

## A Cell Cycle-Specific Requirement for the XRCC1 BRCT II Domain during Mammalian DNA Strand Break Repair

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**XRCC1 protein is essential for viability in mammals and is required for efficient DNA single-strand break repair and genetic stability following DNA base damage. We report here that XRCC1-dependent strand break repair in G<sub>1</sub> phase of the cell cycle is abolished by mutations created within the XRCC1 BRCT domain that interact with DNA ligase III. In contrast, XRCC1-dependent DNA strand break repair in S phase is largely unaffected by these mutations. These data describe a cell cycle-specific role for a BRCT domain, and we conclude that the XRCC1-DNA ligase III complex is required for DNA strand break repair in G<sub>1</sub> phase of the cell cycle but is dispensable for this process in S phase. The S-phase DNA repair process can remove both strand breaks induced in S phase and those that persist from G<sub>1</sub> and can in part compensate for lack of repair in G<sub>1</sub>. This process correlates with the appearance of XRCC1 nuclear foci that colocalize with Rad51 and may thus function in concert with homologous recombination.**

DNA strand breakage can result in chromosomal rearrangement and is a major threat to genetic stability. Of particular threat are breaks that arise from damaged DNA bases, several thousand of which occur spontaneously per cell each day (20). The most common such breaks are single-strand breaks, which are formed as intermediates of base excision repair. The threat to genetic stability from DNA strand breaks that arise from base damage is illustrated by the phenotype of rodent cells that harbor mutations within the DNA repair gene *XRCC1*. *XRCC1* is essential for embryonic development in mice (35), and *XRCC1* mutant mouse or CHO cells that possess little or no *XRCC1* protein exhibit increased frequencies of spontaneous sister chromatid exchange and chromosomal aberration (6, 7, 12, 29, 35, 39). *XRCC1* mutant cells appear unable to efficiently rejoin DNA single-strand breaks resulting from either endogenous base damage (35) or that induced by ionizing radiation or alkylating agents (36, 37, 39). Sequence analysis has not provided any indication of the role of *XRCC1* in single-strand break repair (SSBR). However, biochemical studies have revealed that this protein interacts with DNA ligase III and DNA polymerase  $\beta$  (6–8, 18). Thus, it is possible that *XRCC1* functions as a molecular chaperone or scaffold protein, stabilizing and/or modifying the activity of other polypeptides. For example, the interaction of *XRCC1* with DNA ligase III appears to be required for normal cellular levels of the latter, and reduced levels of DNA ligase III can result in inefficient SSBR during the excision repair of abasic sites *in vitro* (6, 7, 11). This interaction is mediated by a C-terminal BRCT domain in *XRCC1*, designated BRCT II (23, 34). BRCT domains have been identified in more than 40 polypeptides, defining a novel protein superfamily, and typically span 80 to 100 amino acids (5, 10). The function of these structures appears to involve, but may not be restricted to, the mediation of protein-protein interactions (5, 10, 23, 34). In the present study, we have examined the importance of the *XRCC1* BRCT II domain and

its interaction with DNA ligase III to SSBR in Chinese hamster ovary (CHO) cells.

### MATERIALS AND METHODS

**Expression constructs, cell lines, and cell synchrony.** A mutant *XRCC1*<sup>pmBRCT</sup> open reading frame (ORF) was generated by subcloning constructs described previously (34) and was inserted into the mammalian expression vector pcD2E (17). All *XRCC1* ORFs encode a decahistidine tag at the C terminus. *XRCC1* mutant EM9 cells were transfected with pcD2E or pcD2E harboring *XRCC1* by electroporation, and >50 independent G418<sup>r</sup> clones were pooled to generate the cell lines EM9-X, EM9-X<sup>pmBRCT</sup>, and EM9-V. Synchrony in G<sub>1</sub> was achieved via serum starvation and incubation in mimosine (25). Cells were incubated sequentially in  $\alpha$ -MEM plus 10% fetal bovine serum (FBS) for 12 to 15 h,  $\alpha$ -MEM plus 0.1% FBS for 48 h, and  $\alpha$ -MEM plus 10% FBS and 300  $\mu$ M mimosine (Sigma) for 24 h. To maintain G<sub>1</sub> synchrony, mimosine was retained in the medium for up to 7 h. To release into S phase or G<sub>2</sub>, incubation was continued in mimosine-free medium for 5 to 8 h (untreated cells) or 7 to 24 h (ethyl methanesulfonate [EMS]-treated cells). Mammalian cell survival assays were conducted as described previously (7).

**Drosophila melanogaster XRCC1.** A number of *D. melanogaster* EST cDNA clones (GenBank accession no. AA392201, AA246970, AA264299, AA735260, AA817298, and AA142274) were identified in the GenBank EST database (non-redundant database of GenBank non-mouse and non-human EST entries) by using the TBLASTN homology search program with the human *XRCC1* amino acid sequence as the query. Two EST clones were obtained for further analysis: AA392201, which was a full-length cDNA from the IMAGE consortium (IMAGE reference no. EST LD11571/AA392201), and AA142274, which was incomplete, from the Berkeley Drosophila Genome Project (BDGP clone CK350). The encoded ORFs were sequenced completely, and the amino acid sequence of the full-length clone was deposited in EMBL (accession code AJ010073). Comparison of the amino acid sequence of the full-length 1.8-kb ORF with that of human *XRCC1* by using Multiple Alignment Construction and Analysis Workbench (MACAW) revealed significant homology to human *XRCC1* (37.7% identity; 55.2% similarity). The region encoding *XRCC1* was PCR amplified from genomic DNA extracted from *D. melanogaster* (Oregon-R) by using *Taq* polymerase and primers specific to the 5'- and 3'-untranslated regions of the fruit fly clone, AA392201. The product was sequenced directly.

**DNA strand break repair assays.** Strand break repair was assayed by alkaline single-cell agarose gel electrophoresis (24, 31). Cells were harvested ( $\sim 10^5$  per pellet), mixed with low-gelling-temperature agarose (Sigma; type VII), and layered onto agarose-coated glass slides. Slides were maintained in the dark at 4°C to gel and for all further steps. Slides were submerged in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-Cl [pH 7.0], 1% Triton X-100, 1% dimethyl sulfoxide [DMSO]) for 1 h, washed with distilled water, and incubated for 45 min in alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA, 1% DMSO [pH 12.8]). After electrophoresis (25 min, 25 V), air-dried and neutralized slides were stained with SYBR-Green I (FMC BioProducts). Average Comet Tail Moment (24) was scored for duplicate slides (100 cells/slide) by using Comet Assay II software (PerSeptive Instruments). Under alkaline conditions, this assay quantifies DNA strand breaks and alkali-labile abasic sites. To quantitate repair, the

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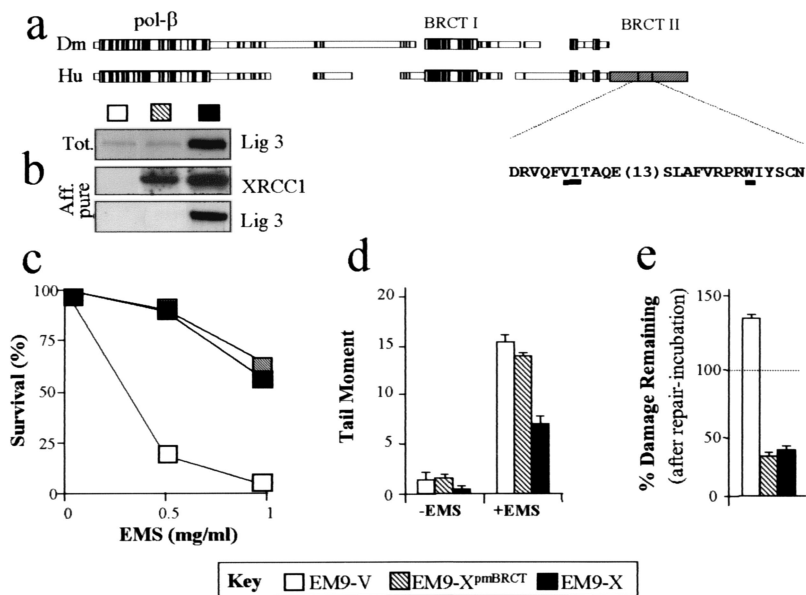


FIG. 1. SSBR independent of XRCC1-DNA ligase III complex. (a) An alignment of human and fruit fly XRCC1 proteins was conducted by MACAW. The ORF is boxed, with gaps for optimal alignment inserted by MACAW. Filled blocks indicate identical residues, and regions of extensive homology are grouped in large blocks. The latter include the N-terminal domain that contains the DNA polymerase  $\beta$  binding site and the internal BRCT I domain. The BRCT II domain in human XRCC1 is hatched, and the positions of the W611D, V584D, and I585D mutations in XRCC1<sup>pmBRCT</sup> are underlined. (b) Total cell extract (top panel) or protein recovered by metal-chelate affinity chromatography (middle and bottom panels) from the indicated cell lines (see Key) was immunoblotted for DNA ligase III (top and bottom panels) or XRCC1 (middle panel). (c) EM9-V cells, EM9-X<sup>pmBRCT</sup> cells, or EM9 cells harboring empty vector (EM9-V) were treated with EMS for 1 h and incubated in drug-free medium for 7 to 10 days. The fraction of surviving cells (percent) is shown. Error bars are within 10% of each value and omitted for clarity. (d) Single-strand breakage (expressed as Tail Moment) present in EM9 transfectants before (–EMS) and immediately after (+EMS) EMS treatment (10 mg/ml; 15 min). (e) EMS-induced single-strand breakage (percent) remaining after 3 h in drug-free medium is shown. Values are the mean ( $\pm$  standard deviation) of at least three experiments.

fraction (percent) of EMS-induced DNA strand breakage remaining after incubation in drug-free medium was calculated by the equation [(mean tail moment<sup>after repair</sup> – mean tail moment<sup>untreated cells</sup>)/(mean tail moment<sup>initial damage</sup> – mean tail moment<sup>untreated cells</sup>)]  $\times$  100.

**Indirect immunofluorescence.** Subconfluent monolayers were resuspended in medium or phosphate-buffered saline (PBS) at  $1 \times 10^5$  to  $5 \times 10^5$  cells/ml. Cells ( $0.1 \times 10^5$  to  $1 \times 10^5$ ) were deposited on glass slides by cytospin and fixed in 50% methanol–50% acetone. PBS-washed slides were incubated sequentially for 1 h in anti-XRCC1 monoclonal antibody (33-2-5), fluorescein isothiocyanate–goat anti-mouse Fab (DAKO; 1/50 dilution in PBS), anti-Rad51 (FBE-1 [2]), or anti-Pol- $\beta$  (7545) polyclonal antibody and Cy3-goat anti-rabbit immunoglobulin G (Sigma; 1/300 or greater dilution). Cells were incubated in antifade and 4',6'-diamidino-2-phenylindole (DAPI) and analyzed with a Zeiss fluorescent microscope at  $\times 400$  magnification. Images were photographed and colored or overlaid by using Adobe Photoshop. Frequencies of focus-positive cells were calculated from  $>100$  cells per data point per slide and from multiple independent experiments. A single clone of EM9-X was used for immunostaining to circumvent the heterogeneity of expression levels observed in pooled transfectants.

## RESULTS

**XRCC1 mediates SSBR by BRCT domain-dependent and -independent mechanisms.** The discovery of an XRCC1 homologue in *D. melanogaster* (Fig. 1a) identifies a number of domains that are highly conserved with the human protein, including an internal BRCT domain (BRCT I) and the amino terminus that binds DNA polymerase  $\beta$  (8, 18). Surprisingly, however, the BRCT II domain that interacts with DNA ligase III in human XRCC1 (23, 34) was absent from two fruit fly cDNA clones, recovered from independent libraries, and from the genomic sequence of these clones (Fig. 1a) (unpublished observations). Although the presence in fruit flies of an XRCC1 homologue that contains a BRCT II domain cannot be excluded, it was considered more likely that BRCT II is evolutionarily a recent addition to the XRCC1 structure and

that the conserved role of this protein does not involve interaction with DNA ligase III.

To directly examine the importance of the BRCT II domain to mammalian SSBR, human XRCC1 in which this structure was disrupted was examined for the ability to correct XRCC1 mutant EM9 cells. Two point mutations were chosen (Fig. 1a): one which removes the single most highly conserved amino acid in BRCT domains and is proposed to abolish correct folding of BRCT II (W611D) (40) and a double mutation that abolishes measurable interaction with DNA ligase III in vitro (V584D-I585D) (34). Expression constructs encoding either wild-type human XRCC1 or the mutated derivative (designated XRCC1<sup>pmBRCT</sup>) were introduced into the XRCC1 mutant CHO cell line, EM9, and G418-resistant transfectants were pooled for analysis. Strikingly, despite the inability of XRCC1<sup>pmBRCT</sup> protein to interact with DNA ligase III in vitro, the resistance of EM9-X<sup>pmBRCT</sup> cells expressing this protein to the alkylating agent EMS was similar to that of EM9-X cells expressing wild-type XRCC1 (Fig. 1c). Two observations support the notion that the XRCC1-DNA ligase III complex was absent from EM9-X<sup>pmBRCT</sup> cells. First, whereas DNA ligase III copurified with XRCC1 from EM9-X cell extract during affinity chromatography, it did not measurably copurify from EM9-X<sup>pmBRCT</sup> cell extract (Fig. 1b, middle and bottom panels). Second, whereas expression of wild-type XRCC1 increased DNA ligase III levels more than sixfold, expression of XRCC1<sup>pmBRCT</sup> failed to increase such levels above those present in EM9-V cells harboring empty vector (Fig. 1b, top panel). Staining with Ponceau S confirmed that equal amounts of total protein were present on these immunoblots (unpublished observations).

SSBR proficiency in the CHO cells after EMS treatment was

measured by alkaline single-cell agarose gel electrophoresis (SCGE). Strand breakage measured in this assay was expressed as the Tail Moment, which is the product of the fraction of DNA that exited the nucleus during electrophoresis multiplied by the distance migrated (24, 31). The DNA strand breakage measured in these experiments reflects the balance between breaks arising from base excision or alkali-labile sites and breaks rejoined by SSBR. The level of DNA strand breakage present in EM9-X cells immediately after EMS treatment was lower than that present in EM9- $X^{pmBRCT}$  cells or EM9-V cells harboring empty vector (Fig. 1d, +EMS). Given that these cell lines differ only in XRCC1 status, and that this protein has only been observed to affect the rate of strand break repair (36, 39), this difference presumably reflects relative SSBR proficiency during the 15-min incubation with EMS. This, in turn, suggests that the BRCT II domain is required for efficient SSBR at early times after base damage. In contrast, DNA strand breakage decreased by 60 to 65% in both EM9-X and EM9- $X^{pmBRCT}$  cells during a subsequent 3-h repair incubation in drug-free medium, suggesting that the BRCT II domain was largely dispensable for SSBR over longer periods (Fig. 1e). In EM9-V cells, the amount of strand breakage appeared to increase by ~35% during repair incubation, presumably reflecting continued base excision, or exonuclease activity, in the absence of any XRCC1-dependent SSBR (Fig. 1e).

**BRCT II-dependent and -independent SSBR processes are cell cycle distinct.** The results described above suggest that the BRCT II domain is important for efficient SSBR at early times after base damage but that XRCC1 can mediate SSBR independently of this structure over longer periods. In an attempt to discriminate these SSBR mechanisms further, we examined whether they exhibited cell cycle specificity. EM9 transfectants were synchronized in late  $G_1$  by a combination of serum starvation and mimosine treatment (24), treated with EMS while arrested in  $G_1$  or after release into S phase, and subsequently incubated for 3 h in drug-free medium to allow time for repair. Whereas the levels of strand breakage present in  $G_1$  and S phase were similar after EMS treatment, that present before EMS treatment was slightly higher in the latter (Fig. 2, middle panels). This may reflect the presence of Okazaki fragments in S-phase cells. As expected, EM9-V cells were deficient, and EM9-X cells were proficient, in SSBR in both cell cycle phases (Fig. 2, right panels). Strikingly, however, EM9- $X^{pmBRCT}$  cells were deficient in SSBR in  $G_1$  but were proficient in SSBR in S phase, in the latter case reducing strand breakage by ~80% (Fig. 2, right panels). To examine whether the inability of XRCC1 $^{pmBRCT}$  protein to restore efficient SSBR to EM9 was due solely to its inability to restore normal levels of DNA ligase III, we compared SSBR proficiencies in EM9-X and EM9- $X^{pmBRCT}$  cells in the presence of specific inhibitors of the 20S proteasome. However, despite increases in DNA ligase III levels in EM9- $X^{pmBRCT}$  of three- to fourfold (~50% of wild-type levels), the proteasome inhibitors did not increase  $G_1$  repair (Fig. 3). The inhibitors similarly did not affect repair in EM9-X cells. Similar results were observed when DNA ligase III levels were increased twofold by expression of the human cDNA (unpublished observations). These results suggest that the BRCT II domain is required during  $G_1$  SSBR for more than the maintenance of DNA ligase III protein levels, although complete restoration of such levels is required to confirm this.

Since EM9- $X^{pmBRCT}$  cells exhibited wild-type levels of EMS sensitivity over the dose range employed (Fig. 1c), it was considered possible that S-phase SSBR can remove not only strand breaks induced in S phase but also those that persist from  $G_1$ . To test this prediction, EM9 transfectants were syn-

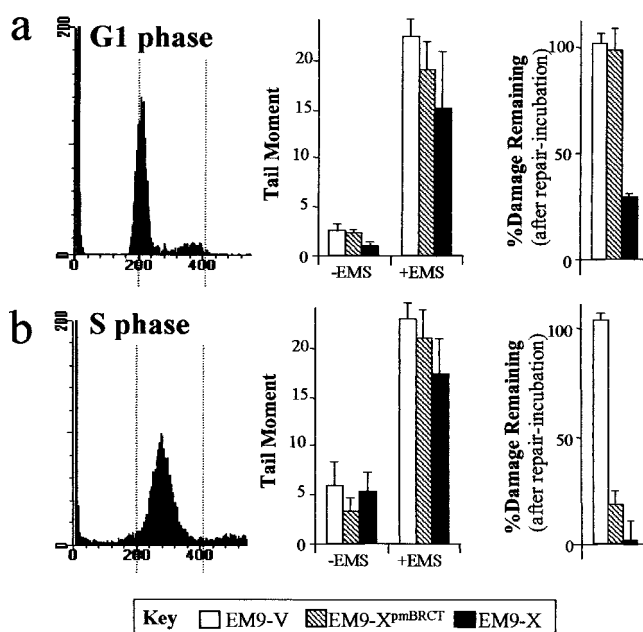


FIG. 2. Cell cycle-distinct SSBR. (Left panels) Flow cytometry profile of  $G_1$  (A)- or S (B)-phase cells; (middle panels) DNA strand breakage before (-EMS) or immediately after (+EMS) treatment of  $G_1$  (a)- or S (b)-phase cells with EMS (10 mg/ml; 15 min); (right panels) EMS-induced strand breakage (percent) remaining in  $G_1$  (A)- or S (B)-phase cells after 3 h in drug-free medium.

chronized in  $G_1$  and treated with EMS for 15 min and then either maintained in  $G_1$  for a further 7 h in drug-free medium or released into S phase for the same period (see Fig. 4a for experimental design). As expected, EM9- $X^{pmBRCT}$  cells failed to reduce EMS-induced DNA strand breakage if maintained in  $G_1$  throughout repair incubation (Fig. 4b). In contrast, however, strand breakage fell by ~70% in EM9- $X^{pmBRCT}$  cells that were released into S phase during repair incubation (Fig. 4b). Control experiments confirmed that EM9-V cells failed to reduce EMS-induced strand breakage in either cell cycle phase (Fig. 4b) and that EM9-X cells did so in both cell cycle phases (unpublished observations). These results confirm that S-phase SSBR can rejoin DNA strand breaks that persist from  $G_1$ .

**S-phase SSBR correlates with XRCC1 foci that partially colocalize with Rad51.** In an attempt to further characterize the role of XRCC1 in S-phase SSBR, the subcellular localization of XRCC1 was examined. Immunostaining of fixed asynchronous EM9-X cells with anti-XRCC1 antibodies revealed nuclei with discrete foci and/or a less-intense speckled staining (Fig. 5A). Both staining patterns were greatly reduced or absent in untransfected EM9 cells. The behavior of the less-intense staining during the cell cycle was difficult to determine. However, the frequency of focus-positive cells appeared to increase in EM9-X cells during progression through S phase but did not increase if cells were maintained in  $G_1$  throughout the experiment (Fig. 5B). These data suggest that the level of XRCC1 foci increases in S phase and are consistent with the participation of these structures in S-phase SSBR. XRCC1 focus-positive cells were also observed in asynchronous populations of EM9- $X^{pmBRCT}$ , the frequency of which was increased ~3-fold 8 h after EMS treatment (Fig. 5C, top panels). EMS-induced increases in focus-positive cells in asynchronous populations of EM9-X were also observed but were slower and less pronounced, with little or no increase observed 8 h after EMS treatment (Fig. 5c, bottom panels) and ~2-fold more

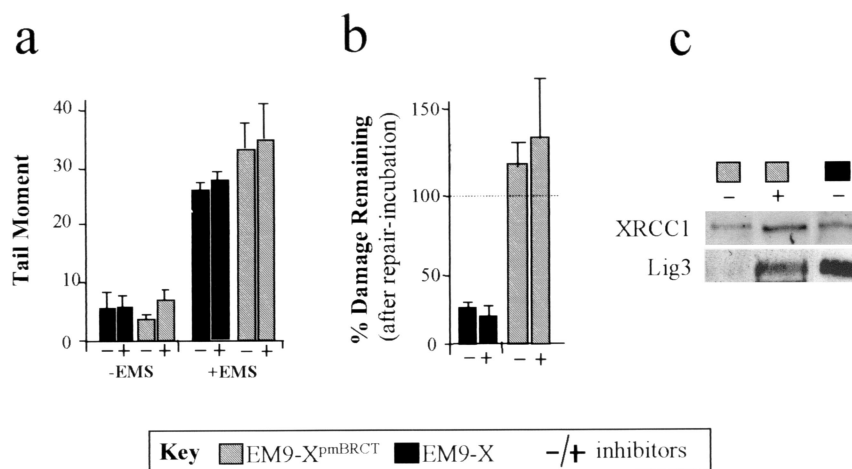


FIG. 3. DNA ligase III protein levels and SSBR proficiency. (A) DNA strand breakage in mock-treated (–EMS) or EMS-treated (+EMS) G<sub>1</sub>-synchronized EM9-X or EM9-X<sup>pmBRCT</sup> cells (see Key). The proteasome inhibitors (Calbiochem) lactacystin (1 μM) and MG-132 (10 μM) were present (+) or absent (–) for 4 h prior to and during EMS or mock treatment. (B) Fraction of EMS-induced strand breakage remaining in G<sub>1</sub>-synchronized cells after a 3-h repair incubation in EMS-free medium in the presence (+) or absence (–) of proteasome inhibitors. Strand breakage present immediately after EMS is set at 100% (dotted line). (C) Immunoblotting of cell extract from EM9-X or EM9-X<sup>pmBRCT</sup> cells (see Key) following synchronization in G<sub>1</sub> and incubation for 4 h in the presence (+) or absence (–) of proteasome inhibitors for XRCC1 or DNA ligase III. Strand break data are the mean (± data range) of two determinations.

observed 24 h after treatment (unpublished observations). The elevated or accelerated assembly of foci in EM9-X<sup>pmBRCT</sup> cells after EMS treatment is also consistent with an involvement of these structures in S-phase SSBR, since these cells are more dependent on the latter process. However, it is also possible that the mutations in XRCC1<sup>pmBRCT</sup> inhibit focus turnover.

The behavior of XRCC1 foci was reminiscent of Rad51 nuclear foci (14, 33). Rad51 is a homologue of the bacterial RecA recombination protein (1, 4, 22, 30). To examine whether the roles of XRCC1 and Rad51 after base damage might be related, we compared the subnuclear distribution of the two proteins in HeLa cells before and after exposure to EMS. XRCC1 and Rad51 partially colocalized both before and

8 h after EMS treatment (Fig. 5D). Two EMS-treated cells are shown (Fig. 5D, right panels), one of which lacks XRCC1 and Rad51 foci and one of which contains a cluster of both. While the level of colocalization depicted in Fig. 5 is representative, the total number and position of nuclear foci varied. These data raise the possibility that XRCC1 and Rad51 fulfill related roles after DNA base damage.

## DISCUSSION

**XRCC1-dependent SSBR in G<sub>1</sub>.** We report here that mammalian SSBR in G<sub>1</sub> phase of the cell cycle is abolished by mutation of the XRCC1 BRCT II. The mutant XRCC1 protein employed in this study combines two mutations, W611D and the double mutation V584D-I585D, but similar results were observed with XRCC1 harboring either of these mutations alone (unpublished observations). It seems unlikely that these mutations exert a dominant-negative effect on XRCC1 activities located outside of the BRCT II domain because a number of such activities (e.g., binding to DNA polymerase β) are normal in XRCC1<sup>pmBRCT</sup> (unpublished observations) and because the mutant protein is still able to mediate SSBR in S phase. Rather, these data suggest that the BRCT II domain is required for SSBR in G<sub>1</sub>, following DNA base damage. The V584D-I585D double mutation prevents measurable interaction of XRCC1 with DNA ligase III in vitro (34). Since the XRCC1-DNA ligase III interaction maintains normal cellular levels of DNA ligase III (6, 7), reduced levels of which result in decreased SSBR during base excision repair in vitro (11), it seems likely that at least one role for BRCT II is to maintain normal levels of the DNA ligase. However, it is unlikely that this is the only role for this domain because increasing the level of DNA ligase III in EM9-X<sup>pmBRCT</sup> cells with proteasome inhibitors did not increase SSBR. It is possible that XRCC1 also chaperones DNA ligase III to sites of base damage and/or modifies its activity, or that the BRCT II domain fulfills a role that is independent of the DNA ligase III interaction.

Interaction with DNA ligase III may be evolutionarily a relatively recent addition to XRCC1 function, since a homologue of XRCC1 identified in fruit flies lacks the BRCT II

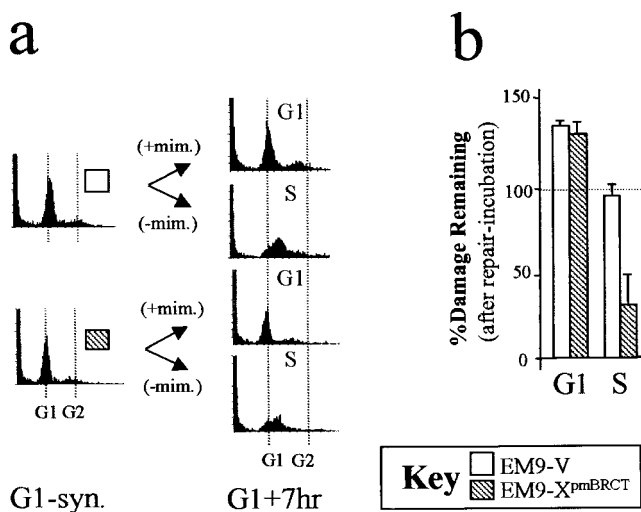


FIG. 4. S-phase SSBR can remove breaks induced in G<sub>1</sub>. (Left and middle panels) Flow cytometry profiles of G<sub>1</sub>-synchronized cells immediately after treatment with EMS (G<sub>1</sub>-syn.) or after 7 h in drug-free medium (G<sub>1</sub>+7hr) in the presence (+mim.) or absence (–mim.) of mimosine to maintain G<sub>1</sub> status or allow entry into S phase, respectively. (Right panel) DNA strand breakage (percent) remaining after 7 h in drug-free medium in the presence (G<sub>1</sub>) or absence (S) of mimosine.

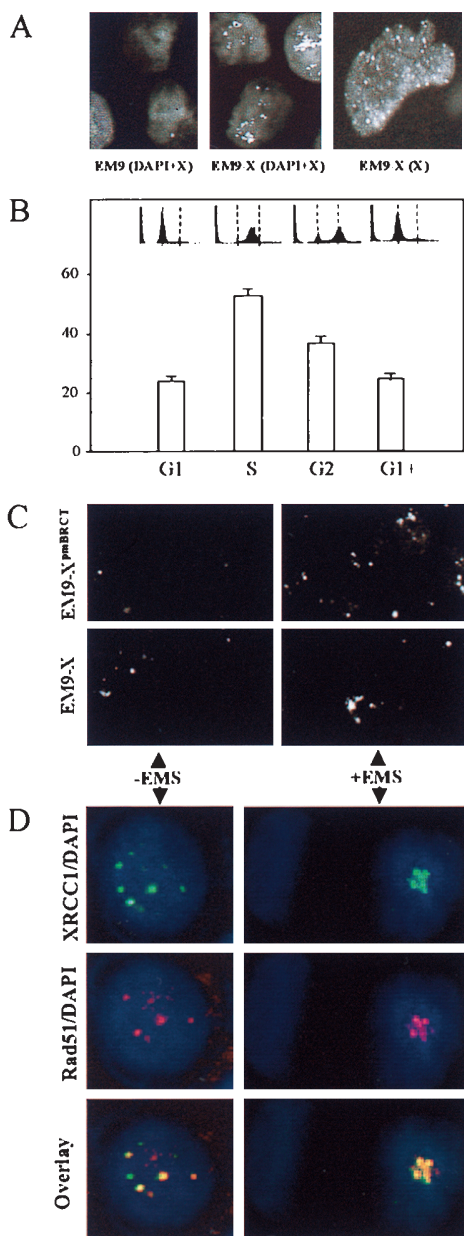


FIG. 5. XRCC1 foci. (A) EM9 (left panel) and EM9-X (middle panel) cells immunostained with anti-XRCC1 monoclonal antibody and DAPI. White spots are XRCC1 foci, and grey background is DAPI-stained nuclear DNA. The right panel shows EM9-X cells immunostained with anti-XRCC1 monoclonal antibody. White spots are foci, and grey background is diffuse or speckled staining. (B) Frequency of XRCC1 focus-positive EM9-X cells following synchronization in G<sub>1</sub>, during transit through S phase or G<sub>2</sub>, or after maintenance in G<sub>1</sub> throughout the experiment (G<sub>1</sub>+). The flow cytometry profiles are shown with dotted lines indicating G<sub>1</sub> and G<sub>2</sub>. (C) Asynchronous EM9-X or EM9-X<sup>pmBRCT</sup> cells immunostained for XRCC1 (white dots) before or 8 h after EMS treatment (2 mg/ml, 1 h). Each panel depicts approximately six cells. (D) Asynchronous HeLa cells were harvested and fixed before (-EMS) or 8 h after (+EMS) EMS treatment (2 mg/ml, 1 h) and stained with DAPI plus anti-XRCC1 monoclonal antibody (green; top panels), DAPI plus anti-Rad51 polyclonal antibody (red; middle panels), or DAPI plus anti-XRCC1 and anti-Rad51 (bottom panels, overlapping antibody signals in yellow).

domain that in human XRCC1 binds the DNA ligase. It is possible that XRCC1-DNA ligase III complex has arisen in higher eukaryotes to supplement SSBR because the larger genomes of these organisms acquire more spontaneous base

damage. Another DNA ligase apparently involved in SSBR following base damage is DNA ligase I (26, 27). DNA ligase I activity was not detected in this study, however, as indicated by the apparent absence of residual SSBR in EM9-V cells (see Fig. 1 to 3). This may indicate that the half-lives of strand breaks generated and repaired by the DNA ligase I base excision repair complex (26) are too short to be detected.

**XRCC1-dependent SSBR in S phase.** Based on the extensive conservation between human and fruit fly XRCC1 outside of the BRCT II domain (Fig. 1a), it seems likely that it is S-phase SSBR that is the evolutionarily conserved role of this protein. Although the BRCT II domain is largely dispensable for mammalian SSBR during S phase, this structure may contribute to the process, since the efficiency of repair in EM9-X<sup>pmBRCT</sup> cells was slightly less than in EM9-X cells (Fig. 2b, right panel). Nevertheless, SSBR mediated by XRCC1 in S phase was sufficient to maintain cellular resistance to an alkylating agent (0 to 1 mg of EMS per ml) in the absence of repair in G<sub>1</sub>. It is likely that the ability of S-phase SSBR to remove strand breaks that persist from G<sub>1</sub> is important in this respect. It is noteworthy that the substrate specificity of SSBR mediated by XRCC1 during S phase may not be restricted to breaks arising from base damage. This is suggested by the observation that XRCC1 mutant cells are hypersensitive to the antitumor agent camptothecin, which breaks DNA in S phase independently of base damage (3, 9, 15, 16, 28).

The conclusion that S-phase SSBR does not require XRCC1-DNA ligase III complex is based on three observations. First, the mutations in XRCC1<sup>pmBRCT</sup> prevent measurable interaction with DNA ligase III in vitro (34) and copurification of the two proteins from cell extract (Fig. 1b, bottom panel). Second, XRCC1<sup>pmBRCT</sup> failed to increase levels of DNA ligase III above those present in EM9 cells transfected with empty vector (Fig. 1b, top panel, compare lanes 1 and 2). This observation is particularly significant because it reflects the amount of complex present in whole cells before lysis. Finally, an XRCC1 deletion mutant lacking the entire DNA ligase III-binding BRCT II domain also confers resistance to EMS (unpublished observations). This observation renders unlikely the caveat that S-phase SSBR mediated by XRCC1<sup>pmBRCT</sup> results from leaky point mutations that allow assembly of a small but undetectable amount of complex. Since S-phase SSBR does not appear to require XRCC1-DNA ligase III complex, what role does XRCC1 play in this process? It is possible that XRCC1 is required during S phase for its interaction with DNA polymerase  $\beta$  (8, 18) or poly(ADP-ribose) polymerase (8, 21), perhaps to chaperone these proteins to sites of damage or to modify their activity.

**XRCC1 nuclear foci.** Immunostaining with anti-XRCC1 monoclonal antibody revealed a speckled distribution of XRCC1 in CHO cell nuclei and also the presence of less frequent, but more intensely staining, foci. Several observations are consistent with the foci being associated with S-phase SSBR. First, the frequency of focus-positive EM9-X cells appeared to increase two- to threefold during progression through S phase. Second, EM9-X<sup>pmBRCT</sup> cells that are more reliant on S-phase SSBR exhibited elevated and accelerated assembly of XRCC1 foci relative to EM9-X cells. Third, the XRCC1 foci partially colocalized with Rad51 protein, which in somatic cells appears to mediate strand break repair specifically in S/G<sub>2</sub> (32). The colocalization of XRCC1 with Rad51 raises the possibility that XRCC1-dependent S-phase SSBR is coordinated or involved with recombination events initiated by strand breaks during replication. Given the frequency at which base damage arises spontaneously (20), XRCC1 and Rad51

might be required routinely during S phase in this scenario, perhaps explaining the similar embryonic-lethal phenotype of *XRCC1*<sup>-/-</sup> and *Rad51*<sup>-/-</sup> nullizygous mice (19, 35, 38). Irrespective of the relationship between XRCC1 and Rad51, it is tempting to speculate that XRCC1 foci reflect the sequestration of this protein into base excision repair and/or strand break repair "factories" through which replicating DNA can translocate.

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