BRCA1-BARD1 Complexes Are Required for p53Ser-15 Phosphorylation and a G1/S Arrest following Ionizing Radiation-induced DNA Damage*

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BRCA1 is a major player in the DNA damage response. This is evident from its loss, which causes cells to become sensitive to a wide variety of DNA damaging agents. The major BRCA1 binding partner, BARD1, is also implicated in the DNA damage response, and recent reports indicate that BRCA1 and BARD1 co-operate in this pathway. In this report, we utilized small interfering RNA to deplete BRCA1 and BARD1 to demonstrate that the BRCA1-BARD1 complex is required for ATM/ATR (ataxia-telangiectasia-mutated/ATM and Rad3-related)-mediated phosphorylation of p53Ser-15 following IR- and UV radiation-induced DNA damage. In contrast, phosphorylation of a number of other ATM/ATR targets including H2AX, Chk2, Chk1, and c-jun does not depend on the presence of BRCA1-BARD1 complexes. Moreover, prior ATM/ATR-dependent phosphorylation of BRCA1 at Ser-1423 or Ser-1524 regulates the ability of ATM/ATR to phosphorylate p53Ser-15 efficiently. Phosphorylation of p53Ser-15 is necessary for an IR-induced G1/S arrest via transcriptional induction of the cyclin-dependent kinase inhibitor p21. Consistent with these data, repressing p53Ser-15 phosphorylation by BRCA1-BARD1 depletion compromises p21 induction and the G1/S checkpoint arrest in response to IR but not UV radiation. These findings suggest that BRCA1-BARD1 complexes act as an adaptor to mediate ATM/ATR-directed phosphorylation of p53, influencing G1/S cell cycle progression after DNA damage.

Damage to genetic material, which occurs as a consequence of exposure to environmental genotoxins or the byproducts of oxidative metabolism, represents a ubiquitous and persistent threat to genomic integrity. In eukaryotes and prokaryotes alike, the deleterious effects of genotoxic stress are countered by a robust DNA damage response pathway that monitors the genome for the presence of abnormal DNA structures, including single-stranded DNA, DNA double strand breaks, and chemically modified DNA bases. The DNA damage response can be reduced to detection, signal transduction, and effector phases, which are analogous to the signaling paradigms of growth factors and their cognate receptors. In mammals, the ATM1 (ataxia-telangiectasia-mutated) and ATR (ATM and Rad3-related) protein kinases function as critical regulators of the cellular DNA damage response (1, 2). ATM and ATR are Ser/Thr-Gln-directed protein kinases with overlapping substrate specificities that are activated in response to distinct, as well as partially overlapping, types of genotoxic stimuli (1, 2). Despite their structural similarity and overlapping substrate specificities, ATM and ATR are functionally nonredundant protein kinases, and this is most convincingly demonstrated by comparing their respective gene knock-out phenotypes. In humans, inactivating mutations in ATM result in the cancer predisposition/neurodegenerative syndrome ataxia-telangiectasia (A-T) (3). Cells from A-T patients or ATM-nullizygous mice are exquisitely sensitive to ionizing radiation (IR) and other agents that induce double strand breaks and fail to activate the IR-induced G1/S or G2/M checkpoints. In addition, A-T cells exhibit radioresistant DNA synthesis, the failure to transiently down-regulate DNA replication in response to IR, which is indicative of an S-phase checkpoint defect. In contrast to ATM−/− mice, which are viable, ATR-deficient mice die early during embryogenesis, and conditional knock-out of ATR gene function in human cells leads to a loss of cellular viability (1). ATR mediates responses to a broad spectrum of genotoxic stimuli, including DNA replication inhibitors (e.g. hydroxyurea), UV radiation, IR, and agents such as cis-platinum that induce DNA interstrand cross-links.

The checkpoint functions of ATM and ATR are mediated, in part, by a pair of checkpoint effector kinases termed Chk1 and Chk2/Cds1 (4). Although structurally distinct, Chk1 and Chk2 are functionally related kinases that phosphorylate an overlapping pool of cellular substrates (5, 6). Chk1 is phosphorylated on two Ser residues (Ser-317 and Ser-345) in an ATM-dependent manner following IR (7) and in an ATR-dependent manner following cellular exposure to hydroxyurea or UV light, two classic ATR stimuli. Chk2 is inductively phosphorylated on multiple Ser/Thr residues, including a regulatory site at Thr-68, by ATM in response to double strand breaks. Substrates for Chk1 and Chk2 include members of the Cdc25 family of protein phosphatases, which are essen-

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1 The abbreviations used are: ATM, ataxia-telangiectasia-mutated; ATR, ATM and Rad3-related; A-T, ataxia-telangiectasia; BrdUrd, bromodeoxyuridine; IR, ionizing radiation; UVC, ultraviolet light wavelength band C; siRNA, small interfering RNA; Gy, gray (unit of measure); GFP, green fluorescent protein; YFP, yellow fluorescent protein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.
tial for S-phase and G2/M-phase cell cycle transitions (1). Like ATM and ATR, Chk1 and Chk2 are nonredundant pro-
tein kinases that regulate distinct and partially overlapping cellular responses to genotoxic stimuli (8).

Although essential for transmission of DNA damage signals, the ATM/ATR and Chk1/Chk2 kinases are not sufficient for checkpoint signaling to occur. Genetic studies using budding and fission yeast have identified gene products that participate in the initiation and propagation of checkpoint signals (4, 9). A critical element of the DNA damage-signaling network is the DNA replication factor C-related protein, Rad17. Studies in yeasts and human cells indicated that Rad17 interacts genetically and biochemically with a heterotrimeric complex of proteins consisting of Rad9, Rad1, and Hus1 (Rad9 complex). The Rad9 complex is structurally, and presumably functionally, related to the PCNA sliding clamp, leading to the hypothesis that hRad17-replication factor C functions as a clamp loader for the Rad9 complex at or near sites of DNA damage (10). Recently, Rad9 has been shown to be required for recruitment of Rad9 complex on damaged DNA and for ATR-dependent phosphorylation of Chk1 after IR (11).

Another protein with a putative adapter/scaffolding function is the tumor suppressor, BRCA1. BRCA1 is a phosphoprotein that is mutated in ~50% of familial breast and ovarian cancers (12). BRCA1 is phosphorylated by ATM and ATR on multiple Ser/Thr residues in response to genotoxic stimuli (13, 14), as well as by Chk2 (15). BRCA1 contains a BRCT motif that is found in a variety of DNA repair and checkpoint proteins (16), including budding yeast ScRad9p. ScRad9p mediates interaction between the ATM/ATR ortholog ScMec1p and its downstream target, the Chk2 ortholog ScRad53p (17). The adapter function of Rad9 is dependent upon prior phosphorylation by ScMec1p. BRCA1 is an ATM/ATR target (14). Therefore, based on this analogy, it is conceivable that ATM/ATR-dependent phosphorylation of BRCA1 generates binding sites for BRCA1-associated factors, which are ATM/ATR substrates. Consistent with this theory, BRCA1 in cells exists as a part of a complex known as BASC (BRCA1-associated genome surveillance complex), which contains many of the known ATM targets including NBS1, BLM, and SMC1 (18). BRCA1 is almost always found complexed with BARD1 in vivo. Based on these studies, we investigated whether BRCA1-BARD1 complexes are required for phosphorylation of ATM/ATR targets after exposure of cells to IR and UV radiation. The present study demonstrates that BRCA1-BARD1 dimers are required for efficient phosphorylation of p53 after exposure of cells to IR and UV radiation, respectively. Furthermore, ATM and ATR-dependent phosphorylation of BRCA1 is necessary for its adapter function. Consequently, we show that the BRCA1-BARD1 complex is required for a G1/S arrest following IR but not UV radiation. These findings highlight the role of the BRCA1-BARD1 complex as an important mediator in the DNA damage response, affecting checkpoint function.

MATERIALS AND METHODS

Cell Culture and Transfection—293T is a human embryonic kidney carcinoma cell line that has been transformed with the SV40 large T-antigen. U2OS is a human osteosarcoma cell line, and MCF-7 is a human breast carcinoma cell line. HeLa is a human cervical carcinoma cell line. HCC1937 is a human breast carcinoma cell line that has been transformed with the SV40 large T-antigen. U2OS is a human osteosarcoma cell line, and MCF-7 is a human breast carcinoma cell line. HCC1937 is a human breast carcinoma cell line harboring a T-antigen. U2OS is a human osteosarcoma cell line, and MCF-7 is a human breast carcinoma cell line that has been transformed with the SV40 large T-antigen. U2OS is a human osteosarcoma cell line, and MCF-7 is a human breast carcinoma cell line that has been transformed with the SV40 large T-antigen. U2OS is a human osteosarcoma cell line, and MCF-7 is a human breast carcinoma cell line that has been transformed with the SV40 large T-antigen.

For immunofluorescence studies, a portion of the cells was taken from the above samples and cytospun to slides prior to lysing. Alternatively, cells were seeded onto coverslips and transfected at 50–60% confluence with 1.5 μg of plasmid DNA with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. In flow cytometry analysis, cells were seeded onto 6-cm dishes and transfected at 50–60% confluence with 5 μg of plasmid DNA with LipofectAMINE according to the manufacturer’s instructions. Cells were harvested and analyzed 72 h post-transfection. When required, cells were either irradiated with a 137Cs γ-ray source or exposed to UVC.

Plasmid Construction—The BRCA1 small interfering RNA (siRNA) construct was generated by annealing the following complementary oligonucleotides and inserted into the pSuper vector as a BglII/HindIII fragment: forward, 5′-GGG GGC CAA GAG ACA GGT TCT GC TGT GTA CTG TGG TAA AAA-3′, and reverse, 5′-AGG TTT TCT CAA AAA GAA AGT AGC AGT TGT AGT TCT GGG AA-3′. The BAR1 siRNA construct was generated in a similar manner with the following oligonucleotides: forward, 5′-ATCC CCG CCT GGT GAA GAA CAA CTA CAA CTT CAA GAG AGT TTC GGA GTA CAA-3′ and reverse, 5′-TTT TCC AAA AGC TGG TGT AGT CTA GCT TGG TAT GCA TCT GGG ATC-3′. The nucleotides in boldface indicate the regions of BRCA1, BARD1, and GFP that were targeted for silencing by siRNA.

Full-length wild-type YFP-tagged BRCA1 has been described previously (21). The QuiClone site-directed mutagenesis kit (Strategene) was used according to the manufacturer’s instructions to introduce alanine point mutations into the YFP-BRCA1 construct to disrupt the DNA damage-induced phosphorylation sites: Ser-1387, Ser-1423, and Ser-1524. To mutate residue serine 1387 to alanine, the following primers were used: forward, 5′-TCA GGG GTC TCA GCT GAG ACC GAC GAG AAC-3′, and reverse, 5′-AAT GTC ACT CTG CAG CCT TCT CAC TCA GAA-3′. To mutate residue serine 1423 to alanine, the following primers were used: forward, 5′-GAT GTC GAG CAT GCT GGG ACC GAC GAC GAG-3′, and reverse, 5′-GTT AGA AGG GTC GGG GCC GGC ATG CCT TGG-3′. To mutate residue serine 1524 to alanine, the following primers were used: forward, 5′-GAA TAC TCA CCT TCA GCT CAC TCA GAC GAG CTC-3′, and reverse, 5′-GAG CTC TCT TGG AGC GGA TTA GGT CCT-3′. The codons in boldface indicate the amino acids mutated to alanine. All plasmid mutations were confirmed by DNA sequencing.

Immunoblotting—Cellular extracts were prepared by resuspending the cells in lysis buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μM aprotinin, 0.2% Triton X-100, and 0.03% Nonidet P-40). After incubating on ice for 30 min, supernatants followed centrifugation at 14,000 × g for 15 min. The protein sample was then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with the appropriate antibodies. The following primary antibodies were used: ATM (Ab-1; Oncogene Research), Chk1Ser-317 (7), Chk1 (Santa Cruz Biotechnology), Chk2Ser-65 (Cell Signaling), Chk2 (Santa Cruz), p53Ser-15 (Cell Signaling), p53 (Novus), p21 (Oncogene Research), and BARD1 (Santa Cruz). Cdc25A (Santa Cruz), BARD1 (Ab-1; Oncogene Research), BRCA1Ser-1387, BRCA1Ser-1423, and BRCA1Ser-1524 (14), BARD1 (provided by Dr. Richard Baer), and γ-tubulin (T9152, Sigma). Antibody bound to the above proteins was detected by incubation with the horseradish peroxidase-conjugated secondary antibody (Sigma). Proteins were visualized using the ECL detection system (Amersham Biosciences). Markers (Bio-Rad) were used as molecular size standards.

Immunofluorescence Microscopy—Cells were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature, washed three times in PBS, and then permeabilized in 0.2% Triton X-100/PBS. Cells were incubated in a blocking buffer of 1% bovine serum albumin/PBS for 45 min before the required primary antibody was applied. BRCA1 was detected with a monoclonal antibody Ab-1 (Oncogene Research) followed by incubation with fluorescein-labeled anti-mouse IgG (Zymed Laboratories Inc.). The phosphospecific antibodies p53Ser-15 (Cell Signaling) and H2AXSer-139 (Upstate Biotechnology) were used to detect the phosphorylated forms of p53 and H2AX, respectively. Antibody bound to p53Ser-15 or H2AXSer-139 was detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Molecular Probes). Cell nuclei were counterstained with the chromosone dye Hoechst 33258 (Sigma). After extensive washing, samples were
mounted onto glass slides with a drop of Vectashield anti-fade mounting reagent (Vector Laboratories). Samples were analyzed with an epifluorescence microscope (Olympus). Images were captured digitally and processed with Adobe Photoshop.

**Bromodeoxyuridine (BrdUrd) Incorporation Assay—**Cells were mock irradiated or exposed to 6 Gy of IR or 50 J/m² UVC. After 16 h, cells were pulsed by 20 nm BrdUrd for 30 min and then harvested by washing with PBS. Cells were fixed in 70% ethanol at −20 °C for at least 1 h. After fixation, the cells were washed with 1% fetal calf serum, PBS and then treated with 2× HCl containing 0.5% Triton X-100 for 30 min at room temperature. The acid was neutralized by resuspending the cell pellet in 0.1 M sodium tetraborate (pH 8.5) and incubating at room temperature for 5 min. Following centrifugation, the cell pellet was resuspended in 100 μl of wash/stain buffer (1% fetal calf serum, 0.5% Tween 20, PBS) and then 10 μl of anti-BrdUrd-FITC antibody (BD Biosciences). Cells were incubated in the dark for 30 min and then washed twice in wash/stain buffer before being stained with 25 μg of propidium iodide/ml and 0.1 mg of RNase A/ml in PBS. Cellular fluorescence was measured by a BD Biosciences FACs Calibur flow cytometer. The data were analyzed using CellQuest software.

**RESULTS**

**BRCA1 and BARD1 Require Heterodimerization to Maintain Stability—**BRCA1 predominantly is found complexed in vivo with its major binding partner, BARD1 (22). These proteins co-localize in nuclear foci during S-phase (23) and following exposure to DNA-damaging agents relocate to sites of DNA repair (24). Furthermore, they co-fractionate in various DNA repair-associated nuclear complexes (25), implicating the BRCA1-BARD1 heterodimer in the DNA damage response. Previous studies have demonstrated that the stability of BRCA1 is dependent on the expression of BARD1 and vice versa (26, 27). Therefore, we sought to confirm these data by using siRNA to specifically inhibit the expression of either BRCA1 or BARD1 in cells. 293T cells were transfected with siRNAs targeting BRCA1 and GFP. Immunoblotting results show that only BRCA1 siRNA specifically decreased BRCA1 expression with or without prior exposure of cells to IR (10 Gy) or UV (50 J/m²). In contrast, GFP siRNA had no apparent effect (Fig. 1A). Similar results were obtained by immunostaining (Fig. 2C). Consistent with published data, BARD1 levels were reduced in BRCA1 siRNA transfected cells compared with control transfected cells (Fig. 1A, GFP siRNA). The same phenomenon applied to BARD1 siRNA, i.e., 293T cells transfected with BARD1 siRNA not only specifically reduced BARD1 expression but also repressed BRCA1 expression, whereas GFP siRNA had no effect on either protein (Fig. 1B). These findings indicate that BRCA1-BARD1 complex formation is essential for mutual stability.

ATM is the primary signal transducer in response to DNA doublestrand breaks caused by exposure to IR. A recent paper illustrated that ATM is activated by autophosphorylation on Ser-1981 in response to IR (28). Therefore, to confirm that BRCA1-BARD1 complexes do not disrupt ATM activity directly or indirectly, we immunoblotted GFP, BRCA1, and BARD1 siRNA cellular extracts with a Ser-1981 phosphospecific antibody as a marker of ATM activity in cells. As expected, ATM Ser-1981 phosphorylation was markedly increased following exposure to IR, but not UV radiation, in control transfected cells (Fig. 1, GFP siRNA). Importantly, phosphorylation of ATM Ser-1981 was not decreased by the depletion of BRCA1 and BARD1 in cells transfected with siRNAs targeting these proteins (see Fig. 1). In contrast, IR-induced phosphorylation of ATM at Ser-1981 was slightly increased in BRCA1 and BARD1 siRNA-transfected cells relative to control cells (GFP siRNA), indicating that BRCA1-BARD1 complexes may set a threshold for ATM autophosphorylation in response to IR. These findings indicate that BRCA1-BARD1 complexes are not required for the activation of ATM.

**BRCA1-BARD1 Complexes Are Required for ATM/ATR-dependent Phosphorylation of p53 Ser-15**—Previous work from our laboratory and those of others (14, 29) has shown that although ATM and ATR phosphorylate the same set of targets in vitro, functionally they are nonredundant kinases in vivo. Rapid IR-induced phosphorylation of downstream targets are catalyzed by ATM in vivo, whereas ATR mediates rapid UVC-induced phosphorylation events. BRCA1 has been shown to bind many ATM/ATR targets as a component of BASC (BRCA1-associated genome surveillance complex) (18). To address whether the BRCA1-BARD1 heterodimer is required for the phosphorylation of ATM/ATR targets, we used siRNA to specifically inhibit the expression of BRCA1 and BARD1 in cells. By immunoblotting we examined the phosphorylation status of p53 (anti-p53Ser-15), Chk2 (anti-Chk2Thr-68), and c-jun (anti-c-JunSer-63) in BRCA1, BARD1, and GFP siRNA-transfected cells before and after exposure to IR (10 Gy) and UV radiation (50 J/m²). In cells transfected with GFP siRNA, phosphorylation of p53 and c-jun increased following exposure to IR and UV radiation, whereas phosphorylation of Chk2 only increased after IR (Fig. 2A). Interestingly, the IR- and UV-induced increase in p53 phosphorylation was significantly less in BRCA1 and BARD1 siRNA-transfected cells after DNA damage (Fig. 2, A and B). Similar results were obtained by immunostaining (Fig. 2C). In contrast, Chk2 and c-jun phosphorylation was not affected by the loss of BRCA1 and BARD1 expression (Fig. 2, A and B). The effect of Chk1 phosphorylation (anti-Chk1Ser-317) was also assessed in GFP and BRCA1 siRNA-transfected cells in the absence and presence of DNA-damaging agents. As expected, Chk1Ser-317 phosphorylation increased following exposure to UV radiation in control transfected cells (Fig. 2A, GFP siRNA). Interestingly, Chk1Ser-317 phosphorylation was not affected in cells depleted of BRCA1 (Fig. 2A). It should be noted that publications from our laboratory and others (7, 40) have shown that phosphorylation of
Chk1Ser-317 is increased following IR in a number of lymphoblastoid cell lines in an ATM-dependent manner; however, we did not observe an increase in Chk1Ser-317 phosphorylation in 293T cells following IR (Fig. 2A). In contrast, as a positive control, we demonstrate that Chk1Ser-317 phosphorylation is markedly increased 1 h post-IR in C3ABR lymphoblastoid cells (Fig. 2A). A recent publication (30) has demonstrated that SV40 transformation can interfere with Chk1 phosphorylation, which would provide an explanation as to why we did not observe an increase in this phosphorylation event in 293T cells. Cdc25A is a downstream target of the Chk1 and Chk2 kinases. Both kinases can mediate the phosphorylation of Cdc25A, stimulating its degradation and resulting in activation of the S-phase checkpoint (31). Therefore, Cdc25A expression was analyzed in GFP, BRCA1, and BARD1 siRNA-transfected cells. As expected, Cdc25A expression decreased in GFP siRNA-transfected cells following DNA damage compared with untreated cells. Consistent with our findings on Chk1/Chk2 phosphorylation, Cdc25A degradation was not affected by the loss of BRCA1 and BARD1 expression (Fig. 2A and B). We conclude that BRCA1-BARD1 dimers are required for ATM/ATR-dependent phosphorylation of p53Ser-15 in response to DNA damage but are dispensable for phosphorylation of various other ATM/ATR targets.

BRCA1 Is Not Required for DNA Damage-induced H2AX Phosphorylation—One of the first proteins phosphorylated by ATM after DNA double strand breaks (32) or by ATR after replication stress (33) is the histone variant H2AX. Phosphorylated H2AX forms nuclear foci at the sites of DNA damage such as MDC1 (34), the MRN complex (Mre11-Rad50-Nbs1), Rad51, and BRCA1 (35, 36). Therefore, to confirm that BRCA1 acts downstream of H2AX, the phosphorylation status of H2AX was examined by immunostaining GFP and BRCA1 siRNA-transfected cells 1 h after exposure to IR or UV radiation with a phosphospecific antibody (anti-H2AXSer-139). Phosphorylation of H2AX was observed in control cells (GFP siRNA-transfected) following IR and UV radiation (see Fig. 2D). As predicted, H2AXSer-139 phosphorylation after DNA damage was not affected by the loss of BRCA1 expression (see Fig. 2D). These findings indicate that H2AX phosphorylation is not dependent on BRCA1 and suggest that BRCA1 acts downstream of H2AX in the DNA damage response.
ATM/ATR-directed Phosphorylation of BRCA1 Is Required for p53Ser-15 Phosphorylation—We have previously reported that BRCA1 is rapidly phosphorylated by ATM and ATR after exposure to IR and UV radiation, respectively. Specifically, residues Ser-1423 and Ser-1524 are phosphorylated in response to IR and UV radiation, whereas Ser-1387 is phosphorylated only following IR (14). By using phosphospecific antibodies, phosphorylated BRCA1 has been detected in DNA damage-induced nuclear foci (14). These foci are thought to be sites of DNA damage/repair (37) and contain many proteins, some of which are ATM/ATR substrates (18). Therefore, site-directed mutagenesis was employed to abolish phosphorylation of YFP-BRCA1 at Ser-1387, Ser-1423, and Ser-1524 by using phosphospecific antibodies recognized wild-type YFP-BRCA1 but not YFP-BRCA1S1387A/S1423A/S1524A (Fig. 3A). In addition, the double mutant YFP-BRCA1S1423A/S1524A was detected only by the anti-BRCA1S1423A/S1524A antibody. To determine whether phosphorylation affects the ability of BRCA1 to form DNA damage-induced foci, these YFP-BRCA1 mutants were transfected into COS-7 cells, synchronized in S-phase, and then visualized by immunofluorescence in the absence of IR and at 1 or 4 h post-IR (6 Gy). S-phase wild-type YFP-BRCA1 nuclear foci were observed in untreated cells, which dispersed following IR and then reappeared 4 h post-IR (Fig. 3B) as reported previously (38). Interestingly, mutations at none of the three phosphorylation sites (Ser-1387, Ser-1423, and Ser-1524) prevented YFP-BRCA1 from forming S-phase or DNA damage-induced nuclear foci (Fig. 3B). Taken together, these data suggest that ATM/ATR-dependent phosphorylation of BRCA1 does not effect dynamic changes in BRCA1 subcellular localization in response to DNA damage.

We next sought to investigate whether BRCA1 phosphorylation is required for its adaptor function by comparing the effect of the BRCA1 phosphorylation mutant S1423A/S1524A to wild-type for its ability to affect p53Ser-15 and Chk2Thr-68 phosphorylation following UV- and IR-induced DNA damage. HCC1937 cells harbor a homozygous pathogenic mutation in the BRCA1 gene that renders the BRCA1 protein inactive (39). Therefore, we used HCC1937 cells to generate three stably transfected cell lines, which included vector, wild-type BRCA1, and BRCA1S1423A/S1524A (20). Consistent with knocking out BRCA1 by siRNA (Fig. 2), p53Ser-15 phosphorylation was reduced in the HCC1937 cell line stably transfected with vector only following UV- and IR-induced DNA damage (Fig. 3C). Importantly, this phosphorylation event was restored in the wild-type BRCA1-complemented HCC1937 cells. In contrast, HCC1937 cells stably expressing phosphorylation mutant BRCA1S1423A/S1524A did not restore p53Ser-15 phosphorylation (Fig. 3C). These findings suggest that phosphorylated BRCA1 is required to mediate efficient ATM/ATR-dependent phosphorylation of p53Ser-15.

siRNA-mediated Repression of BRCA1 Disrupts p21 Induction and the G1/S Checkpoint following IR-induced DNA Damage—Our finding that loss of BRCA1 expression disrupts p53Ser-15 phosphorylation implicates BRCA1 in G1/S checkpoint control because DNA damage-induced phosphorylation of p53Ser-15 is essential for its activity and contributes to a G1/S arrest (41, 42). To establish a role for BRCA1 in the G1/S checkpoint, flow cytometric assays were performed by staining GFP and BRCA1 siRNA-transfected MCF-7 cells with BrdUrd in the absence and presence of the DNA-damaging agents IR (6 Gy) and UV radiation (50 J/m²). In response to UV radiation, incorporation of BrdUrd was dramatically decreased in both GFP and BRCA1 siRNA-transfected cells compared with untreated cells (Fig. 4A), indicating that the depletion of BRCA1 expression does not affect UV-induced G1/S arrest. BrdUrd
BRCA1-BARD1 is Required for p53 Phosphorylation

Fig. 4. siRNA-mediated repression of BRCA1 disrupts the G1/S checkpoint. A, MCF-7 cells were transfected with GFP or BRCA1 siRNA. At 60 h post-transfection, cells were untreated or irradiated with 6 Gy IR or 50 J/m2 UV light and then incubated for 16 h before being pulsed for 1 h with 20 μM BrdUrd. Cells were subsequently stained with anti-BrdUrd-FTTC antibodies and propidium iodide and then analyzed by flow cytometry. The graph illustrates percent relative BrdUrd-positive cells in DNA damage-treated samples compared with untreated samples from two independent experiments. The graph demonstrates that GFP siRNA-transfected cells effectively arrest in G1 after IR or UV radiation; however, BRCA1-depleted cells cannot arrest in G1 following exposure to IR. C, BrdUrd-activated cell sorter profiles of propidium iodide-stained cells of a representative experiment, described in A, are shown and indicate that BRCA1-depleted cells accumulate in G2/M following exposure to IR but not UV light, compared with control cells, which arrest in G1. C, MCF-7 cells were transfected with siRNAs targeting GFP and BRCA1. At 72 h post-transfection, cells were treated with the indicated DNA-damaging agents then incubated for 4 h before cellular extracts were prepared and immunoblotted for p21 levels using anti-p21 antibodies. Cellular extracts were also immunoblotted for γ-tubulin, which served as a loading control.

Incorporation was also reduced by >50% in control cells (GFP siRNA) following exposure to IR compared with untreated cells, indicating an efficient arrest at the G1/S checkpoint. In contrast, BrdUrd incorporation was not significantly affected in BRCA1-depleted cells following exposure to IR compared with untreated cells (Fig. 4A), implicating BRCA1 in the IR-induced G1/S checkpoint. As a result, unlike control cells, which arrested at the G1/S checkpoint, BRCA1-deficient cells accumulated at the next DNA damage checkpoint, G2/M, in the 16-h period after exposure to IR (Fig. 4B). Similar results were obtained in the U2OS cell line (data not shown). Because we observed differences in the ability of BRCA1-depleted cells to arrest at the G1/S checkpoint following exposure to IR and UV radiation, we examined the levels of the CDK inhibitor p21 in GFP and BRCA1 siRNA-transfected cells in response to DNA damage (IR and UV) because it lies downstream and is induced by p53 following its activation. It is interesting to note that in the absence of DNA damage the level of p21 was markedly reduced in BRCA1-depleted cells compared with control cells (Fig. 4C, GFP siRNA). This result is consistent with previous findings implicating BRCA1 in the induction of p21 (43, 44). As expected, p21 levels increased in control cells (GFP siRNA-transfected) 4 h following exposure to IR (Fig. 4C). In contrast, p21 was not induced in BRCA1-depleted cells following IR (Fig. 4C), which is consistent with these cells having a defective G1/S checkpoint. In response to UV radiation, however, neither GFP nor BRCA1 siRNA-transfected cells induced p21, and instead a significant reduction in p21 levels was evident (Fig. 4C), despite their ability to arrest at the G1/S checkpoint. This result is consistent with previously published data demonstrating that cells exposed to UV radiation and not IR are able to arrest at the G1/S checkpoint via a p53- and p21-independent pathway, which is activated by ATR (31). Collectively, these findings support a role for BRCA1 in the IR-but not UV-induced G1/S checkpoint.

Discussion

In the present study, we have demonstrated that the BRCA1-BARD1 tumor suppressor complex acts as an important mediator in the DNA damage response. We show that the BRCA1-BARD1 complex is not required for the activation of ATM kinase function. In addition, BRCA1 is dispensable for the phosphorylation of H2AX, which is rapidly targeted following DNA double strand breaks and replication stress by ATM (32) and ATR (33), respectively. These findings indicate that the BRCA1-BARD1 complex acts downstream in the DNA damage response. A recent study has reported that a subset of ATM and ATR substrates requires BRCA1 for phosphorylation (45).
These substrates include Chk2, CtIP, Nbs1, p53, and c-jun. The findings published by Foray et al. (45) are based on the use of the BRCA1 mutant cell line HCC1937 stably transfected with either vector or wild-type BRCA1. HCC1937 is a tumor cell line that contains mutations in multiple genes other than BRCA1, for example, p53 and PTEN (39). Our experiments, however, using a distinct siRNA methodology to deplete BRCA1 function in cells, are not consistent with the previous study. By using this system, we have demonstrated that BRCA1 is required for p53Ser-15 phosphorylation following UV- and IR-induced DNA damage. In addition our study provides evidence for the first time that ATM/ATR-mediated phosphorylation of BRCA1 at Ser-1423 or Ser-1524 is required for its adaptor function for p53. However, Chk1, Chk2, and c-jun phosphorylation occurs in the absence of BRCA1. Consistent with our findings, earlier studies have reported that the Chk1 and Chk2 mobility shift, which is indicative of phosphorylation, is not affected in the HCC1937 cell line (46, 47). Heterodimerization of BRCA1 and BARD1 protects both proteins from degradation (26, 27), thus explaining why >75% of the cellular pool of BRCA1 is complexed with BARD1 in vivo (22). Therefore, it is not surprising that BARD1 depletion by siRNA also reduced p53Ser-15 phosphorylation and, like BRCA1 siRNA, had no effect on Chk2 and c-jun phosphorylation. These findings suggest that BRCA1-BARD1 complexes act as an adaptor for p53, enabling it to be targeted for ATM/ATR-directed phosphorylation following IR/UV-induced DNA damage. An analogous model consistent with these data has been demonstrated for the budding yeast proteins Rad9 and Rad53 (17, 48). In this case, Rad9 acts as an adaptor for Rad53, enabling it to be phosphorylated by Mec1. However, for Rad9 to recruit Rad53, it must first be phosphorylated by Mec1.

p53 is the main effector target of the G/S checkpoint. It is rapidly turned over in normal cells, and thus for cells to arrest at the G/S border, p53 needs to be stabilized and activated in response to DNA damage. Phosphorylation of p53 at Ser-15 is mediated by ATM rapidly after exposure to IR (49–51) and by ATR rapidly following UV exposure (29). This phosphorylation event is essential for enhancing p53 activity as a transcription factor to induce the CDK2-cyclin E inhibitor, p21, which causes cell cycle arrest (41, 42). Consistent with these findings, the depletion of BRCA1 not only disrupted p53Ser-15 phosphorylation, because BRCA1 depletion does not compromise the UV-induced degradation of Cdc25A and subsequent G/S arrest.

Like BRCA1-BARD1 dimers, other proteins involved in the DNA damage response have also been shown to play an adaptor role, i.e. they present substrates to ATM/ATR for phosphorylation in response to DNA damage. Not a member of the MRN complex, has been shown to be required for the phosphorylation of ATM downstream targets (54–56). Two other proteins, namely MDC1 and 53BP1, have also been implicated as adaptor proteins for ATM-dependent phosphorylation events (34, 57–62). Like BRCA1, Nbs1, MDC1, and 53BP1 contain at least one BRCT domain and are phosphorylated by ATM in response to DNA damage. In addition, all of these proteins are recruited to nuclear foci following DNA damage, suggesting that these foci are concentrated pools of adaptor proteins and ATM/ATR substrates to allow fast and efficient transfer of DNA damage signals to mediate checkpoint control. In conclusion, we provide evidence that the BRCA1-BARD1 tumor suppressor complex acts as an adaptor to mediate G/S checkpoint control following exposure of cells to IR but is dispensable for G/S arrest in response to UV radiation (Fig. 5). Collectively, these findings highlight the importance of the BRCA1-BARD1 complex in the DNA damage response to maintain genomic stability.

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