HDAC1 in the immunoprecipitates was not substantially affected by ionizing radiation. The interaction between ATM and HDAC1 was not detected by immunoprecipitation analysis in AT5BIVA (Fig. 2c) or in AT3BIVA or AT4BIVA (data not shown) cell lines, all of which are derived from AT patients; these cells express HDAC1 at levels similar to MRC5CV1 cells (Fig. 2d). Immunoprecipitates obtained from MRC5CV1 cells with antibodies to IgG as a control did not contain ATM (data not shown). These results demonstrate that ATM associates with HDAC1 in vivo and that the extent of this association is increased by exposure of cells to ionizing radiation.

The ATM-like proteins pATF400, Tra1, and TRR associate with histone acetylase protein complexes (14, 17, 18). In contrast, we have observed that ATM interacts with HDAC1 but not with pCAF (data not shown). To investigate the effect of ionizing radiation on histone acetylase and deacetylase activities, we first monitored the amount of acetylated histone H4, by immunoblot analysis of acid-soluble proteins after irradiation of MRC5CV1 cells. The abundance of acetylated histone H4 was reduced by 80 and 91% at 30 and 60 min after irradiation.
before recovering to 47% of pretreatment levels at 3 h (Fig. 3a).

Equal loading was confirmed by Ponceau staining of the membrane (data not shown). In contrast, the amount of acetylated histone H4 was markedly increased by treating cells with the histone deacetylase inhibitor trichostatin A (TSA). We also measured the effect of ionizing radiation on histone deacetylase activity in both MRC5CV1 and AT cells. The amount of deacetylase activity in MRC5CV1 cells was increased within 30 min of irradiation and thereafter gradually decreased to basal levels by 3 h after treatment (Fig. 3b). The deacetylase activity of AT5BIVA cells was not affected by ionizing radiation. Thus, the radiation-induced decrease in the amount of acetylated histone H4 correlated with the increase in deacetylase activity in MRC5CV1 cells.

To determine whether the HDAC1 associated with ATM exhibits histone deacetylase activity, we incubated nuclear extracts of MRC5CV1 cells with beads coated with GST-ATM(1–300) and then assayed the bead-associated proteins for deacetylase activity. Such beads showed a high level of deacetylase activity, which was inhibited by treatment with TSA (Fig. 4a). Beads coated with GST, GST-1xDeo, or GST-ATM(C117F) retained much less histone deacetylase activity after incubation with MRC5CV1 nuclear extracts than did GST-ATM(1–300)-coated beads. Furthermore, the amount of ATM-associated histone deacetylase activity was increased 30 min after exposure of MRC5CV1 cells to ionizing radiation, as revealed by GST-ATM(1–300) precipitation and immunoprecipitation assays (Fig. 4b); immunoprecipitates prepared with an irrelevant antibody did not exhibit histone deacetylase activity (data not shown).

Taken together, our data indicate that ATM associates with HDAC1 in vivo, that the resulting complex exhibits histone deacetylase activity, and that the extent of this association is increased in cells exposed to ionizing radiation. Although whether the mechanism by which ATM affects the acetylation state of the histone is related to chromatin is the subject of further investigations, the present results reveal a new role for ATM in the cellular response to ionizing radiation-induced DNA damage. Our data with AT cells indicate that mutations in the ATM gene affect the interaction between ATM and HDAC1 and thereby prevent the increase in histone deacetylase activity apparent in normal cells after exposure to ionizing radiation. This observation is consistent with previous studies (31, 32) showing that ATM is associated with chromatin and that decondensation of chromatin increases the radiosensitivity of DNA with respect to formation of double-strand breaks. Therefore, it is also possible that AT cells show an increased susceptibility to radiation-induced DNA damage because of the dysfunction of ATM as a regulator of DNA packaging into chromatin and a monitor of chromosomal integrity.

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