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Advances in Brief

Ataxia Telangiectasia-related Protein Is Involved in the Phosphorylation of BRCA1 following Deoxyribonucleic Acid Damage[1]

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Abstract

The breast/ovarian cancer susceptibility gene *BRCA1* exerts its tumor suppressor function, at least in part, by participating in DNA repair and/or DNA damage-responsive pathways. BRCA1 protein is hyperphosphorylated following various DNA-damaging events. Here, we report that the ataxia telangiectasia mutated protein-related protein kinase (ATR) is involved in the phosphorylation of BRCA1 following gamma radiation and hydroxyurea treatment. We have shown that ATR can phosphorylate several BRCA1 fragments *in vitro* and that a kinase-inactive mutant of ATR interacts with BRCA1 *in vivo*. Taken together, these results suggest that ATR directly phosphorylates BRCA1 following DNA damage.

Introduction

Studies in the last few years suggest that the tumor suppressor BRCA1 has at least two functions: DNA repair and transcriptional regulatory function (for reviews, see Refs. 1–3). The role of BRCA1 in DNA repair was initially proposed based on its association and colocalization with the DNA repair protein Rad51, a human homologue of *Escherichia coli* RecA (4). BRCA1, along with BRCA2, BARD1, and Rad51, localizes in subnuclear foci during the S and early G_2 phases of the cell cycle (4–6). When cells are exposed to DNA-damaging agents, BRCA1 becomes hyperphosphorylated. BRCA1, along with BRCA2, Rad51, and BARD1, temporarily vacates these subnuclear foci (6–8). The phosphorylation status of BRCA1 is tightly regulated by DNA damage (7, 8), suggesting that it is important for BRCA1 function in DNA repair. Recently, DNA damage checkpoint kinases ATM[3] and hChk2/hcds1 have been reported to participate in the phosphorylation of BRCA1 following γ radiation (9, 10). Moreover, these phosphorylation events are important for BRCA1 function in DNA repair (9, 10). Here, we report that ATR, a homologue of ATM, also participates in BRCA1 phosphorylation following DNA damage, suggesting multiple DNA damage checkpoint kinases are involved in the regulation of BRCA1.

Materials and Methods

Cell Lines and Culture Conditions. GM02184D (ATM+/+) and GM03189D (ATM−/−) cells were obtained from Coriel cell depository. Dr. William Cliby (Mayo Clinic, Rochester, MN) kindly provided GM847 and GM847kd cells. GM847kd cells, derived from GM847 cells, produce a dominant negative ATR kinase inactive mutant (ATRkd) under the control of a tet activator, which can be turned on by by the addition of doxycycline in the culture media (11). Cells were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum. For the induction of ATRkd expression in GM847kd cells, doxycycline (1 μg/ml) was added to the medium for 2–4 days before harvesting. Where indicated, cells were exposed to γ radiation from a ^{137}Cs source at a dose of 6.4 Gy/min. After irradiation, cells were returned to the incubator and harvested 1 h later. For HU treatment, 1 mM HU was added to the culture medium and cells were harvested 1 h later. Where indicated, cells were preincubated with various drugs for 30 min before γ radiation or HU treatment. Wortmannin (Sigma Chemical Co.) was dissolved in DMSO and used at final concentration of 100 μM. Caffeine (Sigma Chemical Co.) was dissolved in PBS and used at final concentration of 3 mM. UCN-01 (generously provided by Dr. Edward Sausville, National Cancer Institute, NIH) was dissolved in DMSO and used at final concentration of 1 μM.

Electrophoresis, Immunoprecipitation, and Immunoblotting. Preparation of cell lysates, immunoprecipitation, and immunoblotting were performed as described previously (6). To detect mobility changes of BRCA1 following DNA damage, proteins were separated by electrophoresis using 3–8% NuPAGE Tris-Acetate gels (Novex). Anti-BRCA1 antibodies were described previously (4). Anti-Flag monoclonal antibody M2 was purchased from Sigma Chemical Co.

Kinase Assays. HChk1/hChk2 and ATR kinase assays were performed as described previously (12). Preparation of GST-Cdc25C (residues 200–256) and six GST-BRCA1 fusion proteins (B1F1–B1F6) was performed as described previously (4). B1F1 to B1F6 encode, respectively, GST fusion proteins containing BRCA1 residues 1–324, 260–553, 502–802, 758-1064, 1005–1313, and 1314–1863.

Results

Both ATM and ATR Are Involved in the Phosphorylation of BRCA1 after γ Radiation. Several PI3K-related kinases, including DNA-PK, ATM, and ATR, participate in DNA damage-responsive pathways. We initially examined whether wortmannin, a potent inhibitor of PI3K, would inhibit BRCA1 hyperphosphorylation following γ radiation. As shown in Fig. 1*A*, wortmannin blocked BRCA1 hyperphosphorylation after γ radiation, suggesting that one or more PI3K-related kinases is involved in these phosphorylation events. In agreement with this observation, ATM has recently been shown to phosphorylate BRCA1 following γ radiation (9). But in ATM−/− cells, hyperphosphorylation of BRCA1 still occurs after γ radiation (Fig. 1*B*), suggesting that there are additional kinases involved in BRCA1 hyperphosphorylation. Interestingly, the mobility shift of BRCA1 in ATM-deficient cells is less pronounced than that in wild-type cells (Fig. 1*B*). This raises the possibility that ATM and another kinase(s) may each be responsible for a subset of the phosphorylation events after γ radiation. When the function of ATM is compromised, only ATM-independent phosphorylation events occur due to the function of another kinase(s). This would account for the faster migrating form of BRCA1 in ATM-deficient cells after γ radiation.

The wortmannin inhibition experiments in Fig. 1*A* suggest that an additional PI3K-related kinase may participate in DNA damage-induced phosphorylation of BRCA1. We have previously showed that BRCA1 hyperphosphorylation is normal in DNA-PK-deficient cells (8). The only other known DNA damage-activated PI3K-related kinase is ATR. ATR also localizes to the unsynapsed regions on meiotic chromosomes (13, 14), the same regions that BRCA1 localizes (4).

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[3] The abbreviations used are: ATM, ataxia telangiectasia mutated protein; ATR, ataxia telangiectasia-related protein; HU, hydroxyurea; PI3K, phosphatidylinositol 3-kinase; GST, glutathione-*S*-transferase.

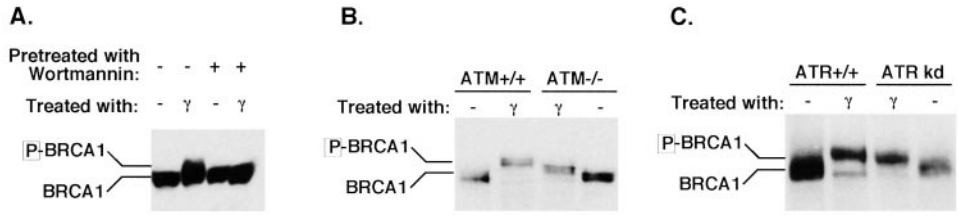

Fig. 1. *A*, wortmannin blocks hyperphosphorylation of BRCA1 after γ radiation. MCF7 cells were incubated with 100 μM wortmannin or DMSO for 30 min before they were irradiated (30 Gy). *B*, BRCA1 is hyperphosphorylated in ATM-deficient cells following γ irradiation. GM02184D (ATM+/+) cells and GM03189D (ATM−/−) cells were irradiated (30 Gy). *C*, ATR is involved in the hyperphosphorylation of BRCA1. GM847 (ATR+/+) cells and GM847kd (ATRkd) cells expressing kinase-inactive mutant of ATRkd) were irradiated (30 Gy). All lysates were prepared 1 h after irradiation. Immunoblots were probed with anti-BRCA1 monoclonal antibody MS110.

Therefore, we examined whether ATR was involved in the hyperphosphorylation of BRCA1 after γ radiation.

There are no known ATR-deficient human cell lines. We examined the role of ATR in BRCA1 phosphorylation using GM847kd cells. In the presence of doxycycline, GM847kd cells produce a dominant negative, kinase-inactive ATR mutant (ATRkd; Ref. 11). Overexpression of this kinase-inactive ATR causes increased sensitivity to DNA-damaging agents (11), suggesting that wild-type ATR function is compromised in these cells. Thus, we examined whether ATR contributed to BRCA1 hyperphosphorylation in these cells. As shown in Fig. 1*C*, a change of BRCA1 migration was still observed in these cells that overproduced ATRkd mutant, presumably due to the action of ATM kinase. However, this change of BRCA1 migration is not as pronounced as that in parental GM847 cells (Fig. 1*C*), suggesting that ATR also participates in the hyperphosphorylation of BRCA1. Thus, both ATM and ATR are involved in the BRCA1 phosphorylation after γ radiation.

ATR Is Required for BRCA1 Hyperphosphorylation after HU Treatment. We have previously shown that various DNA-damaging agents lead to different changes in BRCA1 subnuclear localization. For example, when S phase cells were treated with γ radiation or HU, BRCA1 was hyperphosphorylated (8). However, relocalization of BRCA1 to proliferating cell nuclear antigen foci was only apparent after HU treatment, but not following γ radiation (8). These differences in BRCA1 relocalization may reflect the actions of various DNA damage-activated kinases. Indeed, while ATM is required for the phosphorylation of BRCA1 following gamma radiation (9), it is not required for BRCA1 phosphorylation after UV radiation or HU treatment (8, 9).

We first used caffeine to examine the role of ATR in the phosphorylation of BRCA1 after HU treatment. Caffeine abrogates DNA damage-induced G_2 checkpoint, partially by inhibiting the kinase activity of ATR (12). As shown in Fig. 2*A*, preincubation of cells with caffeine blocked HU-induced BRCA1 hyperphosphorylation (Fig. 2), suggesting that ATR may be involved in BRCA1 phosphorylation following this treatment.

To confirm the role of ATR in BRCA1 phosphorylation, we used GM847kd cells that overproduce a dominant negative form of ATR kinase (11). As shown in Fig. 2*B*, overexpression of kinase-inactive ATR blocked BRCA1 hyperphosphorylation when compared with that in parental GM847 cells, suggesting that ATR is required for BRCA1 phosphorylation after HU treatment.

ATR could directly phosphorylate BRCA1. Alternatively, based on studies of their yeast homologues, ATR can activate its downstream kinases hChk1 or hChk2/hCds1. HChk1 and/or hChk2 could be directly responsible for BRCA1 phosphorylation. Here, we examined the role of hChk1 in the phosphorylation of BRCA1 by using a kinase inhibitor UCN-01 (7-staurosporine). UCN-01 has recently been shown to be a potent inhibitor of hChk1, but not hChk2 (15, 16). Pretreating cells with 1 μM UCN-01 did not block the phosphorylation of BRCA1 after HU treatment (Fig. 2*A*) or γ radiation (data not shown), whereas the same concentration of UCN-01 inhibited the kinase activity of hChk1 *in vitro* (Fig. 2*C*). Thus, these results indicate that hChk1 is not required for BRCA1 phosphorylation following these treatments.

ATR Can Phosphorylate BRCA1 *in Vitro*. To investigate whether ATR can directly phosphorylate BRCA1, we performed *in vitro* ATR kinase assay using six GST-BRCA1 fusion proteins, B1F1–B1F6, as substrates. B1F1 to B1F6 contain overlapping BRCA1 fragments that span the entire BRCA1 open reading frame (4). As shown in Fig. 3*A*, ATR phosphorylated B1F2, B1F4, B1F5, and B1F6, suggesting that ATR could phosphorylate multiple residues in BRCA1. The ATM-dependent phosphorylation sites (9) were mapped to the COOH terminus of BRCA1 (within B1F6). Thus, ATR may phosphorylate certain BRCA1 residues that are distinct from those phosphorylated by ATM.

In some cases, inactive kinase mutants form stable complexes with their substrates. We examined whether we could detect a complex of BRCA1 with mutant ATR overproduced in GM847kd cells. As shown in Fig. 3*B*, BRCA1 coimmunoprecipitated with the Flag-tagged kinase-inactive ATR mutant. As a control, endogenous BRCA1 was not detected in anti-Flag immunoprecipitates using extracts from GM847 cells that did not produce any Flag-tagged ATR.

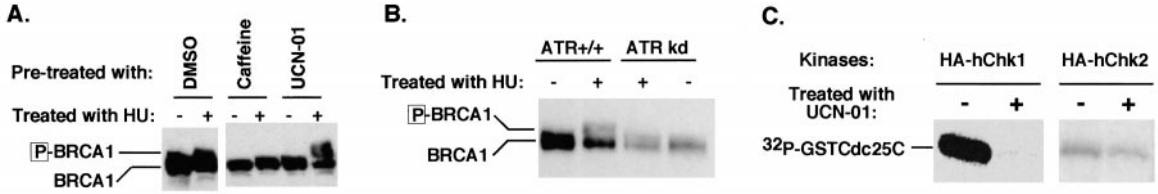

Fig. 2. *A*, caffeine, but not UCN-01, blocks BRCA1 hyperphosphorylation after HU treatment. MCF7 cells were incubated with 3 mM caffeine or 1 μM UCN-01 for 30 min before they were treated with 1 mM HU. Cell lysates were prepared 1 h later, and immunoblots were probed with anti-BRCA1 monoclonal antibody MS110. *B*, ATR is required for the hyperphosphorylation of BRCA1 after HU treatment. GM847 (ATR+/+) cells and GM847kd (ATRkd) cells expressing kinase-inactive ATR (ATRkd) were treated with 1 mM HU for 1 h. Immunoblots were done as above. *C*, UCN-01 inhibits kinase activity of hChk1, but not that of hChk2. Influenza hemagglutinin epitope-tagged hChk1 or hChk2 were immunoprecipitated from lysates of 293T cells that transiently expressed influenza hemagglutinin epitope-tagged hChk1 or hChk2. HA-hChk1 or HA-hChk2 were preincubated with DMSO (-) or 1 μM UCN-01 for 5 min on ice. Kinase reactions were performed using GST-Cdc25C (residues 200–256) as substrates. Phosphorylation of GST-Cdc25C was visualized by autoradiography.

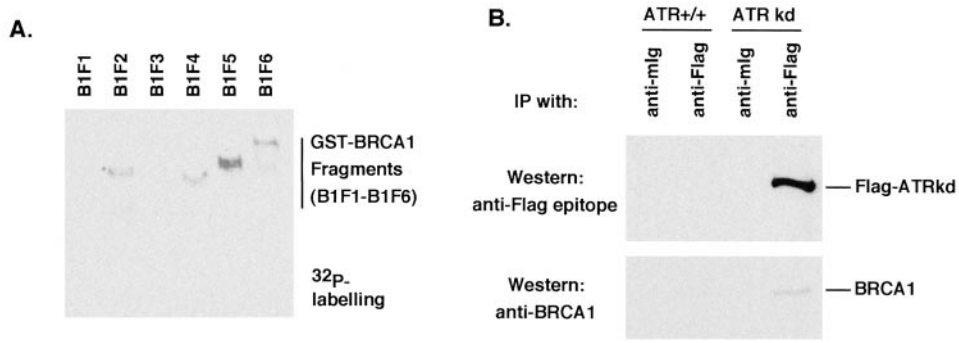

Fig. 3. *A*, ATR can phosphorylate multiple BRCA1 fragments *in vitro*. ATR was immunoprecipitated from HeLa cell extracts. Kinase reactions were performed using 1 μg of GST-BRCA1 fusion proteins as substrates (see "Materials and Methods" for details). Phosphorylation of GST-BRCA1 fragments was visualized by autoradiography. *B*, ATRkd associates with endogenous BRCA1. Lysates of GM847 cells and GM847kd cells expressing Flag-tagged, kinase-inactive ATR (ATRkd) were immunoprecipitated using control rabbit anti-mouse IgG antibody (anti-mIg) or with anti-Flag antibody M2. Immunoblots were probed with anti-BRCA1 antibody MS110 to detect endogenous BRCA1 and with anti-Flag antibody M2 to detect Flag-tagged ATRkd.

Discussion

We have shown that ATR is involved in BRCA1 phosphorylation following HU treatment and γ radiation. In addition, we have detected a complex of BRCA1 with kinase-inactive ATR, suggesting that BRCA1 may be a substrate of ATR. We failed to detect interaction between BRCA1 and endogenous ATR. This may due to the transient nature of enzyme-substrate interactions. In supporting that BRCA1 is a substrate of ATR, we have shown that multiple fragments of BRCA1 can be phosphorylated by ATR *in vitro*. Future experiments will focus on mapping these phosphorylation sites and examining whether these phosphorylation events contribute to the function of BRCA1 in DNA repair.

Following gamma radiation, ATM directly phosphorylates BRCA1 (9). In addition, ATM activates its downstream kinase hChk2/Cds1, which also phosphorylates BRCA1 (10). Thus, we examined whether hChk1, another checkpoint kinase downstream of ATM/ATR, was involved in the phosphorylation of BRCA1. UCN-01 inhibition experiments (Fig. 2 and data not shown) suggest that hChk1 may not be involved in BRCA1 phosphorylation after HU treatment or γ radiation. HChk1 may be downstream of BRCA1. Or hChk1 can be involved in a pathway parallel to that of BRCA1. We may be able to distinguish these two possibilities by examining whether the phosphorylation of hChk1 after DNA damage depends on intact BRCA1 in the cell.

We have shown that ATR is required for BRCA1 phosphorylation following either γ radiation or HU treatment. The ATM/hChk2 pathway only contributes to BRCA1 phosphorylation after gamma radiation; this pathway is not involved in BRCA1 phosphorylation after HU treatment (8, 9). As shown in Fig. 3*A*, at least some of the ATR-dependent phosphorylation sites on BRCA1 are different from the ATM-dependent phosphorylation sites (9). Thus, it is reasonable to speculate that ATR may regulate some aspects of BRCA1 function that are distinct from that of ATM. Once the ATR-dependent phosphorylation sites on BRCA1 are identified, it will be interesting to compare the functions of BRCA1 that carries mutations at these sites with those carrying mutations at ATM-dependent phosphorylation sites. Such experiments may provide insights into the complex regulations of BRCA1 following DNA damage.

Whereas ATM is not involved in the phosphorylation of BRCA1 following UV light radiation (8, 9), ATR is only required for some of these phosphorylation events following low-dose ($\sim$10 J/m^2) but not high-dose UV radiation.[4] It is likely that additional, unknown damage-activated kinase is responsible for these phosphorylation events. Taken together, these results suggest that various DNA-damaging events can mobilize distinct signaling pathways that lead to BRCA1 phosphorylation.

[4] Unpublished observations.

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