Rapid Activation of ATR by Ionizing Radiation Requires ATM and Mre11*

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The ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases are crucial regulatory proteins in genotoxic stress response pathways that pause the cell cycle to permit DNA repair. Here we show that Chk1 phosphorylation in response to hydroxyurea and ultraviolet radiation is ATR-dependent and ATM- and Mre11-independent. In contrast, Chk1 phosphorylation in response to ionizing radiation (IR) is dependent on ATR, ATM, and Mre11. The ATR and ATM/Mre11 pathways are generally thought to be separate with ATM activation occurring early and ATR activation occurring as a late response to double strand breaks. However, we demonstrate that ATR is activated rapidly by IR, and ATM and Mre11 enhance ATR signaling. ATR-ATR-interacting protein recruitment to double strand breaks is less efficient in the absence of ATM and Mre11. Furthermore, IR-induced replication protein A foci formation is defective in ATM- and Mre11-deficient cells. Thus, ATM and Mre11 may stimulate the ATR signaling pathway by converting DNA damage generated by IR into structures that recruit and activate ATR.

Genome maintenance requires coordination of DNA repair with cell cycle events. ATM and ATR, members of the phosphatidylinositol kinase-like kinase family, are crucial regulatory proteins in genotoxic stress response pathways. Ultimately, ATM/ATR signaling halts the cell cycle to permit DNA repair, or in situations where repair systems are overwhelmed they initiate apoptosis (1–3). Although the ATM and ATR kinases phosphorylate some common substrates, they are differentially activated by distinct types of DNA damage. A primary function of the ATR signaling pathway is to monitor DNA replication. When problems arise, ATR phosphorylates and activates the checkpoint kinase (Chk1) as well as many other proteins (4, 5). Replication surveillance by ATR is accomplished by the recruitment of ATR and its accessory protein ATRIP to regions of replication protein A (RPA)-coated single-stranded DNA (ssDNA) that are generated by decoupling of helicase and polymerase activities at damaged replication forks (6–8). Direct binding of ATRIP to RPA-coated ssDNA is required for the stable recruitment of the ATR-ATRIP complex to damaged sites (7, 9). However, there may be alternative mechanisms by which the ATR-ATRIP complex can recognize damaged DNA other than direct ATRIP-RPA-ssDNA binding (9–14).

In contrast, ATM primarily initiates cellular responses to double strand breaks and is recruited to sites of damage by the Mre11-Rad50-Nbs1 (MRN) complex (14–17). Unlike ATR, ATM is not an essential gene, and ATM deficiencies result in a human disease, ataxia-telangiectasia (A-T). Cells from A-T patients are sensitive to IR but not to UV exposure. These observations have led to the general idea that ATM and ATR function in parallel pathways where cellular response to replicative stress and UV radiation is attributed to ATR, whereas ATM mediates cellular responses to IR-induced double strand breaks. However, there are several indications that ATR can also respond to double strand breaks. In A-T cells, p53 phosphorylation in response to IR is delayed but not completely abrogated (18, 19), and ATR is selectively responsible for the late phase of p53 phosphorylation (20). The ATR-ATRIP complex is required for maintaining the G1/M checkpoint in response to IR (6, 21–23). The ATR-ATRIP complex is also recruited to IR-induced intranuclear foci (9, 24). Thus, ATR is activated by double strand breaks, although the prevailing model is that this activation may be a late response compared with ATM signaling.

Here we show that Chk1 phosphorylation in response to IR is rapid and ATR-dependent. ATM- and Mre11-deficient cells display defects in ATR-dependent Chk1 phosphorylation. ATR-ATRIP as well as RPA recruitment to double strand breaks is reduced in ATM- and Mre11-deficient cells. Therefore, ATM and Mre11 stimulate ATR-dependent Chk1 phosphorylation by promoting the conversion of DNA damage generated by IR into structures that recruit the ATR-ATRIP complex.

MATERIALS AND METHODS

Cell Culture and siRNA—All cell lines were maintained in Dulbecco’s modified Eagle’s medium plus 7.5% fetal bovine serum. Transfections of siRNA were performed with Oligofectamine (Invitrogen). The 19-base pair siRNA target sequences used are as follows: ATR, CCUCCGU-GAUGUUGCUUGA; ATM, GCCUCAGGCAGAAAAAGA; ATRIP, GGUCACAGAUUAUAGAU; Mre11, GCUAAUGACUGUAGAU; Rad50, GUCACAGAUUGUAGAAG; and Nbs1, GAA-GAAAGGUGAAACCAG. The 19 base-pair nonspecific control oligonucleotide target sequence is AUGACGUGAACUCAAG. The 19-base pair nonspecific control oligonucleotide target sequence is AUGACGUGAACUCAAG. All RNA oligonucleotides were purchased from Dharmaco (Lafayette, CO).

Drug Treatment and DNA Damage—Hydroxyurea was purchased from Sigma (catalogue no. H8627), dissolved in water at 1 mM, and stored frozen. Aphidicolin was purchased from Sigma (catalogue no. A0781), dissolved in dimethyl sulfoxide at 5 mg/ml, and stored at –80 degrees. Etoposide (4′-demethylepipodophyllotoxin 9-(4,6-d-ethylidenes-β-D-glucopyranoside)) was purchased from Sigma (catalogue no. E1383), dissolved in dimethyl sulfoxide at 10 mg/ml, and stored at –20 degrees. Cells were treated with ionizing radiation from a 137Cs source at a dose...
rate of 1.8 Gy/min. UV radiation was administered with a Stratalinker (Stratagene) after cells were washed one time with PBS.

Proteomics Analysis—S3 HeLa nuclear extracts were generated from untreated and hydroxyurea-treated cells as follows. Briefly, S3 HeLa cells were grown in spinner flasks in Joklik's modified Eagle's medium purchased from Irvine Scientific (Santa Ana, CA) supplemented with 5% newborn calf serum (Invitrogen), sodium bicarbonate (Sigma, catalogue no. S3817), and 1× antibiotic/antimycotic (Invitrogen). Cells were harvested by centrifugation and washed twice with PBS. Cells were resuspended in hypotonic buffer (10 mM Tris-HCl (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT), incubated on ice for 10 min, and homogenized. Resulting nuclei were recovered by centrifugation and resuspended in low salt buffer (20 mM Tris-HCl (pH 7.9), 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.02 M KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and extracted by the addition of high salt buffer (20 mM Tris-HCl (pH 7.9), 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT). The resulting extract was dialyzed in dialysis buffer (20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.5% Igepal CA-630) with Tris-buffered saline (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) in a 1:1 ratio. Proteins were eluted in 8 M urea under conditions that preferentially eluted ATRIP-interacting proteins but not the ATRIP antibody. As a result, ATRIP, although present, was most likely underrepresented in the eluate. Eluates were fractionated by SDS-PAGE, and associated proteins were identified by two-dimensional liquid chromatography coupled tandem mass spectrometry.

Antibodies and Immunoblotting—Cells were lysed in 20 mM Tris (pH 7.5), 250 mM NaCl, 0.5% Igepal CA-630, 0.5% Tween 20, 2.5 mM EGTA supplemented with 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation prior to protein concentration determination (Bio-Rad). The ATRIP antibody has been described previously (6). Antibodies to Chk1 and ATR were purchased from Santa Cruz Biotechnology. Chk1 P-S317 and Chk1 P-S345 antibodies were purchased from Cell Signaling Technology (Beverly, MA). ATM, Nbs1, and Mre11 antibodies were purchased from Novus Biologicals (Littleton, CO). Rad50 antibody was purchased from GeneTex (San Antonio, TX), and ATM P-S1981 antibody was purchased from Rockland Immunchemicals (Gilbertsville, PA).

Immunofluorescence—Immunofluorescence was performed by plating cells directly on glass coverslips. For quantitation of Mre11 and RPA foci in HeLa cells, cells were washed twice with PBS, pre-extracted by a 5-min incubation on ice in MTSB buffer (10 mM PIPES, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 100 mM sucrose, 0.2% Triton X-100) followed by a 2-min incubation on ice in CSB buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.1% Tween 20), washed twice with PBS plus 0.1% Triton X-100, washed twice in PBS, and fixed in methanol prior to permeabilization in Triton X-100 solution. HeLa cells analyzed for
ATRIP foci and all other cells were fixed with paraformaldehyde and permeabilized with Triton X-100 solution as described previously (25). Fluorescein isothiocyanate- and rhodamine red-X-conjugated secondary antibodies were obtained from Jackson Immunoresearch.

RESULTS

Mre11 and ATM Are Required for ATR-dependent Chk1 Phosphorylation in Response to IR but Are Dispensable in Response to UV Radiation and Hydroxyurea—Mre11, Nbs1, and Rad50 were identified in an ATRIP complex using a proteomics screen to identify novel ATRIP-associated proteins. Co-immunoprecipitation and size exclusion fractionation of total HeLa cell lysates suggested that there could be an association between the MRN and ATR-ATRIP complexes (supplemental Fig. 1). However, the percentage of the MRN complex found in ATR or ATRIP immunoprecipitates was small (less than 1%), and the percentage of ATR-ATRIP complex found in immunoprecipitates of the MRN complex was similarly small. Furthermore, the association that occurred in untreated cells was not altered by DNA damage, and we were unable to detect a direct interaction between purified proteins (data not shown). There have been some reports of a connection between the MRN complex and ATR signaling (26–28). Therefore, we decided to investigate the role of the MRN complex in the ATR response to genotoxic stress.

Using RNA interference, we tested the role of Mre11 in ATR signaling to genotoxic stress in HeLa cells. HeLa cells, transfected with nonspecific siRNA oligonucleotides or siRNA targeting Mre11 or ATR mRNA, were exposed to hydroxyurea (1 mM for 5 h), UV radiation (50 J/m² for 2 h), or IR (5 Gy for 1 h). As expected, we found that ATR was required to phosphorylate Chk1 on Ser-317 in response to hydroxyurea and UV radiation, but Mre11 was dispensable (Fig. 1A). We also observed no role for the ATM kinase in the hydroxyurea and UV responses. However, knock-down of Mre11, ATM, and ATR decreased IR-induced Chk1 phosphorylation with ATR knock-down being the most severe and the Mre11 and ATM knock-downs giving intermediate defects (Fig. 1A).

The requirement for both ATM and ATR for Chk1 phosphorylation after IR treatment suggested one of three models: first, ATM and ATR may independently target Chk1; second, ATR may be upstream of ATM; or third, ATM may be upstream of ATR. If ATM and ATR independently targeted Chk1 then we would expect that similar to other ATM substrates, ATM would be required for the early response and ATR for the late response to IR. However, ATR was essential for Chk1 phosphorylation even at the earliest time points we were able to process, wherein the time from initial irradiation to cell lysis was only 15 min (Fig. 1B). Furthermore, if ATM and ATR were acting in parallel pathways then the co-depletion of both ATM and ATR should further decrease Chk1 phosphorylation. However, cells in which both ATM and ATR were depleted showed no further decrease in Chk1 phosphorylation beyond the decrease caused by ATR depletions (supplemental Fig. 2). These results suggest that ATM and ATR function in a common signaling pathway to phosphorylate Chk1 in response to IR. The dependence for both ATM and ATR was not limited to Chk1 on Ser-317 phosphorylation as both were also required for Chk1 Ser-345 phosphorylation (Fig. 1C). Consistent with the current ATM activation models, we saw no requirement for ATR in the activation of ATM because Chk2 Thr-68 and ATM Ser-1981 phosphorylation occurred normally in ATM-depleted cells (Fig. 1, B and C). Thus, these data support the third model in which ATM acts upstream of ATR but only in response to double strand breaks.

The ATM requirement for Chk1 phosphorylation was also observed in A-T cells. A-T cells or A-T cells complemented with the ATM cDNA were harvested at different time points after ionizing radiation exposure (5 Gy) and analyzed for Chk1 phosphorylation. Again, these ATM-deficient cells displayed a defect in Chk1 phosphorylation, whereas Chk1 phosphorylation was restored in A-T cells complemented with functional ATM (Fig. 2).

The involvement of ATM and Mre11 in Chk1 phosphorylation appeared limited to IR treatment. For example, there was no Mre11 requirement for Chk1 activation when cells were treated with the DNA polymerase alpha inhibitor aphidicolin or the topoisomerase II inhibitor etoposide (supplemental Fig. 3A). The requirement for ATM and Mre11 was not unique to HeLa cells because the depletions in U2OS cells yielded similar results. Furthermore, depletion of the other members of the MRN complex, Rad50 and Nbs1, gave results similar to Mre11 depletion (supplemental Fig. 3, A and B). Collectively, these data suggest that the ATM and MRN complex may promote optimal ATR activation but only in response to ionizing radiation.
Mre11 and ATM Enhance RPA and ATR-ATRIP Foci Formation in Response to IR. ATR activation is believed to require the generation of ssDNA coated with the ssDNA-binding protein RPA (29). Therefore, we hypothesize that ATM and Mre11 may be important for converting double strand breaks generated by IR into the DNA structures that recruit and activate ATR. To determine whether ATM and Mre11 were required to generate ssDNA at IR-induced double strand breaks, we monitored RPA and ATRIP foci formation in HeLa cells transfected with nonspecific siRNA oligonucleotides or siRNA targeting Mre11 and ATM. After transfection, cells were either left untreated or treated with 5 Gy of IR and fixed for immunofluorescence 1 h after the exposure. ATRIP and RPA foci were then monitored by indirect immunofluorescence with the indicated antibodies

FIGURE 4. Defects in ATRIP and RPA foci in A-T cells were restored by complementation with ATM. A-T or ATM-complemented A-T cells (38) were exposed to 5 Gy of IR and fixed for immunofluorescence with the indicated antibodies 1 h after exposure. A, representative immunofluorescence images demonstrating ATRIP, RPA34, and Mre11 foci are shown. These images also demonstrate the colocalization of these proteins at IR-induced foci. B, quantitation of ATRIP and RPA34 foci in A-T or ATM-complemented A-T cells. Error bars represent standard error between experiments. DAPI, 4’-6-diamidino-2-phenylindole.

To confirm that these results were not specific to HeLa cells transfected with siRNA oligonucleotides, RPA and ATRIP foci formations were also monitored in A-T cells and A-T cells complemented with functional ATM. Consistent with the defect observed in ATM-depleted HeLa cells, A-T cells displayed defects in both ATRIP and RPA foci formations in response to IR exposure relative to A-T cells complemented with functional ATM (Fig. 4B). Only 10% of cells without ATM contained ATRIP foci and 15% contained RPA foci. In contrast, 32.5 and 46% of A-T cells complemented with functional ATM contained ATRIP and RPA foci, respectively. In addition, Mre11 foci formation occurred independently of ATM as seen by no observable difference in the percentage of cells containing Mre11 foci in A-T and ATM-complemented A-T cells. Taken together, these results suggest that both Mre11 and ATM contribute to optimal activation of ATR by promoting the generation of RPA-coated ssDNA and thereby promoting the recruitment of ATR-ATRIP complexes to the sites of double strand breaks.

DISCUSSION

Our results demonstrate that Chk1 is rapidly phosphorylated in response to IR in an ATR-dependent manner. ATM- and Mre11-deficient cells display defects in Chk1 phosphorylation suggesting that the ATR, ATM, and Mre11 proteins function in a common signaling pathway. The requirement for both Mre11 and ATM is unique to IR-induced ATR-dependent Chk1 phosphorylation and dispensable in ATR-dependent phosphorylation of Chk1 in response to UV radiation and agents that induce replicative stress. Moreover, our data also suggest that Chk1 is largely an ATR substrate, as Chk1 phosphorylation is abro-

FIGURE 5. Model of ATR and ATM signaling pathways. APH, aphidicolin.
ATM-dependent ATR Activation

gated in ATR-deficient cells, which contain active ATM, and depletion of both ATM and ATR does not further impair Chk1 phosphorylation beyond that observed by ATR depletion alone. Thus, we have identified a novel role for ATM upstream of the ATR signaling pathway (Fig. 5).

One explanation for the role of Mre11 and ATM in the ATR-dependent response to IR is that both are required for an intact ATM signaling pathway. Mre11 is required for optimal ATM activation (17, 30, 31). Activation of ATM results in the phosphorylation of several proteins at double strand breaks sites including RPA (32, 33), which can be important for ATR recruitment and/or activation and subsequent Chk1 phosphorylation. Alternatively, ATM along with the Mre11 nuclease may be involved in processing double strand breaks into the structures that support ATR signaling. Our analysis of RPA and ATRIP localization is consistent with this second model because we observed a reduction in RPA and ATR-ATRIP recruitment to foci in ATM- and Mre11-deficient cells.

Recently, the recruitment of the Caenorhabditis elegans ATR homolog, AT1-1, to the site of DNA damage generated by IR is found to be RPA- and Mre11-dependent (34). In a separate study, the involvement of the Mre11-Rad50-Xrs2 complex in chromatin remodeling at double strand breaks in the Saccharomyces cerevisiae is also reported (35). These observations and our results support a model in which ATM and Mre11 are required to process double strand breaks into ssDNA, thereby facilitating RPA binding. The increased RPA-ssDNA generated by ATM and Mre11 promotes ATR-ATRIP recruitment, which results in Chk1 phosphorylation and augments cell cycle checkpoint activation. This model would explain a dual requirement for ATM and ATR to initiate the G2/M checkpoint in response to IR as well as the requirement for ATR to maintain the checkpoint (23). In the absence of ATM and Mre11 function, ATR activation would proceed although with reduced kinetics. This model reconciles the prevailing idea that ATR is required for the late response to IR with our data indicating that rapid Chk1 activation by IR is ATR-dependent. The main data supporting the late activation of ATR model come from experiments done in A-T cells showing delayed phosphorylation of substrates such as p53 in response to IR when ATM is absent (18–20). We suggest that the lack of ATM in these experiments delayed both direct ATM-dependent substrate phosphorylation and maximal ATR activation.

During the preparation of this manuscript, Jazayeri et al. (36) published results consistent with our findings that both ATM and Mre11 enhance IR-induced ATR-dependent Chk1 phosphorylation, at least in part, through enhancing RPA and ATR recruitment to double strand breaks. It will be important to determine how ATM and Mre11 generate the RPA-coated ssDNA. Interestingly, Mre11 is phosphorylated in response to double strand breaks and this phosphorylation activates its nuclease activity (37). Perhaps, ATM promotes an Mre11-dependent nuclease activity to generate stretches of ssDNA that recruit and activate ATR. Alternatively, ATM and Mre11 may help recruit other nucleases that process the double strand breaks to reveal ssDNA. In any case, our results have important implications for how double strand breaks are sensed by the ATM and ATR checkpoint kinases.

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