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DNA-PK-Dependent G₂ Checkpoint Revealed Following Knockdown of ATM in Human Mammary Epithelial Cells

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Abstract

Members of the phosphatidylinositol 3-kinase related kinase (PIKK) family, in particular the ataxia-telangiectasia mutated (ATM) kinase and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), regulate cellular responses to DNA double strand breaks (DSBs). Increased sensitivity to ionizing radiation (IR) in DNA-PKcs or ATM deficient cells emphasizes their important roles in maintaining genome stability. Furthermore, combined knockout of both kinases is synthetically lethal, suggesting functional complementarity. In the current study, using human mammary epithelial cells with ATM levels stably knocked down by >90%, we observed an IR-induced G₂ checkpoint that was only slightly attenuated. In marked contrast, this G₂ checkpoint was significantly attenuated with either DNA-PK inhibitor treatment or RNAi knockdown of DNA-PKcs, the catalytic subunit of DNA-PK, indicating that DNA-PK contributes to the G₂ checkpoint in these cells. Furthermore, in agreement with the checkpoint attenuation, DNA-PK inhibition in ATM-knockdown cells resulted in reduced signaling of the checkpoint kinase CHK1 as evidenced by reduced CHK1 phosphorylation. Taken together these results demonstrate a DNA-PK-dependent component to the IR-induced G₂ checkpoint in addition to the well-defined ATM-dependent component. This may have important implications for chemotherapeutic strategies for breast cancers.

Introduction

Cellular responses to DNA double strand breaks (DSBs) rely heavily upon three members of the phosphatidylinositol 3-kinase related kinase (PIKK) family, the ataxia-telangiectasia (A-T) mutated kinase (ATM), the AT and Rad3-related kinase (ATR), and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). While ATM and ATR act as initiators of DNA damage checkpoint signaling (1), DNA-PKcs has a critical role in DSB repair via nonhomologous end-joining (NHEJ) (2). The importance of each of these proteins in maintaining genomic stability is underscored by the increased sensitivity to DNA damage observed with reduced activity of any one of these kinases (3-5).

In response to DSBs, ATM and ATR halt cell cycle progression through activation of checkpoint signaling. The canonical model of DNA damage checkpoint activation involves ATM and ATR initiating two distinct signaling pathways dependent on the type of DNA lesion present (6). After ionizing radiation (IR)-induced DSBs, ATM signals through CHK2 to initiate a cell cycle checkpoint. Alternatively, in response to DSBs associated with replication forks, ATR signaling through CHK1 is the predominant checkpoint signaling pathway. However, a

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growing body of evidence indicates overlap and crosstalk between these signaling pathways (7-11)

DNA-PKcs, through its kinase activity and as a scaffolding protein at the DSB site, contributes to the process of NHEJ, the major pathway for repair of DSBs in mammals (2). The essential NHEJ components include DNA-PK (composed of the heterodimeric Ku70/80 subunit and DNA-PKcs), DNA ligase IV, Artemis, and XRCC4 (12). Upon damage, Ku70/80 is believed to bind DNA and recruit DNA-PKcs to the DSB, consequently stimulating its kinase activity (2). The endonuclease Artemis is involved in DNA end processing, and NHEJ is completed with the joining of DNA ends by XRCC4 and DNA ligase IV (2). Studies correlating DNA-PK activity and drug sensitivity suggest that DNA-PK may contribute to radio- and chemo-resistance of tumor cells (12). In light of this, DNA-PK inhibition is being studied as a means to modulate resistance to standard cancer treatments (12).

Similar to the coordinated crosstalk and overlapping activities of ATM and ATR, there is some evidence of functional complementarity between ATM and DNA-PK. This complementarity is strongly indicated by the observation that mice deficient in ATM and DNA-PKcs are embryonic lethal (13). Additionally, there is some substrate redundancy in the kinase activities of DNA-PK and ATM, such as the phosphorylations of H2AX, RPA, and even DNA-PKcs itself after DNA damage (14-17). However, in spite of these observations, the extent of functional overlap between ATM and DNA-PK and whether DNA-PK plays a role in cell cycle checkpoint functions remains unclear. In the present study we used RNA interference to create and characterize several novel human mammary epithelial cell lines with reduced ATM levels that are isogenic with the parental lines. Surprisingly, using these cells with reduced amounts of ATM, we identified a DNA-PK-dependent, ATM-independent component of the IR-induced G₂ cell cycle checkpoint. To our knowledge, this is the first evidence showing a role for DNA-PK in activating DNA damage-induced G₂ checkpoint signaling. This observation may have significance in the development of new chemotherapeutic approaches.

Materials and Methods

Chemicals and Reagents

Nocodazole was purchased from Sigma-Aldrich (St. Louis, MO) and resuspended in DMSO at a concentration of 1 mg/ml. Stock solutions were stored at -20° C. Interferon α and calyculin A were purchased from Sigma-Aldrich (St. Louis, MO). Calyculin A was resuspended in DMSO and stored at -20° C. Blasticidin was purchased from Invitrogen (Carlsbad, CA), resuspended in water, and stored at -20° C. Bleomycin, wortmannin, and NU7026 were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

The immortalized human mammary epithelial cell line hTERT184 was derived from the normal human mammary epithelial 184 cell line (18,19) and was a kind gift from J. Carl Barrett (NCI, Bethesda, MD). Cells were grown in defined MEGM media (Cambrex, Rockland, ME) supplemented with 5 μ g/ml transferrin (Sigma, St. Louis, MO), 10⁻⁵ M isoproterenol (Sigma, St. Louis, MO), and 250 μ g/ml geneticin (Invitrogen, Carlsbad, CA) in a humidified 2% CO₂ incubator. Two additional hTERT immortalized human mammary epithelial cell lines, ME16C and HME-CC, were a kind gift from Dr. Charles Perou and were grown as described (20).

Clonogenic Assays

Exponentially growing cells were allowed to attach overnight before being exposed to either γ -radiation using a ¹³⁷Cs source or UV radiation using a UV Stratalinker 1800 (Stratagene, La

Jolla, CA). After 10 days, cells were stained with 0.1% coomassie blue, 5% acetic acid, and 30% methanol. Colonies containing greater than 100 cells were counted. Cell fraction surviving treatment was normalized to survival of control cells.

Western blotting

Treated or control cells were harvested by scraping into 2X SDS-PAGE Sample Buffer (62.5 mM Tris-HCL (pH = 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue), sonicated for 15 sec, and heated at 99° C for 5-10 min. Aliquots representing equal amounts of protein from each lysate were separated on a 6%, 7.5%, or 10% SDS-PAGE gel and analyzed by western blot analysis. Antibodies to phospho-CBK1 (Ser345), phospho-CBK2 (Thr68), PKR, phospho-PKR (Thr451), EIF2 α , and phospho-EIF2 α (Ser51) were from Cell Signaling Technology (Beverly, MA). Antibodies to CBK1, CBK2, and DNA-PK were from Stressgen (San Diego, CA). The antibody to phospho-ATM (Ser1981) was from Rockland Immunochemicals (Gilbertsville, PA). The antiserum to total ATM was generated in our laboratory (21); alternatively, we also used anti-ATM purchased from Bethyl (Montgomery, TX). The antibody to phospho-DNA-PKcs (Thr2609) was from Abcam (Cambridge, MA). Equivalent loading and protein transfer were confirmed by Ponceau stain and western blot using either a β -actin antibody (Sigma, St. Louis, MO) or a β -tubulin antibody (Sigma, St. Louis, MO). Primary antibodies were detected using a peroxidase-conjugated secondary antibody and enhanced chemoluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Quantitation of bands in Western blots was performed using ImageQuant TL v.2005 software (GE Healthcare).

Phosphorylated Histone H3 Immunostaining for Mitotic Fraction

Cells were harvested and fixed in 70% ethanol and stored at -20° C. For analysis, cells were immunostained using a mouse phospho-histone H3 (Ser10) monoclonal antibody (Cell Signaling, Beverly, MA) followed by FITC-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The mitotic percentage of treated samples was calculated as percentage of control. Mean mitotic percentages were then determined for 2 or 3 independent experiments.

RNA Interference and Lentiviral Infection

The BLOCK-IT Lentiviral RNAi Expression System (Invitrogen, Carlsbad, CA) was used to create stable ATM knockdown hTERT184 cells. The following target sequences were used to express double-stranded oligonucleotides encoding ATM shRNAs (note: the first three sequences were obtained using the Invitrogen website and the last sequence was obtained from Dharmacon and initially screened using siRNA):

#1 5'GCATTGAGATTCCAAACAAGGCGAACCTTGTGTTGGAATCTGAATGC 3'

#2 5'GCAACCCAATTAATATCAAAGCGAACTTTGATATTAATTGGGTTGC 3'

#4 5'GCAAGCAGCTGAAACAAATAACGAATTATTTGTTTCAGCTGCTTGC 3'

#5 5'GCAAGCAGCTGAAACAAATTTGCGAAAAATTTGTTTCAGCTGCTTGC 3'

The following lacZ target sequence was used as a control oligo:

5'GCTACACAAATCAGCGATTTGCGAAAAATCGCTGATTTGTGTAC 3'

Viral supernatants were generated by transfecting 293FT cells with 6 μ g total DNA (4.5 μ g packaging mix + 1.5 μ g DNA) using Eugene 6 reagent (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's instructions. After 48 h, supernatant was collected and used to infect mammary epithelial cells. Cells were infected in the presence of polybrene (6 μ g/ml) (Sigma-Aldrich, St. Louis, MO) for 24 h, then media was changed and cells were

allowed to grow for an additional 24 h. Pooled and clone populations were then selected using 4 µg/ml of blasticidin.

For experiments in which either ATM or DNA-PKcs was knocked down transiently, we used siRNAs from Ambion targeting ATM (Cat. # 16804, siRNA ID # 1309) or DNA-PK (Cat. # 51331, siRNA ID # 844) (Austin, TX). As a negative control, a non-targeting siRNA was used (siCONTROL non-targeting siRNA #1, Dharmacon, Lafayette, CO). Cells were seeded overnight and then transfected with siRNA for 48 h using the TransIT-TKO reagent according to the manufacturer's instructions (Mirus Bio Corp., Madison, WI). Cells were then treated with 2 Gy IR, incubated for 2 h, and harvested for analysis.

Statistical analysis

Statistical analyses were performed with Student's t-test using StatView software version 5.0 (SAS Institute Inc., Cary, NC). Two-side p values < 0.05 were considered statistically significant.

Results

Human mammary epithelial cells expressing reduced levels of ATM are sensitized to IR and bleomycin sulfate and have an attenuated IR-induced G₂ checkpoint

As a first step in examining the DNA damage response in human mammary epithelial cells, we ascertained the survival response of hTERT184 cells to IR using a clonogenic assay. This allowed us to identify a suitable dose for subsequent analyses (Figure 1A). We also examined DNA damage signaling pathways activated after IR in hTERT184 cells. The introduction of DNA double strand breaks leads to autophosphorylation of ATM on serine 1981 and ATM activation (22). ATM can then phosphorylate numerous targets, including p53 and CHK2, and initiate cell cycle arrest (23). As expected, ATM was phosphorylated in response to IR, as were the known ATM targets p53 and CHK2, on serine 15 and threonine 68, respectively (data not shown).

After initially using siRNA to transiently reduce ATM protein levels, we sought to increase the knockdown efficiency by creating hTERT184 cell lines stably expressing reduced levels of ATM. Therefore, we generated lentiviral shRNA constructs encoding four different ATM-targeting shRNAs. ATM mRNA (data not shown) and protein levels were found to be reduced by TaqMan analysis and Western blot analysis, respectively. When expressed using lentivirus, three of these sequences were equally effective in knocking down ATM protein (Figure 1D). The ATM protein levels were reduced in the cell lines expressing ATMshRNA#1 (hTERT184-ATM1), ATMshRNA#2 (hTERT184-ATM2), ATMshRNA#4 (hTERT184-ATM4) and ATMshRNA#5 (hTERT184-ATM5) to 6%, 19%, 3%, and 5%, respectively, of the levels in the control cell line (hTERT184-lacZ). In addition, we found no evidence for activation of the interferon response after shRNA expression (24), as measured by PKR and EIF-2 α phosphorylation (Supplemental Figure 1). Taken together, these data strongly suggest that the lentiviral shRNA sequences were specific for ATM and that the reduced expression of ATM was not due to non-specific anti-viral responses.

A-T cells are characterized by hypersensitivity to agents such as IR and radiomimetic drugs (25). Likewise, ATM knockdown hTERT184 cells were sensitized to both IR (Figure 1A) and bleomycin sulfate (Figure 1B). Clones isolated from the hTERT184-ATM1 knockdown line were sensitized to IR to the same degree as the pooled population (data not shown). Importantly, ATM deficient cells were not sensitized to killing by UV irradiation, a DNA damaging agent that generates DNA lesions that are believed to signal predominately through ATR (Figure 1C).

In addition to radiosensitization, we and others have shown that cells lacking ATM have a defective IR-induced G₂ checkpoint (26,27). Histone H3 is phosphorylated early in mitosis and therefore an antibody against phosphorylated histone H3 (P-H3) can be used to assess the IR-induced G₂ checkpoint delay of entry into mitosis (28). As a positive control, we initially treated cells with nocodazole, an agent known to arrest cells in M phase. Cells treated with 0.2 µg/ml nocodazole for 21 h displayed a substantial increase in P-H3 staining (Supplemental Figure 2). Treatment of parental and lacZ hTERT184 cells with a sub-lethal dose of 2 Gy IR (Figure 1A) induced a robust G₂ checkpoint as seen by a dramatic reduction in P-H3 positive cells 2 h after IR exposure (Figure 2A and Supplemental Figure 2). Surprisingly, treatment of ATM knockdown hTERT184 cells with 2 Gy IR resulted in only a minor attenuation of the G₂ checkpoint (Figure 2A). These results suggest that the G₂ checkpoint seen following exposure to 2 Gy IR has an ATM-independent component.

Knockdown of ATM in human mammary epithelial cells diminishes IR-induced CHK2 phosphorylation but does not affect CHK1 phosphorylation

To investigate the DNA damage-induced signaling that resulted in a strong G₂ checkpoint response in spite of severely reduced levels of ATM (~5% of control levels), we looked at the activation of the downstream effectors, CHK1 and CHK2. Examination of checkpoint signaling in hTERT184-ATM5 cells showed greatly diminished phosphorylation of CHK2 following IR exposure, as expected from the reduction of ATM levels (Figure 2B). Furthermore, we found CHK2 to be slightly phosphorylated after UV and this phosphorylation did not significantly change after knockdown of ATM (Figure 2B). This observation of CHK2 phosphorylation despite severely reduced levels of ATM is in agreement with previous studies showing that CHK2 can be phosphorylated independent of ATM at high doses of IR, UV, or hydroxyurea (29).

Recent studies have shown that ATM can act upstream of ATR to induce CHK1 phosphorylation after IR (7,9,10). ATM and meiotic recombination 11 (MRE11) were shown to be required for ATR recruitment to sites of DNA damage (7,9,10). Jazayeri and colleagues further showed that the exonuclease activity of MRE11 was responsible for processing of DNA double strand breaks to replication protein A (RPA)-coated single strand DNA leading to ATR recruitment and CHK1 phosphorylation (9).

From this model, one would predict that loss of ATM should compromise IR-induced phosphorylation of CHK1. We assessed the induction of CHK1 phosphorylation in hTERT184 parental, hTERT184-lacZ control, and hTERT184-ATM5 knockdown cells after IR treatment. IR-induced levels of CHK1 phosphorylation at serine 345 in the ATM knockdown cells were not substantially different from control hTERT184-lacZ cells (Figure 2B). This again suggested that the G₂ DNA damage checkpoint response observed despite reduced levels of ATM (Figure 2A), was mediated in an ATM-independent manner, likely involving CHK1 signaling. As expected, treatment of cells with UV radiation caused robust and ATM-independent induction of CHK1 phosphorylation in all three cell lines (Figure 2B).

Transient transfection of human mammary epithelial cells containing reduced levels of ATM with ATM siRNA only slightly attenuates the IR-induced G₂ checkpoint

The two ATM knockdown hTERT184 cell lines (hTERT184-ATM1 and hTERT184-ATM5) that were screened for radiosensitization and G₂ checkpoint attenuation displayed a similar phenotype, strongly suggesting these effects were due to specific loss of ATM and not due to an off-target effect. However, since only a minor attenuation of the G₂ checkpoint was observed, it was possible that residual amounts of ATM were still present in the knockdown lines, providing sufficient DNA damage response signaling after 2 Gy IR to elicit a G₂ arrest. To address this, we transiently transfected both the hTERT184-lacZ line and the hTERT184-

ATM5 knockdown line with siRNA against ATM in an attempt to further reduce ATM levels in the hTERT184-ATM5 cell line. The sequence of this siRNA was different from the original sequences used in the lentiviral constructs. As shown in Figure 3A, this siRNA was effective at knocking down ATM in the control hTERT184-lacZ cells to approximately 25% of the starting protein levels. In contrast, even after a long exposure, ATM in the hTERT184-ATM5 stable line is essentially undetectable. We did find levels of phosphorylated CHK2 to be slightly further reduced in the “double knockdown” from the already reduced levels after IR treatment observed in the hTERT184-ATM5 knockdown line alone (Figure 3A). In contrast to the hTERT184-ATM5 stable knockdown line with significantly reduced levels of ATM and IR-induced CHK2 phosphorylation, transient transfection of the control hTERT184-lacZ line did not alter IR-induced CHK2 phosphorylation, presumably because enough ATM was still present to phosphorylate CHK2 (Figure 3A - compare ATM levels in hTERT184-lacZ ATM siRNA treated lane with hTERT184-ATM5 ATM siRNA treated lane).

We performed flow cytometry analysis to assess the IR-induced G₂ checkpoint after transiently knocking down ATM. Transient knockdown of ATM in the control hTERT184-lacZ line did not affect the IR-induced G₂ checkpoint (Figure 3B). Again, this was likely due to residual levels of ATM persisting after transient knockdown and failing to reach a “threshold” level for noticeable reduction in ATM function. Transient transfection of ATM siRNA into the ATM knockdown line hTERT184-ATM5 slightly increased the attenuation of the IR-induced G₂ checkpoint (Figure 3B). Although we cannot entirely eliminate the possibility that a very small amount of ATM was still present in the “double knockdown”, these results together with the results above strongly support activation of an ATM-independent aspect of the G₂ checkpoint following exposure of these mammary epithelial cells to IR.

Pre-treatment of human mammary epithelial cells with wortmannin attenuates the IR-induced G₂ checkpoint

To gain insight into the mechanism of the IR-induced G₂ checkpoint in human mammary epithelial cells with severely reduced ATM function, we treated cells with the PIKK inhibitor wortmannin. At the concentrations used, wortmannin has stronger inhibitory activity against ATM and DNA-PK than against ATR (30). Pretreatment of both hTERT184-lacZ cells and hTERT184-ATM5 cells with 5 μ M wortmannin resulted in an attenuation of the IR-induced G₂ checkpoint (Figure 4A). Furthermore, pretreatment with 20 μ M wortmannin resulted in strong attenuation of the IR-induced G₂ checkpoint in both the hTERT184-lacZ and hTERT184-ATM5 cell lines. These results are consistent with wortmannin inhibiting both ATM and DNA-PK and suggested that DNA-PK may be involved in the IR-induced G₂ checkpoint in human mammary epithelial cells.

Inhibition or reduced expression of DNA-PK attenuates the IR-induced G₂ checkpoint in human mammary epithelial cells containing reduced levels of ATM

To investigate more specifically the potential involvement of DNA-PK in the G₂ checkpoint or whether the wortmannin effect on the G₂ checkpoint was due simply to further inhibition of ATM, we used the competitive and highly specific inhibitor of DNA-PK, NU7026. NU7026 has a 60-fold greater potency against DNA-PK versus PI3-kinase and is virtually inactive against ATM and ATR (31). To verify that NU7026 was not inhibiting ATR, we pretreated cells with 25 μ M NU7026 before UV exposure. We found that NU7026 did not substantially inhibit UV-induced ATR-dependent phosphorylation of CHK1 (Supplemental Figure 3A). Furthermore, pretreatment with 25 μ M NU7026 did not affect IR-induced ATM autophosphorylation (Supplemental Figure 3B), strongly suggesting that ATM was not a target of NU7026. As shown in Figure 4B, pretreatment of both hTERT184-lacZ and hTERT184-ATM5 cells with 10 μ M NU7026 had no effect on attenuation of the IR-induced G₂ checkpoint. In contrast, whereas pretreatment with 25 μ M NU7026 did not significantly affect the G₂

checkpoint of hTERT184-lacZ cells, pretreating hTERT184-ATM5 cells with 25 μ M NU7026 dramatically attenuated the IR-induced G₂ checkpoint (Figure 4B). These data, taken together with the wortmannin data, suggest that in human mammary epithelial cells, IR treatment activates both an ATM-dependent pathway and a pathway that involves DNA-PK.

The involvement of DNA-PK in the G₂ checkpoint damage response to IR was further confirmed with RNAi knockdown of DNA-PKs. Transient transfection of the hTERT184-lacZ and hTERT184-ATM5 cell lines with siRNA targeting DNA-PKs resulted in a substantial decrease in DNA-PKs protein levels to approximately 20% and 45% of the untreated levels, respectively (Figure 4C). Knockdown of DNA-PKs in the hTERT184-lacZ line did not substantially affect the IR-induced G₂ checkpoint (Figure 4D). In contrast, knockdown of DNA-PKs in hTERT184-ATM5 cells was accompanied by an approximately 2.5 fold increase in the attenuation of the IR-induced G₂ checkpoint (Figure 4D).

To address whether this involvement of DNA-PK in the G₂ checkpoint was a characteristic of human mammary epithelial cells in general and not unique to the hTERT184 cells, we created stable ATM knockdown cell lines as before using two independent hTERT immortalized human mammary epithelial cell lines, ME16C and HME-CC (20). As shown in Figure 5A, ATM protein levels were significantly reduced in both cell lines following stable expression of ATMshRNA#1, ATMshRNA#4, and ATMshRNA#5, and to a lesser extent, ATMshRNA#2, as was seen with the hTERT184 cells (Figure 1D). To assure that our results were not unique to the hTERT184-ATM5 line, we analyzed the G₂ checkpoint response to DNA damage in the ME16C and HME-CC cells expressing the ATMshRNA#1 (ME16C-ATM1 and HME-CC-ATM1, respectively). As seen in Figure 5B and 5C, respectively, the HME-CC-ATM1 and the ME16C-ATM1 cells show a strong G₂ checkpoint arrest following treatment with IR that was only slightly attenuated relative to their lacZ control cells. However, treatment with the potent DNA-PK inhibitor NU7026 caused a release from the G₂ checkpoint in a manner similar to that seen in the hTERT184-ATM5 cells (Figure 5B and 5C).

Inhibition of DNA-PK in human mammary epithelial cells with reduced ATM levels results in reduced IR-induced CHK1 phosphorylation

The molecular signaling mechanisms to IR-induced DNA damage were investigated with all three mammary epithelial cell lines. In all three cell lines expressing the LacZ control, ATM signaling was activated following IR, as reflected by phosphorylation of the ATM substrate CHK2 (Figures 6A, B, and C). This phosphorylation of CHK2 was not inhibited by the DNA-PK inhibitor NU7026.

Knockdown of ATM levels in both the hTERT184-ATM5 and the HME-CC-ATM1 cells (Figures 6 A and B) resulted in reduced phosphorylation of CHK2 following IR-treatment, supporting the conclusion that ATM function was significantly reduced in these cells. In contrast, the ME16C-ATM1 cells still showed strong CHK2 phosphorylation following IR (Figure 6C), suggesting either that an alternate pathway is phosphorylating CHK2 or that the levels of ATM in these cells is sufficiently high to respond to IR-induced DNA damage.

DNA-PKs, itself, was phosphorylated on Thr2609 following IR-treatment in the hTERT184-lacZ cells, and this phosphorylation increased in the presence of the inhibitor NU7026 (Figure 6A). Thr2609 phosphorylation was also observed, but to a lesser extent in hTERT184-ATM5 cells. With treatment of NU7026 phosphorylation at this site was undetectable in hTERT184-ATM5 cells. This indicates both ATM-dependent and DNA-PK-dependent contributions to Thr2609 phosphorylation, consistent with previously published results showing that this particular residue can be phosphorylated by ATM and DNA-PK (16, 17).

We then investigated the phosphorylation of the DNA damage effector kinase CHK1 in response to IR-induced damage in all three cell lines. In each cell line, when ATM was present at endogenous levels in the LacZ controls, CHK1 phosphorylation was induced by IR treatment (Figure 6A, B, and C). This was independent of the presence of DNA-PK inhibition with NU7026. However, in each ATM-knockdown cell line, the induction of CHK1 phosphorylation by IR was reduced or eliminated when DNA-PK was inhibited with NU7026 (Figure 6A, B and C). Together, the results from Figures 4, 5, and 6 show that DNA-PK inhibition in these ATM-knockdown cells results in attenuation of the G₂ DNA damage checkpoint and reduced signaling through CHK1, suggesting that DNA-PK contributes to the activation of the G₂ DNA damage checkpoint response and that the mechanism of this DNA-PK effect is via CHK1 signaling.

It is interesting to note that in each of the lacZ control cell lines, treatment with NU7026 in combination with IR resulted in greater levels of IR-induced phospho-CHK1 and CHK2 (and phospho-DNA-PKcs in hTERT184-lacZ cells) than was seen when NU7026 was absent. This observation is consistent with a previous report showing enhanced activation of CHK1 and CHK2 after DNA-PK inhibition (32) and may be reflective of interplay between PIKK DNA damage response signaling pathways.

In summary, our data in multiple human mammary epithelial cells lines with reduced ATM levels show a DNA-PK-dependent, IR-induced G₂ checkpoint that involves CHK1 signaling. While this DNA-PK-dependent effect was only evident in lines with reduced ATM, it may be present but obscured by ATM signaling in our lacZ control cell lines. Thus, it appears that the enforcement of the G₂ checkpoint response to IR-induced DNA damage in human mammary epithelial cells is an integrated response of ATM-dependent signaling and ATM-independent, DNA-PK-dependent signaling.

Discussion

We have developed novel, isogenic human mammary epithelial ATM knockdown cell lines that facilitated the discovery of a DNA-PK-dependent and CHK1-mediated component in the IR-induced G₂ checkpoint in these cells. To our knowledge, this is the first report investigating the G₂ DNA damage checkpoint in human mammary epithelial cells with reduced ATM levels.

Our focus on mammary epithelial cell lines was motivated by previous reports suggesting that mutations in the *ATM* gene resulting in reduced ATM expression may contribute to breast cancer (33-36). Thus, we sought to explore the mechanisms by which human mammary cells respond to DNA damage and how reduced ATM levels affect and alter these responses. With respect to clonogenic survival, we found that human mammary epithelial cells expressing reduced levels of ATM are sensitized to IR similar to the sensitization of fibroblasts from individuals with A-T (37). In addition, these cells were also sensitized to bleomycin sulfate but not UV radiation. Much to our surprise, reduced expression of ATM in these mammary epithelial cells caused only a minor defect of the IR-induced G₂ checkpoint after exposure to 2 Gy radiation, less than the attenuation generally observed following a comparable dose exposure of fibroblast or lymphoblast cells from individuals with A-T (26,38).

Modulation of RNA interference pathways to “knockdown” gene expression in cells rarely results in complete loss of the protein of interest. To address the potentially confounding issue of residual ATM expression in our stable ATM knockdown cell lines, we transiently transfected the stable hTERT184-ATM5 and hTERT184-lacZ lines with siRNA targeting ATM, in an effort to reduce any residual amounts of ATM which could contribute to the G₂ checkpoint observed after 2 Gy IR. Transient transfection of ATM siRNA did not attenuate the IR-induced G₂ checkpoint in hTERT184-lacZ cells, probably because ATM levels were only reduced to

approximately 25% of control levels. Transient transfection of ATM siRNA into the hTERT184-ATM5 stable knockdown line only slightly increased the attenuation of the IR-induced G₂ checkpoint. While we cannot exclude the possibility that extremely low levels of ATM contributed to the arrest seen in ATM knockdown cells, we have reduced levels of ATM protein below 10% of control levels and it appears that an additional pathway exists that contributes to the G₂ checkpoint in the presence of reduced levels of ATM in these human mammary epithelial cells.

Cellular responses to DNA damage include not only the activation of cell cycle checkpoints to prevent cell cycle progression but also activation of DNA repair pathways (3). While DNA-PK plays a critical role in double-strand break repair, it also appears to play an active role in apoptosis induction after excessive DNA damage (39). In addition, cells lacking DNA-PK have double strand break repair defects and are sensitized to radiation (5). However, the role or roles DNA-PK may play in cell cycle checkpoint functions remain unresolved.

Our studies using wortmannin suggested that DNA-PK was involved in the IR-induced G₂ checkpoint response in human mammary epithelial cells. Indeed, use of the DNA-PK inhibitor NU7026 compromised the IR-induced G₂ checkpoint in the ATM knockdown cells after 2 Gy IR. A role for DNA-PK in the IR-induced G₂ checkpoint was confirmed using RNAi to reduce the levels of DNA-PK, which resulted in a significant attenuation of the G₂ checkpoint. Our data further show that this DNA-PK-dependent signaling is associated with phosphorylation of CHK1. Although CHK1 has been clearly shown to play an important role in the G₂ checkpoint (40-42) and to interact with DNA-PKcs (43), this is the first demonstration of a DNA-PK-dependent, CHK1-mediated G₂ checkpoint response.

The apparent redundancy of DNA-PK with regards to what was initially regarded as ATM-dependent IR-induced G₂ checkpoint responses is further supported by several reports from *in vivo* mouse studies that provide evidence for ATM and DNA-PK having complementary functions. Gurley and colleagues found that *scid/scid* (mutated gene encoding DNA-PKcs) *ATM*^{+/-} and *scid*^{+/+}, *ATM*^{-/-} embryos developed normally while *scid/scid*, *ATM*^{-/-} embryos died early in embryogenesis, indicating redundant functions and/or synergistic interaction between mutations in these two related kinases (13). Gladdy and colleagues further showed that, in fact, *scid/scid*, *ATM*^{-/-} embryos failed to undergo normal organogenesis and this was due to increased p53-independent apoptosis (44). In addition, Lee and colleagues reported that DNA-PK activity is regulated in a cell cycle-dependent manner and further showed that cells from *scid/scid* mice underwent permanent G₂/M arrest after irradiation (45). In line with these results, inhibition of DNA-PK by DNA-PK-specific inhibitors also caused G₂/M accumulation in response to irradiation and topoisomerase II poisons (46,47) and DNA-PKcs deficient cells were found to accumulate in G₂/M after IR (48). It is interesting that these studies showed that loss of DNA-PK led to G₂/M accumulation in response to DNA damage (45-47). It was concluded that DNA-PKcs deficient cells had an intact IR-induced G₂ checkpoint (48). It is important to note, however, that in our work the DNA-PK-dependent component of the IR-induced G₂ checkpoint was only apparent in cells containing reduced levels of ATM.

The mechanism of how loss of ATM and/or DNA-PK may contribute to breast cancer remains unclear. Inhibition or loss of DNA-PK activity would almost certainly cause the persistence of DNA double strand breaks that would activate ATM-dependent cell cycle checkpoint mechanisms. Loss of ATM may lead to continual cycling and further chromosome aberrations ultimately leading to genomic instability. On the other hand, DNA-PK, in addition to its known role in DNA repair, may be actively involved in DNA damage checkpoint signaling. Loss of both ATM and DNA-PK would compromise direct activation of signaling pathways leading to cell cycle arrest. In addition, it was recently shown that both DNA-PK and ATM can interact with and phosphorylate Artemis, which has been implicated in having a role in the cellular

response to DNA damage (49). These results are intriguing and it is possible that Artemis may play a role in DNA damage checkpoint responses in human mammary epithelial cells as well.

In summary, we have shown that human mammary epithelial cells have both ATM-dependent and DNA-PK-dependent pathways to activate checkpoint responses to DNA damage. Loss of both components may compromise genomic integrity, leading to chromosomal damage. Consistent with this notion, it is interesting that a recent report found an association between reduced DNA-PK activity in peripheral blood lymphocytes (PBL) from patients with sporadic cases of uterine, cervical, and breast cancer (50). Thus, sporadic breast tumors may arise from multiple mutations affecting independent pathways. It will be interesting to determine if, in addition to loss of ATM (36), the corresponding breast tumors also contain reduced DNA-PK activity. Alternatively, it will be interesting to determine whether those breast tumors that show no altered ATM levels or function might have alterations in DNA-PK function. A better understanding of the involvement of ATM and DNA-PK in breast cancer may provide better therapeutic strategies for treating mammary tumors. Clearly, the precise role DNA-PK plays in DNA damage responses in normal human mammary epithelial cells requires further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Clonogenic survival shows that stable knockdown of ATM in hTERT184 cells sensitizes cells to IR and bleomycin sulfate but not UV radiation treatment. hTERT184 cells and derivative lines were treated with the indicated amounts of **A. IR**, **B. bleomycin sulfate** or **C. UV radiation** and colonies allowed to grow for 10 days. Results are the mean $\pm$ SEM of at least 3 independent experiments in duplicate plates. **D.** Human mammary epithelial hTERT184 cells were infected with lentiviruses encoding shRNAs against ATM (#1, 2, 4, and 5) and stable transfectants selected in 4 μ g/ml blasticidin. Western blot analysis of ATM levels was performed with β -tubulin used as a loading control.

Figure 2. ATM knockdown hTERT184 cells demonstrate a G₂ checkpoint response following IR and IR-induced phosphorylation of CHK1 and CHK2 in ATM knockdown cells

A. hTERT184-lacZ, -ATM5, and -ATM1 cells were treated with 2 Gy IR and collected and fixed 2 h after IR treatment. Cells were stained with a phospho-serine10 histone H3 antibody to assess the G₂ checkpoint by flow cytometry. Mean mitotic percentages $\pm$ s.d. of 3 independent experiments. **B.** Western blot analysis of phosphorylation of CHK1 and CHK2 after IR in hTERT184 and derivative cells. Cells were treated with 2 Gy IR, cultured for an additional 2 h and then analyzed for phospho-CHK1 (Ser345), phospho-CHK2 (Thr68), total CHK1 and total CHK2 levels. β -actin was used as a loading control. As a positive control for

CHK1 phosphorylation, cells were also treated with 50 J/M² UV radiation followed by culturing for an additional 2 h. (con = control)

Figure 3. Transient transfection of hTERT184-ATM5 stable knockdown cell line with ATM siRNA only slightly attenuates the G₂ checkpoint response

A. hTERT184-lacZ and hTERT184-ATM5 cells were transfected with a control siRNA or a siRNA against ATM for 48 h, exposed to 2 Gy IR and then cultured for an additional 2 h. Western blot analysis of ATM, phospho-Chk2 (Thr68), and total Chk2 levels was performed. β-actin was used as a loading control. **B.** Cells treated as in A., but 2 h after IR, cells were collected and fixed. Flow cytometry was performed using a phospho-histone H3 (Ser10) antibody to assess the G₂ checkpoint. Data shown are the mean mitotic percentages ± s.d. of 2 independent experiments. (con = control, si-con = control siRNA)

Figure 4. Wortmannin, the DNA-PK inhibitor NU7026, and DNA-PKcs siRNA attenuate the IR-induced G₂ checkpoint response in hTERT184 cells

A, B. Cells were pretreated with the indicated amounts of wortmannin or NU7026 1 h prior to 2 Gy IR treatment. Cells were collected and fixed 2 h after IR. Flow cytometry was performed using a phospho-histone H3 (Ser10) antibody to assess the IR-induced G₂ checkpoint. Data shown are the mean mitotic percentages $\pm$ s.d. of 2 or 3 independent experiments. **C.** hTERT184-lacZ and hTERT184-ATM5 cells were transfected with a control siRNA (si-con) or a siRNA against DNA-PKcs (DNA-PKsiRNA) for 48 h, then treated with 2 Gy IR and cultured for an additional 2 h. Western blot analysis of DNA-PK with β -actin a loading control. **D.** Cells were treated as in C., but 2 h after IR, cells were collected and fixed. Flow cytometry was performed using a phospho-serine 10 histone H3 antibody to assess the IR-induced G₂ checkpoint. Data shown are the mean mitotic percentages $\pm$ s.d. of 3 independent experiments. *, $p < 0.05$ relative to transfection with control siRNA.

Figure 5. DNA-PK inhibition with NU7026 attenuates the IR-induced G₂ checkpoint response in HME-CC and ME16C human mammary epithelial cells

A. Human mammary epithelial ME16C and HME-CC cells were infected with lentiviruses encoding shRNAs against ATM (#1, 2, 4, and 5) and stable transfectants selected in 4 μg/ml blasticidin. Western blot analysis of ATM and DNA-PKcs levels were performed with β-actin used as a loading control. **B, C.** HME-CC and ME16C cells, respectively, were pretreated with the indicated amounts of NU7026 1 h prior to 2 Gy IR treatment. Cells were collected and fixed 2 h after IR. Flow cytometry was performed using a phospho-histone H3 (Ser10) antibody to assess the IR-induced G₂ checkpoint. Data shown are the mean mitotic percentages ± s.d. of 3 independent experiments.

Figure 6.

IR-induced CHK1 phosphorylation is inhibited by the DNA-PK inhibitor NU7026 in hTERT184, HME-CC, and ME16C cells with reduced ATM levels. Western blot analysis of phosphorylation of CHK1 and CHK2 after IR in **A.** hTERT184, **B.** HME-CC, and **C.** ME16C cells. Cells were pretreated with 25 μ M of NU7026, as indicated, 1 h prior to 2 Gy IR treatment, cultured for an additional 2 h and then analyzed for phospho-CHK1, phospho-CHK2, total CHK1 and total CHK2 levels. Total DNA-PK_{cs} and phospho-DNA-PK levels are shown in hTERT184 lines. β -actin was used as a loading control.