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BRCA1 Modulates Ionizing Radiation-Induced Nuclear Focus Formation by the Replication Protein A p34 Subunit

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Abstract Mutations in BRCA1 account for a significant proportion of familial breast and ovarian cancers. BRCA1 has been implicated in DNA damage responses including double-strand break (DSB) repair. However, its exact role in DSB repair and its functional relationship with other known repair proteins remain to be elucidated. In this study, we carried out a cytological analysis of the effect of BRCA1 on damage-induced nuclear focus formation mediated by the replication protein A (RPA). RPA is a multi-functional protein that participates in both DNA replication and various types of DNA repair including DSB repair. Following ionizing radiation (IR), RPA and BRCA1 formed punctate nuclear staining patterns that co-localized with each other, consistent with the implicated roles of both proteins in the same repair process. The number of damage-induced RPA foci in BRCA1-deficient cells, however, was significantly greater than that in BRCA1-positive cells. Moreover, the effect of BRCA1 on the RPA staining pattern appeared to be specific for IR but not ultraviolet (UV) irradiation. These data suggest that BRCA1 plays an important role in processing the RPA-associated intermediates during DSB repair. J. Cell. Biochem. 84: 666–674, 2002. © 2001 Wiley-Liss, Inc.

Key words: ionizing radiation; double strand break repair; nuclear staining; BRCA1; replication protein A

Mutations in BRCA1 account for 45% of hereditary breast cancers and more than 80% of the cases where both hereditary breast- and ovarian-cancers occur (breast–ovarian cancer syndrome) [Rahman and Stratton, 1998]. The human *BRCA1* gene encodes a 1,863-amino acid protein with a highly conserved RING finger domain at the amino terminus and two repeats of the BRCT domain at the carboxyl terminus. While most cancer-predisposing mutations of BRCA1 result in gross truncation of the protein, 5–10% of the disease-associated mutations lead to single amino acid substitutions.

The exact biochemical function of the BRCA1 protein remains to be elucidated, however, several lines of evidence suggest that BRCA1 is involved in regulation of multiple nuclear processes following DNA damage, including DNA repair and transcription [Zhang et al., 1998; Scully and Livingston, 2000]. BRCA1 is a nuclear protein that is phosphorylated in a cell cycle-dependent manner [Chen et al., 1996; Ruffner and Verma, 1997]. It forms discrete S-phase nuclear foci that co-localize with the repair/recombination protein RAD51 [Scully et al., 1997b]. Following DNA damage, BRCA1 becomes hyperphosphorylated and co-localized with proliferating cell nuclear antigen (PCNA), a well-characterized replication and repair protein [Scully et al., 1997a]. BRCA1-deficient mouse embryonic stem cells are defective in transcription-coupled repair of oxidative DNA damage and double strand break-induced homologous recombination [Gowen et al., 1998; Moynahan et al., 1999; Xu et al., 1999]. Consistent with its role in DNA repair, BRCA1 physically associates with several repair proteins such as RAD51 [Scully et al., 1997b], RAD50/MRE11/NBS1 [Zhong et al., 1999; Wang et al., 2000], and MSH2/MSH6 [Wang et al., 2000]. While mounting evidence has implicated

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BRCA1 in DNA damage response, it is not clear how BRCA1 facilitates double-strand break (DSB) repair in vivo. In particular, the functional relationship between BRCA1 and the repair machinery remains to be established.

Among the multiple repair proteins characterized so far, replication protein A (RPA) is a multi-functional, single strand (ss) DNA-binding protein complex composed of 70-, 34-, and 11-kDa subunits [Wold, 1997]. It facilitates DNA-unwinding and DNA synthesis in the initiation and elongation stages of DNA replication. RPA is also involved in DSB repair, nucleotide excision repair (NER), and homologous recombination (HR) [Wold, 1997]. While RPA's role as a ssDNA binding protein in DNA synthesis is most likely common to all of these various chromosomal events, it may play additional roles that are distinct in different forms of DNA repair. For example, RPA interacts with RAD52 and/or RAD51 [Firmenich et al., 1995; Park et al., 1996; Golub et al., 1998] and stimulates homologous pairing and DNA strand exchange steps during DSB repair and homologous recombination [Sung, 1994; Benson et al., 1995; Sugiyama et al., 1997; Gupta et al., 1998]. On the other hand, RPA facilitates recognition of pyrimidine dimers during NER by interacting with excision repair proteins such as XPA and XPG [He et al., 1995; Lee et al., 1995; Matsuda et al., 1995]. Thus, the interactions between RPA and specific components of the different repair machineries are consistent with the functions of RPA in stimulating multiple repair pathways.

To examine the relationship between BRCA1 and RPA in the cellular response to DNA damage, we analyzed the damage-dependent RPA focus formation in BRCA1-replete and BRCA1-deficient cells. Our work shows that, in response to ionizing radiation, RPA and BRCA1 co-localize with each other in the nucleus, supporting an intimate functional link between the two proteins. The current study also indicates that the presence of functional BRCA1 modulates the kinetics of the DNA damage-induced RPA focus formation. Interestingly, the effect of BRCA1 on the RPA nuclear distribution only occurs following ionizing radiation, not ultraviolet irradiation. Thus, our findings support the notion that BRCA1 is required for the processing of DSB repair intermediates as represented by RPA nuclear foci.

MATERIALS AND METHODS

Cell Culture

HCC1937 cells were grown in RPMI1640 medium supplemented with 15% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. T24, ES-2, and SK-OV-3 cells were grown in McCoy's medium containing 10% fetal bovine serum. MCF7 and HeLa cells were grown in Dulbecco's modified Eagle's medium containing high glucose supplemented with 10% fetal bovine serum.

Immunofluorescence Staining and Image Analysis

Monolayer cells growing on glass coverslips were washed twice with PBS and fixed with methanol-acetone (1:1) at −20°C for 10 min. The fixed cells were rehydrated and washed three times with PBS containing 1% goat serum and processed for cell staining. The following antibodies were used for immunostaining: (i) polyclonal anti-RPA antibody (a generous gift from Dr. Bruce Stillman); (ii) monoclonal anti-RPA34 antibody (Ab-3 from Oncogene, Inc.; p34-2, p34-19, p34-20 from Dr. Bruce Stillman); (iii) monoclonal anti-RPA70 antibody (p70-16 from Dr. Bruce Stillman); and (iv) monoclonal anti-BRCA1 antibody (Ab-1, Oncogene).

Indirect immunostaining was performed by first incubating cells in primary antibodies in a humidified chamber at room temperature for 1 h. Following three washes in PBS containing 1% goat serum, the coverslips were incubated for 1 h with Texas Red- and/or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Vector Laboratories, at 1:100 dilution). Coverslips were washed four times with PBS, and mounted with a drop of mounting medium (90% glycerol, 10% PBS buffered with 0.5 M carbonate/bicarbonate buffer to pH 9.0, 0.1% *p*-phenylenediamine).

Conventional immunofluorescence microscopy was performed on a Nikon Microphot-SA microscope with Improvision Openlab software. Images from the same experiments were captured with a fixed exposure time for a direct comparison of the image intensity. The photographs shown in this report are representatives of results from multiple experiments. Confocal immunofluorescence microscopy was performed on a Zeiss LSM 410 inverted laser scan microscope equipped with 63×/1.3 N.A. objectives and HeNe and argon ion lasers.

RESULTS

DNA Damage-Induced Co-localization of RPA With BRCA1

Given the implicated functions of both RPA and BRCA1 in DSB repair, we sought to compare the nuclear localization patterns of these two proteins following ionizing radiation (IR). A bladder carcinoma cell line, T24, was chosen for the initial work because it had been used in previous studies of BRCA1 function in damage response [Zhong et al., 1999]. Either mock- or IR-treated cells were immunostained with anti-BRCA1 and anti-RPA34 antibodies. As shown in Panel A of Figure 1, a sub-population of untreated cells gave rise to a punctate nuclear staining pattern for BRCA1, whereas the anti-RPA34 antibody gave more homogeneous nuclear staining, with some faint dots in the nuclei (Panel B). No obvious co-localization of the BRCA1 and RPA-34 was observed in the untreated cells (Panel C). In contrast, a large number of the cells displayed punctate staining patterns for both BRCA1 and RPA-34 following ionizing radiation (Panels D and E). Furthermore, most of the damage-induced RPA foci were co-localized with those of BRCA1 (Panel F). This result supports the notion that both proteins are re-localized to the damaged chromosomal region to support DSB repair.

Distinct RPA Nuclear Staining Pattern in a BRCA1-Deficient Cell Line

To verify the damage-induced co-localization of RPA-34 and BRCA1 observed in T24 cells, we used a BRCA1-deficient cell line, HCC1937, which carries both alleles of a BRCA1 cancer-predisposing mutation that results in both truncation and reduced expression of the protein [Tomlinson et al., 1998]. Consistent with previous findings [Zhong et al., 1999], the BRCA1 staining in HCC1937 cells was much dimmer than that in T24 cells. More importantly, no distinct punctate staining of BRCA1 was observed in IR-treated HCC1937 cells (compare Panels G and J in Fig. 1). On the other hand, the same IR-treated cells still exhibited punctate nuclear staining for RPA, suggesting that nuclear redistribution of RPA-34 per se does not require functional BRCA1. However, the number of nuclear RPA-34 foci in the IR-treated HCC1937 cells was significantly higher than that in T24 cells (compare Panel K with E; also see below for quantitation). Moreover, the size of the RPA-34 foci was smaller in HCC1937 cells. These findings suggest that, while the appearance of the damage-induced RPA-34 foci is BRCA1-independent, the number and size of RPA-34 foci following IR is affected by BRCA1.

To confirm the damage-triggered punctate staining pattern of RPA in HCC1937 cells, we also used several other RPA-specific antibodies for immunostaining. Three different antibodies raised against the RPA-34 subunit gave rise to a similar speckled pattern in IR-treated HCC1937 cells (Fig. 2; Panels E–G), with the number of foci significantly higher than that in T24 cells (data not shown). A monoclonal antibody against the RPA-70 subunit, however, revealed fewer prominent RPA nuclear dots than did the anti-RPA-34 antibodies (Panel H of Fig. 2).

HCC1937 is the only well-characterized BRCA1-deficient cell line. To verify the difference in RPA staining observed between HCC1937 and T24 cells, we also included several other established cell lines that carry wild-type copies of the BRCA1 gene (Fig. 3). These include MCF7 (breast cancer), SK-OV-3 and ES-2 (ovarian cancer), and HeLa (cervical cancer). In the absence of DNA damage, all cell lines tested showed relatively homogeneous nuclear staining for RPA-34 (Panels A–C, G–I of Fig. 3). Following IR treatment, a fraction of the cell population in each cell line exhibited a speckled pattern similar to that observed in T24 cells (Panels E,F,J–L). However, there were significantly fewer damage-induced RPA-34 nuclear dots in these BRCA1-positive cell lines than those in HCC1937 cells (compare Panel D with E,F,J–L). To quantitatively assess RPA-34 focus formation among the different cell lines, the number of damage-induced RPA-34 foci per cell and the percentage of cells displaying the speckled pattern were counted from the IR-treated cell population. As shown in Figure 4A, IR treatment resulted in an average of 90 nuclear dots in the nucleus of an HCC1937 cell (column 1). In contrast, the same treatment only yielded fewer than 20 nuclear dots per cell in all of the BRCA1-replete cell lines (columns 2–6 in Fig. 4A). In addition, more than 75% of the IR-treated HCC1937 cells showed the speckled staining pattern, compared to 10–40% for the other cell lines (Fig. 4B). Taken together, these results clearly demonstrate the distinct RPA-34 nuclear staining pattern in HCC1937 cells following DNA damage.

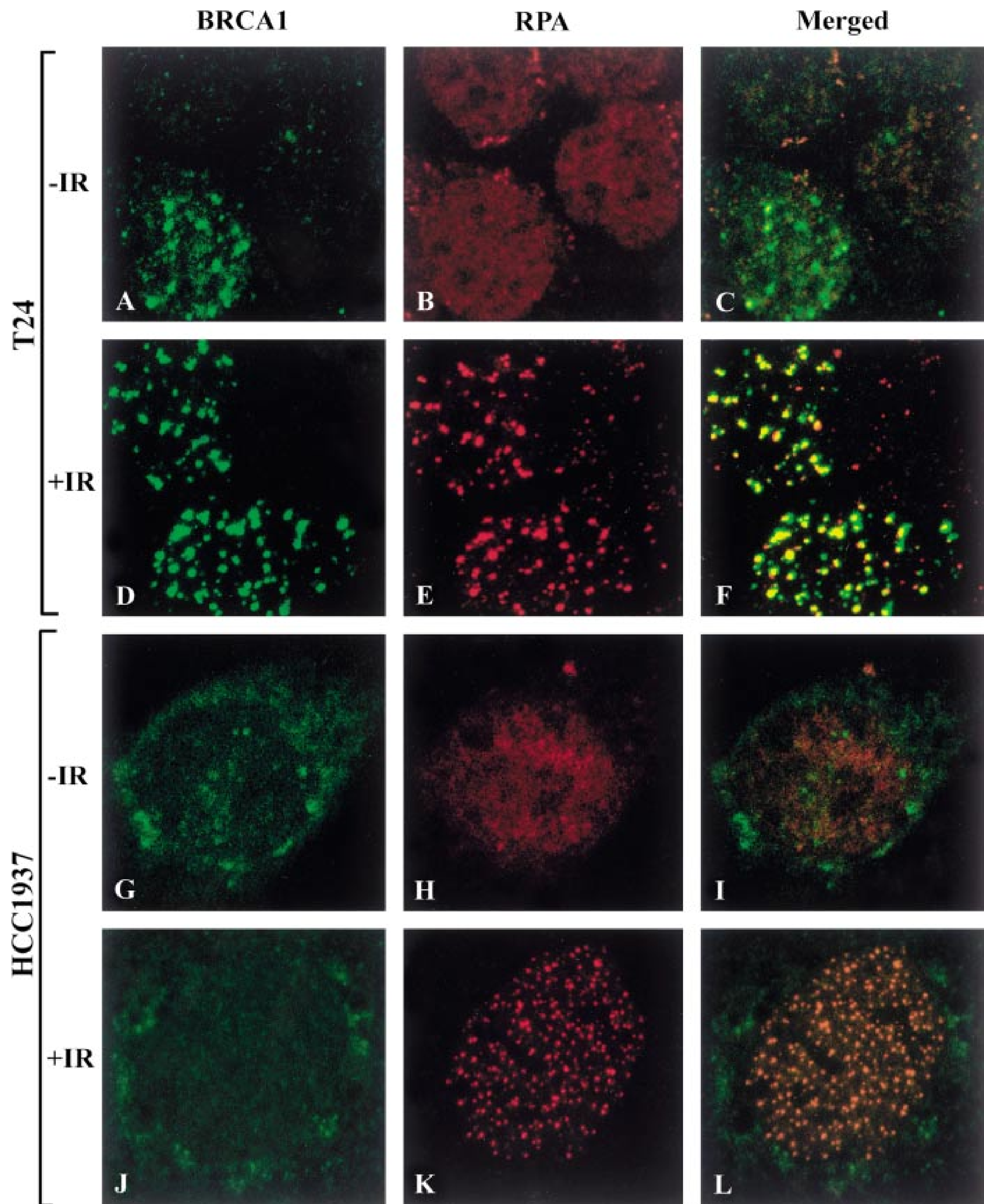

Fig. 1. Co-localization of BRCA1 and RPA following γ-irradiation. Asynchronous, logarithmically growing T-24 (**A–F**) or HCC1937 (**G–L**) cells were mock-treated (A–C and G–I) or irradiated with 12 Gy (D–F and J–L) and incubated for 24 h prior to fixation and staining. The cells were stained with a mouse monoclonal anti-BRCA1 antibody (Ab-1 from Oncogene) followed by a fluorescein isothiocynate (FITC) conjugated anti-mouse antibody, and with a polyclonal anti-RPA34 antibody followed by a Texas Red-conjugated anti-rabbit antibody. Confocal image was captured by a Zeiss LSM inverted laser scan microscope. Representative cells stained for BRCA1 (green), RPA (red), and merged images are shown.

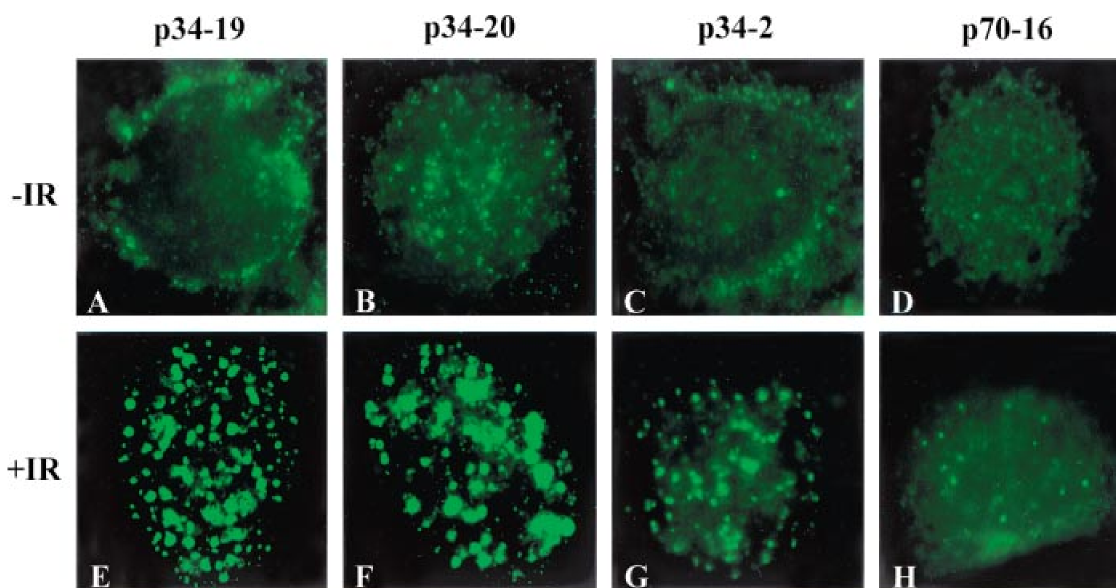

Fig. 2. RPA immunostaining with different RPA antibodies. HCC1937 cells were mock-treated (**A–D**) or irradiated with 12 Gy (**E–H**). Twenty-four hours after irradiation, cells were fixed and subsequently stained with various anti-RPA34 antibodies (p34-19, -20, -2) and one anti-RPA70 antibody (p70-16). An FITC-conjugated secondary antibody was used for indirect immunostaining.

Persistent RPA Focus Formation in BRCA1-Deficient Cells Following IR

To further examine the characteristics of RPA focus formation in BRCA1-positive and BRCA1-deficient cells, we compared the dose response and time course of the RPA-34 nuclear staining in T24 and HCC1937 cells. As shown in Figure 5A, the number of RPA-34 foci per cell in both cell lines increased steadily in response to increasing doses of IR (2–20 Gy). However, the focus number in HCC1937 cells was consistently higher than that in T24 cells at each IR dose applied. For instance, exposure to 20 Gy of ionizing radiation triggered an average of 100 RPA-34 foci per HCC1937 cell, whereas the same treatment only resulted in 25 foci per T24 cell. We also examined the time course of RPA-34 focus formation following irradiation. In this case, very different response curves between the two cell lines were observed (Fig. 5B). The focus numbers in the BRCA1-deficient HCC1937 cells rose to approximately 90 in the first 12 h post-irradiation and maintained at that level for the first 24 h. In contrast, the number of damage-induced foci in T24 cells steadily decreased during the same period. Overall, our study shows that the IR-dependent RPA-34 foci in HCC1937 cells are more abundant and sustained over a longer period of time than those in BRCA1-replete cell lines.

RPA Nuclear Staining in Response to UV Damage

In addition to ionizing radiation, other DNA damaging agents such as ultraviolet (UV) and alkylating chemicals also trigger changes in BRCA1 post-translational modification and its nuclear re-distribution, implicating BRCA1 in response to multiple types of DNA damage. Furthermore, RPA is also known to participate in different DNA repair pathways, including DSB repair and NER. To determine the effect of different DNA damaging agents on RPA nuclear staining, HCC1937 and T24 cells were treated with either IR or UV. As shown in Figure 6, both types of irradiation induced RPA-34 punctate nuclear staining in a dose-dependent manner. Consistent with the results from the previous experiments, IR resulted in a greater number of RPA-34 nuclear speckles in HCC1937 than in T24 cells (e.g., compare Panels O and P). In contrast, the UV treatment yielded a similar number of RPA-34 foci in the two cell lines at all doses tested (e.g., compare Panels M and N). These results support the notion that the abnormality of RPA-34 focus number in HCC1937 cells may reflect specific defects in

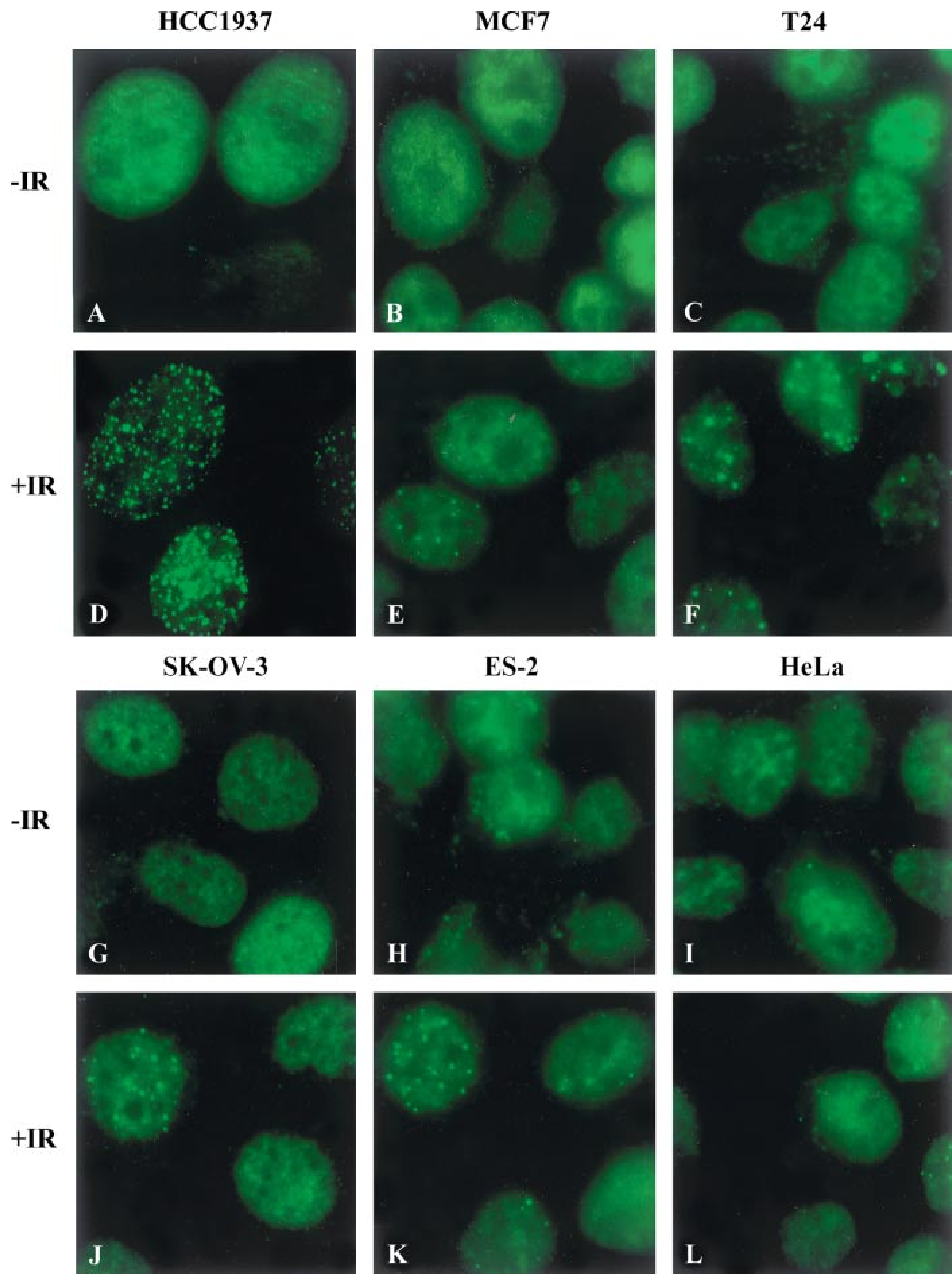

Fig. 3. Comparison of damage-induced RPA nuclear staining patterns in various cell lines. BRCA1-positive (MCF7, T24, SK-OV-3, ES-2, and HeLa) or BRCA1-deficient cells (HCC1937) were mock-treated (**A–C**, **G–I**) or irradiated with 12 Gy (**D–F**, **J–L**) and allowed to recover for 24 h before fixation and immunostaining. The cells were stained with an anti-RPA34 antibody (mAb p34-20) followed by staining with an FITC-conjugated anti-mouse antibody. All photographs are representatives of multiple experiments.

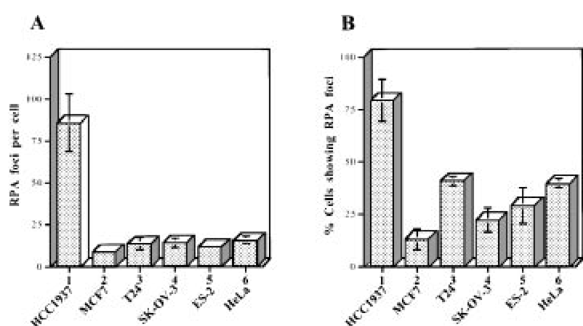

Fig. 4. Quantitation of the difference in RPA focus formation between BRCA1-deficient and BRCA1-replete cells. **A**: The number of the IR-induced RPA foci per cell nucleus was quantified in different cell lines. **B**: Percentage of cells showing the speckled RPA staining pattern was determined for each cell line following IR treatment. The numbers represent averages of three independent experiments. At least 100 cells were counted in each case. Error bars indicate standard deviation.

DSB repair as a result of the lack of a functional BRCA1 gene.

DISCUSSION

In this report, we describe the relationship between the nuclear staining patterns of RPA-34 and BRCA1 following DNA damage. In keeping with the known functions of BRCA1 and RPA in DSB repair, both proteins undergo dramatic nuclear redistribution in response to ionizing radiation. Moreover, our study shows that the damage-induced RPA-34 and BRCA1 foci colocalize in the nuclei of BRCA1-positive cells, suggesting that they work in proximity to repair the damaged sites in the genome. A cell line deficient in BRCA1 displayed a significant increase in the RPA nuclear dots after exposure to IR, whereas the RPA punctate staining after exposure to UV did not appear to depend upon BRCA1. The damage-specific effect of BRCA1 on RPA nuclear staining suggests that the two proteins functionally interact with each other during DSB repair.

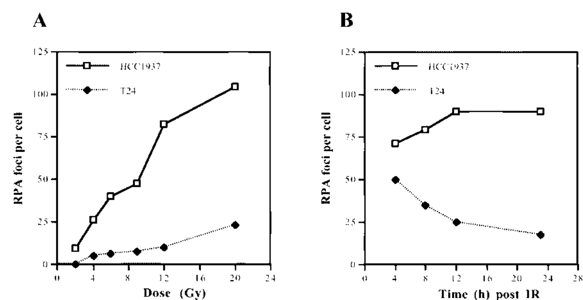

Fig. 5. Quantification of radiation-induced RPA foci. **A**: T24 (◆) and HCC1937 cells (□) were irradiated with 2, 4, 6, 9, 12, and 20 Gy of IR and incubated for 24 h prior to fixation and staining. **B**: T24 (◆) and HCC1937 cells (□) were irradiated with 12 Gy of IR and allowed to recover for 4, 8, 12, and 24 prior to fixation and staining. The cells were stained with a mouse monoclonal anti-RPA-34 antibody (p34-20) and an FITC-conjugated secondary antibody. At least 100 cells were counted for RPA foci at each dose or time point.

In keeping with a role of BRCA1 in DSB repair, it has been shown that IR-induced double strand breaks in HCC1937 cells are removed at a slower rate than in BRCA1-replete cells [Scully et al., 1999]. Our finding that HCC1937 cells are still capable of redistributing RPA-34 molecules to damaged DNA argues that at least part of the repair process, i.e., recruitment of RPA to DNA lesions, may still be operational. However, the significant increase of RPA-34 focus number in HCC1937 cells is consistent with an active role of BRCA1 in facilitating a specific step after RPA recruitment. The repair process may be stalled in the absence of a functional BRCA1 gene, and as a consequence, the RPA-associated repair intermediates may start to accumulate. In support of this notion, the time course study showed that the RPA-34 focus number in HCC1937 cells increased steadily during the first 24 h post damage, whereas the number in the BRCA1-positive cells (T24) followed the opposite trend (Fig. 5). The exact function of BRCA1 in DSB repair remains to be elucidated. However, recent studies indicate that BRCA1 can mediate reconfiguration of chromatin structure [Hu et al., 1999; Bochar et al., 2000], an activity that is likely to be required both for its role in DNA repair and in transcriptional regulation. Therefore, it is conceivable that a BRCA1-dependent chromatin-remodeling step may be required to increase chromatin accessibility and promote the completion of the repair process.

RPA is required for different types of excision repair. Although the ssDNA binding activity of RPA is almost certain to be universally important in the gap-filling DNA synthesis step of various DNA repair, its interaction with specific components of the repair machinery may confer additional roles to RPA in different repair pathways. In agreement with this notion, we found that BRCA1 modulates RPA-34 nuclear staining in response to IR, but not UV. The repair-specific effect of BRCA1 on RPA staining may be due to their interactions with the components of the DSB repair machinery, such as RAD51 and RAD52 [Firmenich et al., 1995;

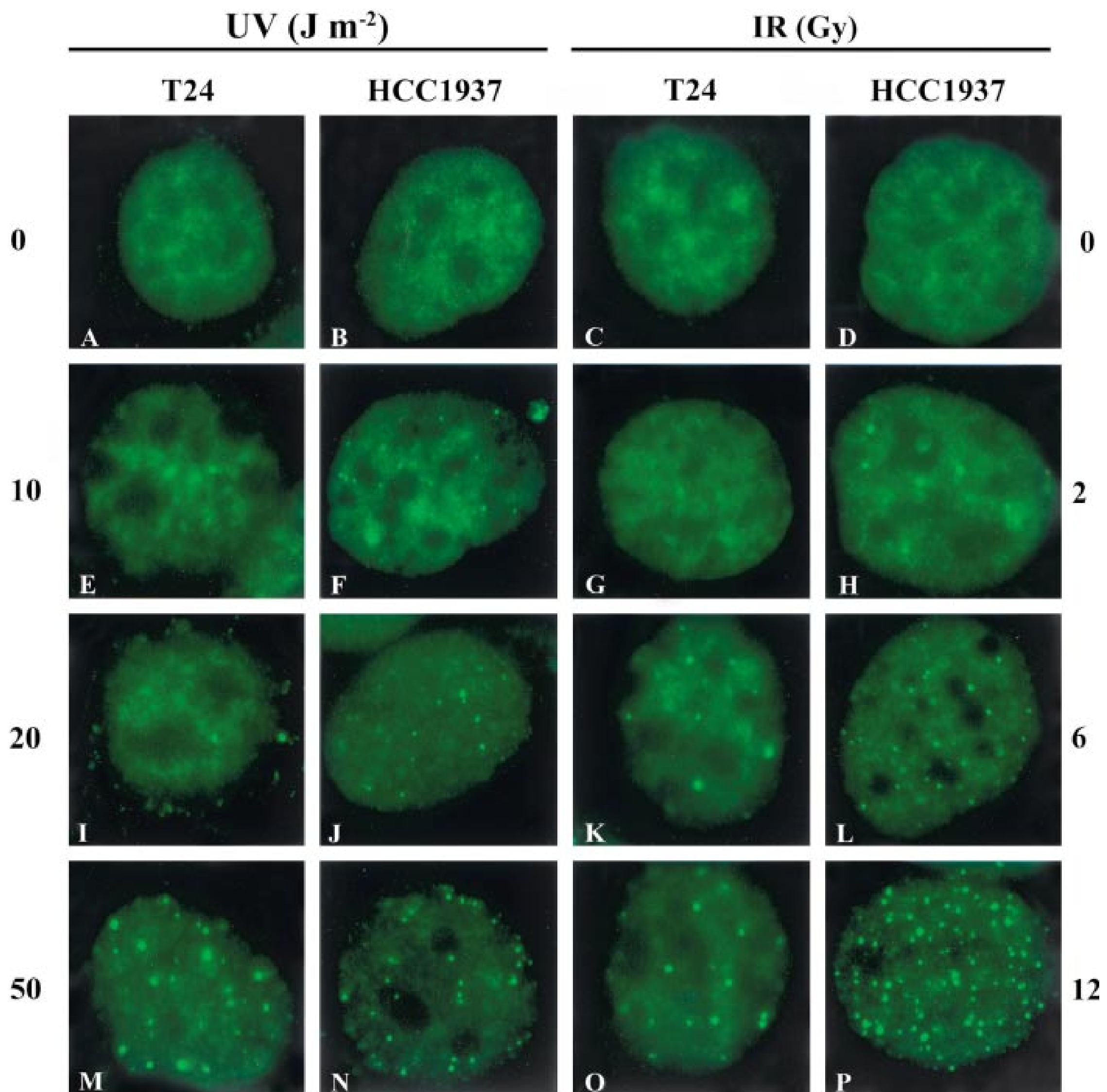

Fig. 6. The different effects of IR and UV on RPA staining pattern. T-24 and HCC1937 cells were irradiated with different doses of UV (10, 20, and 50 J/m^2) or IR (2, 6, and 12 Gy). The zero point refers to the unirradiated cells. The cells were stained with an anti-RPA34 antibody (p34-20) followed by an FITC-conjugated anti-mouse antibody. All photographs are representatives of multiple experiments.

Park et al., 1996; Scully et al., 1997b; Golub et al., 1998].

Our study with different RPA antibodies showed that those against RPA-34 revealed a more prominent damage-induced RPA punctate staining than the one against RPA-70. One possible explanation could be that the epitope specifically recognized by the anti-RPA70 antibody might have been masked in the damage-induced RPA nuclear foci. Alternatively, the difference in focus number may reflect different amounts of the RPA subunits present at the damaged sites. It is known that a sub-population of RPA-34 is not associated with RPA-70. In addition, there have been reports that the trimeric RPA complex may dissociate at certain stages of the cell cycle [Murti et al., 1996; Treuner et al., 1999]. In particular, RPA-34 was reported to be associated with chromatin after disintegration of the trimeric complex. Thus, it is conceivable that the stalled repair machinery in HCC1937 cells may trigger disintegration of the RPA complex, leaving RPA-34 at the damaged sites. Future studies on RPA composition during DNA repair may shed additional light on this issue.

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