Ataxia Telangiectasia Mutated (ATM) Is Essential for DNA-PKcs Phosphorylations at the Thr-2609 Cluster upon DNA Double Strand Break*§

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The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is rapidly phosphorylated at the Thr-2609 cluster and Ser-2056 upon ionizing radiation (IR). Furthermore, DNA-PKcs phosphorylation at both regions is critical for its role in DNA double strand break (DSB) repair as well as cellular resistance to radiation. IR-induced DNA-PKcs phosphorylation at Thr-2609 and Ser-2056, however, exhibits distinct kinetics indicating that they are differentially regulated. Although DNA-PKcs autophosphorylates itself at Ser-2056 after IR, we have reported here that ATM mediates DNA-PKcs phosphorylation at Thr-2609 as well as at the adjacent (S/T)Q motifs within the Thr-2609 cluster. In addition, our data suggest that DNA-PKcs- and ATM-mediated DNA-PKcs phosphorylations are cooperative and required for the full activation of DNA-PKcs and the subsequent DSB repair. Elimination of DNA-PKcs phosphorylation at both regions severely compromises radioresistance and DSB repair. Finally, our result provides a possible mechanism for the direct involvement of ATM in non-homologous end joining-mediated DSB repair.

Among the different types of DNA damage, DNA double strand break (DSB) is the most detrimental type of DNA lesion. Unattended DSBs often lead to cell death, genomic instability, and tumorigenesis (1–3). In mammalian cells, DSBs are predominantly repaired by the non-homologous end joining (NHEJ) pathway, which reconnects the broken DNA ends together directly without using a homologous DNA template as homologous recombination does (4). The NHEJ pathway is governed by DNA-dependent protein kinase (DNA-PK) which is composed of the DNA end-binding Ku70/80 heterodimer and the catalytic subunit DNA-PKcs kinase. The intrinsic kinase activity of DNA-PKcs is essential for cellular resistance to radiation and NHEJ-mediated DSB repair (5, 6), most likely through phosphorylation of NHEJ components, including DNA-PKcs itself.

DNA-PKcs is rapidly autophosphorylated in vitro upon activation and is phosphorylated in vivo after ionizing radiation (IR). Many in vitro and in vivo phosphorylation sites of DNA-PKcs have been identified thus far, including the Thr-2609 cluster (7–9), Ser-2056 (10), and the recently identified C′-terminal phosphorylation sites (11). The majority of these phosphorylation sites are of the (S/T)Q motif (serine or threonine followed by a glutamine residue) commonly present in many DNA damage repair proteins and are the cognate substrates of phosphoinositide kinase-related protein kinases, including DNA-PKcs, ATM, and ATR (ATM-Rad3-related) kinases (12–14). Similar to its kinase activity, DNA-PKcs phosphorylation is also required for NHEJ-mediated DSB repair. Particularly, mutations at the Thr-2609 cluster severely compromise the ability of DNA-PKcs to restore the radioresistance and DSB repair defects in DNA-PKcs-deficient cells (7, 15).

We have previously demonstrated that, among the many phosphorylation sites identified, phosphorylations of DNA-PKcs at Thr-2609 and Ser-2056 occur in vivo upon IR (7, 10). Although DNA-PKcs autophosphorylation is responsible for IR-induced Ser-2056 phosphorylation, our data suggest that ATM is likely the kinase mediating IR-induced DNA-PKcs phosphorylation at Thr-2609 and other phosphorylation sites within the Thr-2609 cluster (10). ATM and DNA-PKcs are the major kinases activated upon IR and are both required for cellular resistance to radiation. Although DNA-PKcs plays a direct role in DSB repair, ATM plays a more general role in activating the DNA damage response, which spans many signaling pathways including the cell cycle checkpoints (16). Recent evidence suggests that ATM is spe-
cifically required for the repair of slow kinetics DSBs (17, 18). The possible involvement of ATM in phosphorylating DNA-PKcs could further unveil the role of ATM in DSB repair. To clarify the role of ATM in mediating DNA-PKcs phosphorylation after IR, we show here that ATM is indeed the major contributor for IR-induced DNA-PKcs phosphorylation at the Thr-2609 cluster.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—Most of the cell lines were maintained in α-minimal essential medium supplemented with 10% fetal calf serum and penicillin/streptomycin, including normal human skin fibroblasts and GM02052C ATM-deficient primary fibroblasts, SV40-transformed A-T (AT5BIVA) and wild-type (1BR3) fibroblasts, and Chinese hamster ovary wild-type (AA8) and DNA-PKcs-deficient (V3) cell lines. ATM-proficient (BT) and ATM-deficient (L3) human lymphoblastoid cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum as previously described (19). Retroviral vector-mediated expression of small hairpin RNA against ATM was performed as previously described (20). In brief, HeLa and MCF-7 cells were infected with previously 293T-packaged retroviral particles and selected with 10 μg/ml puromycin to achieve a stable cell line. The small hairpin green fluorescent protein plasmid was used as a control (20). Cells subjected to ATM inhibitor Ku55933 (21) or DNA-PKcs inhibitor Nu7441 (22) were pre-incubated with the drugs for 30–60 min prior to IR. The kinase inhibitors were gifts to Y. Shiloh from Kudos, Inc. and were gifts to D. Chen from Ryss Laboratory.

Immunofluorescent Staining and Antibodies—Immunofluorescent staining was performed as previously described (7, 10). Anti-pS2056 and anti-pT2609 phosphospecific rabbit polyclonal antibodies were also described previously (7, 10). Anti-pT2647 was prepared by immunizing New Zealand White rabbits with keyhole limpet hemocyanin-conjugated phosphopeptide AGQIRAT[PO3]QQHQDFC (Thr-2638) or QHDFTLT[PO3]Q-TADGRC (Thr-2647) and was affinity-purified through a phosphopeptide-conjugated Sepharose CL-4B column (Pierce). Anti-pS2612 and anti-pT2638 phosphospecific rabbit polyclonal antibodies were custom synthesized by Bethyl Laboratories. Anti-DNA-PKcs 25-4 monoclonal antibody (NeoMarkers) and anti-γH2AX monoclonal antibody (Upstate Biotechnology) are commercially available.

Clonogenic Survival and γH2AX Foci Analysis—Clonogenic survival was performed as previously described (7). Analysis of γH2AX foci analysis was performed as previously described (23).

Laser Micro-irradiation—Laser micro-irradiation was carried out on a Zeiss Axiovert200 inverted microscope equipped with a MicroPoint laser system (Photonic Instruments, Inc.). In brief, MicroPoint laser (365 nm) was coupled directly to the epifluorescence path and was focused through a 63× objective to yield a spot size of ~1 μm. The output of the laser was set at 60% to generate detectable foci of YFP-tagged DNA-PKcs in live cells.

RESULTS

We have previously reported that IR-induced DNA-PKcs phosphorylation at Thr-2609 and Ser-2056 may be subjected to differential regulation due to their distinct kinetics and dosage response to IR (7, 10). Although DNA-PKcs autophosphorylation is responsible for IR-induced Ser-2056 phosphorylation (10), our data suggest that ATM may be required for Thr-2609 phosphorylation, as IR-induced DNA-PKcs phosphorylation at Thr-2609 was significantly reduced in A-T fibroblasts as compared with that of ATM-complemented cells (7). The possible role of ATM in mediating IR-induced Thr-2609 phosphorylation was further analyzed in SV40-transformed human fibroblast cell lines. Wild-type fibroblast cell line 1BR3 and A-T fibroblast cell line AT5 were subjected to 10 Gy of x-ray and were analyzed for IR-induced DNA-PKcs phosphorylation at Ser-2056 and Thr-2647. D, ATM-proficient (BT) and ATM-deficient (L3) human lymphoblastoid cell lines were subjected to a similar analysis as in C.
suggesting that ATM is required for IR-induced DNA-PKcs phosphorylation at Thr-2609.

In close proximity and downstream from Thr-2609, five additional phosphorylation sites were identified in 

\[ \text{in vitro} \] 

autophosphorylated DNA-PKcs (8), four of which are in (S/T)Q motifs. Phosphorylation at these sites is also required for the function of DNA-PK in DSB repair, as alanine substitution at the entire Thr-2609 cluster of phosphorylation sites led to severe radiosensitivity and defective NHEJ (15), whereas T2609A mutation alone only caused modest radiosensitivity (7). It is highly likely that phosphorylation at the other sites within the Thr-2609 cluster are being regulated in a similar fashion as Thr-2609 phosphorylation \textit{in vivo}. This hypothesis is supported by analyzing DNA-PKcs phosphorylation at Thr-2647 (Fig. 1B). Using a phosphospecific antibody against Thr-2647, we noticed that IR also induces DNA-PKcs phosphorylation at this site. Similar to Thr-2609 phosphorylation, IR-induced DNA-PKcs phosphorylation at Thr-2647 is ATM-dependent as it was robust in 1BR3 cells and diminished in AT5 cells in either Western blotting (Fig. 1B) or immunofluorescent (supplemental Fig. 2) analyses.

To examine whether ATM dependence is a general characteristic of the Thr-2609 cluster, IR-induced phosphorylation of this cluster was further analyzed in ATM-proficient and -deficient primary skin fibroblasts and lymphoblastoid cell lines. The cells were irradiated with either 1 or 10 Gy of IR followed by Western blotting analysis of DNA-PKcs phosphorylations. DNA-PKcs phosphorylation at Ser-2056 could be consistently detected in all cell lines after 1 or 10 Gy of IR (Fig. 1, C and D). However, although the phosphorylation at Thr-2647 was detected in ATM-proficient cells (HSF and BT), it was markedly reduced and barely detectable after 10 Gy in ATM-deficient cells (Fig. 1, C and D, GM02052C and L3). The dependence of ATM to DNA-PKcs phosphorylation at the Thr-2609 cluster was also examined using the RNA interference approach to knock down the ATM expression (20) in HeLa and MCF7 cells. Stable expression of small hairpin RNA against ATM mRNA in HeLa cells drastically reduced the steady-state protein level of ATM as compared with that of control cells expressing small hairpin RNA against green fluorescent protein (Fig. 2A). Silencing ATM in HeLa cells led to
considerable attenuation of IR-induced DNA-PKcs phosphorylation at Thr-2609 and Thr-2647, whereas DNA-PKcs autophosphorylation at Ser-2056 was not affected (Fig. 2A). Similarly, small hairpin RNA-mediated inhibition of ATM expression in MCF7 cells blocked IR-induced phosphorylation of Thr-2609 and Thr-2647 in these cells as well (Fig. 2B), suggesting that IR-induced DNA-PKcs phosphorylation at the Thr-2609 cluster is generally dependent on ATM but not on DNA-PKcs itself.

To demonstrate that the kinase activity of DNA-PKcs is expendable for IR-induced DNA-PKcs phosphorylation at the Thr-2609 cluster, we analyzed DNA-PKcs phosphorylation in Chinese hamster ovary (CHO) V3 cells complemented with wild-type DNA-PKcs or kinase-dead mutant DNA-PKcs. Yellow fluorescent protein (YFP) fusion of wild-type DNA-PKcs (YFP-WT) and kinase-dead mutant (YFP-KD) were stably transfected into CHO V3 cells. Expression of YFP-WT (but not YFP-KD) restores the radioresistance phenotype to V3 cells, suggesting that YFP-WT fusion protein is functional in the NHEJ pathway for DSB repair. We have demonstrated previously that laser micro-irradiation induces DNA-PKcs phosphorylation at sites of localized laser damage (10). Using the YFP fusion approach coupled with laser micro-irradiation, we observed that both wild-type and kinase-dead mutant DNA-PKcs are rapidly accumulated at laser damage sites, indicating that the kinase activity of DNA-PKcs is not required for the initial accumulation of DNA-PKcs at damage sites (Fig. 3). However, the kinase activity of DNA-PKcs is required for Ser-2056 autophosphorylation at laser micro-irradiation as Ser-2056 phosphorylation was diminished in V3 YFP-KD cells compared with that in V3 YFP-WT cells. In contrast, laser-induced DNA-PKcs phosphorylation at Thr-2609 and Thr-2647 remained detectable in V3 YFP-KD cells (Fig. 3B), confirming that the kinase activity of DNA-PKcs is expendable for the Thr-2609 cluster phosphorylation in vivo.

Because both DNA-PKcs and ATM play important roles in radioresistance, several inhibitory small molecules have been recently developed to target specifically DNA-PKcs (22) and ATM (21) and presumably serve as radiosensitizers for cancer radiotherapy. The role of ATM and DNA-PKcs in IR-induced Thr-2609 cluster phosphorylation was examined using these inhibitors to further confirm the role of ATM in mediating the phosphorylation at the Thr-2609 cluster. HeLa cells were incubated with either the ATM inhibitor Ku55933 (21) or the DNA-PKcs kinase inhibitor Nu7441 (22) prior to IR and were subsequently analyzed for IR-induced DNA-PKcs phosphorylation using Western blotting analysis. As shown in Fig. 4A, the DNA-PKcs inhibitor Nu7441 specifically blocked DNA-PKcs autophosphorylation at Ser-2056 but not the phosphorylation at Thr-2609 and Thr-2647. Conversely, the ATM inhibitor Ku55933 blocked the phosphorylation at Thr-2609 and Thr-2647 but not autophosphorylation at Ser-2056 (Fig. 4A). In addition, we noticed that ATM inhibitor Ku55933 attenuated IR-induced DNA-PKcs phosphorylation at Thr-2612 and Thr-2638, which are included at the Thr-2609 cluster (Fig. 4B). The effect of Ku55933 and Nu7441 on DNA-PKcs phosphorylation was tested also after laser micro-irradiation. The results showed that Ku55933 preferentially blocks Thr-2609 phosphorylation, whereas Nu7441 preferentially blocks Ser-2056 phosphorylation (Fig. 4C), further confirming that ATM is the key kinase mediating DNA-PKcs phosphorylation at the Thr-2609 cluster in response to IR.

It is likely that, in response to IR, phosphorylation of DNA-PKcs at Ser-2056 by itself and at the Thr-2609 cluster by ATM blocks Ser-2056 phosphorylation (Fig. 4C). In addition, we noticed that ATM inhibitor Ku55933 preferentially blocks Ser-2056 phosphorylation but does not phosphorylation at Thr-2609 and Thr-2647. Conversely, the ATM inhibitor Ku55933 blocks the phosphorylation at Thr-2609 and Thr-2647 but not autophosphorylation at Ser-2056 (Fig. 4A). In addition, we noticed that ATM inhibitor Ku55933 attenuated IR-induced DNA-PKcs phosphorylation at Thr-2612 and Thr-2638, which are included at the Thr-2609 cluster (Fig. 4B). The effect of Ku55933 and Nu7441 on DNA-PKcs phosphorylation was tested also after laser micro-irradiation. The results showed that Ku55933 preferentially blocks Thr-2609 phosphorylation, whereas Nu7441 preferentially blocks Ser-2056 phosphorylation (Fig. 4C), further confirming that ATM is the key kinase mediating DNA-PKcs phosphorylation at the Thr-2609 cluster in response to IR.

It is likely that, in response to IR, phosphorylation of DNA-PKcs at Ser-2056 by itself and at the Thr-2609 cluster by ATM are both required for full activation of DNA-PKcs activity. Incomplete phosphorylation at either Ser-2056 or the

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**FIGURE 4. Inhibition of ATM (but not DNA-PKcs) attenuates IR-induced DNA-PKcs phosphorylations at the Thr-2609 cluster.** A, HeLa cells were mock-treated or pretreated with 10 μM ATM inhibitor Ku55933 (Ku) or DNA-PKcs inhibitor Nu7441 (Nu) for 30 min. The cells were then irradiated (10 Gy, 30 min of recovery) and analyzed for DNA-PKcs phosphorylation at Ser-2056, Thr-2609, and Thr-2647. B, mock-treated and Ku55933- or Nu7441-treated HeLa cells were irradiated (10 Gy, 30 min of recovery) and analyzed for DNA-PKcs phosphorylation at Ser-2612 and Thr-2638. C, CHO V3 cells expressing YFP fusion of wild-type DNA-PKcs (YFP-WT) were mock-treated or pretreated with 10 μM Ku55933 or Nu7441 for 30 min and then subjected to laser micro-irradiation. Ten minutes later the cells were fixed and immunostained for γH2AX and DNA-PKcs phosphorylations at Ser-2056 or Thr-2609 as indicated.

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4 N. Uematsu and D. J. Chen, unpublished results.
ATM Phosphorylates DNA-PKcs at the Thr-2609 Cluster

Thr-2609 cluster may impair the NHEJ pathway of DSB repair. To test this hypothesis, we asked whether alanine substitutions at both the Thr-2609 cluster and Ser-2056 would further increase radiosensitivity and impair DSB repair. CHO V3 cells complemented with wild-type DNA-PKcs or various phosphorylation mutants (Fig. 5A) were analyzed for clonogenic survival and kinetics of γH2AX foci formation after IR. In clonogenic survival analysis, we consistently observed increasing, although subtle, radiosensitivity in V3 cells complemented with the 7A mutant DNA-PKcs (alanine substitutions at both Ser-2056 and Thr-2609 cluster) as compared with that of V3 cells complemented with 6A mutant DNA-PKcs (alanine substitutions at Thr-2609 cluster alone) (Fig. 5B). The slight increase of radiosensitivity in 7A mutant was further analyzed in two more independent V3-7A cell lines and was compared with that of additional V3-6A mutant cell lines (supplemental Fig. 3B).

Although the differences of radiosensitivity between the 6A and 7A cell lines are small, the result is reproducible and showed that 7A cell lines are slightly more radiosensitive than 6A cell lines.

To further explore the difference of DNA-PKcs phosphorylation at Ser-2056 and Thr-2609 cluster in DSB repair, we analyzed the regression of γH2AX foci kinetics after IR (23) in various V3-complemented cell lines. CHO V3-complemented cells were subjected to 1 Gy of IR. The cells were harvested at different time points and underwent immunofluorescent staining for γH2AX nuclear foci. The results showed that regression of IR-induced γH2AX foci in V3-2A cells (S2056A/T2609A) was slower than that of V3-S2056A or V3-T2609A cells (Fig. 5C). Additionally, the regression of γH2AX foci was also further attenuated in V3–7A cells as compared with that in V3–6A cells (Fig. 5C). This observation was repeated in additional independent V3–6A and V3–7A cell lines with similar results (supplemental Fig. 3C), indicating that DNA-PKcs phosphorylation at the Ser-2056 and Thr-2609 clusters contribute distinctively to radioresistance and DSB repair, and both are required for these processes. This also provides evidence that the coordination between DNA-PKcs and ATM-mediated phosphorylation events is essential for the optimal DSB repair.

DISCUSSION

Both ATM and DNA-PKcs are members of the phosphoinositide 3-kinase-related kinases (14, 25), and are the major kinases activated upon the induction of DNA double strand breaks. Defects in either ATM or DNA-PKcs kinase activity result in a radiosensitive phenotype. DNA-PKcs is known to play a direct role in DSB repair and is the key component of the NHEJ repair pathway, whereas ATM plays a key role in general damage signaling and cell cycle regulation (16). Evidence is emerging that ATM specifically plays an important role in the slow component of DSB repair (17, 18). This component of the DSB repair process may require both the ATM and the Artemis protein for mediating an end-processing step before the ends of DNA strands can be rejoined by NHEJ (17, 18).

Here, we present further evidence for the involvement of ATM in DSB repair and direct cross-talk between ATM and DNA-PKcs. ATM is required for IR-induced DNA-PKcs phosphorylation at the Thr-2609 cluster (Figs. 1–4). Furthermore, ATM-mediated DNA-PKcs phosphorylation at the Thr-2609 cluster is critical for the radioresistance and DSB repair activities of DNA-PKcs. Our results demonstrate that alanine substitution at the entire Thr-2609 cluster of phosphorylation sites (6A mutant) or the Thr-2609 cluster together with Ser-2056 (7A mutant) lead to severe radiosensitivity and defective DSB repair (Fig. 5 and supplemental Fig. 3). A similar result was reported previously (15). The dominant negative effects of 6A and 7A mutants is likely because of their occupation at the broken DNA ends but failure to support or even to prevent DSB repair via NHEJ. This assertion is supported by our recent evidence that 6A or 7A mutants stay much longer at laser micro-irradiation-induced DSBs than wild-type DNA-PKcs.4 In contrast, in the absence of DNA-PKcs, DSBs could be repaired by homologous recombination or slowly by the residual activity of the NHEJ machinery. It is important to point out that, although
the difference in clonogenic survival between the 6A and 7A mutants is small, it is consistently reproducible (Fig. 5 and supplemental Fig. 3). The difference between 6A and 7A mutants is also supported by γH2AX foci kinetics analysis showing that DSB repair activity in V3–7A cells is further reduced than that in V3–6A cells, suggesting that DNA-PKcs phosphorylation, mediated by ATM at the Thr-2609 cluster and by DNA-PKcs itself at Ser-2056, are both required for the optimal DSB repair activity after IR. As shown recently by Goodarzi et al. (26), DNA-PKcs phosphorylation at the Thr-2609 cluster is required for the localization of Artemis endonuclease activity. These two lines of evidence provide a mechanism for the direct involvement of ATM in DSB repair; ATM-mediated DNA-PKcs phosphorylation at the Thr-2609 cluster is required to stimulate the endonucleolytic activity of Artemis that trims otherwise non-ligatable DNA ends before they can be rejoined by NHEJ. Although the foci of DNA-PKcs phosphorylated at the Ser-2056 or Thr-2609 clusters overlap with γH2AX foci, which mark the damaged sites, alanine substitution at these putative phosphorylation sites does not affect the recruitment of DNA-PKcs to these sites.4 Instead, it is possible that DNA-PKcs phosphorylation at the Thr-2609 cluster creates a docking site for Artemis, enhancing its recruitment to DSBs, and the interaction between the phosphorylated Thr-2609 cluster and Artemis may stimulate Artemis endonucleolytic activity. In addition to the potential interaction with Artemis, DNA-PKcs phosphorylation at the Thr-2609 cluster might also serve as a docking site for molecules containing BRCA1 C-terminal or Forkhead-associated domains, which interact specifically with the phosphorylated (S/T)Q motif (27–29). Similarly, we have also found that the protein phosphatase 5 (PP5) interacts with DNA-PKcs at the Thr-2609 cluster region (30).

The collaboration between ATM and DNA-PKcs may be essential for the proper initiation of DSB signaling (e.g. γH2AX formation) and the processing of detrimental DSBs to ensure proper repair and ultimately to preserve the genome integrity after DNA damage. Indeed, double knock-out of the genes encoding ATM and DNA-PKcs in mice is embryonically lethal (31). The cross-talk between ATM and DNA-PKcs may not be limited to one direction, in which DNA-PKcs is phosphorylated by ATM. Notably, ATM expression is dependent on the activity of DNA-PKcs, as deficiency of DNA-PKcs results in down-regulation of ATM (24). ATM and DNA-PKcs are key molecules in the response of mammalian cells to IR-induced DNA damage. The cross-talk between them is probably a central axis in this response.

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