UV-induced Ataxia-telangiectasia-mutated and Rad3-related (ATR) Activation Requires Replication Stress*

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Ataxia-telangiectasia-mutated and Rad3-related (ATR) plays an essential role in the maintenance of genome integrity and cell viability. The kinase is activated in response to DNA damage and initiates a checkpoint signaling cascade by phosphorylating a number of downstream substrates including Chk1. Unlike ataxia-telangiectasia-mutated (ATM), which appears to be mainly activated by DNA double-strand breaks, ATR can be activated by a variety of DNA damaging agents. However, it is still unclear what triggers ATR activation in response to such diverse DNA lesions. One model proposes that ATR can directly recognize DNA lesions, while other recent data suggest that ATR is activated by a common single-stranded DNA (ssDNA) intermediate generated during DNA repair. In this study, we show that UV lesions do not directly activate ATR in vivo. In addition, ssDNA lesions created during the repair of UV damage are also not sufficient to activate the ATR-dependent pathway. ATR activation is only observed in replicating cells indicating that replication stress is required to trigger the ATR-mediated checkpoint cascade in response to UV irradiation. Interestingly, H2AX appears to be required for the accumulation of ATR at stalled replication forks. Together our data suggest that ssDNA at arrested replication forks recruits ATR and initiates ATR-mediated phosphorylation of H2AX and Chk1. Phosphorylated H2AX might further facilitate ATR activation by stabilizing ATR at the sites of arrested replication forks.

In response to genotoxic stress, cells activate DNA damage checkpoints that induce cell cycle arrest. ATM1 (ataxia-telangiectasia-mutated) and ATR (ataxia-telangiectasia-mutated and Rad3-related), members of the phosphatidylinositol 3-kinase superfamily, play a central role in regulating these DNA damage checkpoints in mammalian cells. Several findings showed that the two kinases act in distinct but partially overlapping pathways. While ATM appears to be activated mainly by ionizing radiation-induced DNA double strand breaks, ATR has been shown to regulate checkpoint responses after treatment of cells with a variety of DNA damaging agents including UV irradiation (1, 2). Both proteins are able to bind DNA in vitro and are rapidly targeted to DNA in response to DNA damage. Moreover, ATR has been shown to bind with higher affinity to UV-damaged than to undamaged DNA (3). It has been suggested that ATM and ATR might be activated through interaction with DNA and DNA-associated checkpoint/repair proteins and act as DNA damage sensors (4–7).

However, this direct sensing model was challenged by recent observations that ATM is activated by damage-induced rapid intermolecular autophosphorylation prior relocation to sites of DNA breaks (8, 9). Similarly, instead of interaction with primary DNA lesions, ATR activation seems to require single-stranded DNA (ssDNA) coated with replication protein A. The recruitment of ATR to damage sites appears to be mediated by an ATR-interacting protein that forms a stable complex with the vast majority of ATR in human cells (10). However, it is still remains to be determined whether ssDNA regions that arise during the repair of UV lesions or other DNA lesions are sufficient for ATR activation. Studies using a Xenopus cell-free system suggest a requirement for DNA replication in ATR activation (11, 12), but such a cell-free system might not support sufficient DNA repair. Moreover, a recent study describes UV-induced ATR activation in nonreplicating human cells (13), suggesting that ATR activation is independent of DNA replication. Given the essential role of ATR in genome maintenance (14), we investigated the activation of ATR in response to UV and CPT (camptothecin) treatment. Our results indicate that ssDNA lesions induced by the repair of UV lesions or CPT treatment are not sufficient to elicit detectable recruitment of ATR to chromatin in vitro or to induce ATR-mediated phosphorylation of Chk1. ATR activation is only observed in the context of replication arrest, suggesting that ssDNA at stalled replication forks generate the signals required for ATR activation.

Experimental Procedures

Antibodies—The rabbit polyclonal anti-phospho-Chk1-S317 and anti-phospho-Chk1-S345 antibodies were purchased from Cell Signaling, and the monoclonal anti-Chk1 antibody (G4) was obtained from Santa Cruz. The goat anti-ATR (N-19) and rabbit-anti-ATR (Ab-2) antibodies were purchased from Santa Cruz and Oncogene, respectively. The monoclonal antibodies specific for BrdUrd (BrdUrd-FITC) and thymidine dimers were purchased from BD Biosciences and Karna Biomedical Co., respectively, while monoclonal anti-6-4 photoproduct (6-4PP) antibody was generously provided by Dr. Tsukasa Matsunaka (Kanazawa University, Japan). Monoclonal anti-β-actin antibody AC15 was obtained from Sigma, and the mouse monoclonal anti-H2AX antibody was raised against a C-terminal H2AX peptide phosphorylated at Ser139.

Cell Lines—HeLa and U2OS cells were purchased from the ATCC. 

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Fig. 1. UV lesions are not sufficient to induce ATR chromatin binding and Chk1 phosphorylation. A, XPC cells were irradiated with 40 J/m² UV and co-immunostained 30 min later with either anti-6-4PP and anti-Chk1-S317P antibodies or anti-γH2AX and anti-ATR antibodies. To specifically detect chromatin-bound ATR, cells were subjected to a short detergent extraction prior fixation and staining as described under “Experimental Procedures.” B, U2OS cells were either mock-treated or irradiated with 40 J/m² UV in the presence or absence of a 3-μm filter and 30 min later, after detergent extraction, co-stained with anti-thymidine dimer (CPD) and anti-ATR antibodies. C, U2OS cells were either mock- or UV (40 J/m²)-treated and co-stained 30 min later with anti-thymidine dimer and anti-Chk1-S317P antibodies.

The XPC-deficient cells XP4PA were a generous gift from R. Legerski (University of Texas). The Hus1+/p21−/− and Hus1−/−/p21−/− fibroblasts were generously provided by P. Leder and R. Weiss (Harvard Medical School, Boston). The H2AXlox/lox and H2AXlox/lox ES cells were a generous gift from F. Alt and C. Bassing (Howard Hughes Medical Institute, Harvard Medical School, Boston).

UV and Drug Treatment—Cells were pulse-labeled with 25 μM BrdUrd for 1 h prior to UV irradiation (40 J/m²) or CPT (1 μM) treatment. For micropore UV irradiation, a 3-μm isopore polycarbonate filter (Millipore) was placed on top of the cell monolayer prior UV irradiation. The cells were fixed or harvested 30–45 min after treatment as indicated.

FACS Analysis—HeLa cells were synchronized with a 20-h nocodazole block (1 μg/ml) followed by mitotic shake off and release into nocodazole-free culture medium. At various time points later, the cells were trypsinized and fixed with 70% EtOH overnight at −20 °C. After a PBS wash, the cells were incubated for 30 min with 10 μg/ml RNase A in citrate buffer and stained with 25 μg/ml propidium iodide for an additional 30 min at 37 °C. Cell cycle analysis was done on a FACScan (BD Biosciences).

Immunofluorescent Staining—Cells grown on coverslips were fixed with 3% paraformaldehyde plus 0.5% Triton X-100 for 30 min on ice. In the case of ATR staining, fixation was preceded by a short detergent extraction step where the cells were incubated on ice for 5 min in 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40. For detection of incorporated BrdUrd or photoslesions, fixed cells were de-natured with 2 M HCl for 10 min at 37 °C followed by a PBS rinse to remove the HCl. The cells were blocked with 5% milk for 30 min at room temperature prior incubation with the primary antibodies diluted in 5% milk or 10% fetal calf serum + 0.1% Triton (ATR) at the following dilutions: cyclobutane-pyrimidine dimer (CPD) and 6-4PP (1:100), BrdUrd-FITC (1:20), ATR (1:1000), Chk1-S317P (1:300), γ-H2AX (hybridoma supernatant). After a 1-h incubation at room temperature, the cells were washed twice with PBS and incubated for 45 min at room temperature with secondary antibodies (FITC-conjugated goat anti-mouse IgG at 1:200), rhodamine-conjugated goat anti-rabbit IgG at 1:250, or rhodamine-conjugated donkey anti-goat IgG at 1:500 dilution, Jackson ImmunoResearch). Cells were counterstained with 4',6-diamidino-2-phenylindole and viewed with a Nikon ECLIPSE E800 fluorescence microscope.

Immunoblotting—Cells were lysed in Frackelton buffer (10 mM Tris-HCl, 30 mM NaP2O7, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, pH 7.1) supplemented with 0.5 mM Na3VO4, 10 mM β-glycerophosphate, and protease inhibitor mixture (Roche Applied Science). The samples (80 μg of protein) were diluted in SDS sample buffer and loaded on a 3–8% or 10% polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk for at least 30 min prior overnight incubation with anti- phospho-Chkl-S345 antibody (at 1,500 dilution) or anti-ATR (at 1:1000 dilution) at 4 °C. The blots were washed with a 1:1 dilution of TBST (10 mM Tris-Cl, pH 8, 150 mM, 0.2% Tween) and PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, and visualized by chemiluminescence using the Supersignal kit purchased from Pierce. For reblotting with anti-phospho-Chkl-S317 (at 1,500 dilution), anti-Chkl, and anti-actin (at 1,500 dilutions each), the membrane was stripped with 7 M guanidine for 30 min.

RESULTS AND DISCUSSION

UV Lesions Are Not Directly Recognized by ATR—UV radiation produces two major classes of DNA lesions, the CPDs and 6-4PPs, which comprise 75 and 25% of the damage products, respectively. Both types of lesions distort the DNA helix and, if unrepaired, lead to DNA transcription and DNA replication arrest (15). In humans, repair of these photoslesions depend on the nucleotide excision repair (NER) pathway, a multistep
process that involves recognition of the lesion by the XPC-hHR23B complex, opening of the DNA helix around the lesion, and gap-filling DNA synthesis (16).

To test whether primary unprocessed UV lesions can activate ATR as suggested earlier (3), we analyzed ATR chromatin binding and ATR-mediated phosphorylation of Chk1 (17, 18) in UV-irradiated XPC-deficient cells that lack NER activity. As shown in Fig. 1A, UV-induced 6-4 photoproducts were equally detected in all of the UV-irradiated cells. In contrast, ATR retention and Chk1 phosphorylation were only observed in a subpopulation of these UV irradiated cells (Fig. 1A and data not shown). Since XPC-deficient cells are defective in the first step of NER, our findings suggest that primary UV lesions cannot directly initiate ATR activation in vivo.

We next asked whether ssDNA generated during the normal repair of UV lesions could trigger ATR activation. We irradiated U2OS cells with 40 J/m² UV and allowed the cells to recover and repair the UV lesions for 30 min at 37 °C. Previous reports had shown that XPG and ERCC1-XPF, the endonucleases involved in the NER incision reaction, are enriched at the sites of UV lesion within 15 min after UV radiation (16). As shown in Fig. 1, A and B, binding of ATR to chromatin as well as phosphorylation of Chk1 at Ser317 were confined to a subpopulation of UV-irradiated cells, resulting in a staining pattern similar to that observed in NER-deficient XPC cells. In the cells that did show ATR retention, this retention was restricted to sites of UV lesions as assessed by local UV irradiation through a 3-μm isopore polycarbonate filter (Fig. 1B). Together these results indicate that neither unprocessed UV lesions nor ssDNA generated in the context of UV repair are sufficient to provoke ATR activation.

**ATR Activation in Response to UV and CPT Treatment Requires Replication Stress—**ATR is known to be activated in response to replication stress (1, 2). To examine whether induction of replication stress is the critical step in UV-induced ATR activation, we pulse-labeled U2OS and XPC-deficient cells with BrdUrd prior UV irradiation. Co-staining with anti-phospho-Chk1 and anti-BrdUrd antibodies revealed that ATR-mediated phosphorylation of Chk1 is restricted to S phase cells (Fig. 2).

To further assess the role of replication in ATR activation, we synchronized HeLa cells in the G1 and S phase using nocodazole block and subsequent release of cells from mitosis. The synchronized G1 or S phase cells were either mock-treated or exposed to UV irradiation or CPT treatment. CPT is a specific topoisomerase I (topo I) inhibitor that traps topo I in a covalent enzyme-DNA complex and blocks religation of the topo I-created DNA nicks (19). While all S phase cells showed strong recruitment of ATR to chromatin in response to UV or CPT treatment, no chromatin binding was observed in G1 phase cells (Fig. 3, A and B). Likewise, ATR-mediated phosphorylation of Chk1 at Ser317 and Ser345 was confined to S-phase cells (Figs. 3B and 4, B and C). Since ATR as well as Chk1 protein level are not overtly reduced in G1 compared with S phase cells (Fig. 4B), our findings strongly suggest that replication stress is the major signal for ATR activation.
ATR Activation Requires DNA Replication

Together our results indicate that ssDNA formed either during the repair of UV lesions or introduced by drug treatment is not sufficient to initiate relocalization of ATR to chromatin and to trigger the checkpoint signaling cascade. ATR activation in response to UV irradiation is only observed in the context of replication arrest. It is possible that only ssDNA generated at the stalled replication fork is of sufficient length to recruit ATR in response to UV or CPT treatment. In vitro data suggest that the binding of ATR-interacting protein to replication protein A-coated ssDNA is length-dependent with a threshold length between 50 and 75 nucleotides (10). Notably, repair of UV lesions by NER usually involves the excision of 24–31 nucleotides (27). Thus, it is unlikely that normal NER repair would generate sufficient ssDNA to activate ATR pathway. Of course, our data do not rule out that ATR might be activated in some specific situation (for example, nonreplicating cells) if the repair process would create ssDNA intermediates of appropriate length.

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Fig. 5. H2AX is required for ATR accumulation but not Chk1 phosphorylation. A, wild-type H2AX (H2AXfloX/floX) and H2AX-deficient (H2AX–/–) ES cells were UV-irradiated (40 J/m2) and allowed to recover for 30 min prior to detergent extraction and subsequent costaining with anti-ATR and anti-γ-H2AX antibodies. B, H2AXfloX/floX and H2AX–/– ES cells were pulse-labeled with 25 μM BrdUrd prior to UV irradiation (40 J/m2). 30 min later, cells were directly fixed and stained with anti-Chk1-S345P and anti-BrdUrd antibodies. C, whole cell extracts from mock- or UV-treated wild-type H2AX and H2AX-deficient cells were immunooblotted with anti-Chk1-S345P, anti-Chk1, anti-ATR, and anti-actin antibodies.

H2AX Is Required for ATR Accumulation but Not Chk1 Phosphorylation—We had described earlier that histone H2AX is phosphorylated by ATR in response to HU (hydroxyurea) or UV treatment (20). Moreover, phosphorylation of H2AX in response to ionizing radiation has been shown to be critical for the accumulation of various DNA damage response proteins at sites of DNA lesions (21–25). To determine whether H2AX is required for the chromatin association of ATR and/or Chk1 activation, we compared UV-induced ATR retention and Chk1 phosphorylation in H2AXfloX/floX and H2AX–/– cells. As shown in Fig. 5A, ATR accumulation at sites of replication fork arrest was severely affected in the absence of H2AX, while ATR protein levels were similar in H2AXfloX/floX and H2AX–/– cells (Fig. 5C). Surprisingly, phosphorylation of Chk1 appeared to be similar in UV-irradiated H2AXfloX/floX and H2AX–/– cells (Fig. 5, B and C) suggesting that, described previously for other DNA damage response proteins (22), H2AX is required for the accumulation but not initial recruitment of ATR to chromatin following replication stress. Interestingly, recent data imply that the kinase activity of ATR regulates its translocation to sites of DNA damage (26). Therefore, we speculate that ATR-dependent phosphorylation of H2AX supports ATR retention at sites of replication stress and might contribute to cellular responses following replication stress.
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