

Involvement of Brca1 in S-Phase and G₂-Phase Checkpoints after Ionizing Irradiation

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Cell cycle arrests in the G₁, S, and G₂ phases occur in mammalian cells after ionizing irradiation and appear to protect cells from permanent genetic damage and transformation. Though Brca1 clearly participates in cellular responses to ionizing radiation (IR), conflicting conclusions have been drawn about whether Brca1 plays a direct role in cell cycle checkpoints. Normal Nbs1 function is required for the IR-induced S-phase checkpoint, but whether Nbs1 has a definitive role in the G₂/M checkpoint has not been established. Here we show that Atm and Brca1 are required for both the S-phase and G₂ arrests induced by ionizing irradiation while Nbs1 is required only for the S-phase arrest. We also found that mutation of serine 1423 in Brca1, a target for phosphorylation by Atm, abolished the ability of Brca1 to mediate the G₂/M checkpoint but did not affect its S-phase function. These results clarify the checkpoint roles for each of these three gene products, demonstrate that control of cell cycle arrests must now be included among the important functions of Brca1 in cellular responses to DNA damage, and suggest that Atm phosphorylation of Brca1 is required for the G₂/M checkpoint.

The cellular responses to DNA damage induced by ionizing radiation (IR) include activation of cell cycle checkpoints that delay progression of cells through the cell cycle (6, 7). IR-induced checkpoints are active at the transition from the G₁ phase to the S phase, in the S phase, and at the transition from the G₂ phase to mitosis (G₂/M). The surveillance mechanisms responsible for initiating these checkpoints appear to facilitate maintenance of the integrity of the genome, presumably because they ensure that damaged DNA templates are neither replicated nor segregated into the daughter cells until they are repaired. The option to undergo apoptosis can be considered as one of the checkpoint endpoints, and a failure of the cell to die under appropriate circumstances can lead to inappropriate survival of cells with altered genomes. Failure of these mechanisms to adequately monitor the state of DNA or to signal for repair or apoptosis after DNA has been damaged is a hallmark of most cancer cells (7).

The regulatory network of proteins involved in cell cycle checkpoints has been the focus of numerous studies, and the protein Atm plays a central role in this network in mammalian cells (16, 19). The *ATM* gene is mutated in the autosomal recessive disease ataxia-telangiectasia (A-T). Patients with A-T display a complex phenotype of clinical abnormalities, including progressive cerebellar ataxia, telangiectasias, predisposition to cancer, and hypersensitivity to IR (11). Cells from A-T patients show defects in cell cycle checkpoints and hypersensitivity to IR. Since Atm activity is required for optimal induction and phosphorylation of p53 protein following IR, the G₁ checkpoint is affected in A-T cells (3, 9, 23). A lack of an IR-induced S-phase checkpoint results in persistent DNA synthesis at early time points after IR (radioresistant DNA syn-

thesis [RDS]), and cells derived from patients with A-T and Nijmegen breakage syndrome (NBS) exhibit this abnormal phenotype (22). Atm phosphorylation of serine 343 in Nbs1, the altered protein in NBS, has recently been shown to be required for this IR-induced S-phase checkpoint (13, 27). Though additional substrates of Atm kinase have been found, including Brca1, Chk2, CtIP, and Mdm2 (5, 10, 12, 15), Atm phosphorylation of these targets has not been definitively functionally linked to the IR-induced S-phase or G₂/M cell cycle checkpoints.

Accumulated data have demonstrated an important role for Brca1 in cellular responses to IR (20); however, conflicting conclusions have been drawn about whether Brca1 plays a direct role in cell cycle checkpoints. Though deletion of exon 11 from Brca1 in primary murine cells affects a G₂/M checkpoint and causes mitotic abnormalities (25), normal IR-induced G₂/M and S-phase checkpoints have been reported for the Brca1-defective human tumor cell line HCC1937 (21). Similarly, though normal Nbs1 function is clearly required for the IR-induced S-phase checkpoint, a definitive role for Nbs1 in the G₂/M checkpoint has not been established. Results reported here demonstrate that Nbs1 is not required for the G₂/M checkpoint whereas Brca1 function is required for both the IR-induced S-phase and G₂/M checkpoints. Further, Atm phosphorylation of serine 1423 in Brca1 is implicated in the G₂/M checkpoint signaling pathway.

MATERIALS AND METHODS

Cell culture and irradiation. Epstein-Barr virus-immortalized lymphoblastoid cell lines from healthy persons (GM0536; NIGMS Human Mutant Cell Repository, Camden, N.J.) and from persons who were homozygous for the *NBS1* mutation (NBS7078A) (4) were cultured in RPMI 1640 supplemented with 15% fetal bovine serum. Simian virus 40-transformed human fibroblast lines from a healthy A-T heterozygote or from a patient with A-T (GM0637 or GM9607, respectively; NIGMS Human Mutant Cell Repository) and the Brca1-mutant human breast cancer cell line HCC1937 and HeLa cells (both from the American Type Culture Collection, Manassas, Va.) were all grown as monolayers in Dul-

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becco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. AT22IJET cells, generously provided by Yossi Shiloh (Tel Aviv University), were maintained in 100 μ g of hygromycin B (Life Technologies, Rockville, Md.) ml^{-1} . Note that the *ATM* heterozygous GM0637 cells are indistinguishable from cells with two wild-type *ATM* alleles in the RDS assay (data not shown). All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2 . Radiation from a ^{137}Cs source was delivered at a rate of approximately 120 cGy/min.

Immunofluorescent detection of phosphorylated histone H3. Cells were harvested 1 to 1.5 h after irradiation, washed with phosphate-buffered saline (PBS), and fixed in suspension (concentration, 10^6 cells per ml) by the addition of 2 ml of 70% ethanol and by incubation at -20°C for as long as 24 h. After fixation, the cells were washed twice with PBS, suspended in 1 ml of 0.25% Triton X-100 in PBS, and incubated on ice for 15 min. After centrifugation, the cell pellet was suspended in 100 μ l of PBS containing 1% bovine serum albumin (BSA) and 0.75 μ g of a polyclonal antibody that specifically recognizes the phosphorylated form of histone H3 (Upstate Biotechnology, Lake Placid, N.Y.) and incubated for 3 h at room temperature. The cells were then rinsed with PBS containing 1% BSA and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulinG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted at a ratio of 1:30 in PBS containing 1% BSA. After a 30-min incubation at room temperature in the dark, the cells were washed again, resuspended in 25 μ g of propidium iodide (PI) (Sigma, St. Louis, Mo.)/ml and 0.1 mg of RNase A (Sigma)/ml in PBS, and incubated at room temperature for 30 min before the fluorescence was measured. Cellular fluorescence was measured by using a Becton Dickinson (San Jose, Calif.) FACSCalibur flow cytometer/cell sorter.

RDS assay. Inhibition of DNA synthesis after irradiation was assessed as previously described (13, 17). Briefly, cells in the logarithmic phase of growth were prelabeled by culturing in DMEM containing 10 nCi of [^{14}C]thymidine (NEN Life Science Products, Inc., Boston, Mass.) for approximately 24 h; this prelabeling provides an internal control for cell number by allowing normalization for total DNA content of samples. The medium containing [^{14}C]thymidine was then replaced with normal DMEM, and the cells were incubated for another 24 h. When cells were to be transiently transfected, they were incubated in normal DMEM for 6 h after the [^{14}C]thymidine-containing medium was discarded. Cells were irradiated, incubated for 30 to 45 min, and then pulse-labeled with 2.5 μ Ci of [^3H]thymidine (NEN Life Science Products)/ml for 15 min. Cells were harvested, washed twice with PBS, and fixed in 70% methanol for at least 30 min. After the cells were transferred to Whatman filters and fixed sequentially with 70% and then 95% methanol, the filters were air dried and the amount of radioactivity was assayed in a liquid scintillation counter. The resulting ratios of ^3H counts per minute to ^{14}C counts per minute, corrected for those counts per minute that were the result of channel crossover, were a measure of DNA synthesis.

Transfection of wild-type and mutated *BRCA1* genes into HCC1937 cells. Transfection of HCC1937 cells with the gene encoding hemagglutinin-tagged wild-type *Brcal* (generously provided by D. Livingston, Dana Farber Cancer Institute, Boston, Mass.) or mutant (alanine substitution at serine 1423) *Brcal* and transfection of HeLa cells with kinase-inactive *ATM* (2, 13) were performed in the logarithmic phase of growth with Lipofectamine (Life Technologies). The efficiency of transfection was assessed by cotransfection with a green fluorescent protein (GFP) reporter vector and analyzing for GFP expression by flow cytometry 36 h after transfection. The efficiency of transfection in multiple assessments was always between 90 and 97% (data not shown). For assessment of *Brcal* expression in the transfectants, the cells were harvested 48 h after transfection and total cellular lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in an 8% polyacrylamide gel. Expressed transfected *Brcal* proteins were detected by Western blot analysis with an antihemagglutinin monoclonal antibody (Roche Molecular Biochemicals, Indianapolis, Ind.).

RESULTS

***Brcal* participates along with *Atm* and *Nbs1* in the IR-induced S-phase checkpoint.** The IR-induced S-phase checkpoint in mammalian cells is thought to primarily represent an inhibition of replicon initiation and is measured as a transient decrease in [^3H]thymidine incorporation at early time points (30 to 90 min) after irradiation (18). The absence of this IR-induced S-phase arrest, referred to as a RDS, has been previously reported for cells from both A-T and NBS patients (22).

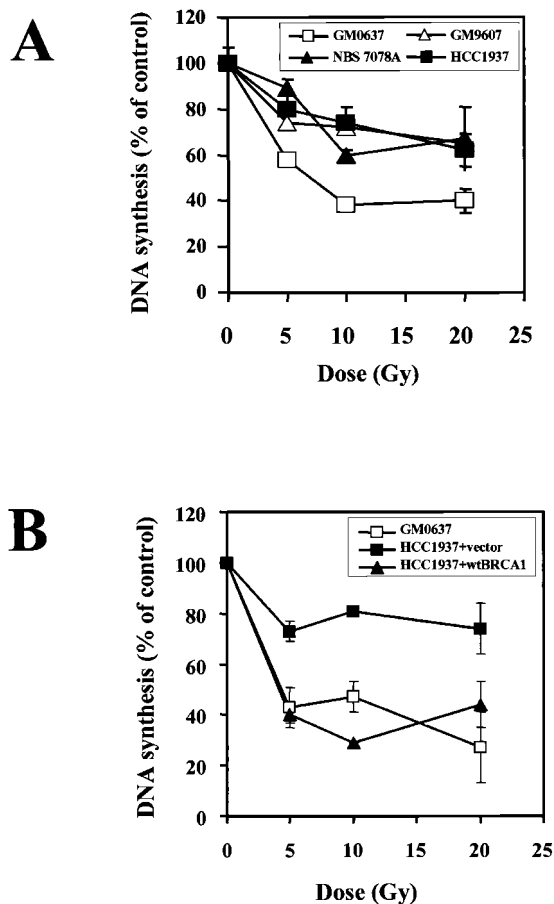


FIG. 1. *Atm*, *Nbs1*, and *Brcal* are all involved in the S-phase checkpoint activated by IR. (A) Replicative DNA synthesis was assessed 30 min after various doses of ionizing irradiation in control cells (GM0637) and cells defective in *Atm* (GM9607), *Nbs1* (NBS7078A), or *Brcal* (HCC1937) function. (B) Replicative DNA synthesis was assessed 30 min after various doses of ionizing irradiation in control cells (GM0637) and in HCC1937 cells transfected with either empty vector or wild-type *BRCA1*. Error bars represent the averages of at least triplicate samples. If the error bar is not visible, the standard error is smaller than the symbol.

One study has reported a normal S-phase checkpoint in *Brcal*-null cells (21), but no experimental details or data were provided to support this conclusion. In our hands, the S-phase arrest after irradiation over an IR dose range from 5 to 20 Gy in the *Brcal*-null cell line HCC1937 was defective and was indistinguishable from cells lacking *Atm* or *Nbs1* (Fig. 1A).

To demonstrate that this abnormality was due to a lack of *Brcal* function and not some other genetic alteration in this cell line, the HCC1937 cells were transfected with a full-length *BRCA1* cDNA and the complemented cells were assessed for this checkpoint. Flow cytometric analysis of GFP expression indicated that the transfection efficiency was greater than 90%, and expression of the wild-type *Brcal* was easily detected by Western blot analysis. Because transient overexpression of *Brcal* can induce apoptosis, the cells were irradiated 36 h after transfection, a time point when wild-type *Brcal* was expressed and the number of apoptotic cells was minimized. *Brcal* trans-

fection by itself did not affect the [3 H]thymidine incorporation in nonirradiated cells. However, the IR-induced S-phase checkpoint was restored in the HCC1937 cells following complementation with Brca1 (Fig. 1B). These results demonstrate that, in addition to the previously reported involvement of Atm and Nbs1, normal Brca1 function is also required for the IR-induced S-phase checkpoint.

Development of a facile assay to evaluate the G₂-to-M transition after irradiation in mammalian cells by flow cytometry. Rapid assessment of the progression of cells from G₂ into mitosis is made difficult by the fact that both G₂ and mitotic cells have a 4N DNA content and thus are not simply distinguishable from each other by standard PI staining and flow cytometry. Historically, the lack of the IR-induced G₂ checkpoint in A-T cells has been assessed by performing mitotic spreads and counting the mitotic cells remaining in the culture at various times after IR (17, 26). Using such an assay, the number of mitotic figures is markedly diminished within 30 min after IR in cells from healthy individuals while cells from A-T patients continue to exhibit mitoses. However, this type of assay is both cumbersome to perform and difficult to quantitate accurately. These difficulties could be circumvented by having a facile way to distinguish the G₂ cells from the mitotic cells in the population of cells with a 4N DNA content. Since histone H3 is phosphorylated exclusively during mitosis, an antibody that specifically recognizes the phosphorylated form of histone H3 can be used to identify the mitotic cells and thus distinguish G₂ cells from mitotic cells in a flow cytometric assay (8). Costaining of cells with PI to assess DNA content and an anti-phospho-histone H3 antibody demonstrates that the mitotic cells can be distinguished from G₂ cells in the 4N population of cells (Fig. 2A, top row, first column). Note that we have used this assay in a variety of different mammalian cell types and the 1.5% to 3% of cells in mitosis at any given time has been very reproducible (Fig. 2B and data not shown). Within 30 to 60 min after irradiation, a significant decrease in the number of cells in mitosis was evident (Fig. 2A, top row, second column, and B). The decrease of the mitotic percentage is time dependent and dose independent (data not shown). In normal cells, the number of mitotic cells will drop 80% within an hour (Fig. 2B). These effects after IR are qualitatively the same as those seen when mitotic entry is assessed by counts of mitotic spreads (data not shown), but counts of mitotic figures in such analyses are difficult to accurately quantitate. Thus, this flow cytometric assay provides a facile and quantitative way to assess the IR-induced G₂ checkpoint and the genetic determinants that control it.

Participation of Atm and Brca1, but not Nbs1, in the IR-induced G₂ checkpoint. Cells from A-T patients have previously been shown to lack IR-induced G₂ arrest (1, 17), and this flow cytometric assay confirmed this abnormality. While irradiation of normal cells (GM0536) resulted in an approximately 75% decrease in the number of mitotic cells, there was little to no decrease in the percentage of A-T cells (GM9607) in mitosis 60 min after irradiation (Fig. 2). This checkpoint abnormality was observed in a large number of different A-T cell lines (data not shown). Inhibition of Atm function in HeLa cells by transfection of a dominant-negative construct of *ATM* similarly demonstrated the loss of the G₂ checkpoint (Fig. 2, HeLa+kdATM). In contrast to the cells with defective Atm

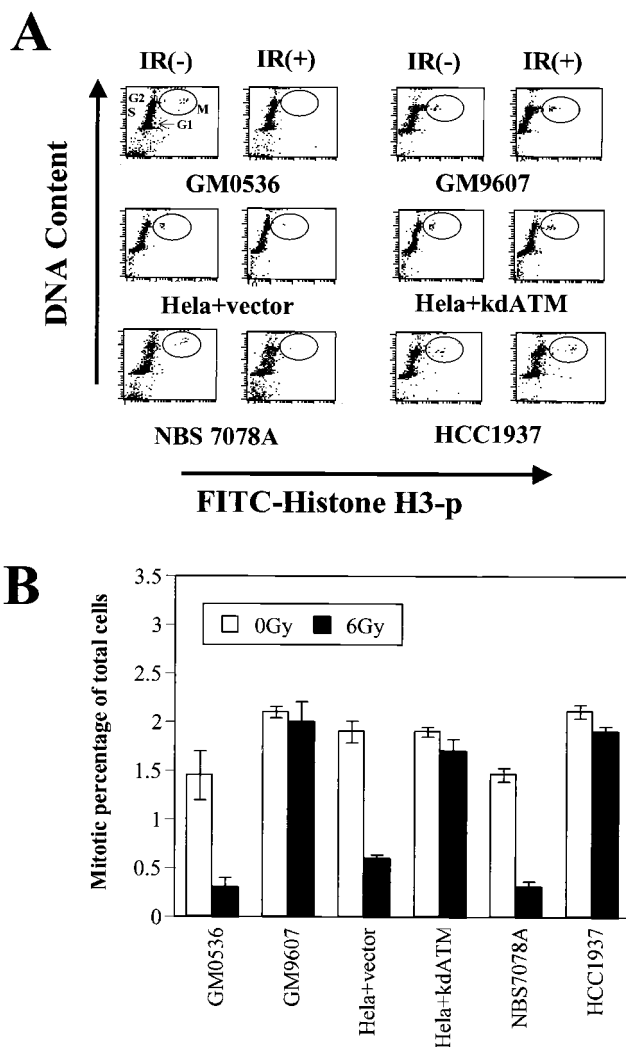


FIG. 2. Atm and Brca1, but not Nbs1, are required for the IR-induced G₂ checkpoint. (A) Flow cytometric profiles of cell cycle distribution before [IR(-)] and 1 h after [IR(+)] IR using a combination of staining for DNA content (y axis) and for histone H3 phosphorylation (x axis). The 4N population of cells is distinguished as either in G₂ or in mitosis (encircled, M). Control cells (GM0536 and HeLa+vector) and cells defective in Nbs1 (NBS7078A) exhibit a marked decrease in the number of mitotic cells after IR, while cells defective in Atm (GM9607 and HeLa+kdATM) or Brca1 (HCC1937) continue to enter mitosis after IR. (B) Mitotic cells as a percentage of total cells before and after IR (quantitation of data shown in panel A). The error bars represent the variability after combining the results of at least three different experiments for each cell line.

function, cells from an NBS patient (NBS7078A) exhibited a normal IR-induced G₂ checkpoint (Fig. 2). Thus, in contrast to its critical role in the S-phase checkpoint, Nbs1 function does not appear to be required for the G₂ checkpoint. The Brca1-null cell line HCC1937 exhibited a G₂/M checkpoint abnormality similar to that seen in the A-T cells (Fig. 2). In order to assess whether this abnormality in HCC1937 cells was due to their altered Brca1 function, wild-type Brca1 was reintroduced into the cells by transient transfection. Transfection of Brca1 did not affect cell cycle distribution in nonirradiated cells.

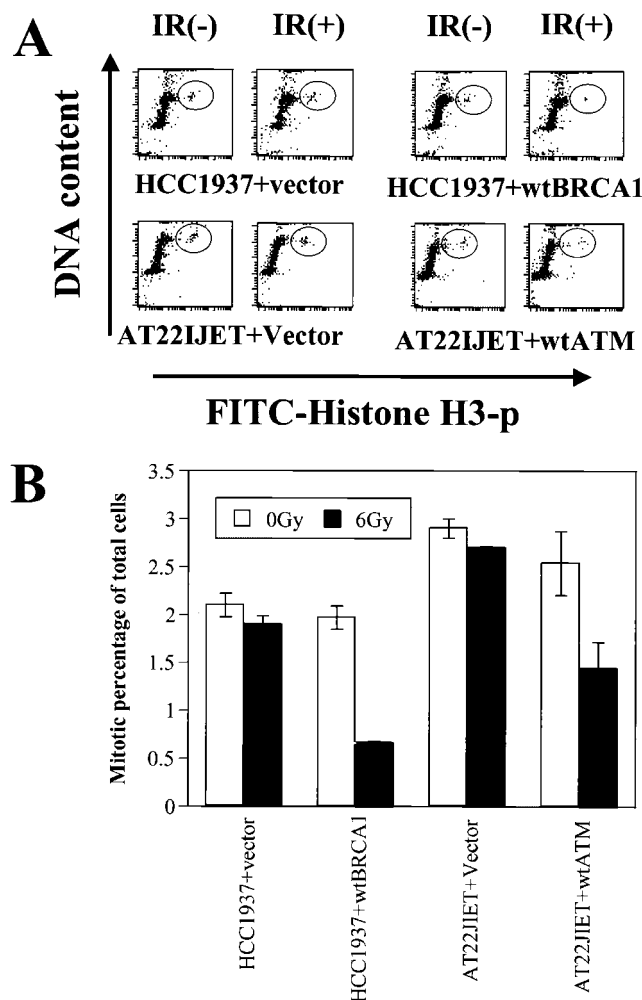


FIG. 3. Introduction of wild-type Brca1 complements the G_2/M checkpoint defect in HCC1937 cells. (A) Assessment of the G_2/M checkpoint as in Fig. 2A, comparing HCC1937 cells transfected with either control vector or wild-type *BRCA1*. A-T cells transfected with control vector or complemented with wild-type *ATM* are shown for comparison. (B) Quantitation of data shown in panel A, averaged over three experiments.

However, restoration of Brca1 function restored the IR-induced G_2 arrest in these cells (Fig. 3), thus demonstrating a role for Brca1 in this checkpoint pathway.

Serine 1423 of Brca1 is important for the IR-induced G_2/M checkpoint but not the S-phase checkpoint. Since similar cell cycle checkpoint defects were found in cells defective in Atm and Brca1, and since Brca1 is phosphorylated by Atm *in vitro* and *in vivo* (5, 24), we examined the impact of mutating a major Atm phosphorylation site in *BRCA1* (serine 1423 to alanine). While the mutated Brca1 still rescued the S-phase checkpoint defect in HCC1937 cells (Fig. 4B), the G_2/M checkpoint defect was not complemented (Fig. 4C and D). The failure to complement the G_2/M checkpoint is not attributable to a lack of expression of the mutant Brca1, since the S-phase checkpoint was rescued in the same cells (Fig. 4B) and since the mutant Brca1 is expressed at levels similar to those of the wild-type protein (Fig. 4A). These results implicate Atm phos-

phorylation of serine 1423 of Brca1 in the regulation of the IR-induced G_2/M checkpoint.

DISCUSSION

Exposure of cells to DNA-damaging agents can result in perturbations of cell cycle progression and in cell death. The molecular mechanisms controlling these endpoints has important implications for both cancer causation and tumor responses to cytotoxic therapies. Patients carrying mutations in both alleles of the *ATM* or *NBS1* gene are at high risk for the development of lymphoid malignancies (22), while patients with heterozygous mutations in *BRCA1* have a markedly increased risk of developing breast or ovarian cancer (14). Cells defective in the function of any of these three gene products exhibit decreased viability after exposure to ionizing irradiation (21, 22). Atm function is also required for the arrests of cell cycle progression in the G_1 , S, and G_2 phases typically seen after the exposure of the cells to ionizing irradiation (9, 16), and Nbs1 function has been reported to be required for the IR-induced S-phase checkpoint but not for the G_1 checkpoint (13, 27). However, conclusions about the role of Nbs1 in the G_2 checkpoint and the importance of Brca1 in any of these checkpoints have been left somewhat ambiguous from previous reports.

Here, examination of the cell cycle arrests following IR demonstrated that cells defective in any of these three gene products lack the rapid, but transient, arrest typically seen in the S phase. Similar to the previously reported complementation of the S-phase checkpoint defects in Atm-null and Nbs1-null cells with their respective missing gene products (13, 28), introduction of wild-type *BRCA1* into Brca1-null cells restored the IR-induced S-phase checkpoint. This implicates Brca1 in the IR-induced S-phase checkpoint for the first time. Though our data suggest that Atm phosphorylation of serine 1423 of Brca1 is not an important step in the IR-induced S-phase delay, this does not mean that Atm phosphorylation of Brca1 is not important for this checkpoint. Since Atm phosphorylates other sites in Brca1 (5), one or more of these other sites could be important for this function. This could be addressed by mutating each phosphorylation site individually and examining them for a failure to complement RDS.

Using a facile flow cytometric assay that distinguishes G_2 cells from mitotic cells, we were also able to confirm that the progression of cells from G_2 into mitosis is halted at very early times after irradiation and to demonstrate that this IR-induced cell cycle arrest requires the function of Atm and Brca1 but not Nbs1. This result confirms the earlier suggestion from studies with mouse cells (25) that Brca1 participates in the IR-induced G_2/M checkpoint. Finally, since mutation of serine 1423 in Brca1, a major phosphorylation site by Atm (5, 24), abolished complementation of the G_2/M checkpoint, Atm phosphorylation of this site is implicated in the G_2/M checkpoint pathway. This is the first target of Atm that has been shown to affect this checkpoint. Though the mechanism by which Brca1 affects cell cycle progression in G_2 remains to be elucidated, the potential involvement of serine 1423 phosphorylation in this process provides a starting point for subsequent investigations.

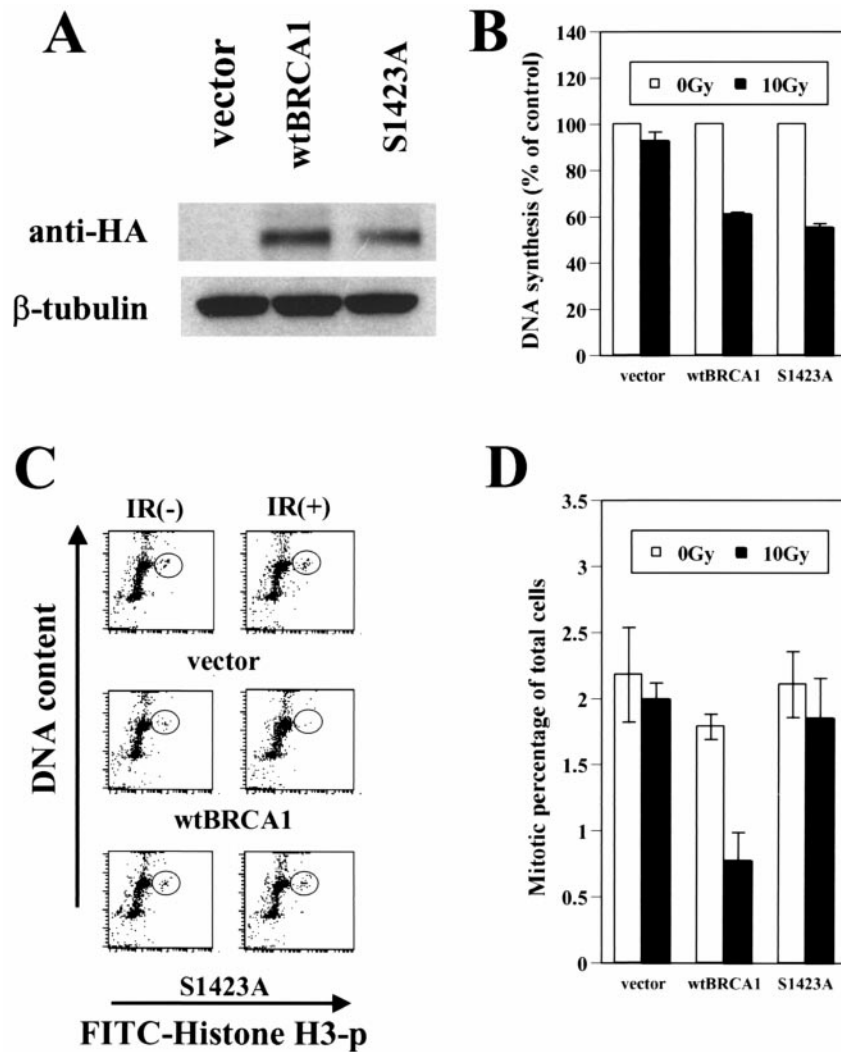


FIG. 4. *BRCA1* mutated at serine 1423 complements the S-phase checkpoint but not the G_2/M checkpoint. (A) Expression of transduced hemagglutinin (HA)-tagged Brca1 (wild type and Brca1 with an alanine substitution at serine 1423) was assessed by immunoblotting with anti-HA antibody. β -Tubulin levels are shown as a loading control. (B) Replicative DNA synthesis was assessed 30 min after 10 Gy of ionizing irradiation in the complemented HCC1937 cells. Error bars represent the averages of at least triplicate samples. (C) Assessment of the G_2/M checkpoint comparing HCC1937 cells complemented with either control vector, wild-type Brca1, or Brca1 with serine 1423 mutated to alanine (S1423A). (D) Quantitation of data shown in panel C, averaged over three experiments.

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