THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 31, Issue of August 4, pp. 23899–23903, 2000

Printed in U.S.A.

The Breast Cancer Susceptibility Gene *BRCA1* Is Required for Subnuclear Assembly of Rad51 and Survival following Treatment with the DNA Cross-linking Agent Cisplatin*

Received for publication, April 21, 2000, and in revised form, June 6, 2000
Published, JBC Papers in Press, June 7, 2000, DOI 10.1074/jbc.C000276200

Anamitra Bhattacharyya‡, Uy S. Ear‡, Beverly H. Koller§, Ralph R. Weichselbaum‡, and Douglas K. Bishop‡¶

From the ‡Department of Radiation & Cellular Oncology, University of Chicago, Chicago, Illinois 60637 and the §Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

Mutations in breast cancer tumor susceptibility genes, *BRCA1* and *BRCA2*, predispose women to early onset breast cancer and other malignancies. The Brca genes are involved in multiple cellular processes in response to DNA damage including checkpoint activation, gene transcription, and DNA repair. Biochemical interaction with the recombinational repair protein Rad51 (Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. M. (1997) *Cell* 90, 425–435), as well as genetic evidence (Moynahan, M. E., Chiu, J. W., Koller, B. H., and Jasin, M. (1999) *Mol. Cell* 4, 511–518 and Snouwaert, J. N., Gowen, L. C., Latour, A. M., Mohn, A. R., Xiao, A., DiBiase, L., and Koller, B. H. (1999) *Oncogene* 18, 7900–7907), demonstrates that Brca1 is involved in recombinational repair of DNA double strand breaks. Using isogenic *Brca1*$^{+/+}$ and *brca1*$^{-/-}$ mouse embryonic stem (ES) cell lines, we investigated the role of Brca1 in the cellular response to two different categories of DNA damage: x-ray induced damage and cross-linking damage caused by the chemotherapeutic agent, cisplatinum. Immunoflourescence studies with normal and *brca1*$^{-/-}$ mutant mouse ES cell lines indicate that Brca1 promotes assembly of subnuclear Rad51 foci following both types of DNA damage. These foci are likely to be oligomeric complexes of Rad51 engaged in repair of DNA lesions or in processes that allow cells to tolerate such lesions during DNA replication. Clonogenic assays show that *brca1*$^{-/-}$ mutants are 5-fold more sensitive to cisplatinum compared with wild-type cells. Our studies suggest that Brca1 contributes to damage repair and/or tolerance by promoting assembly of Rad51. This function appears to be shared with *Brca2*.

Germ line mutations in *BRCA1* or *BRCA2* genes result in a marked increase in the risk of early onset breast and ovarian cancers (5–10). *BRCA1* and *BRCA2* appear to have multiple functions including roles in transcriptional regulation (11–14) and cell cycle checkpoint control (15–20).[1] Brca1 and Brca2 both have transcription activation functions (11, 13); Brca1 co-activates transcription with p53 (21, 22). Recently it has been demonstrated that Brca1 participates in homologous recombinational repair pathways (2, 3). These observations are consistent with earlier work that demonstrated interaction of Brca1 and Brca2 with the recombinational repair protein Rad51, in addition to studies showing that *brca1* and *brca2* mutants are phenotypically similar to *rad51* mutants (1, 4, 23–26).

In addition to contributing to recombinational repair of double strand breaks (DSBs),[2] *BRCA1* has also been implicated in other DNA repair pathways. Mutational analysis has shown a role for *BRCA1* in transcription-coupled base excision repair of oxidative DNA damage (27, 28). Furthermore, a recent study reported biochemical interactions between Brca1 and proteins required for DNA-end joining, nucleotide mismatch repair, DNA replication, and signal transduction in response to damage (29). This study also identified interactions between Brca1 and other proteins thought to be involved in recombinational repair. Although these results raise the possibility that *BRCA1* contributes to multiple cellular DNA damage responses, the specific mechanisms through which *BRCA1* contributes to these processes remain to be determined.

Studies primarily in yeast have indicated that Rad51 promotes homology-dependent repair of DNA DSBs. The strand exchange activity of Rad51 catalyzes the exchange of genetic information between a damaged DNA molecule and an undamaged template copy (30, 31). Similarly, studies have shown that the human Rad51 possesses DNA strand-exchange activity (32). Immunostaining analysis of yeast and mammalian cells undergoing DNA repair and recombination have revealed the presence of visible subnuclear assemblies of Rad51 (33, 34). The properties of Rad51 foci indicate that they are multimeric nucleoprotein complexes engaged in recombinational repair (33–38). In mammalian cells, *rad51* "knock-out" mice have been shown to display embryonic lethality and sensitivity to ionizing radiation indicating a role in mediating genome stability (26).

Rad51 plays a central role in mediating homologous recombination events and can promote strand-exchange alone *in vitro*. However, its strand-exchange activity requires various accessory factors. For example, one category of accessory factor promotes assembly of Rad51 into the helical protein-DNA filaments needed for strand exchange. In yeast, biochemical (39–42) and cytological (36) observations indicate that RPA, Rad55, Rad57, and Rad52 proteins promote the assembly of Rad51 during yeast meiotic recombination. Thus, one model for Rad51 assembly at sites of damage is that formation of the initial RPA nucleoprotein complex at single-stranded DNA tracts provides the necessary structural "platform" for Rad51 to be recruited to

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¶ To whom correspondence should be addressed: University of Chicago Medical Center, Room O-055, 5841 S. Maryland Ave., MC1105, Chicago, IL 60637. Tel.: 773-702-9211; Fax: 773-702-1968; E-mail: dbishop@midway.uchicago.edu.

[1] B. H. Koller, unpublished observations.

[2] The abbreviations used are: DSB, double strand break; ES, embryonic stem; RPA, replication protein A; Gy, gray.

the damage repair complex (36). This model for Rad51 assembly is supported in mammalian cells by cytological and biochemical co-localization of RPA and Rad51 foci following DNA damage (43). In addition, the Xrcc3 protein (44, 45) is a likely candidate for a Rad51 assembly factor based on genetic (45, 46) and cytological observations (35).[3] Hence formation of Rad51 complexes at sites of damage is dependent upon at least two criteria: (*a*) formation of a DNA substrate (*e.g.* single-stranded DNA tracts) upon which (*b*) assembly factors form and facilitate recruitment of Rad51.

Here we report that in mouse ES cells, *Brca1* is required for formation of subnuclear Rad51 complexes in response to cellular damage by ionizing radiation or cisplatinum treatment. Accordingly, cells lacking normal *Brca1* function are more sensitive to ionizing radiation (27, 47, 48) and cross-linking agents (Ref. 49 and this work) compared with normal cells. Our findings are in contrast to those reported recently, in which a role for *BRCA2* in damage-induced assembly of Rad51 was detected but an equivalent role for *BRCA1* was not found (24). We propose that both *Brca1* and *Brca2* contribute to recombinational repair by promoting the assembly of Rad51 at the sites of DNA damage.

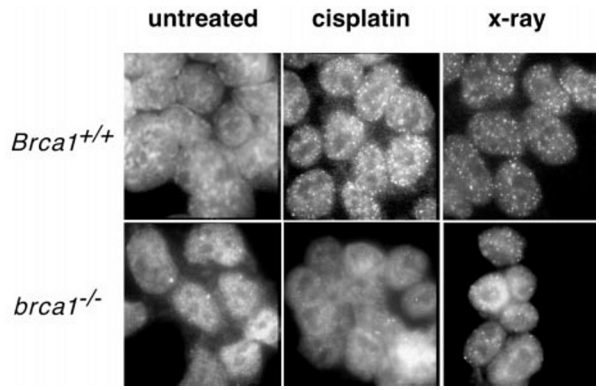

FIG. 1. **Formation of Rad51 subnuclear foci in response to DNA damage induced by x-ray and cisplatinum treatment.** Mouse $Brca1^{+/+}$ and $brca1^{-/-}$ ES cells were damaged with either x-rays or cisplatinum. Induction of Rad51 foci was analyzed following damage by immunostaining cells with α-Rad51 serum and then counterstaining with the DNA-specific stain 4,6-diamidino-2-phenylindole to highlight nuclei. Representative nuclei are displayed from $Brca1^{+/+}$ and $brca1^{-/-}$ mutant ES cells from either untreated, x-ray (9 Gy)-, or cisplatinum (10 μM)-treated cells.

EXPERIMENTAL PROCEDURES

Cell Lines—$Brca1^{+/+}$ (E14Tg2a) and $brca1^{-/-}$ isogenic mouse embryonic stem (ES) cell lines (50) were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, nonessential amino acids, glutamine, penicillin/streptomycin, and murine leukemia inhibitory factor (ESGRO, Life Technologies, Inc.) and plated on 0.1% gelatinized 100-mm^2 tissue culture plates.

DNA Damage by X-rays and Cisplatinum—Exponentially growing cultures in 100-mm^2 dishes were x-irradiated with a Maxitron generator (General Electric) at a dose rate of 114 cGy/min. Dishes were returned to the incubator immediately after treatment. For dose-response studies, cells were incubated for 3 h after irradiation before being harvested as described previously (35). For cisplatinum dose-response experiments, cultures were washed twice in serum-free medium and then incubated for 1 h in serum-free medium containing varying concentrations of cisplatinum (Bristol Laboratories). Dishes were washed three times in serum-free medium, and complete medium was added. Cultures were then placed at 37 °C for 3 h, at which time a single-cell suspension was obtained with trypsin/EDTA and the cells were prepared for immunostaining.

Immunostaining and Microscopy—Cells were immunostained as described previously (35). Samples consisted of focus counts from 50 unselected nuclei. The Kruskal-Wallis test was used to determine the statistical significance of observed differences between samples. Color images that combine fluorescein and 4,6-diamidino-2-phenylindole staining patterns were generated by converting grayscale images to pseudocolor and then merging the patterns electronically using I.P. Lab Spectrum software (Signal Analytics Corp., Vienna, VA).

Western Analysis—Samples were prepared as described previously (35). The anti-HsRad51 IgG (a generous gift from Dr. Akira Shinohara) and anti-CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies were used at concentrations of 0.5 and 0.3 μg/ml, respectively. Secondary antibodies (goat anti-rabbit and goat anti-mouse peroxidase conjugates, Santa Cruz Biotechnology) were used at a 1:2000-fold dilution. Signals were detected by chemiluminescence (Renaissance, NEN Life Science Products).

Clonogenic Survival Assays—For cisplatinum treatment, cells were exposed to drug for 1 h at 37 °C, in liquid medium as described above, replated (at 400 and 4000 cells/plate), and allowed to grow. 10–12 days later the colonies were fixed and stained with crystal violet, and surviving cells were scored. Colonies that contained >50 cells were counted as survivors. All survival experiments were performed in triplicate, and the means of the surviving fraction of cells were determined. The number of colonies were normalized for plating efficiency, which was 93 and 74% for the $Brca1^{+/+}$ and $brca1^{-/-}$ cell lines, respectively.

Cell Cycle Analysis—Cycling *Brca1* wild-type or mutant cells were either untreated or incubated with 10 μM cisplatinum under conditions described above. Cells were returned to growth for 3 h in medium with full serum and then harvested, washed in phosphate-buffered saline, and fixed in cold 70% ethanol while vortexing to ensure disaggregation of cell clumps. After storage on ice for 30 min, cells were washed twice in phosphate-buffered saline. Cells were then treated with RNase A (Sigma) for 30 min at 37 °C followed by addition of propidium iodide (Sigma) for 30 min on ice. Samples were analyzed immediately using a Becton-Dickinson FACS analyzer, and further data processing was accomplished using CellQuest software (Becton-Dickinson).

RESULTS AND DISCUSSION

Mouse $brca1^{-/-}$ ES Cells Are Defective in Rad51 Focus Formation following X-ray or Cisplatinum Treatment—We employed an isogenic pair of mouse ES cell lines, bearing either wild-type $Brca1^{+/+}$ or a $brca1^{-/-}$ mutant (deleted for exon 11, which encodes 60% of the Brca1 gene) (50) to investigate the role of *Brca1* in assembly of the recombinational repair protein Rad51. X-rays induce many types of DNA damage including single and double strand DNA breaks. Cisplatinum induces formation of inter- and intrastrand cross-linked adducts (Ref. 35 and references therein). To determine if *Brca1* function is required for Rad51 focus formation following induction of damage with these two agents, cycling $Brca1^{+/+}$ and $brca1^{-/-}$ cells were exposed to varying doses of x-rays or cisplatinum, as described above. Cells were fixed and stained with anti-HsRad51 antibody, and nuclei were visualized by fluorescence microscopy (Fig. 1). Consistent with earlier work in other mammalian tissue culture cells (1, 34, 35), examination of $Brca1^{+/+}$ cells revealed a dramatic increase in the number of subnuclear Rad51 foci in response to both ionizing radiation and cisplatinum treatment (Fig. 1, *top panel*). In contrast, the *brca1* mutant displayed relatively few Rad51 foci even after relatively high doses (Fig. 1, *bottom panel*; Fig. 2*A*). These results suggest that Brca1 is required for normal subnuclear assembly of Rad51 protein in response to DNA damage by x-rays or cisplatinum. While the $brca1^{-/-}$ cell line was defective relative to the wild-type control cell line, we did observe induction of a small number of Rad51 foci in response to x-rays in the *brca1* mutant (Fig. 2*A*). The *brca1* mutant displayed a mean-induced level of 4.7 foci/nucleus with compared 21.7 foci/nucleus in wild type after x-irradiation (9 Gy).

Brca1 Is Required for Resistance to Cisplatinum—The same mouse brca1$^{-/-}$ ES line examined here was previously shown to be more sensitive to x-rays than its isogenic $Brca1^{+/+}$ progenitor at doses higher than 3 Gy (27). In addition, *BRCA1*-deficient human cells have also been demonstrated to be sensitive to ionizing radiation (48, 51). A recent study has shown

[3] S. Takeda, unpublished observations.

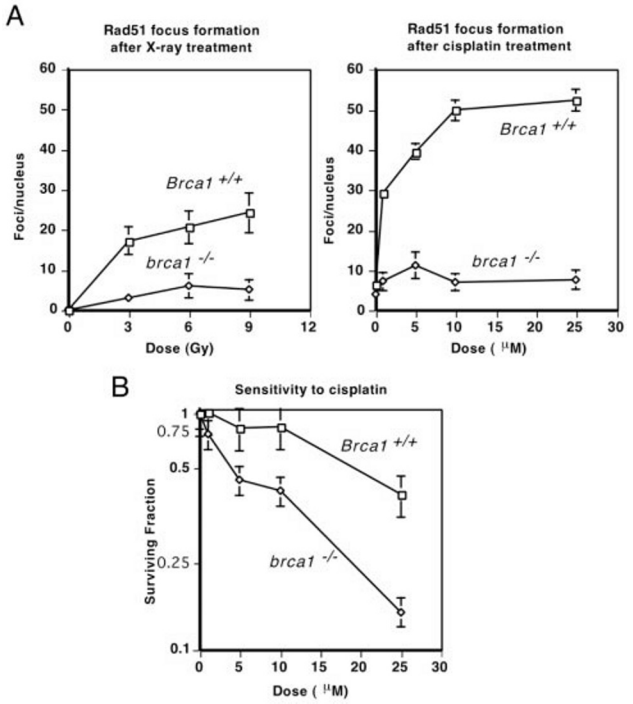

FIG. 2. **Analysis of Rad51 foci formation and sensitivity to cisplatinum-induced DNA damage.** *A*, x-ray and cisplatinum dose-response analysis of Rad51 focus formation in *Brca1* wild-type and mutant cell lines. Cells were damaged as described under "Experimental Procedures," and cells returned to growth for 3 h; subsequently cells were fixed and stained with anti-Rad51 antibody. Images were taken of 50 unselected nuclei, and the number of Rad51 foci were scored. The mean number of Rad51 foci/nucleus at each dose, from several experiments, was determined and plotted. *B*, sensitivity of *Brca1* wild-type and mutant ES cell lines toward cisplatinum treatment was analyzed in a clonogenic survival assay as described under "Experimental Procedures." Survival curves for ES cells exposed to cisplatinum treatment are shown. Following treatment, cells were seeded onto 100-mm gelatinized plates and grown for 10–12 days, after which time cells were stained with crystal violet. The number of colonies obtained with untreated cells was corrected for plating efficiency and normalized to 100% survival.

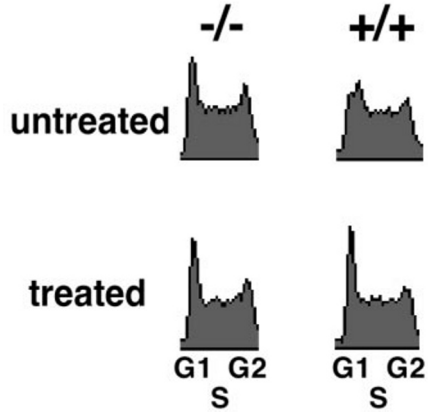

FIG. 3. **Cell cycle analysis of cisplatinum-treated *Brca1*$^{+/+}$ and *brca1*$^{-/-}$ cell lines.** The cell cycle distribution of *Brca1* wild-type and mutant ES cells before and after treatment with 10 μM cisplatinum was performed by FACS analysis as described under "Experimental Procedures." Cells were returned to growth for 3 h post-treatment and then harvested for analysis. The relative amounts of G_1, S, and G_2 cell populations were quantitatively determined by a FACS gate. Quantification of cell distributions are shown in Table I.

that in cisplatinum-resistant MCF-7 cells *BRCA1* is up-regulated, suggesting that *BRCA1* also contributes to cellular resistance to cisplatinum (49). To compare the relative effects of drug dose on cellular resistance and Rad51 focus formation and also to provide more direct evidence implicating *Brca1* in cisplatinum resistance, we performed clonogenic survival assays. In *Brca1*$^{+/+}$ cells, Rad51 foci were induced at doses of cisplatinum that are tolerated by most cells. The number of foci induced by the drug reaches a plateau value at about 10 μM, which corresponds to the maximum dose tolerated without substantial loss of cell viability (Fig. 2, *A* and *B*). Higher doses of the drug resulted in a dramatic decline in viability and no further induction of Rad51 foci. The *brca1*$^{-/-}$ mutant line was more sensitive to cisplatinum than the wild-type cell line. The dose of cisplatinum needed to kill 50% of cells was 20 μM in wild-type and 4 μM for the *brca1*$^{-/-}$ mutant indicating that the mutant is 5-fold less resistant to cisplatinum than wild-type cells. These results are consistent with the hypothesis that Brca1 makes a contribution to cisplatinum and radiation resistance through its effect on Rad51 focus formation.

The Failure of brca1 Mutant Cells to Produce Rad51 Foci Cannot Be Explained by Accumulation of Cells in G_1—Previous work suggested that CHO cells do not form Rad51 foci in response to x-rays in the G_1 phase of the cell cycle but can form such foci in S and G_2 phases.[4] Furthermore, analyses in isogenic ES cell lines have suggested a role for Brca1 in G_2/M checkpoint control.[5] These results raised the possibility that the effect of the *brca1* mutation on damage-induced Rad51 foci might be mediated indirectly through an effect on cell cycle progression. We therefore tested the possibility that *brca1* mutant cells do not form Rad51 foci because cisplatinum treatment causes the mutant cells to accumulate in G_1. Flow cytometric analysis of *Brca1* wild-type and mutant ES cells was carried out following treatment with 10 μM cisplatinum. This analysis revealed that, 3 h after treatment with cisplatinum, the fraction of cells in G_1 was 34.5% for the mutant compared with 27.4% for wild type (Fig. 3, Table I). This difference was too small to account for the difference in the fraction of cells that failed to form foci after treatment (86% in the mutant *versus* 34% in wild type), thus the role of Brca1 in Rad51 assembly cannot be explained as an indirect effect of perturbation of progress through the cell cycle.

Brca1 Is Not Required for Maintaining Normal Levels of Rad51 Protein—To test if the number of Rad51 foci formed in *Brca1* wild-type and mutant cells treated with radiation or cisplatinum damage results from changes in Rad51 protein levels, Western blot analysis was carried out (Fig. 4). Rad51 levels were normalized against CDK2 protein, which is present throughout the cell cycle and whose steady-state levels increase only modestly (less than 2–3-fold) in S and G_2/M (52). We observed little or no difference in steady-state Rad51 protein levels in wild-type or mutant cells untreated or treated with radiation or cisplatinum (Fig. 4, *A* and *B*). Therefore, the changes observed in the number of Rad51 foci observed cytologically with x-irradiation and cisplatinum treatment is not associated with a corresponding change in Rad51 steady-state protein levels. The results also indicate that the *brca1* defect in Rad51 focus formation results from a failure to redistribute Rad51 to subnuclear foci rather than from a failure to express normal levels of protein.

Brca1 and Cisplatinum-induced Damage—Cisplatinum forms two types of adducts with DNA: intrastrand and interstrand nucleotide cross-links (53). In contrast to other cross-linking agents, the most abundant cisplatinum adducts formed

[4] U. S. Ear, D. Hari, R. R. Weichselbaum, and D. K. Bishop, unpublished data.

[5] A. Pace and B. H. Koller, unpublished observations.

TABLE I
Cell cycle analysis of cisplatin-treated Brca1$^{+/+}$ and brca1$^{-/-}$ cell lines

The cell cycle distribution of *Brca1* wild-type and mutant mouse ES cells before and after treatment with 10 μM cisplatin was performed by FACS analysis as described under "Experimental Procedures." Cells were returned to growth for 3 h post-treatment and then harvested for analysis. The relative amounts of G_1, S, and G_2 cell populations were quantitatively determined by a FACS gate.

Cell-line	Cell cycle stage	Untreated	Cisplatin
		%	
Brca1$^{+/+}$	G_1	36.8	27.4
	S	48.4	59.7
	G_2	14.8	13
brca1$^{-/-}$	G_1	30	34.5
	S	56.1	60.6
	G_2	14	5

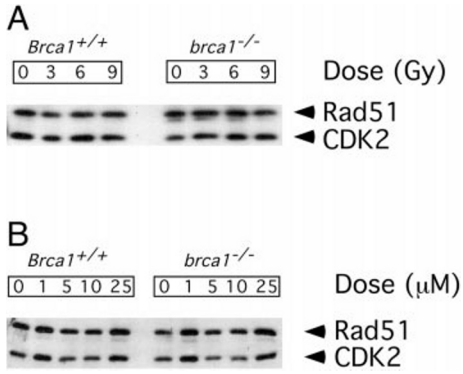

FIG. 4. **Western blot analysis reveal that steady-state Rad51 protein levels are unaffected in *Brca1$^{+/+}$* and *brca1$^{-/-}$* cells in response to damage.** Whole cell lysates were prepared from asynchronously growing *Brca1$^{+/+}$* and *brca1$^{-/-}$* cell lines exposed to x-rays (0, 1, 3, 6, 9 Gy) (*A*) or cisplatinum (0, 1, 5, 10, 25 μM) (*B*). Lysates were subjected to Western blot analysis with anti-Rad51 antibody, 40 μg of total protein was loaded in each lane. Protein levels were normalized to the steady-state levels of CDK2 protein (using anti-CDK2 antibody), which is present throughout the cell cycle.

are the 1,2-d(GG) intrastrand lesions comprising 60–70%, while the 1,2-d(AG) interstrand lesion constitutes approximately 20–30% (53). The ability of cisplatinum to form interstrand cross-links is shared with other damaging agents including mitomycin C, chloronitrosoureas (54, 55), nitrogen mustards (56), and members of the psoralen family (53, 57). The intrastrand cross-links formed by cisplatinum are unusual in that they are refractory to repair via the nucleotide excision repair and translesion synthesis pathways (58–60). This refractivity likely results from the binding of high mobility group proteins to the adducts (58–60).

Bacterial and yeast studies demonstrate that repair of interstrand cross-links requires both participation of nucleotide excision repair proteins and recombinational repair proteins (61–63). Nucleotide excision repair proteins are responsible for lesion recognition and for single strand incision and/or DSB formation at the sites of damage. Recombinational repair proteins are responsible for repairing the intermediates formed by the nucleotide excision repair proteins acting on interstrand cross-links. The intermediates acted on by recombinational repair proteins may include DSBs formed by incision of both strands at the lesion, daughter strand gaps caused when replicative polymerases are blocked by lesions, or DNA ends formed when polymerases encounter single strand incisions. In the first case, recombinational repair can be employed to accurately "heal" the DSB using a homologous duplex as a donor of sequence information; in the latter two cases, recombinational repair can be used to accurately restore a functional replication fork. Recombinational repair is also important for restoring replication forks when unrepaired intrastrand cross-links are encountered by polymerase (Refs. 64 and 65 and references therein). As mentioned above, the intrastrand cross-links formed by cisplatinum are refractory to excision and bypass repair pathways and are thus likely to cause replication fork damage.

Brca1 has been implicated in two types of repair, base excision repair of oxidative damage (thymine glycol) (27) and recombinational repair (2, 48). Thus, Brca1 could promote Rad51 assembly by promoting recognition and incision at the sites of cisplatinum-induced lesions, which in turn leads to Rad51 assembly. The alternative possibility is that Brca1 is involved in directing assembly of Rad51 at the sites of ssDNA regions that form at incision-induced DSBs or at sites of blocked replication forks. We view the alternative possibility as more likely in the case of cisplatinum-induced damage for the following several reasons. First, the nucleotide excision repair mechanism, shown previously to promote excision of cisplatinum-induced damage, appears to be functional in *brca1* mutants (27). In contrast, recombinational repair of DSBs is defective in these cells (2). Other observations suggesting that the defect in Rad51 assembly is not an indirect consequence of an incision defect indicate that cisplatinum blocks the replicative DNA polymerase and that such blocks normally induce Rad51 assembly. Specifically, cisplatinum treatment increases the duration of S-phase in CHO cells by slowing the rate of DNA synthesis (66). Treatment with hydroxyurea blocks DNA synthesis and causes accumulation of Rad51 foci (1) as does treatment with aphidicolin, a drug that directly inhibits DNA polymerase α.[6] Taken together these observations lead us to favor a model in which Brca1 contributes to cisplatinum resistance, at least in part, by promoting assembly of Rad51 at cisplatin-damaged replication forks.

Brca1, Brca2, and Rad51—We have demonstrated here that Brca1 promotes assembly of Rad51 after treatment with cisplatinum and ionizing radiation, a function that could account for the role of Brca1 in conferring cellular resistance to these treatments (27, 48, 51). In contrast to our results with mouse ES cells, no defect in Rad51 assembly was detected in the *BRCA1*-defective human tumor line HCC1937 (24). In the same study a *Brca2* mutant cell line was found to be defective in Rad51 assembly (24). It is possible that an interspecies difference in Brca1 function was responsible for the difference between our results and those of the previous study. We did find evidence that damage-induced Rad51 foci form in mouse ES cells, albeit at a reduced efficiency. Such a Brca1-independent mechanism could be more active in human cells than in murine cells, thereby accounting for the observed difference. Alternatively, an undefined genetic difference between the two *brca1*-defective cell lines may have been responsible for the different observations in the two studies. In this context, we note that the *brca1* mutant line used in our study was derived by a targeted mutation and is thus closely related to the parent *Brca1$^{+/+}$* control line. Finally, it is possible that the immunostaining conditions used in our experiments are particularly sensitive to a structural difference between Rad51-containing structures that form in *Brca1$^{+/+}$* and those that form in *brca1$^{-/-}$* cells. Further studies are needed to determine if human Brca1 contributes to Rad51 assembly, but our results raise the possibility that both Brca1 and Brca2 promote repair of DNA damage by facilitating assembly of Rad51 complexes.

Our observations in mouse ES cells are similar to previous observations in hamster *XRCC3*-defective cells (35), human

[6] R. Casanova and D. K. Bishop, unpublished observations.

BRCA2-deficient cells (24), and mouse *rad54* mutant fibroblasts (67). Recent work in a chicken B-cell lymphoma line adds Xrcc2, Rad51B, and Rad51C to the growing list of factors that play a role, either directly or indirectly, in assembly of Rad51 in response to DNA damage.[7] The large number of proteins required suggests that damage-dependent Rad51 assembly is a highly regulated process.

Acknowledgments—We thank Anne Koons and Steve Gasior for technical assistance with flow cytometric analysis. We also thank Brian Orelli, Phil Connell, and Jeremy Grushcow for critical comments on the manuscript.

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[7] S. Takeda, personal communication.