Cell, Vol. 90, 425–435, August 8, 1997, Copyright ©1997 by Cell Press

Dynamic Changes of BRCA1 Subnuclear Location and Phosphorylation State Are Initiated by DNA Damage

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Summary

BRCA1 localizes to discrete nuclear foci (dots) during S phase. Hydroxyurea-mediated DNA synthesis arrest of S phase MCF7 cells led to a loss of BRCA1 from these structures. Ultraviolet light, mitomycin C, or gamma irradiation produced a similar effect but with no concurrent arrest of DNA synthesis. BARD1 and Rad51, two proteins associated with the BRCA1 dots, behaved similarly. Loss of the BRCA1 foci was accompanied by a specific, dose-dependent change(s) in the state of BRCA1 phosphorylation. Three distinct DNA damaging agents preferentially induced this change in S phase. The S phase BRCA1 phosphorylation response to DNA damage occurred in cells lacking, respectively, two DNA damage-sensing protein kinases, DNA-PK and Atm, implying that neither plays a prime role in this process. Finally, after BRCA1 dot dispersal, BRCA1, BARD1, and Rad51 accumulated, focally, on PCNA⁺ replication structures, implying an interaction of BRCA1/BARD1/Rad51 containing complexes with damaged, replicating DNA. Taken together, the data imply that the BRCA1 S phase foci are dynamic physiological elements, responsive to DNA damage, and that BRCA1-containing multiprotein complexes participate in a replication checkpoint response.

Introduction

BRCA1 is a tumor suppressor gene that maps to human chromosome 17q 21.3 (Futreal et al., 1994; Hall et al., 1990; Miki et al., 1994; Neuhausen and Marshall, 1994; Smith et al., 1992). When one copy of *BRCA1* is inactivated in the germ line, affected individuals are predisposed to developing breast, ovarian, and other malignant tumors (reviewed in Feunteun and Lenoir, 1996). Until recently, there has been little understanding of how its product operates as a tumor suppressor or in any other capacity.

BRCA1 is an 1863 residue nuclear polypeptide which appears in discrete, nuclear foci (dots) during S phase (Chen et al., 1996; Scully et al., 1996, 1997a). These structures contain at least two other proteins, Rad51 and BARD1, both of which form complexes with BRCA1 in vivo (Scully et al., 1997a; Wu et al., 1996; R. Baer, personal communication and data presented below).

The *BRCA1* gene is widely expressed in developing embryos, with a marked preference for replicating cells (Lane et al., 1995; Marquis et al., 1995). It is essential for early embryonic proliferation and development (Gowen et al., 1996; Hakem et al., 1996; Liu et al., 1996). Recently, its full-length product was found to interact, directly or indirectly, with Rad51, a major participant in eukaryotic double-strand break repair and homologous recombination (Shinohara et al., 1992; Baumann et al., 1996; Scully et al., 1997a). BRCA1/Rad51 interactions have been identified in both mitotic and meiotic cells (Scully et al., 1997a), where Rad51 contributes to meiotic recombination (Ashley et al., 1995; Bishop, 1994; Terasawa et al., 1995). These observations imply that BRCA1 and Rad51 communicate physiologically and further suggest that BRCA1 functions in the maintenance of genome integrity.

In keeping with these findings, Sharan et al. (1997) have reported that another familial breast cancer tumor suppressor gene product, BRCA2, can interact with Rad51, and that murine embryos lacking wild-type *BRCA2* exhibit radiation sensitivity. Collectively, these data suggest that loss of functional BRCA1 or BRCA2 are mutagenic events, and, thereby, accelerate neoplastic transformation. Interestingly, BRCA1 and BARD1 each contain a C-terminal "BRCT" domain, which is found in many DNA repair and cell cycle checkpoint proteins (Bork et al., 1997; Callebaut and Mornon, 1997; Koonin et al., 1996). The generic function of the BRCT domain is not clear. However, this segment of BRCA1 has both transactivation (Chapman and Verma, 1996; Monteiro et al., 1996) and growth suppression properties (Humphrey et al., 1997) and may play a part in docking BRCA1 onto the RNA polymerase II holoenzyme (Scully et al., 1997b).

Although these observations are consistent with a role for BRCA1 in DNA repair and the maintenance of genome stability, there is little evidence that speaks to a dynamic function of BRCA1 in this regard. Here we report that BRCA1/Rad51/BARD1 containing S phase nuclear foci are sensitive to the integrity of the genome, undergoing a major structural change in the face of genotoxic insult. This response to DNA damage is accompanied by a specific change in BRCA1 phosphorylation and by the relocation of BRCA1, BARD1, and Rad51 to sites of "abnormal" (nonduplex) DNA structure in S phase cells. These findings suggest that BRCA1 participates in an S phase, DNA damage-dependent cell cycle checkpoint response.

Results

Disruption of BRCA1 S Phase Nuclear Foci by DNA Damage

A proportion of BRCA1 is localized to nuclear foci in S phase cells. These structures were not detected in

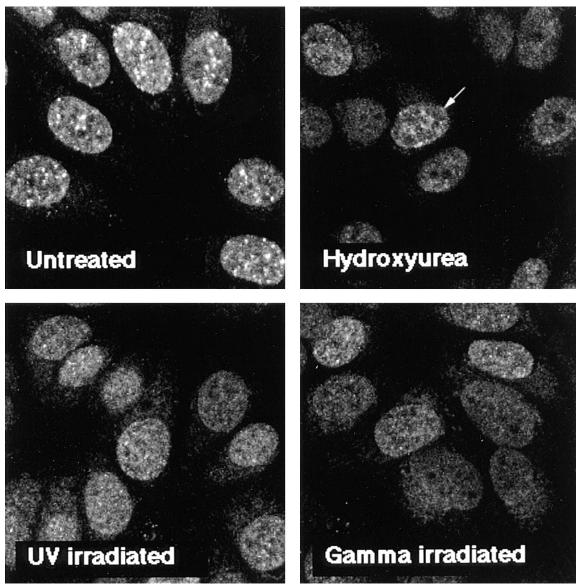

Figure 1. DNA Damage Disperses BRCA1 S Phase Focal Staining

S phase MCF7 cells were treated with DNA damaging agents, as indicated. Immunostaining for BRCA1 was performed using mAb MS13. Cells received either no treatment, HU 1 mM, UV 10 Jm^{-2}, or 5000 Rads and were harvested 1 hr later. The arrow indicates a rare cell in an HU-treated culture which retains some focal staining for BRCA1.

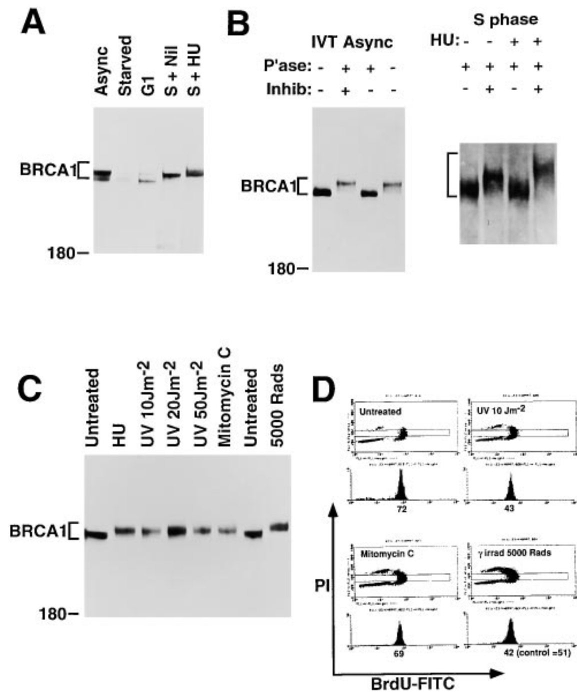

Figure 2. Specific Phosphorylation of BRCA1 following DNA Damage

(A) Cell cycle variation in BRCA1 gel mobility. MCF7 extracts were immunoblotted for BRCA1 using mAb MS110. Migration of BRCA1 is indicated. Async, asynchronous culture (58% G1, 30% S); starved, cells after 24 hr of serum starvation (90% G1, 2.5% S); G1, cells 12 hr after release into high serum (86% G1, 7% S); S + Nil, cells 24 hr after release into high serum (40% G1, 54% S); S + HU, identically treated S phase cells (in parallel) cultured after 24 hr of serum refeeding in HU for 1 hr before harvesting.

(B) Changes in mobility are due to changes in phosphorylation of BRCA1. BRCA1 IPs were treated with λ-phosphatase (see Experimental Procedures) ± phosphatase inhibitors, as indicated, and then immunoblotted for BRCA1. IVT, in vitro translated wild-type BRCA1. Left panel, MCF7 cells were asynchronous. Right panel, similar treatment of S phase MCF7 cells ± HU, as indicated. BRCA1 species are bracketed.

(C) Phosphorylation of BRCA1 in S phase after DNA damage. S phase MCF7 cells were exposed to the treatments shown for 1 hr prior to harvesting. Cell extracts were then immunoblotted for BRCA1.

(D) Cell cycle analysis on samples from (C). BrdU staining and cell cycle FACS analysis were performed as described in Experimental Procedures. To quantitate BrdU incorporation in S phase cells, a FACS gate was used to exclude G1 and G2 populations. Under each panel, the histogram gives the mean BrdU fluorescence intensity of gated (S phase) cells, in arbitrary units. HU-treated S phase cells, which had arrested DNA synthesis, had a mean BrdU fluorescence intensity of 13 in the same experiment.

multiple cell lines during G1, when a less intense nucleoplasmic BRCA1 immunostaining signal was observed (Scully et al., 1997a). They can be detected using many different BRCA1-specific Abs, raised to distinct epitopes, using any of several different fixation methods, or in living cells containing green fluorescent protein (GFP)-tagged BRCA1 (Scully et al., 1996; R. S., D. M. L., J. A. DeCaprio, and P. A. Silver, unpublished observations). Further, BRCA1 foci exist in mouse fibroblast nuclei as shown by immunofluorescence with anti-murine BRCA1 (X. Wu and D. M. L., unpublished observations). Hence, they are general BRCA1 phenomena.

Since BRCA1 is suspected of playing a role in genome integrity maintenance, we asked whether the S phase BRCA1 dots were altered in S phase cells after DNA damage and/or when DNA synthesis is interrupted. Hydroxyurea (HU) was used to induce DNA synthesis arrest of S phase cultures of the human breast cancer cell line, MCF7. BRCA1 immunostaining of HU-treated cells, performed with any of three different BRCA1 monoclonal antibodies, revealed overt dispersal of BRCA1 nuclear foci (Figure 1). Given the likelihood that HU treatment of S phase cells mimics DNA damage (Allen et al., 1994; Carr, 1995; Sanchez et al., 1996; Sun et al., 1996), we asked whether other DNA damaging agents affect the integrity of the S phase BRCA1 dots. Treatment with ultraviolet (UV) irradiation, mitomycin C, or gamma irradiation also led to dispersal of the dots within 1 hr (Figure 1 and data not shown). Untreated or mock-treated S phase cells revealed typical BRCA1 dots (Figure 1 and data not shown). Thus, dispersal of BRCA1 S phase foci might represent a cellular response to DNA damage. A few cells retained BRCA1 foci after HU or UV treatment (e.g., cell indicated by an arrow in Figure 1). The nature of these foci is discussed below (Figures 5 and 6 and accompanying text).

Cell Cycle–Specific Phosphorylation of BRCA1

In an effort to understand the mechanisms governing the migration of BRCA1 into and out of S phase foci, we sought biochemical correlates of the different BRCA1 nuclear immunofluorescence patterns. Immunoblotting for p220 BRCA1 in asynchronous MCF7 cells revealed a doublet (Figure 2A). Each band of the doublet reacted

with BRCA1 monoclonal antibodies (mAbs), MS13, MS110, SG11, and AP16 (data not shown and Figure 2B). Serum-starved MCF7 cells contained reduced levels of BRCA1 (Figure 2A). Cells released into G1 for 12 hr produced an enrichment of the faster migrating band of the doublet (Figure 2A). In contrast, 24 hr after release into high serum, when most cells were in S phase, there was a further increase in BRCA1 protein level, represented primarily by the slower migrating form of the protein (Figure 2A). Similar observations concerning the migration of G1 and S phase associated forms of BRCA1 have been made by Ruffner and Verma (1997).

A parallel culture of S phase MCF7 cells was treated with HU for 1 hr. Immunoblotting revealed the presence of a form of BRCA1 that was not detected in untreated cycling cells but which migrated more slowly than the BRCA1 present in untreated S phase cells (Figure 2A). Thus, endogenous ''p220'' BRCA1 was detectable in at least three different forms: a rapidly migrating, G1-associated form; a more slowly migrating, S phase form; and an even more slowly migrating form, noted in HU-treated S phase cells.

Thus, BRCA1 might undergo regulated post-translational modifications, such as phosphorylation. Consistent with this, phosphatase treatment of BRCA1 immunoprecipitates (IPs) altered the gel mobility of BRCA1 (Figure 2B). IPs of BRCA1 from asynchronous MCF7 cells, using the C-terminal mAb, SG11, were aliquoted into three fractions. The first was treated with λ-phosphatase in the presence of phosphatase inhibitors; the second with λ-phosphatase in the absence of inhibitors; and the third was left untreated. IPs were immunoblotted using the N-terminal BRCA1 mAb, MS110. Phosphatase treatment in the absence of inhibitors resulted in collapse of the BRCA1 doublet into a single band which comigrated with in vitro synthesized, clonal BRCA1 (Figure 2B). Phosphatase treatment in the presence of inhibitors did not perturb the BRCA1 doublet relative to untreated IPs (Figure 2B), ruling out nonspecific effects of the phosphatase preparation. Similarly, phosphatase treatment of BRCA1 IPs, prepared from HU-treated S phase cells, led to its comigration with the phosphatase-treated BRCA1 species detected in naive S phase cells (Figure 2B). These results strongly suggest that the differential gel mobility of the three forms of BRCA1, noted in G1, S phase, and HU-treated S phase cells, is due to differential phosphorylation.

HU treatment of S phase cells, therefore, led to three measured events: DNA synthesis arrest, dispersal of BRCA1 foci, and phosphorylation of BRCA1. DNA synthesis arrest following brief (2 hr) HU exposure was found to be reversible. Removal of HU after this time led to the resumption of full DNA synthesis. Furthermore, both the BRCA1 foci and the faster migrating S phase BRCA1 band reappeared, while the slower HU-associated band disappeared (data not shown). Therefore, within the time limits of this experiment, all three effects of HU were reversible.

Phosphorylation of BRCA1 after DNA Damage in S Phase without Arrest of Scheduled DNA Synthesis

Exposure of S phase MCF7 cells to UV irradiation, mitomycin C, or gamma irradiation was found to retard the migration of the S phase BRCA1 band, similar to the effect of HU treatment (Figure 2C). This effect, coupled with the above noted dispersal of BRCA1 S phase foci (Figure 1), indicated a similarity between the effect of these DNA damaging agents and HU. However, in contrast to HU-treated cells, the response of S phase cells to two of these three DNA damaging agents did not include acute DNA synthesis arrest. Specifically, mitomycin C–treated and gamma-irradiated S phase MCF7 cells showed no impairment of BrdU incorporation compared with untreated controls, at a time when BRCA1 had already undergone the relevant DNA damage-induced phosphorylation (Figures 2C and 2D). UV treatment led to a dose-dependent inhibition of BrdU uptake, with only a modest impairment of DNA synthesis detectable in cells treated with 10 Jm^{-2}, but near total DNA synthesis arrest seen at 50 Jm^{-2} (Figure 2D and data not shown). Ten joules per square meter did, however, lead to the supershift of the S phase band, as seen following treatment with HU, mitomycin, or gamma irradiation (Figure 2C).

The finding of continued BrdU incorporation into S phase cells that had sustained acute DNA damage could be interpreted as unscheduled DNA synthesis (i.e., repair synthesis) in the context of an arrest of scheduled DNA synthesis. Although some repair process might be expected to be occurring at this time (e.g., to permit resolution of abnormal DNA structures at replication forks), the data are incompatible with the idea that scheduled DNA synthesis itself had ceased. First, BrdU incorporation during repair synthesis should be much less efficient than during normal DNA replication (Li et al., 1996), whereas near normal DNA synthesis levels were noted after acute exposure to mitomycin C, gamma irradiation, or 10 Jm^{-2} UV. Second, if the BrdU incorporation detected were a manifestation of repair synthesis alone, a higher density of DNA lesions should produce a higher level of BrdU incorporation. However, the reverse was true for UV treatment, where increasing doses led to progressive impairment of BrdU incorporation efficiency. Therefore, 1 hr after treatment with either UV irradiation (10 Jm^{-2}), mitomycin C, or gamma irradiation, scheduled DNA synthesis had not yet ceased. Therefore, DNA damage-associated BRCA1 phosphorylation can occur in S phase cells without arrest of scheduled DNA synthesis.

Although three DNA damaging agents and HU had disparate effects upon scheduled DNA synthesis, the feature common to all these treatments is their ability to induce DNA lesions, rather than their effect on the replication machinery per se. HU treatment might be predicted to produce, at least transiently, ''abnormal'' (i.e., nonduplex) DNA structures at arrested replication forks. The simplest model to explain these phenomena would be one in which ''abnormal'' DNA structures, generated in S phase, trigger a signaling cascade, one outcome of which is specific BRCA1 phosphorylation.

Time Course of the Response to UV Irradiation

These results suggest a relationship between DNA damage-associated phosphorylation of BRCA1 and dispersal of the BRCA1 dots. This was explored further, using UV as the stimulus. The phosphorylation status

of BRCA1 was followed at 10-min intervals following a pulse of 10 Jm^{-2}, administered to S phase MCF7 cells. A significant alteration in BRCA1 gel mobility was apparent 20–30 min after treatment (Figure 3A). In a similar experiment, the time course of dispersal of BRCA1 foci was followed at 5-min intervals, by scoring, at each time point, four randomly selected confocal microscopic fields for the percentage of cells containing BRCA1 foci. Significant dispersal of BRCA1 foci was not detected until 25 min after the UV pulse (Figure 3B). Thus, at this UV dose (and also at higher doses, data not shown), there was a close temporal correlation between damage-induced phosphorylation of BRCA1 and dispersal of the BRCA1 foci.

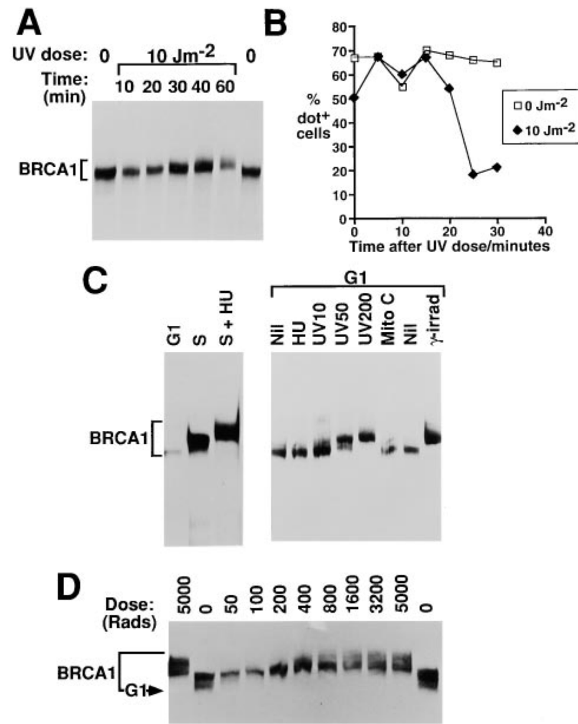

Figure 3. Time Course and Cell Cycle Specificity of the BRCA1/DNA Damage Response

(A) Time course of phosphorylation change after UV irradiation. S phase enriched MCF7 cells were exposed to 10 Jm^{-2} UV light, harvested at the time points indicated, and immunoblotted for BRCA1. The shift in BRCA1 migration is seen ~20–30 min after UV exposure.

(B) Time course of BRCA1 focus dispersal following UV exposure. In a protocol identical to that employed in A, S phase MCF7 cells were exposed to 10 Jm^{-2} UV light (filled diamonds), or mock treated (open squares), and were harvested at the time points indicated. Each coverslip was stained for BRCA1, and cells in four randomly selected confocal fields were scored for the presence or absence of BRCA1 nuclear foci. One hundred and fifteen to 185 cells were scored per time point. Results are presented as the percentage of cells scoring positive for BRCA1 foci.

(C) S phase specificity of the BRCA1 damage response. MCF7 cells were released from serum starvation into G1 for 7 hr. After treatment with DNA damaging agents, as shown, cells were harvested 1 hr later (while still in G1) and immunoblotted for BRCA1. Extracts of S phase MCF7 and HU-treated S phase cells were analyzed in parallel to show the relative migration of the G1, G1/damage, S and S/damage forms of BRCA1. Note that HU, mitomycin C, and low-dose UV treatment (10 Jm^{-2}) each failed to shift the G1 form of BRCA1 under conditions in which the S phase form had undergone damage-induced phosphorylation (compare with Figure 2C).

(D) BRCA1 gel migration change after ionizing radiation. Asynchronously growing MCF7 cells were exposed to metered doses of gamma irradiation, harvested 1 hr later, and then immunoblotted for BRCA1. The lower (G1) band of BRCA1, indicated with an arrow, was noted to disappear at 50 Rads, whereas the S phase band shifted only at doses above 200 Rads.

Hydroxyurea, Mitomycin C, and UV Treatments Preferentially Target BRCA1 in S Phase

The data, noted above, raise the question of whether BRCA1 is targeted for phosphorylation by DNA damage only in S phase. The migration pattern of MCF7 in asynchronous or G1-enriched cells provided a means to address this question. We had noted (Figure 2A) that there is a faster migrating form of p220 BRCA1 enriched in G1 MCF7 cells and detectable in asynchronous cultures. Asynchronous MCF7 cells were subjected to treatment with HU, UV, or gamma irradiation. One hour later they were harvested and immunoblotted for BRCA1. Consistently, HU treatment or low-dose UV (10 Jm^{-2}) treatment induced the predicted BRCA1 gel shift of the upper (S phase correlated) but not the lower (G1 correlated) BRCA1 band (data not shown). In contrast, gamma irradiation (5000 Rads) appeared to displace both forms of BRCA1. This implied that low-dose UV or HU treatment might produce phosphorylation of BRCA1 in S phase but not in G1.

To test this notion directly, we prepared G1-synchronized MCF7 cells by serum starvation followed by 7 hr of incubation in high serum. These synchronized cells were then exposed to HU, UV, mitomycin C, or gamma irradiation and harvested 1 hr later, while still in G1. Immunoblotting for BRCA1 in unperturbed control G1 cells revealed the presence of the faster-migrating, G1 form of BRCA1, albeit at levels lower than in S phase cells (Figure 3C, left panel). Strikingly, neither HU, mitomycin C, nor low-dose UV treatment (10 Jm^{-2}) led to a change in the mobility of the G1 band (Figure 3C, right panel). Under identical conditions, the S phase band shifted (compare Figures 2C and 3C). Higher doses of UV led to a dose-dependent shift in the G1 form of BRCA1 (Figure 3C) as did gamma irradiation (5000 Rads).

This preferential S phase targeting of BRCA1 for phosphorylation, following HU, low dose UV, or mitomycin C, could be interpreted in two ways. First, the sensor(s) of abnormal DNA structure, or their subsequent amplificatory cascades, might operate differently between S phase and G1. Second, the S phase preference for BRCA1 phosphorylation after UV/mitomycin C could be an attribute of the BRCA1 protein itself, rather than of the signals impinging on it. A hint that the former might be correct came from examination of the response to gamma irradiation. When asynchronous MCF7 cells were exposed to a range of doses of gamma irradiation, the emergence of BRCA1 species migrating slower than the S phase band was apparent only at doses above 200 Rads (Figure 3D). In contrast, exposure to 50 Rads was sufficient to displace the G1 form of the protein (Figure 3D). Therefore, gamma irradiation appeared to

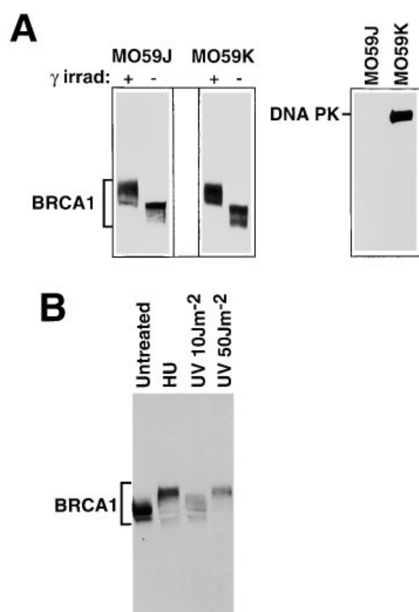

Figure 4. BRCA1 Damage Response in DNA-PK and ATM Mutant Cell Lines

(A) Asynchronously growing human glioma cell lines, MO59J (DNA-PK null) and MO59K (DNA-PK wild type) were exposed to 5000 Rads, or mock treated, and harvested 1 hr later. Immunoblotting for BRCA1 is shown in the left panel, and for DNA-PK in the right panel.

(B) Response of AT fibroblasts to HU and UV treatment. Asynchronous cultures of the AT primary human diploid fibroblast culture, GM02052B, were exposed to DNA damaging agents as shown, harvested 1 hr later, and immunoblotted for BRCA1.

provide an exception to the above-noted preference for S phase in signaling from DNA damage to BRCA1. This suggests that cell cycle specificity in the BRCA1 DNA damage response is a property of the particular sensors of and signaling arising from DNA damage, rather than of the BRCA1 protein itself.

DNA-Dependent Protein Kinase and the Ataxia Telangiectasia Gene Product Are Not Required for DNA Damage-Induced BRCA1 Phosphorylation in S Phase

Genetic and biochemical approaches suggest a role for the PIK family of nuclear protein kinases in linking the detection of DNA damage to cell cycle responses (Bentley et al., 1996; Cimprich et al., 1996; Hari et al., 1995; Hartley et al., 1995; Keith and Schreiber, 1995; Morrow et al., 1995; Savitsky et al., 1995). This family of proteins includes the catalytic subunit of DNA-dependent protein kinase (p460 DNA-PK), Atm, and Atr. Initially, we asked whether a functional copy of DNA-PK was necessary for detection of the BRCA1 DNA damage-phosphorylation response. The human glioma cell line, MO59, has two derivatives, one of which (MO59K) is wild-type for p460 DNA-PK. The other (MO59J) does not express its gene (Lees-Miller et al., 1995). Using gamma irradiation as the stimulus, we asked whether the two cell lines could each respond to DNA damage by phosphorylating BRCA1. Indeed, the two cell lines mounted indistinguishable responses to gamma irradiation (Figure 4A and data not shown). Immunoblotting of whole cell extracts was used to confirm the expression of p460 DNA-PK in MO59K cells and its absence from MO59J cells (Figure 4A). BRCA1 phosphorylation after HU or UV treatment was also detected in both cell lines, and the response to each of these stimuli was indistinguishable between MO59J and MO59K cells (data not shown). These results exclude p460 DNA-PK as a necessary component of the DNA damage-BRCA1 phosphorylation pathway.

To investigate a potential role for Atm, we analyzed primary cultures of Atm homozygous mutant fibroblasts. Both HU and UV exposure elicited a clear retardation in the migration of the S phase BRCA1 band (Figure 4B). A similar response to gamma irradiation was also noted (data not shown), although a quantitative effect of Atm on the DNA damage-BRCA1 signaling pathway has not yet been ruled out.

Recruitment of BRCA1 to PCNA- and DNA-Containing Nuclear Structures after DNA Damage in S Phase

Close examination of the BRCA1 immunostaining pattern after HU treatment or UV irradiation revealed that the frequency of cells, within S phase cultures, containing BRCA1 nuclear foci, although substantially reduced, was not zero (Figures 1 and 3B). In a small proportion of cells, there were characteristic small, punctate BRCA1 dots. In yet others, a qualitatively different BRCA1 focal pattern was detected (see below). The reasons for the presence of these different BRCA1 focal staining patterns became clear when cells were double stained for BRCA1 and proliferating cell nuclear antigen (PCNA), as detailed below.

Under some fixation conditions, PCNA immunostaining is seen only in cells synthesizing DNA, and given that its staining pattern changes during S phase, it can be used as an S phase temporal marker (Bravo, 1986; Bravo and Macdonald-Bravo, 1987). In early/mid–S phase cells, PCNA immunostaining is in a multifocal/diffusely nuclear pattern. In late S phase, the staining pattern changes dramatically and becomes ''nodular.'' Importantly, throughout S phase, the immunostaining pattern of BrdU incorporation into replication forks clearly overlies the PCNA stain (Bravo and Macdonald-Bravo, 1987; Nakamura et al., 1986; Figure 5A). We confirmed, by the use of a mimosine block and release protocol, that these changes in PCNA morphology are similarly correlated with the stage of S phase in MCF7 cells (data not shown).

In synchronized cells, BRCA1 foci first appear in S phase. This raised the question of whether BRCA1 foci coincide with PCNA foci. This was addressed using two-color immunostaining followed by confocal microscopy. Images in Figure 5B depict unperturbed, S phase MCF7 cells doubly stained for BRCA1 (green, fluorescein isothiocyanate [FITC]) and PCNA (red, rhodamine). In repeated experiments, the two immunostaining patterns were found to be distinct, even when the PCNA pattern resembled the nodular one reported for late S phase cells (Bravo and Macdonald-Bravo, 1987; Nakamura et al., 1986). Thus, in conventionally cycling S phase cells, there was no immunocytochemical indication that

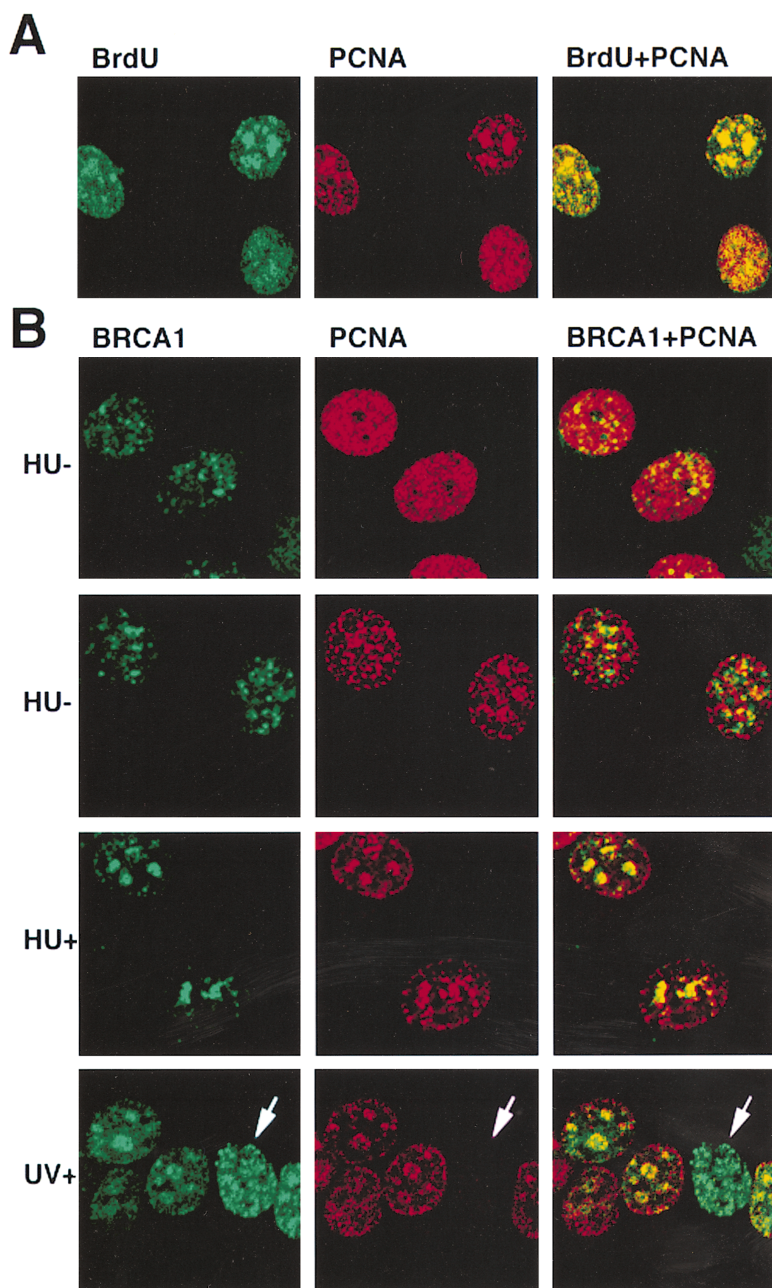

Figure 5. Recruitment of BRCA1 to Replication Structures after HU or UV Treatment

(A) In S phase cells, PCNA immunostaining can be used to locate sites of DNA synthesis. Panels depict S phase MCF7 cells, pulsed with BrdU prior to fixation, double stained with anti-BrdU mAb (green), and anti-PCNA Ab (''AK'' serum, red). Where colocalization of the two images is seen, the signal appears in the right hand panel as a yellow signal.

(B) Recruitment of BRCA1 to replication structures after HU treatment. In untreated S phase cells (''HU−''), BRCA1 foci (mAb MS13, green) were not significantly coincident with PCNA (AK Ab, red) in either early S phase (upper row) or late S phase (second row). In contrast, in HU-treated cells (''HU+,'' third row), BRCA1 colocalizes extensively with PCNA in late S phase nuclei, as shown by the yellow overlap signal of green and red. Similar results were obtained in UV treated cells (''UV+,'' lowest row). The arrow points to a cell carrying BRCA1 dots and no PCNA staining. This may be a G2 cell.

BRCA1 focally accumulates at replication forks. Further, a small proportion of cells was noted to be positive for BRCA1 foci and negative for PCNA. This population was found to be enriched in late S phase cultures (data not shown), suggesting that the presence of BRCA1 foci is also a feature of some G2 cells.

In contrast, when HU- or UV-treated S phase cultures were similarly examined, a striking colocalization of the BRCA1 staining pattern and the PCNA staining pattern was noted in those rare, late S phase cells in which PCNA immunostaining was clearly nodular or focal (Figure 5B and data not shown). In the majority of S phase nuclei, where the PCNA pattern was diffuse, the BRCA1 stain was also diffuse (data not shown). The overt relocation of BRCA1 to $PCNA^{+}$ structures after DNA damage suggests that BRCA1 is recruited to replication forks after HU or UV treatment of S phase cells. By contrast, the small subset of nuclei scoring positive for BRCA1 dots but negative for PCNA (presumed G2 cells, as noted above) were not perturbed by either HU or UV treatment (e.g., ''UV^{+}'' panel in Figure 5B, cell indicated by an arrow).

Colocalization of BRCA1, BARD1, and Rad51 before and after DNA Damage in S Phase

Two proteins associated with BRCA1 in S phase foci—Rad51 and BARD1—were examined during the DNA damage response. Consistent with the described physical interaction between BRCA1 and BARD1 (Wu et al., 1996), BARD1 immunostaining, reflected by binding of multiple antibodies to BARD1, colocalized with BRCA1 in S phase nuclear dots (Figure 6A). This result was

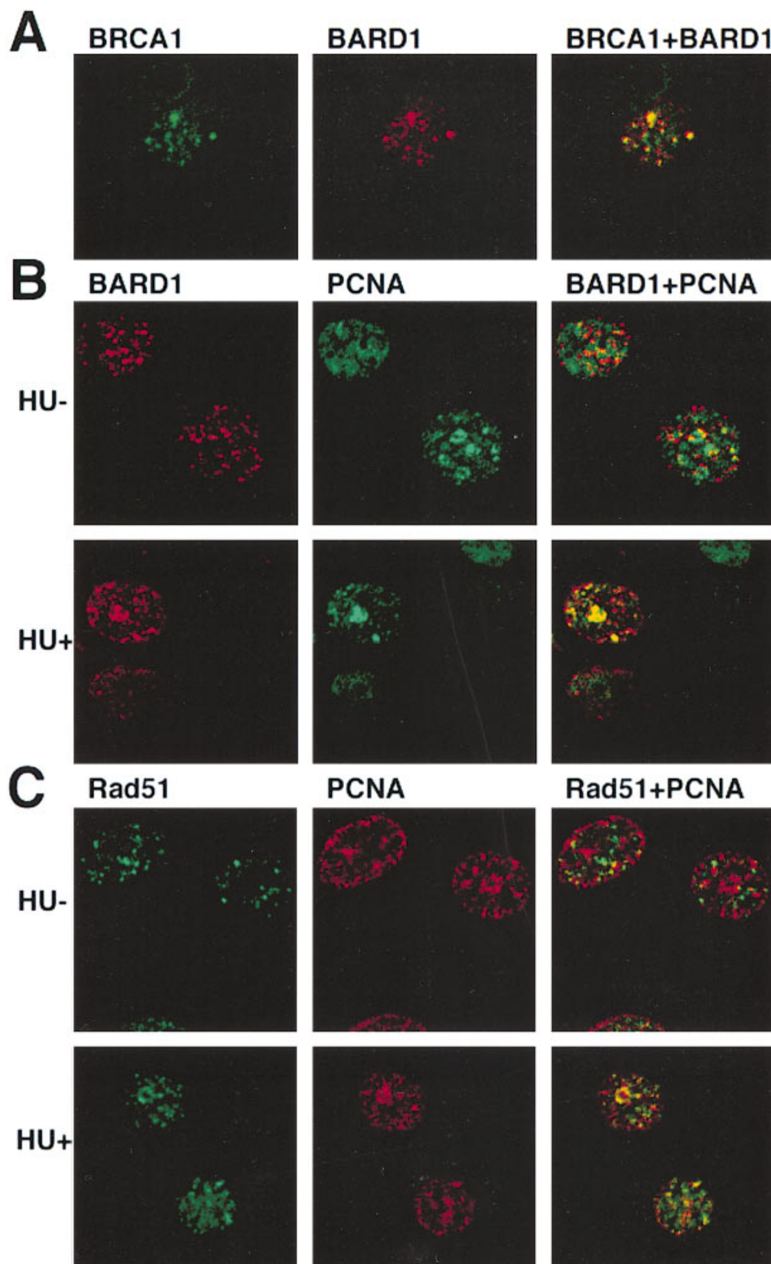

Figure 6. Recruitment of BARD1 and Rad51 to Replication Structures after DNA damage

(A) Colocalization of BRCA1 and BARD1 in S phase nuclear foci. Untreated S phase MCF7 cells, stained with BRCA1 mAb MS13 (green, FITC) and BARD1 Ab (rhodamine, red). Where the two nuclear dot signals overlap, a yellow signal was detected.

(B) Recruitment of BARD1 to replication structures after HU treatment. The upper panel depicts untreated S phase MCF7 cells ("HU−") double stained for BARD1 (using affinity purified polyclonal antiserum to BARD1, red) and PCNA (using mAb PC10, green). No significant colocalization of the green and red signals was noted. The lower panel shows the same, two-color immunstaining experiment, performed on HU-treated MCF7 cells ("HU+"). Where BARD1 and PCNA signals overlap, a yellow color was noted in the right-hand panel.

(C) Recruitment of Rad51 to replication structures after HU treatment. Similar treatments as for (B). Cells were double stained for Rad51 (using affinity purified polyclonal antiserum to Rad51, green) and PCNA (using "AK" antiserum, red), as described in Experimental Procedures. After HU treatment ("HU+"), but not in untreated cells ("HU−"), significant colocalization of Rad51 and PCNA is seen as a yellow overlap.

first obtained by Richard Baer and coworkers (personal communication). S phase (PCNA$^+$) nuclei were examined for BARD1 and Rad51 before and after HU or UV treatment. As was noted for BRCA1, undamaged cells revealed no significant colocalization of either Rad51 or BARD1 with PCNA (Figures 6B and 6C, see HU−). After HU or UV exposure, however, colocalization was seen on PCNA nodules (Figures 6B and 6C, see HU+). In the majority of S phase nuclei, where the PCNA stain was diffuse, Rad51 and BARD1 stains were also found to have become diffuse (data not shown). Thus, BRCA1 and two known associated proteins, BARD1 and Rad51, both concentrate in PCNA-containing, replicating structures after DNA damage. Like BRCA1 dots, BARD1 and Rad51 foci appeared to persist into G2 (data not shown). In addition, as was noted above for BRCA1 foci in G2, the BARD1/Rad51 G2 foci were not perturbed by either UV or HU treatment (data not shown).

These findings strengthen the notion that BRCA1 relocalizes to replication forks after DNA damage, since it does so in the company of two known physiological partners. Hence, either multiprotein BRCA1-containing complexes leave the dots after DNA damage, or the underlying subnuclear structure which constitutes the substance of the S phase foci, itself, undergoes disassembly after DNA damage.

Discussion

These experiments identify the p220 BRCA1 protein as a participant in a DNA damage response of cycling cells, thereby fulfilling the prediction that BRCA1 participates

in the maintenance of genome integrity (Scully et al., 1997a). Within 1 hr of treatment of cells with various DNA damaging agents, two effects were noted in the behavior of the BRCA1 protein. First, S phase cells lost the characteristic BRCA1 nuclear foci. Second, the protein underwent a specific change(s) in phosphorylation. Third, BRCA1 was now associated with PCNA/replicating DNA-containing structures. The timing of these events was closely correlated, suggesting that they are different manifestations of the same cellular response. Taken together, these findings allow one to construct a functional model of BRCA1 behavior, at least during S phase.

First, the BRCA1 dots, which clearly contain BRCA1-containing complexes, given the colocalization of both Rad51 and BARD1, appear to be dynamic physiological structures. Their integrity is, at a minimum, tied to the integrity of the genome. DNA damage leads to signals, transmitted over a measurable period of time, which result in the loss of BRCA1 containing protein/protein complexes from these structures, if not the loss of the structures themselves. These signals do not depend upon the cessation of DNA synthesis for accurate transmission and are, hence, not a specific result of replication arrest. Whether the dots are active in the absence of genome damage, playing an as yet unknown role in the replication process (and/or in postreplication events) or whether they are simply repositories of proteins that are active in the damage (and possibly other stress) response(s) is not clear. That BRCA1 appears to disperse from the dots after genome damage strongly suggests that BRCA1 itself plays a role in the response to DNA damage. Such a conclusion strongly supports the earlier speculation put forward on the occasion of the first detection of BRCA1/Rad 51 complexes (Scully et al., 1997a). Thus, the BRCA1/Rad51/BARD1 nuclear dots are an example of multiprotein-containing nuclear structures whose integrity is modified by modifiers of genome integrity.

Second, in parallel with the loss of the BRCA1 dots, DNA damage led to a specific alteration in the state of BRCA1 phosphorylation. The timing of the two events was similar, and both events were reversible in HU-treated cells, implying that they are linked and that both are reflections of the existence of unrepaired DNA damage. These findings indicate that BRCA1 is a substrate of one or more kinases activated specifically by DNA damage. They, therefore, place BRCA1 on an S phase DNA damage-initiated signaling pathway. G1 cells were able to respond with specific BRCA1 phosphorylation events following DNA damage, but there were clear differences in the substance of the responses between G1 and S phase cells. Whether the protein contributes to the enaction of both G1 and S (and possibly G2) checkpoint responses remains to be seen.

One class of genes implicated in such signaling pathways encode the "PIK" kinases, Atm, Atr, and p460 DNA-PK, each of which shows extensive conservation between yeast, drosophila and human (Bentley et al., 1996; Cimprich et al., 1996; Hari et al., 1995; Hartley et al., 1995; Keith and Schreiber, 1995; Morrow et al., 1995; Savitsky et al., 1995). Genetic analysis has suggested a role for Atm in multiple cell cycle checkpoints (Painter and Young, 1980; reviewed in Elledge, 1996). The yeast homologs of Atr, rad3^{sp}, and MEC1^{sc}, have been clearly implicated in S phase and other DNA damage checkpoints (Bentley et al., 1996; Paulovich and Hartwell, 1995). DNA-PK functions in double-stranded break repair and VDJ recombination (reviewed in Lieber et al., 1997). In addition, the products of these genes are protein kinases and they interact with yet other protein kinases. This combined evidence suggests that the "PIK" kinases are signal transducers, e.g., linking DNA damage with cell cycle events (reviewed in Elledge, 1996). Our observations on BRCA1 suggest that its phosphorylation after DNA damage might be an assay for the activity of one or more "PIK" kinases. There are data in the literature consistent with a model in which BRCA1 and Atr and, possibly, Atm interact on meiotic chromosomes (Keegan et al., 1996; Scully et al., 1997a).

The availability of tissue from ataxia-telangiectasia patients has provided cultured primary cells lacking Atm function. For each modality of DNA damage examined—HU treatment, UV, or ionizing radiation—S phase BRCA1 mobility slowed within 1 hr of exposure. Thus, Atm is not absolutely required for S phase DNA damage-induced phosphorylation of BRCA1. Whether the same is true for G1 cells is unclear at present. Similarly, p460 DNA-PK deficient cells responded normally to this same spectrum of DNA damaging agents. This implies that DNA-PK is not an absolute requirement for the S phase effect as well. The components of the S phase DNA damage/BRCA1 signaling pathway, therefore, remain to be identified. Based upon what is known from analyses of Drosophila and yeast, Atr must be considered a potential participant in this pathway. At present, however, there are no cell lines known to be functionally null for Atr.

Finally, BRCA1 appears to relocalize to replicating DNA structures after DNA damage. The interpretation of these observations can only be speculated upon at present. HU and UV induced the same relocalization behavior in BRCA1 (also in Rad51 and BARD1), again suggesting that the responses provoked by these two agents have fundamental similarities. One interpretation of these phenomena is that, in each case, a DNA repair process is initiated at the replication fork. In the case of UV-induced damage, DNA replication may be accompanied by a recombinational DNA damage response (Fornace, 1983; Friedberg et al., 1995). In the case of HU treatment, the replication fork likely contains a high density of "abnormal," or nonduplex, DNA structures, which might provoke a DNA damage response. If such speculations hold true, one might deduce that BRCA1 has an affinity for sites of specialized DNA structure, a conclusion supported by its localization to the axial element of the developing synaptonemal complex (Kleckner, 1996; Scully et al., 1997a).

If BRCA1 is recruited to certain abnormal DNA structures as part of a DNA damage response, a role for BRCA1 in DNA repair seems likely. This might or might not be linked to an inferred role of BRCA1 in transcription regulation, as evidenced by its transactivation domain and by its stable association with the RNA polymerase II holoenzyme (Scully et al., 1997b). Two paradigms, which are not mutually exclusive, could be considered.

First, BRCA1 may play a DNA repair role, even in the context of the RNA polymerase II holoenzyme, perhaps analogous to some components of TFIIH (reviewed in Bhatia et al., 1996). Second, BRCA1 may be truly bifunctional (or multifunctional), serving as both a factor in the processing of abnormal DNA structures and as a participant in the signaling which results in the activation of certain genes which follow DNA damage. The p53 tumor suppressor protein likely operates in such a bifunctional manner (reviewed in Ko and Prives, 1996).

How do these observations reflect upon the function of the BRCA1 dots in undamaged cells? One might speculate that the BRCA1 S/G2 phase dots are sites specialized for the processing of replicating or replicated DNA. It is worth noting, at this point, that BRCA1 is active during both the mitotic and meiotic cell cycle and interacts with developing synaptonemal complexes (Scully et al., 1997a). Given the functional parallel between meiotic interhomolog and mitotic intersister interactions (Kleckner, 1996), one wonders whether function in the BRCA1 dots is connected with intersister interactions.

Similarly obscure at present is the relationship that might exist between the mechanisms governing the behavior of BRCA1 in a replication checkpoint pathway and the tissue specificity of its role in tumor suppression. The connection may become clearer from a better understanding of the biology of breast and ovarian epithelium.

Experimental Procedures

Tissue Culture Methods

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (FBS). MCF7 cells were synchronized as described previously (Scully et al., 1997a). For late G1 synchronization, mimosine (200 μM final concentration) was added to MCF7 cells for 16 hr. Release into S phase produced tight synchrony through this segment of the cycle, allowing preparation of early or late S phase cultures.

DNA Damaging Agents

Cells were exposed to genotoxic agents and, unless otherwise stated, harvested 1 hr later. HU (Sigma) treatment was added to a final concentration of 1 mM. Mitomycin C (Sigma) was added to a final concentration of 20 μg/ml. UV doses were delivered in a single pulse using a Stratalinker (Stratagene). Prior to pulsing, medium was removed, being replaced immediately after treatment. Gamma irradiation was delivered using a Gammacell 1000 apparatus.

Immunoblotting and Immunoprecipitation of BRCA1

Cell extracts were prepared in EBC buffer (50 mM Tris, pH 8, 120 mM NaCl, 0.5% Nonidet P-40 [NP-40]), with the addition of 50 mM NaF, 1 mM sodium orthovanadate, 100 μg/ml polymethylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin, and 10 μg/ml leupeptin. One hundred micrograms of whole cell extract were loaded per lane. To detect changes in the mobility of p220 BRCA1, prolonged 5 or 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used (e.g., 100 V for 16 hr). Transfer to nitrocellulose was performed using a semidry transfer method (Novablot, Pharmacia), in 50 mM Tris base, 40 mM glycine, 0.37 g/l SDS, 20% methanol (for 3 hr at 1.5 mA/cm2). After blocking with 5% nonfat dried milk in TBS-T (20 mM Tris, pH 8, 0.9% NaCl, 0.05% Tween 20) with sodium azide (0.1%), the primary antibody, MS110 (Scully et al., 1996; Oncogene Science), was used at 2 μg/ml in PBS/1% nonfat dried milk/0.1% azide, for 1 hr at room temperature. The secondary antibody was peroxidase-conjugated goat anti-mouse IgG (H+L, Jackson Immunoresearch), at 1:10,000 in 1% nonfat milk/TBS-T. Signals were developed by ECL (Amersham). IP of BRCA1 was performed as described previously (Scully et al., 1997a).

Phosphatase Treatment of Immunoprecipitates

BRCA1 IPs were washed in extraction buffer in the absence of phosphatase inhibitors. Parallel samples were resuspended in λ-phosphatase buffer (New England Biolabs) either in the presence or absence of the phosphatase inhibitors, NaF (50 mM final concentration) and sodium orthovanadate (2 mM final concentration). After heating samples to 30°C for 1 min, 500 U of λ-phosphatase (New England Biolabs) was added to each sample, followed by incubation at 30°C for 1 hr. Samples were separated by SDS-PAGE and immunoblotted for BRCA1. In the same experiments, in vitro translation of the BRCA1 cDNA was performed using a TNT kit (Promega).

Antibodies, Immunostaining, and Confocal Microscopy

Cells were fixed for 10 min in PBS-buffered 3% paraformaldehyde/2% sucrose solution, followed by 5 min permeablization on ice in Triton buffer (0.5% Triton X-100 in 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM MgCl, 300 mM sucrose). Alternatively, to visualize replication forks using PCNA Ab, methanol acetone (70%:30% v/v) fixation for 15 min at −20°C was performed. The latter coverslips were air dried and rehydrated in PBS prior to immunostaining. Methanol/acetone fixation produced poor results with the Rad51 Ab. To visualize replication forks in this case, cells were permeablized in Triton buffer (above) prior to paraformaldehyde fixation, to elute away the soluble PCNA fraction (a modification of Li et al., 1996).

BRCA1 was visualized using mAbs- MS13, MS110, or AP16 (Scully et al., 1996). PCNA was visualized using AK antiserum at 1:5000, or with PCNA mAb PC10 (Santa Cruz) at 1:100. BARD1 was visualized using an affinity-purified rabbit polyclonal antiserum to residues 141–388 of the gene product. This was shown to colocalize with BARD1-specific mAbs, confirming the identity of the signal. Cross-reactivity between this antiserum and BRCA1 protein was sought but not detected. Rad51 Ab was generated by immunization of rabbits with GST-Rad51 fusion protein. After absorption of anti-GST Abs, affinity purification was performed by standard methods using an aminolink column (Pierce) coupled to GST-Rad51. All secondary antibodies used were species-specific fluorochrome-conjugated Abs from Jackson Immunoresearch, used at 1:200 throughout.

Two-color immunostaining for BrdU and PCNA was performed as follows. Methanol-acetone fixed cells were stained with PCNA antiserum "AK" (from R. Ochs), followed by secondary Ab. After post-fixing in paraformaldehyde/sucrose solution (above) for 10 min at room temperature, cells were incubated for 5 min in 2 N HCl to expose incorporated BrdU. After multiple phosphate-buffered saline (PBS) washes, fluorescein isothiocyanate (FITC)-conjugated anti-BrdU mAb (Becton Dickenson) was used to develop a BrdU incorporation signal.

All antibody incubations were performed at 37°C for 20 min. Under the conditions used, no significant signal attributable to secondary antibody, alone, was detected. All images were collected by confocal microscopy (Zeiss) and processed using Adobe Photoshop software.

Cell Cycle Analysis

Cells were pulsed with BrdU (Boehringer-Mannheim) for 10 min prior to harvesting. Aliquots of harvested plates were trypsinized, neutralized, washed in PBS, and fixed in cold 70% ethanol while vortexing. After storage on ice, cells were vortexed into 2 N HCl/0.5% Tween-20. After 30 min of incubation, cells were washed twice in PBS/HEPES, pH 7.4, to restore physiological pH, and incubated with 50 μl of PBS/1% BSA/0.5% Tween-20 and 20 μl of FITC-conjugated anti-BrdU mAb (Becton-Dickenson), for 30 min at room temperature. After further washes, cells were incubated in 70 μM propidium diiodide dissolved in 38 mM sodium citrate, in the presence of DNAase-free RNAase (2.5 μg/ml final concentration, Boehringer-Mannheim) for 30 min at 37°C. Samples were analyzed immediately thereafter by FACS (Becton-Dickenson).

Acknowledgments

We are indebted to numerous colleagues whose generosity in sharing reagents and thoughts made this work possible. In particular, we

thank Dr. Richard Baer for generously making available antibodies to BARD1 and for informing us of his results revealing colocalization of BARD1 and BRCA1 in dots; Drs. David Weaver and Kurt Auger for gifts of cell lines; Dr. David Hill for antibody to DNA-PK; and Drs. Myles Brown, James DeCaprio, Mark Ewen, William Kaelin, and Richard Kolodner for critical and stimulating discussions.

Received June 11, 1997; revised July 1, 1997.

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