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Distinct RAD51 Associations with RAD52 and BCCIP in Response to DNA Damage and Replication Stress

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Abstract

RAD51 has critical roles in homologous recombination (HR) repair of DNA double-strand breaks (DSB) and restarting stalled or collapsed replication forks. In yeast, Rad51 function is facilitated by Rad52 and other “mediators.” Mammalian cells express RAD52, but BRCA2 may have supplanted RAD52 in mediating RAD51 loading onto ssDNA. BCCIP interacts with BRCA2, and both proteins are important for RAD51 focus formation after ionizing radiation and HR repair of DSBs. Nonetheless, mammalian RAD52 shares biochemical activities with yeast Rad52, including RAD51 binding and single-strand annealing, suggesting a conserved role in HR. Because RAD52 and RAD51 associate, and RAD51 and BCCIP associate, we investigated the colocalization of RAD51 with BCCIP and RAD52 in human cells. We found that RAD51 colocalizes with BCCIP early after ionizing radiation, with RAD52 later, and there was little colocalization of BCCIP and RAD52. RAD52 foci are induced to a greater extent by hydroxyurea, which stalls replication forks, than by ionizing radiation. Using fluorescence recovery after photo bleaching, we show that RAD52 mobility is reduced to a greater extent by hydroxyurea than ionizing radiation. However, BCCIP showed no changes in mobility after hydroxyurea or ionizing radiation. We propose that BCCIP-dependent repair of DSBs by HR is an early RAD51 response to ionizing radiation-induced DNA damage, and that RAD52-dependent HR occurs later to restart a subset of blocked or collapsed replication forks. RAD52 and BRCA2 seem to act in parallel pathways, suggesting that targeting RAD52 in BRCA2-deficient tumors may be effective in treating these tumors.

Introduction

DNA double-strand breaks (DSB) are a critical form of DNA damage formed by various mechanisms. Once a DSB has occurred, it can be repaired by two primary pathways: nonhomologous end joining (NHEJ) or homologous recombination (HR; ref. 1). NHEJ is an error-prone process that is most active during G₁ and early S, whereas HR has less risk of producing errors during repair and is most active in S and G₂ phases of the cell cycle (2,3). RAD51-mediated HR also has critical roles in restarting stalled or collapsed replication forks (4–6), and in higher eukaryotes, RAD51 function is essential for cell viability (2).

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In the yeast *Saccharomyces cerevisiae*, HR is mediated by the Rad52 group of proteins that includes Rad51, Rad52, Rad54, and two Rad51 paralogs (7). Yeast Rad51 (ScRad51) and mammalian RAD51 play a central role in HR by forming nucleoprotein filaments on ssDNA that perform the homology search and strand exchange reactions (8–10). To ensure proper assembly of ScRad51 filaments, accessory factors such as yeast Rad52 (ScRad52) are needed (11,12). In addition to facilitating ScRad51 filament formation, ScRad52 may be directly involved in single-strand annealing and the promotion of DNA homologous pairing (11,12). ScRad52 is essential for HR-mediated repair of DSBs, and *rad52* mutants show marked radiosensitivity (7), but the specific roles of mammalian RAD52 in HR and radiation resistance has been elusive. Mammalian RAD52 has retained similar biochemical activities as ScRAD52 *in vitro* (13), suggesting a conserved function through evolution. However, knockout of *RAD52* reduces HR-mediated gene-targeting efficiency in mouse ES cells by only 30% to 40% and has little effect on radiation resistance (14). In addition, Rad52 is required for Rad51 focus formation in yeast (15) but not in mammalian cells (16). Knockout of *RAD52* in chicken DT40 cells reduced gene targeting to a greater degree (3–10-fold depending on the target locus), but there was no change in radioresistance (17). One intriguing model is that RAD52 function in mammals is performed in part by another RAD51-interacting protein, BRCA2, which is not present in yeast.

BRCA2 has two regions that bind RAD51, including eight BRC repeats and a COOH-terminal domain. Expression of individual BRC repeats significantly reduces the ability of RAD51 to form nuclear foci after ionizing radiation and impairs HR-mediated DSB repair (18). The COOH-terminal domain coordinates HR activity with cell cycle regulation (19,20). A conserved region proximate to the COOH terminus of BRCA2, sometimes referred as the BRCA2 DNA/DSS1 Binding Domain (BRCA2-DBD), forms a structure similar to the ssDNA binding region of replication protein A (RPA), which is displaced from ssDNA during RAD51 binding. The corresponding BRCA2-DBD of *Ustilago maydis* BRCA2 ortholog Brh2 has been suggested to play a critical role in loading RAD51 by mediating intermolecular Brh2 dimerization (21). The human BRCA2-DBD has been shown to bind DSS1, BUBR1, ABP-280/filamin-A, and BCCIP (22–24). Importantly, BCCIP binding to BRCA2 facilitates RAD51 nuclear focus formation and HR repair of DSBs (25,26).

BCCIP is a BRCA2 and CDKN1A (p21Waf1/Cip1)-interacting protein (22). The *BCCIP* gene is alternatively spliced to produce two commonly expressed isoforms, BCCIP α and BCCIP β (27). These isoforms contain identical NH₂-terminal regions of 258 amino acids including an NH₂-terminal acidic domain and the internal conserved domain but have distinct COOH-terminal domains. In this report, “BCCIP α ” and “BCCIP β ” indicate the specific isoforms, and “BCCIP” denotes both isoforms. Previously, it has been shown that BCCIP coprecipitates and colocalizes with BRCA2 and RAD51 (25). BCCIP knockdown by siRNA also reduces BRCA2 and RAD51 focus formation, reduces HR repair of DSBs, and causes the accumulation of spontaneous DNA damage (25,26). These results indicate a critical role for BCCIP in BRCA2- and RAD51-dependent HR (25,26).

Because RAD52 interacts with RAD51, and RAD51 colocalizes and immunoprecipitates with BCCIP (25,28,29), we investigated the potential interactions between RAD52 and BCCIP. We found no evidence of physical interaction between RAD52 and BCCIP, and that RAD52 and RAD51 foci colocalize much later than RAD51-BCCIP foci. Furthermore, BCCIP is present in constitutive nuclear foci, and its mobility remains constant after ionizing radiation or hydroxyurea treatment, whereas RAD52 mobility is reduced by ionizing radiation and sharply reduced by hydroxyurea. These results implicate the BCCIP/BRCA2 complex and RAD52 in distinct modes of RAD51 regulation, with BCCIP/BRCA2 involved in an early DSB repair response and RAD52 likely involved in a late response at stalled or collapsed replication forks.

Materials and Methods

Plasmid DNA and cell culture

Human *RAD52* was cloned into the retroviral Myc or enhanced green fluorescent protein (EGFP)-tagged pLXSP vector. HT1080 cells were cultured in α MEM (BioWhittaker) supplemented with 10% fetal bovine serum (Nova-Tech, Inc.), 1% penicillin/streptomycin (Life Technologies Bethesda Research Laboratories), and 20 mmol/L glutamine and incubated at 37°C. The pLXSP-myc-RAD52 and pLXSP-EGFP-RAD52 constructs were then stably expressed in HT1080 cells as described previously (22). HT1080 cells were transiently transfected with a Bio-Rad electroporator, set at 400 mV and 250 μ F, with the pEYFP vector or the same vector, including cDNAs for either BCCIP α or β .

Antibodies, immunostaining, and scoring of foci/colocalized foci

Mouse anti-RAD51 monoclonal (Cal-Biochem), mouse anti-Myc monoclonal (BD-Biosciences), rabbit anti-RAD52 polyclonal (Santa Cruz), γ H2AX (Ser¹³⁹; Upstate), and rabbit RAD51 polyclonal antibodies (EMD Biosciences) were purchased from the indicated sources. BCCIP antibodies were reported previously (22). HT1080 cells expressing Myc-RAD52 were grown on coverslips in supplemented α MEM medium. The cells were treated with 8 Gy of ionizing radiation using a Cs-137 γ -irradiator or 10 mmol/L hydroxyurea for 6 to 8 h in supplemented α MEM. After ionizing radiation, at 0, 0.25, 2, 4, 6, and 8 h, the cells were permeabilized with 0.5% Triton X-100 on ice for 5 min, fixed with 4% phosphate-buffered paraformaldehyde solution (PBPS) for 10 min, and incubated in 3% bovine serum albumin for at least 30 min at room temperature. The cells were incubated with the appropriate primary antibodies overnight at 4°C then with secondary antibodies at room temperature for 1 h (FITC-conjugated donkey anti-mouse and rhodamine-conjugated goat anti-rabbit from Jackson Immuno-research). The cells were plated on slides using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) and sealed, and slides were imaged using an Axioscope wide-field fluorescent microscope affixed with filter sets for DAPI, FITC, and rhodamine to detect total foci. Argon 488 nm and HeNe 543 nm lasers were used in conjunction with filter sets specific for FITC and rhodamine. The pinhole was set to equal for each channel and to never exceed an image slice thickness of 1 μ m. The total number of foci was counted for 20 cells per experiment and performed in duplicate, stained with both antibodies, using an Axioscope or Bio-Rad widefield fluorescent microscope with filter sets for FITC and rhodamine. The number of colocalized foci per cell was also determined in 20 cells per experiment using a Zeiss LSM510 confocal microscope. The number of total foci per cell for each time point was multiplied by the percentage of colocalized foci to calculate an average of total foci colocalized per cell.

Fluorescence recovery after photo bleaching

All live cell experiments were performed on a 37°C stage of an inverted Zeiss LSM 510 Confocal microscope. EGFP and EGFP fusion proteins were imaged using a 488-nm line of a 200-mW argon laser and a $\times 63$, 1.25 numerical aperture objective. The filter sets used included a 488 dichroic beamsplitter, a 490 dichroic mirror, and a 505 to 530 bandpass filter. A 2- μ m (± 0.25 μ m) strip was photo bleached using 100% power of the 488 laser line for 10 iterations. After bleaching, the strip region was subsequently monitored with minimal laser intensity (0.5–3%, fixed within each experiment). Images were analyzed using the Slidebook Software package to determine mean intensity level of the strip, width of the strip, and the number of seconds of each measurement. The intensities and the corresponding time and width data were imported into Microsoft Excel for further analysis. The diffusion coefficient was determined by fitting the data into an equation for one-dimensional diffusion (30):

$$I_t = I_{\text{final}} \times \left[1 - \left[w^2 (w^2 + 4\pi D t)^{-1} \right] \right]^{1/2} \quad (1)$$

or

$$D = \left[(w^2) (I_t^2) \right] / \left[4\pi t (I_f^2 - I_t^2) \right] \quad (2)$$

where I_t is the intensity at time, I_{final} is the maximum intensity after recovery from bleaching, w is the width of the strip bleached, t is time corresponding to I_t , and D is the diffusion coefficient. The intensities at each time point of each sample were normalized to prebleach and postbleach levels, termed the relative fluorescence (RF). The prebleach and postbleach normalizations were accomplished using the equations at each time point, similar to those described by Essers et al. (31):

$$\text{RF to Prebleach at time } t = (I_t - I_0) / (I_{\text{prebleach}} - I_0) \quad (3)$$

$$\text{RF to Post - bleach at time } t = (I_t - I_0) / (I_{\text{final}} - I_0) \quad (4)$$

The prebleach and postbleach RF values were then plotted on graphs with values between 0 and 1 as a function of time in seconds.

Cells expressing enhanced yellow fluorescent protein (EYFP)-tagged BCCIP or EGFP-tagged RAD52 were subjected to the fluorescence recovery after photo bleaching (FRAP) analysis described. The cells were exposed to mock treatment, 8 Gy ionizing radiation or 10 mmol/L hydroxyurea. RAD52 was imaged for FRAP analysis at 6 to 8 h after mock or initial treatment and BCCIP was imaged for FRAP analysis 3 to 5 h after mock or initial treatment.

EGFP-RAD52 focus formation

HT1080 cells stably expressing EGFP-RAD52 were cultured in supplemented α MEM. The cells were either untreated or treated with 8 Gy ionizing radiation or 10 mmol/L hydroxyurea. After 7 h, the cells were washed thrice, fixed with 4% PBPS, and washed three more times. The cells were then mounted in Vectashield with DAPI and sealed. Using a Bio-Rad wide-field fluorescent microscope, the cells were evaluated for the total number of foci per cell. All cells were counted, including those with low signal, because the EGFP-tagged RAD52 was stably expressed and visible in all cells. The results were graphed, showing all cells counted for each treatment.

Results

Ionizing radiation induced RAD51 foci form before RAD52 foci

Nuclear focus formation by RAD51 and other DNA repair proteins can serve as an indicator of their participation in DNA repair processes (15,32). To investigate the roles of RAD52 and BCCIP in RAD51 focus formation, we compared the time courses of RAD52, BCCIP, and RAD51 after exposure to 8 Gy γ -irradiation (Fig. 1). Because γ H2AX is a marker of DNA DSBs (33), these foci were also examined. Consistent with prior results (25), an average of 25 to 30 BCCIP nuclear foci are observed per cell before irradiation (Fig. 1A), ~90% of cells have more than five nuclear BCCIP foci, and there was no increase in BCCIP foci after irradiation

(Fig. 1B). Within 15 minutes after ionizing radiation, γ H2AX foci increase to a maximum level similar to that of BCCIP foci, and then gradually decrease. The number of RAD51 foci reaches a maximum ~2 hours after irradiation and remains at this level during the remaining 6-hour time course (Fig. 1A). There were fewer RAD51 foci than γ H2AX foci, consistent with the idea that some DSBs are repaired by NHEJ. Thus, BCCIP foci are constitutive, and γ H2AX foci form before RAD51 foci. Intriguingly, the number of RAD52 foci does not increase until 4 to 6 hours postirradiation, at least 2 to 4 hours after RAD51 foci reached plateau. In addition, there are more RAD51 foci than RAD52 foci. This indicates that RAD52 focus formation in HT1080 cells is not a prerequisite for most RAD51 focus formation in response to ionizing radiation, particularly within the first 4 hours. However, some RAD51 foci colocalize with RAD52 foci at later times after ionizing radiation (see below). BCCIP foci are present before ionizing radiation and precede the formation of γ H2AX and RAD51 foci, RAD51 foci precede RAD52 foci, and down-regulation of BCCIP inhibits RAD51 focus formation (25). Irradiation with 2 Gy has similar kinetics as 8 Gy but fewer foci (Supplementary Fig. S2A and B). Thus, the majority of RAD51 focus formation in response to γ -irradiation likely requires a functional BCCIP/BRCA2 complex but not RAD52.

Colocalization of RAD51, RAD52, BCCIP, and γ H2AX

To further investigate the functional interactions among RAD51, RAD52, BCCIP, and γ H2AX, we scored the colocalization of RAD51 foci with BCCIP, γ H2AX, and RAD52, and also γ H2AX foci with BCCIP and RAD52 at various times after 8 Gy ionizing radiation. Focus colocalization is most accurately assessed by confocal microscopy. However, because this provides a view of only a narrow slice of the nucleus (1 μ m of a ~10- μ m thick nucleus), not all foci are detected. To estimate the total number of colocalized foci per cell, we determined the percentage of colocalized foci by confocal microscopy (Supplementary Fig. S1) and then multiplied this value by the total number of foci measured by wide-field microscopy (Fig. 1). As shown in Fig. 1C, Fig. 2A, and B, RAD51 colocalization with BCCIP reaches a maximum at 2 hours postirradiation and then declines steadily during the remaining 6 hours of the time course, reflecting the removal of DNA damage as a result of repair. RAD51 colocalization with γ H2AX increases until 4 hours after irradiation and decreases slightly during the remaining 4 hours. In contrast, there is little colocalization of RAD51 with RAD52 until 6 hours after irradiation, much later than with BCCIP and γ H2AX. BCCIP colocalization with γ H2AX increased immediately after ionizing radiation, then decreased slightly at the 2 hour time point, was maximal at 4 hours after ionizing radiation, and then decreased during the remainder of the time course (Fig. 1D). This complex pattern may reflect the fact that γ H2AX foci increase rapidly in number after ionizing radiation, but BCCIP foci remain constant, and because many DSBs marked by γ H2AX are shunted to repair by NHEJ. Similarly, there are fewer RAD51 foci than γ H2AX foci (Fig. 1A). These data suggest that a subset of RAD51 foci first associate with BCCIP and γ H2AX within 15 minutes. Much later, another subset of RAD51 foci may associate with RAD52. This is further validated by taking into account that BCCIP and RAD52 show very low colocalization (Fig. 1C and Fig. 2C). It is not clear if these associations reflect the transfer of RAD51 from an early BCCIP repair complex to a late RAD52 complex or whether distinct populations of RAD51 interact with these repair factors at different times, perhaps at different types of DNA lesions. The kinetics of RAD51 colocalization with BCCIP and RAD52 after 2 Gy is similar to 8 Gy (Supplementary Fig. S2C and D).

RAD52 focus formation in response to replication fork blockage

RAD52 foci can be induced by irradiation (Fig. 1 and Fig. 2; ref. 29). In addition to repairing radiation-induced DSBs, HR is also important for resolving blocked replication forks. Although biochemical studies clearly implicate mammalian RAD52 in homologous recombinational repair of ionizing radiation-induced DNA damage (34–36), this view is challenged by the mild phenotype exhibited by cells and mice with RAD52 defects (14,17)

and by the marked delay in the colocalization of RAD52 and RAD51 foci after ionizing radiation (Fig. 1C and Fig. 2B). We hypothesized that RAD52 may have a more important role in resolving blocked replication forks than in repairing DSBs directly produced by ionizing radiation. In this view, the late association of RAD52 with RAD51 may reflect late encounters of replication forks with unresolved ionizing radiation-induced DNA damage such as base damage or single-strand breaks. To test this, we stably expressed EGFP-RAD52 in HT1080 cells and then treated the cells with 8 Gy γ -irradiation or 10 mmol/L hydroxyurea. Seven hours after these treatments, the cells were fixed and RAD52 nuclear foci were scored. As shown in Fig. 3A and B, RAD52 foci were strongly induced by hydroxyurea (significantly more than in irradiated cells by *t* test; $P = 0.001$), although cell survival measured by colony formation assay showed that the hydroxyurea treatment resulted in substantially higher cell survival than ionizing radiation treatment (44% versus 2.8% cell survival; see Fig. 3C). These data confirm a role for RAD52 in response to replication fork blockage and suggest that this role is more dominant than its role in repairing ionizing radiation-induced DSBs. In addition, there is strong colocalization of RAD52 with RAD51 in hydroxyurea-treated cells (Fig. 3D), supporting the idea that RAD52 functions in HR-mediated fork restart similar to that seen in yeast (15,37).

RAD52 mobility is reduced more in cells treated with hydroxyurea than ionizing radiation

The formation of DNA damage-induced nuclear foci by repair factors reflects their migration to damage sites, and in theory, the sequestration of proteins at these foci will limit their nuclear mobility. Therefore, a change in the relative mobility of RAD52 in response to irradiation or replication fork blockage can help characterize its response to these treatments. To determine RAD52 mobility, we used FRAP technology. Cells expressing EGFP-RAD52 were photobleached, and the relative intensity of RAD52 protein diffusing back into the bleached area was quantified (Fig. 4). A representative image series for the FRAP analysis is shown in Supplementary Fig. S3. Cells in untreated controls and after ionizing radiation or hydroxyurea treatment were evaluated for protein diffusion rates and the amount of mobile and immobile protein present. In untreated cells, the RAD52 signal recovers to 60% of the prebleach value within 8 to 10 seconds (Fig. 4A). In cells that received an 8 Gy ionizing radiation dose, the signal recovered to the same 60% level, and there was a slight extension in the recovery period to ~12 seconds (Fig. 4B). In contrast, hydroxyurea-treated cells required ~40 seconds for the RAD52 signal to recover to a maximum level that was only 40% of the prebleach signal (Fig. 4C). Based on the data shown in Fig. 4 and data not shown, we calculated relative diffusion constants (Fig. 4D). As another control, we measured the diffusion of EGFP, which is significantly lower in molecular weight than the EGFP-RAD52 fusion protein. As expected, EGFP had a higher relative diffusion constant than the EGFP-RAD52 fusion protein, and EGFP mobility was unchanged by γ -irradiation or hydroxyurea treatment. Interestingly, irradiation reduced RAD52 mobility by ~2-fold, but hydroxyurea treatment had a much stronger effect, reducing RAD52 mobility by 10-fold. The greater reduction in RAD52 mobility by hydroxyurea and the reduction in the mobile fraction from 60% to 40%, indicates that RAD52 is highly responsive to replication fork blockage, and that this response is greater than to ionizing radiation-induced damage. In contrast to RAD52, neither BCCIP α nor BCCIP β showed altered mobility in response to ionizing radiation or hydroxyurea (Fig. 5). The BCCIP recovery signal decreases between 0.4 and 0.6 consistently for all samples. This seems lower than for RAD52, although EYFP shows a greater deal of intrinsic photobleaching than does EGFP. Notably, EYFP recovers to ~0.5 (Fig. 5). The lack of BCCIP mobility agrees with the prior observation that ionizing radiation treatment does not result in increased numbers of BCCIP foci (Fig. 1A).

Discussion

In the present study, we show that two RAD51-associated proteins, RAD52 and BCCIP, display distinct nuclear dynamics after DNA damage, including focus formation and the timing of their colocalization with RAD51. Our data suggest that after ionizing radiation-induced DNA damage, there may be two modes of RAD51 focus formation or two types of RAD51 foci (Fig. 6), one reflecting early acting, BCCIP and BRCA2-dependent repair, and a later response that involves RAD52. Thus, shortly after irradiation, RAD51 is recruited to a fraction of DSBs and associates with pre-existing BCCIP foci, whereas at later times, additional RAD51 foci form that are associated with RAD52. BCCIP mobility is unchanged after ionizing radiation, and γ H2AX colocalizes with BCCIP, implying that BCCIP is part of a constitutive “repairosome” assembly to which damaged DNA, marked by γ H2AX, is recruited and includes BRCA2 (25). Further validation of this concept has been reported, which indicates that BRCA1 and BRCA2 form colocalized nuclear foci regardless of DNA damage (38), and we previously reported that BCCIP is in complex with BRCA2 (25). This idea shows striking similarity, in a mammalian system, to that proposed by Lisby et al., based on evidence in yeast, in which multiple DSBs are processed in a single repair center (39).

Yeast *rad52* Δ mutants show dramatic sensitivity to ionizing radiation (7) and fail to form Rad51 foci after ionizing radiation (15). These results illustrate the importance of HR in yeast radioresistance and the critical role for ScRad52 in mediating Rad51-dependent HR. In contrast, mammalian cells with RAD52 defects show mild or no radiosensitivity. Although this can be explained in part by the greater role for NHEJ in mammalian cell radioresistance, more direct measures of HR, such as gene targeting and ionizing radiation-induced RAD51 focus formation, indicate that RAD52 has a less dominant role in the homologous recombinational repair of DSB damage in mammalian cells (14,16). Yeasts lack BRCA2, and it has been suggested that BRCA2 may have replaced RAD52 during evolution as a RAD51 mediator in higher eukaryotes, specifically mediating the exchange of RPA with RAD51 on ssDNA at resected DSBs (40).

It is known that ionizing radiation-induced RAD51 focus formation is abrogated in cells with defects in either BCCIP or BRCA2 (25,41). Previously, we showed that BCCIP also colocalizes and coimmunoprecipitates with RAD51, but it is not yet known if this association is direct or mediated through BRCA2 or another protein. In addition, siRNA down-regulation of BCCIP diminishes ionizing radiation-induced RAD51 focus formation (25). We propose that BCCIP, in association with BRCA2, is a prerequisite for early RAD51-dependent repair of ionizing radiation-induced DSBs. Although RAD51 interacts with both BCCIP and RAD52 (25,28, 29), we have been unable to show an interaction between BCCIP and RAD52 by coimmunoprecipitation.³ This is consistent with the finding that RAD51 colocalizes with BCCIP and RAD52 at different times after ionizing radiation damage (Fig. 1C, Fig. 2A, and B), and it further suggests a possibility that BCCIP and RAD52 may be involved in the formation of distinct subset of RAD51 foci after irradiation. The key role for BCCIP in DSB repair by HR and the lack of a BCCIP-RAD52 interaction further supports the idea that RAD52 does not have a central role in early RAD51-dependent repair of DSBs that are directly produced by ionizing radiation in mammalian cells.

However, it is curious that mammalian RAD52 shares key biochemical properties with ScRad52, including the ability to interact with and load RAD51, anneal ssDNA, and stimulate homologous pairing by RAD51 (13,28), yet the protein does not seem to play a role in early RAD51-dependent repair of DSBs that are directly induced by ionizing radiation. In addition to DSB repair, HR also has important roles in restarting stalled or collapsed replication forks

³H. Lu and Z. Shen, unpublished results.

(6,42). Our findings that hydroxyurea induces RAD52 foci, which colocalize with RAD51, and reduces RAD52 mobility to a greater extent than ionizing radiation, despite significantly greater killing with ionizing radiation than hydroxy-urea (Fig. 3 and Fig. 4), all suggest that RAD52 has important roles in HR-mediated replication fork restart. This model can also explain why RAD52 foci are formed at late times after ionizing radiation–induced DNA damage. Ionizing radiation produces a wide range of DNA lesions including DSBs, single-strand breaks, base damage, and crosslinks (43). Many of these lesions can block replication; however, ionizing radiation also activates checkpoint responses, including the intra-S checkpoint. The intra-S checkpoint prevents replication initiation at late-firing origins (34). The lack of significant RAD52 focus formation at early times after ionizing radiation may reflect the relatively low frequency of stalled forks (despite high levels of initial DNA damage) because only a fraction of log-phase cells are in S phase and a small fraction of replicons are active at a time. Several hours after ionizing radiation, the intra-S checkpoint is deactivated and late-firing origins may initiate replication. In addition, at late times, cells in other phases of the cell cycle (chiefly G₁) will progress to S phase. When the active forks encounter unrepaired lesions, they may stall and collapse, leading to recruitment of RAD52 and other HR proteins to promote fork restart. Alternatively, RAD52 may be involved in repair complex DNA damage that cannot be processed by the initial RAD51-dependent process.

The lesions that remain at late times after ionizing radiation may remain unrepaired because they are difficult to repair or because high levels of DNA damage, such as that caused by 8 Gy ionizing radiation, exceed the cellular DNA repair capacity. DNA lesions refractory to repair could trigger a “last ditch” response in which RAD52 mediates DSB repair via nonconservative (and RAD51-independent) HR by single-strand annealing. Although we cannot rule out a role for RAD52 at lesions refractory to repair, we propose that RAD52 has a role in restarting collapsed replication forks because RAD52 foci are strongly induced by hydroxyurea, which causes fork stalling and collapse. Fork collapse results in one-ended double-stranded DNA, termed double strand ends (DSE). A role for mammalian RAD52 in replication fork restart may be evolutionarily conserved from yeast to humans. Indeed, spontaneous RAD52 foci are seen in a fraction of S phase yeast cells, and these foci have been attributed to RAD52 acting at replication forks that encounter endogenous DNA damage (37). A recent study identified a class of yeast RAD52 mutants that are deficient in DSB repair but proficient in spontaneous and UV-induced HR, suggesting that a significant fraction of spontaneous HR initiates at lesions other than DSBs and DSEs (i.e., single-strand nicks and gaps). Interestingly, RAD52 seems to play a key role in break-independent spontaneous HR in yeast.

How might mammalian RAD52 function at replication forks? The simplest answer is that it loads RAD51 onto ssDNA. In this view, BRCA2 and RAD52 each act as RAD51 mediators but at distinct lesions arising in replication-independent (DSB) and replication-dependent (DSE) contexts, respectively, as described in the model presented in Fig. 6. This model is consistent with the finding that BRCA2 is required for ionizing radiation–induced RAD51 focus formation but that RAD51 foci can form independently of BRCA2 in S phase (41) and that RAD51 is preferentially bound to the nuclear matrix in an ataxia telangiectasia Rad3–dependent fashion during the intra-S checkpoint (44). As in yeast, spontaneous HR in mammals is thought to initiate at collapsed replication forks (45). These results provide further support for the model that BRCA2 and RAD52 play complementary roles in the repair of DSBs and DSEs, respectively. BRCA2 has also been implicated in stabilizing stalled replication forks (46). This role for BRCA2 does not exclude a parallel role for RAD52, and the two proteins may collaborate to promote replication fork restart or they may operate on different subsets of lesions. RAD51, as the central recombinase, is an essential protein in mammalian cells, and it is likely that its essential role is in restarting stalled/collapsed replication forks. Yeast Rad51, on the other hand, is not essential, but this probably reflects less frequent encounters with spontaneous blocking lesions during replication of this relatively

small genome. In higher eukaryotes, defects in RAD52 or the RAD51 paralog, XRCC3, are tolerated quite well individually, but the double knockout in chicken DT40 cells is lethal (47). This lethality, such as that of RAD51 defects, probably reflects redundant roles for RAD52 and XRCC3 in replication fork restart. A similar redundancy between RAD52 and BRCA2 would suggest that BRCA2-deficient tumors may be effectively treated by abolishing RAD52 function, particularly in combination with induced DNA damage from ionizing radiation or replication-blocking chemotherapeutics such as AraC and hydroxyurea. Because RAD52 is nonessential in mammalian cells, targeting RAD52 should have little effect on normal cells, analogous to poly(ADP)ribose polymerase inhibition (48,49).

In summary, we present three key findings regarding the role of RAD52 in DNA damage repair and blocked replication: (a) RAD52 foci form late after ionizing radiation compared with RAD51 and γ H2AX foci, and RAD51 colocalizes with BCCIP much sooner than with RAD52; (b) stalled replication is a potent inducer of RAD52 foci, and it sharply reduces RAD52 mobility; and (c) unlike RAD52, ionizing radiation and hydroxyurea do not reduce the mobility of BCCIP. These results suggest that BCCIP is part of a constitutive “repair center” to which BRCA2 and ionizing radiation–induced DSBs are recruited for RAD51-dependent repair by HR, that RAD52 mediates RAD51 function at stalled or collapsed replication forks in a complementary pathway, and that targeting RAD52 in BRCA2-deficient tumors could substantially improve treatment efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Dynamics of RAD51, RAD52, BCCIP, and γ H2AX focus formation and colocalization after 8 Gy of ionizing radiation (IR) treatment. Cells were treated with 8 Gy ionizing radiation, the number of foci per cells were scored, and the percentage of colocalized foci was determined by confocal microscopy at the indicated times (see Supplementary Fig. S1). These percentages were multiplied by the total number of foci per cell (A) to yield the total number of colocalized foci per cell ($\pm$ SE). A, the average numbers of foci per cell were scored by using immunofluorescence microscopy. Points, mean; bars, SE. B, the percentage of cells with at least five foci per cell as determined by immunofluorescence microscopy. C, colocalization of RAD51 with RAD52, BCCIP, or γ H2AX. D, colocalization of RAD52 with BCCIP or γ H2AX, and BCCIP with γ H2AX.

Figure 2.

Distinct temporal colocalization of RAD51, BCCIP, and RAD52 after ionizing radiation. Shown are representative images of cells 2 or 6 h after 8 Gy ionizing radiation. *A*, RAD51 colocalized with BCCIP at 2 h after ionizing radiation but not after 6 h. *B*, RAD51 is independent of RAD52 at 2 h after ionizing radiation but colocalizes with RAD52 after 6 h. *C*, RAD52 does not colocalize with BCCIP after ionizing radiation.

Figure 3.

RAD52 responds more robustly to hydroxyurea (HU)-induced replication stress than to ionizing radiation. **A**, representative images of cells expressing EGFP-RAD52 and untreated, treated with 8 Gy ionizing radiation or 10 mmol/L hydroxyurea. Cells were fixed 7 h after treatment. **B**, average numbers of RAD52 foci per cell for control, ionizing radiation-treated (8 Gy), and hydroxyurea-treated (10 mmol/L). **C**, percentage survival of HT-1080 cells expressing RAD52 after 8 Gy ionizing radiation or 10 mmol/L hydroxyurea ($\pm$ SE). All values were normalized to 100% of untreated control. **D**, a representative image of RAD52 and RAD51 colocalization after 10 mmol/L hydroxyurea treatment for 6 h.

Figure 4.

RAD52 is immobilized by replication stress more than by ionizing radiation. A to C, fluorescence recovery curves of EGFP-RAD52 in untreated controls or cells treated with 8 Gy ionizing radiation or 10 mmol/L hydroxyurea. D, calculated diffusion constants ($\pm$ SE) for EGFP or EGFP-RAD52 in treated and untreated cells. All samples were imaged 6 to 8 h after mock or initial treatment.

Figure 5. BCCIP mobility is not altered by replication stress or ionizing radiation. *A*, FRAP curves as described in Fig. 4A to C for BCCIP and EYFP control. *B*, calculated BCCIP diffusion constants ($\pm$ SE).

Figure 6.

A model of the spatio-temporal interactions of RAD51 with BCCIP/BRCA2 and RAD52.

Left, ionizing radiation-induced DSBs rapidly associate with MRN/ATM and then are recruited to repair centers where BCCIP/BRCA2 facilitates RAD51 loading and HR repair.

Right, ionizing radiation-induced single-strand damage is encountered by replication forks after the intra-S checkpoint is released, leading to fork stalling and/or collapse to DSEs. RAD52 is proposed to act at these late structures (independently of BRCA2 and BCCIP) to facilitate RAD51-dependent fork restart.