ATM and DNA-PK Function Redundantly to Phosphorylate H2AX after Exposure to Ionizing Radiation

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ABSTRACT

H2AX phosphorylation is an early step in the response to DNA damage. It is widely accepted that ATM (ataxia telangiectasia mutated protein) phosphorylates H2AX in response to DNA double-strand breaks (DSBs). Whether DNA-dependent protein kinase (DNA-PK) plays any role in this response is unclear. Here, we show that H2AX phosphorylation after exposure to ionizing radiation (IR) occurs to similar extents in human fibroblasts and in mouse embryo fibroblasts lacking either DNA-PK or ATM but is ablated in ATM-deficient cells treated with LY294002, a drug that specifically inhibits DNA-PK. Additionally, we show that inactivation of both DNA-PK and ATM is required to ablate IR-induced H2AX phosphorylation in chicken cells. We confirm that H2AX phosphorylation induced by DSBs in nonreplicating cells is ATR (ataxia telangiectasia and Rad3-related protein) independent. Taken together, we conclude that under most normal growth conditions, IR-induced H2AX phosphorylation can be carried out by ATM and DNA-PK in a redundant, overlapping manner. In contrast, DNA-PK cannot phosphorylate other proteins involved in the checkpoint response, including chromatin-associated Rad17. However, by phosphorylating H2AX, DNA-PK can contribute to the presence of the damage response proteins MDC1 and 53BP1 at the site of the DSB.

INTRODUCTION

Three related phosphatidylinositol 3′-kinase-like kinases play important roles in the response to DNA damage (1). ATM [ataxia telangiectasia (A-T) mutated protein], which is defective in the hereditary disorder A-T, is a central component of the signal transduction pathway that responds to DNA double-strand breaks [DSBs (2)]. ATM is activated by DSBs and phosphorylates key proteins that lead to cell cycle checkpoint arrest and/or apoptosis. ATM substrates include Chk1, Chk2, Rad17, NBS1, BRCa1, BLM, SMC1, 53BP1, and MDC1 (3–7). Consistent with a role of ATM in cell cycle checkpoint control, A-T cells show impaired G1-S, S, and G2-M checkpoint arrest after exposure to ionizing radiation [IR (8–10)]. ATR (ataxia telangiectasia and Rad3-related protein) phosphorylates many of the same proteins phosphorylated by ATM. Current evidence suggests that ATR, in contrast to ATM, responds to stalled replication forks and bulky lesions (11–14).

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is distinct from the former phosphatidylinositol 3′-kinase-like kinases because it does not appear to function in signal transduction damage response pathways but rather functions in DNA nonhomologous end joining, a pathway for DSB repair (Ref. 15; for a review, see Ref. 16).

Thus, cell cycle checkpoint arrest and p53 phosphorylation occur normally in DNA-PKcs-deficient cells (17).

An early response to DSBs is phosphorylation of a variant form of the histone H2A designated H2AX. Phosphorylated H2AX (called γ-H2AX) can be visualized as foci by immunofluorescence using phosho-specific antibodies (18, 19). H2AX foci colocalize with foci of other proteins, including NBS1, 53BP1, MDC1, and BRCA1 (3, 6, 18, 20). Although the initial recruitment of these proteins appears to be γ-H2AX independent, their retention as foci at longer times post-irradiation does not occur in cells lacking H2AX, leading to the suggestion that γ-H2AX plays a critical role in the retention of repair factors at the sites of DSBs (21, 22). Serine 139, which lies within an ATM consensus sequence, is the major H2AX residue phosphorylated by ATM (23). We have also observed failure to phosphorylate H2AX in A-T lymphoblastoid cell lines [LCLs (24)]. Together, these data have led to the widely held belief and logical conclusion that γ-H2AX formation in response to DSBs is an early step in the ATM-dependent signal transduction process. However, another study reported defective H2AX phosphorylation in the DNA-dependent protein kinase (DNA-PK)-defective tumor line M059J (18). Here, we have addressed the roles of ATM, ATR, and DNA-PK in H2AX phosphorylation after exposure to IR. We demonstrate that IR-induced H2AX phosphorylation can be carried out by ATM or DNA-PK in a redundant, overlapping manner.

MATERIALS AND METHODS

Cell Culture. 1BR3 are control primary fibroblasts. AT7BI and AT1BR are A-T primary fibroblast cell lines (25), F02-98 is an ATR-defective fibroblast derived from a Seckel Syndrome patient (14). 5B7IVA is a SV40-transformed A-T fibroblast line. M059J and M059k are glioma cell lines lacking and expressing DNA-PKcs, respectively (26). Cells were cultured in MEM supplemented with 20% (primary cells) or 15% (transformed lines) FCS, penicillin, and streptomycin as described previously (27). Sweig is a control EBV transformed LCL; AO and LB541 are A-T LCLs (24). LCLs were grown in RPMI 1640 supplemented with 15% FCS, penicillin, and streptomycin. DNA-PKcs−/− and ATM−/− mouse embryo fibroblasts (MEFs) were derived as described previously (28, 29). Chicken DT40 cells were grown in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 1% antibiotics, and 100 μM β-mercaptoethanol as described previously (30).

Treatment with DNA-Damaging Agents. Irradiation was performed using a 150 Cs source (8.5 Gy/min). X-irradiation (95 kV, 25 mA, –6 Gy/min) was used for the experiments involving DT40 chicken cells. When present, LY294002 (200 μM) was added to the cells 30 min before treatment with the relevant agent. LY294002 was purchased from Sigma-Aldrich (Poole, United Kingdom).

Antibodies. Anti-pS3Ser15 and anti-Rad17pSer645 were purchased from Cell Signaling Technology (Beverly, MA) and New England Biolabs (Hitchin, United Kingdom), respectively. Anti-H2AXSer139 antibodies were obtained from Upstate Technology (Buckingham, United Kingdom). The anti-53BP1 mouse monocular antibodies were as described previously (31). The anti-MDC1 (NH2-terminal FHA domain) rabbit polyclonal antibodies were kindly provided by Dr. M. Goldberg and Prof. S. Jackson. Antiirabbit and antimouse secondary antibodies were purchased from Dako (Glostrup, Denmark).

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**Immunofluorescence.** Cells were fixed in 3% paraformaldehyde and 2% sucrose PBS for 10 min at room temperature and permeabilized in 20 mM HEPES (pH 7.4), 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, and 0.5% Triton X-100 (Sigma-Aldrich) for 5 min at 4°C. Thereafter, coverslips were washed in PBS before immunostaining. Primary antibody incubations were performed for 40 min at 37°C at 1:100 dilutions (1:800 for anti-γ-H2AX) in PBS supplemented with 2% bovine serum fraction V albumin (Sigma-Aldrich) and followed by washing in PBS. Incubations with antinouse tetramethylrhodamine isothiocyanate and FITC or with antirabbit FITC secondary antibodies (Sigma-Aldrich) were performed at 37°C at 1:100 dilutions in 2% bovine serum fraction V albumin for 20 min. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich; data not shown) for 10 min at 4°C. Coverslips were mounted in Vectashield (Vector Laboratories, Peterborough, United Kingdom). Foci were counted using a fluorescence microscope. Foci present in 200 cells were counted in each experiment. The error bars represent the SD of the mean. A minimum of three experiments were carried out where error bars are shown. For experiments involving the DT40 cells, cells in suspension were spotted onto glass slides coated with 0.1% gelatin, fixed, and permeabilized with 100% ice-cold methanol for 30 min and ice-cold acetone for 1 min. Further analysis was as described previously (32).

**DNA-PK Kinase Assay.** Whole cell extracts were prepared by freezing and thawing as described previously (33). Cell extract was mixed with pre-washed and prewashed DNA-cellulose beads (Sigma; 5 mg/reaction) and incubated for 30 min at 4°C. After two washes in Z-buffer [25 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM MgCl₂, 1 mM DTT, 1% NP40, and 20% glycerol], the beads were resuspended in the same buffer and used for the kinase assay. The beads were incubated with 1 μL of p53-derived substrate (Promega) and 0.52 μCi of [γ-32P]ATP for 15 min at 30°C, and then the reaction was stopped by the addition of 30°C acetic acid. Reactions were spotted on P81 cation exchange paper to bind the peptide substrate, washed three times with 15% acetic acid, and then dried. Radioactivity retained on the filters was quantified by liquid scintillation counting.

**RESULTS**

IR-Induced H2AX Phosphorylation Occurs Efficiently in Human Cells Lacking ATM or DNA-PK. To examine H2AX phosphorylation, we used phospho-specific anti-H2AX(Ser139) antibodies for immunofluorescence and counted the number of foci formed per cell. The time course of γ-H2AX formation after exposure to 2 Gy was examined in two A-T primary fibroblast cell lines (AT1BR and AT7BI), an immortalized A-T cell line (AT5BIVA), and DNA-PKcs-deficient M059J cells. Although the foci formed with slightly slower kinetics in the three A-T cell lines, by 30 min postirradiation, a similar number of foci were observed in A-T and control cell lines (Fig. 1A). M059J (DNA-PKcs−/−) and the parental line, M059K (DNA-PKcs+/−), a glioma cell line, gave a more robust response relative to the primary fibroblast cell lines, but a similar peak of foci was observed in both lines (Fig. 1A). The elevated number of foci in these glioma cell lines relative to primary cell lines is most likely explained by the fact that the cells are hyperdiploid (modal chromosome number approximately 110), allowing the formation of more DSBs (34). Foci both form and commence disappearing more rapidly in the M059K (DNA-PKcs+/−) cells relative to the fibroblasts. By 1 h postirradiation, the number of foci in M059K cells had already decreased, whereas foci were lost more slowly in DNA-PKcs−/− M059J cells, indicative of their impaired DSB repair (26). With the fibroblasts, foci remained at a similar level until around 60 min postirradiation, most likely representing a steady state between continued formation and disappearance due to DSB repair. With prolonged incubation, the number of foci decreased, and this occurred to similar extents in A-T and control cells over a 3-h period examined (data not shown).

Examination of foci formation at 30 min after exposure to different doses of IR also demonstrated a similar response in the A-T and DNA-PKcs−/− cell lines relative to their control counterpart (Fig. 1B). At higher doses, the response ceased to be linear, probably due to inaccuracy in scoring >60 foci/cell. Taken together, these results show that foci form efficiently in human cells lacking either ATM or DNA-PK.

**H2AX Phosphorylation Is Ablated in Human A-T Fibroblasts Treated with a DNA-PK-Inhibiting Drug.** One explanation for these findings is that ATM and DNA-PKcs function redundantly to phosphorylate H2AX. To examine this possibility, we measured γ-H2AX formation in the presence of the drug LY294002, a specific inhibitor of DNA-PKcs, in cells lacking ATM (35–37). Whereas IR-induced foci form normally in the presence of LY294002 in IB, M059K (DNA-PKcs+/−), and M059J (DNA-PKcs−/−) cells, foci were barely detectable in the two A-T fibroblast cell lines (AT1BR and AT7BI) examined (Fig. 1C). To confirm that LY294002 does not affect ATR, we exploited a recent finding that F02-98 cells, which have a mutational change in ATR leading to aberrant splicing and...
impaired ATR activity, show dramatically reduced UV-induced γ-H2AX formation, demonstrating that this event is ATR dependent (14). LY294002 did not impair UV-induced H2AX phosphorylation in 1BR3 cells, demonstrating that ATR remains active in the presence of the drug (data not shown). To verify that LY294002 does not impair ATM function, we examined the impact of the drug LY294002 on IR-induced p53 and Rad17 phosphorylation by immunofluorescence using phospho-specific antibodies (anti-pSer15 and anti-Rad17Ser645) and observed similar levels of phosphorylated p53 and Rad17 in control and drug-treated control cells but impaired phosphorylation in AT1BR (data not shown). Taken together, these findings demonstrate that LY294002 does not impair either ATM or ATR activity. To verify that LY294002 does impair DNA-PK activity, we first carried out a DNA-PK kinase assay in the presence of the drug (data not shown). To verify that LY294002 does not impair UV-induced H2AX phosphorylation (14). LY294002 did not impair UV-induced H2AX phosphorylation in human cells, we observed slightly slower induction of γ-H2AX after IR treatment. We show that DNA-PK, another phosphatidylinositol 3-kinase that is inhibited by LY294002 can phosphorylate H2AX after IR treatment. We show that DNA-PK, but not ATM or ATR, is inhibited by LY294002.

IR-Induced H2AX Phosphorylation Occurs Efficiently in MEFS Lacking ATM and Is Ablated by Drug-Induced Inhibition of DNA-PK. An earlier study carried out on primary and transformed MEFs reported that H2AX phosphorylation is ATM dependent (23). To verify that our findings were not specific to human cells, which have 50-fold higher levels of DNA-PK activity compared with mouse cells, we also examined transformed ATM−/− and DNA-PKcs−/− MEFs for induction of γ-H2AX foci. Similar to our findings with human cells, we observed slightly slower induction of γ-H2AX foci in ATM−/− MEFs compared with control and DNA-PKcs−/− MEFs, but by 15 min postirradiation, a similar level of foci was observed (Fig. 2). Addition of LY294002 ablated H2AX foci formation in ATM−/− MEFs, identical to the results found with human fibroblasts (Fig. 2). Thus, our findings suggest that in rodent cells, as in human cells, DNA-PK has a redundant, overlapping function with ATM in phosphorylating H2AX.

Analysis of IR-Induced γ-H2AX Foci in LCLs. In contrast to these results, we have previously reported the lack of H2AX phosphorylation in an A-T LCL after exposure to IR (24). In initial experiments, we observed, as had been seen previously, the lack of γ-H2AX foci formation in A-T LCLs (24). These experiments were carried out with LCLs that had grown to saturation after several days of growth without medium change. To examine whether this might be a factor influencing the response, we monitored IR-induced γ-H2AX formation in two A-T LCLs replenished by the addition of fresh medium postirradiation in parallel to cells maintained in growth saturating conditions. No significant IR-induced H2AX phosphorylation was observed in the two A-T LCLs [AO (ATM−/−) and LB541 (ATM−/−)] that were maintained in their original medium postirradiation (Fig. 3A). In contrast, efficient γ-H2AX formation similar to that observed in control cells was observed in both A-T LCLs when the medium was changed immediately after irradiation (Fig. 3A). The addition of LY294002 inhibited this H2AX phosphorylation. To examine whether DNA-PK is down-regulated in growth-saturated LCLs, we monitored DNA-PK activity in extracts prepared under both conditions (Fig. 3B). Although the level of DNA-PK activity was slightly lower when held under nongrowing conditions, the difference was small and unlikely to explain the dramatic impact on H2AX phosphorylation. Thus, the inability of DNA-PK to phosphorylate...
H2AX in growth-saturated LCLs cannot be explained simply by down-regulation of DNA-PK levels or activity.

To verify whether proliferation status might influence IR-induced H2AX phosphorylation in primary fibroblasts, we compared H9253-H2AX foci formation in primary fibroblasts that were actively growing with fibroblasts maintained in plateau phase for 2 weeks. H9253-H2AX foci formation occurred to a similar extent in all samples (Fig. 3C).

Taken together, we conclude that ATM and DNA-PK can phosphorylate H2AX in a redundant, overlapping manner in response to DSBs. However, under certain growth conditions (e.g., growth-inhibited LCLs), DNA-PK does not appear to play a major role.

ATM and DNA-PK Contribute to IR-Induced H2AX Phosphorylation in Chicken Cells. To substantiate that it is DNA-PK rather than another phosphatidylinositol 3'-kinase inhibited by LY294002 that overlaps with ATM in H2AX phosphorylation, we sought to examine cells lacking both DNA-PK and ATM and exploited chicken DT40 cells because double mutant cell lines lacking Ku70 and ATM have been described previously (30). Because DNA-PK activation requires Ku binding to DNA ends, Ku70 mutants are devoid of DNA-PK activity. We therefore examined foci formation in ATM−/−, Ku70−/−, and ATM−/−Ku70−/− cells at 2 h posttreatment with 1 and 2 Gy of X-rays in the presence and absence of the drug LY294002 using human anti-phospho-H2AX antibody. By Western blotting, we verified that human anti-H2AX antibodies cross-react with chicken H2AX in a manner inducible by IR (Fig. 4A). To allow a quantitative examination of H2AX phosphorylation, we examined foci formation as carried out with human and mouse cells (Fig. 4B). All three chicken cell lines had a high background of H9253-H2AX foci relative to that observed with mammalian cells, which we attribute to ATR-dependent events (Fig. 4B). After irradiation, there was an increase in the number of cells with >5 foci/cell in the ATM−/− and Ku70−/− single mutant cells that was not observed in the ATM−/−Ku70−/− double mutants. The slightly greater increase in γ-H2AX foci in the Ku70−/− cells relative to ATM−/− cells possibly reflects the inability of Ku-defective cells to repair their DSBs relative to ATM−/− cells during the 2-h incubation period. Furthermore, LY294002 inhibited the radiation-induced γ-H2AX foci formation in ATM−/−, Ku70−/−, and ATM−/−Ku70−/− cells (Fig. 4A).

Fig. 3. Ataxia telangiectasia (A-T) lymphoblastoid cell lines (LCLs) form H2AX foci normally if growth is stimulated after irradiation; they fail to form foci if they are maintained under growth-saturating conditions. A, control (Sweig) and two A-T (AO and LB541) LCLs were grown to near saturation and exposed to 10 Gy of γ-rays. LY294002 was added 30 min before irradiation. After irradiation, cells were either maintained in their original medium (with or without LY294002) or centrifuged and placed in fresh medium (with or without LY294002). Cells were immunostained for γ-H2AX (right panels) and counterstained with 4',6-diamidino-2-phenylindole (left panels). Unirradiated cells were also treated with LY294002, and the endogenous level of foci was identical to that observed in untreated cells (data not shown). It is more difficult to quantify foci formation in LCLs as compared with fibroblasts, but the H2AX phosphorylation is readily visible by the clear elevated fluorescent signal. B, DNA-dependent protein kinase activity present in extracts obtained from cells used in A. DNA-dependent protein kinase was pulled down using DNA cellulose beads, and the kinase activity was measured using a p53-derived peptide as substrate. The results represent the average of two experiments. The counts produced by the control (peptide alone) were subtracted from the results. C, examination of H2AX phosphorylation in growing and plateau phase-arrested human fibroblasts. Human control (1BR3) and A-T (AT7BI) fibroblasts were either maintained under actively growing conditions or grown to and maintained in plateau phase for 2 weeks before irradiation and scoring of γ-H2AX foci. Numbers represent the number of foci/cell.
diation-induced γ-H2AX foci in ATM−/− cells but not in the cell line lacking Ku, consistent with our conclusion that the impact of LY294002 on γ-H2AX foci formation is due to its inhibition of DNA-PK. Taken together, we conclude that ATM and DNA-PK jointly contribute to H2AX phosphorylation in vertebrate cells.

p53 and Rad17 Phosphorylation Occurs Normally in M059J Cells. Recently, we have shown that BRCA1 is required for a subset of ATM and ATR phosphorylation events but is dispensable for both H2AX and Rad17 phosphorylation (38). Because Rad17 and H2AX are located on the chromatin, we proposed that BRCA1 might be required for ATM-dependent phosphorylation of non-histone-bound downstream substrates. Although previous studies have shown convincingly that DNA-PK is not required for p53 phosphorylation, the role of DNA-PK in phosphorylating chromatin-bound substrates has not been studied (17). The overlapping role of DNA-PK in phosphorylating H2AX, therefore, prompted us to examine its ability to phosphorylate Rad17. We observed normal phosphorylation of Rad17 and p53 in M059J (DNA-PKcs−/−) and M059K (DNA-PKcs+/+) cells (Fig. 5). These findings are consistent with the dependency of Rad17 and p53 phosphorylation on ATM (39–41) and demonstrate that whereas DNA-PK can phosphorylate H2AX, it is not able to phosphorylate these other components of the signaling pathway. It is worth noting that previous studies have reported that M059J cells have low ATM activity (42). The present results show that the level of ATM in M059J is sufficient to efficiently phosphorylate H2AX, Rad17, and p53.

DNA-PK Can Impact on the Presence of Damage Response Proteins at DSBs. In addition to H2AX, other proteins, including the MRE11/RAD50/NBS1 complex, MDC1, and 53BP1 accumulate in foci at the sites of DSBs (3–6, 20, 43–45). Recent evidence has demonstrated that phosphorylated H2AX is required for the initial recruitment of these proteins but is required for their retention as foci (22). By 60 min post-IR, foci formation of these proteins appears to be ATM independent but γ-H2AX dependent (22). To examine the contribution of DNA-PK to this process, we examined the formation of MDC1 and 53BP1 foci at 1 h post-IR in 1BR3, AT1BR, M059J, and M059K cells. In control cells, both 53BP1 and MDC1 changed from diffuse nuclear staining with the occasional (1–3) large foci in the absence of irradiation to abundant small nuclear foci after radiation exposure. 53BP1 and MDC1 foci formed normally in M059J and AT1BR but were abolished in drug-treated AT1BR (Fig. 6). These findings consolidate previous reports that retention of these two proteins as foci post-irradiation is ATM independent (22). Our results suggest that this is because there is DNA-PK-dependent H2AX phosphorylation, thereby demonstrating that DNA-PK can contribute to the retention of these complexes at the sites of DSBs in the absence of ATM.

DISCUSSION

To gain insight into the early events initiated by DNA damage, we examined which phosphatidylinositol 3′-kinase contributes to H2AX phosphorylation after exposure to IR. Because γ-H2AX foci are required for the stable formation of NBS1, 53BP1, MDC1, and BRCA1 foci at the damage sites, this represents an important step that determines subsequent events in the signal transduction pathway (3, 6, 18, 20–22).

Firstly, we show that inactivation of ATM and a second phosphatidylinositol 3′-kinase that is inhibited by the drug LY294002 is required to ablate IR-induced H2AX phosphorylation. Consistent with reported data that LY294002 is a specific DNA-PK inhibitor, we show that in vivo DNA-PK is inhibited by LY294002, whereas both ATM and ATR remain active. We substantiate the notion that DNA-PK is the inhibited kinase by using the DT40 model system to examine chicken cells lacking both ATM and DNA-PK activity. Thus, we conclude that ATM and DNA-PK function redundantly to phosphor-

![Fig. 5. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is dispensable for p53 and Rad17 phosphorylation. Control (M059K) and DNA-PKcs-deficient (M059J) cells were exposed to 10 Gy of γ-rays and examined for immunofluorescence using phospho-specific anti-p53Ser15 and anti-Rad17Ser645 antibodies 2 h postirradiation. 4′,6-Diamidino-2-phenylindole staining showed the presence of nuclei in all fields (data not shown). Phosphorylation of both proteins occurred normally in M059J cells, demonstrating that DNA-PKcs does not contribute to these phosphorylation events and that ATM is active in M059J cells.](image-url)
Phosphorylation was similar to that observed in DNA-PKcs. We observed a substantial increase after IR treatment, and the induced level of background level of foci was elevated in M059J cells, we observed a slightly after IR (18). In our experiments using 2 Gy, although the DNA-PKcs activity (42). However, nonproliferating (plateau phase) A-T human fibroblasts demonstrate which protein phosphorylates H2AX when both proteins are present. Interestingly, DSBs undergo repair and initiate ATM-dependent phosphorylation events concomitantly, indicating that the DNA-PK complex and ATM are both localized to or activated by the double-stranded DNA ends noncompetitively. The slightly slower kinetics of induction of γ-H2AX foci in ATM-deficient human and mouse cells indicates that ATM may play the dominant role, at least at early times postirradiation. Additionally, we observed ATM-dependent H2AX phosphorylation in growth-saturated A-T LCLs. However, nonproliferating (plateau phase) A-T human fibroblasts phosphorylate H2AX efficiently, suggesting that this observation is not linked to proliferation per se. DNA-PK was not markedly down-regulated under these conditions, suggesting that there may be factors influencing its access to H2AX that remain to be elucidated. Nonetheless, this was only one of many conditions examined where H2AX phosphorylation was found to be specifically ATM dependent. Thus, we conclude that under most normal conditions (actively growing and plateau phase human fibroblasts, growing MEFs and LCLs, and in chicken cells), the two kinases function redundantly, although there are kinetic and growth conditions where ATM predominates.

Two previous studies have addressed which phosphatidylinositol 3'-kinase-like kinase is responsible for IR-induced H2AX phosphorylation (18, 23). Our findings disagree with the study of Burma et al. (23), which concluded that ATM is the responsible kinase. It is possible that the growth status of the cells could explain this result. Nonetheless, our findings show clearly that DNA-PK can contribute to H2AX phosphorylation after IR. The other study reported a very high background of γ-H2AX foci in M059J cells that increased only slightly after IR (18). In our experiments using 2 Gy, although the background level of foci was elevated in M059J cells, we observed a substantial increase after IR treatment, and the induced level of phosphorylation was similar to that observed in DNA-PKcs−/− M059K cells. It has been shown previously that M059J cells carry a mutational change in ATM and have reduced ATM as well as DNA-PKcs activity (42). However, here we observed efficient Rad17 and p53 phosphorylation (Fig. 5), indicating that ATM is functional and capable of efficiently phosphorylating its substrates. Nonetheless, certain conditions might facilitate exposure of the dual phosphatidylinositol 3'-kinase-like kinase requirement in M059J. Paull et al. (18) used two-dimensional gel analysis to examine H2AX phosphorylation and observed decreased phosphorylation in M059J cells (18). This procedure required high doses of IR, and it is possible that the reduced ATM activity in M059J cells could not fully achieve the extensive phosphorylation required, thereby exposing a role for DNA-PK. Although our results are substantially different, our conclusion is similar: namely that ATM and DNA-PK both contribute to H2AX phosphorylation in response to DSBs. Furthermore, the fact that H2AX phosphorylation is reduced after high radiation doses in M059J cells supports our contention that DNA-PK can contribute to H2AX phosphorylation. We also show that ATR does not contribute to IR-induced H2AX phosphorylation in primary fibroblasts, at least over the time scale examined. It is likely, however, that at longer times postirradiation after replication of IR-induced lesions, a contribution of ATR may occur. Our findings do not eliminate this possibility but do show, importantly, that ATR is not activated by DSBs in G1-phase cells.

Activation of ATM by DSBs results in phosphorylation of substrates further downstream that include Chk2, NBS1, CtIP, BLM, and p53. None of these events are observed in A-T cell lines, demonstrating that DNA-PK does not contribute significantly to this aspect of the checkpoint response. Consistent with this, it has been shown that DNA-PKcs-defective cells show checkpoint arrest and p53 phosphorylation (17). We show that Rad17, which, like H2AX, is located on the chromatin, is also efficiently phosphorylated in DNA-PK-defective cells but not in A-T cells. The fact that DNA-PK phosphorylates H2AX but not other substrates demonstrates the constraints of phosphorylation events in vivo. More recently, another aspect of the DNA damage response has been described in which the role of ATM appears to be redundant, namely, the recruitment of proteins that form as foci that overlap with γ-H2AX foci (4, 6, 21, 22). The proteins that fall into this class include MCD1, 53BP1, MRE11/RAD50/NBS1, and BRCA1. We show that at 1 h post-IR treatment, MDC1 and 53BP1 foci are not visible when both DNA-PK and ATM activities are inhibited but are visible in cells lacking either DNA-PK or ATM alone. Because this correlates with the abolition of γ-H2AX foci...
formation, this is consistent with the model that retention of these proteins as foci is dependent on H2AX phosphorylation but not ATM activity. Interestingly, a recent study has suggested that H2AX is not required for the recruitment of these proteins to the DSB but is required for their retention (22). Our findings, therefore, provide evidence that DNA-PK plays a redundant, overlapping role with ATM in the retention of these proteins as foci at the damage site by contributing to H2AX phosphorylation.

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