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Deficient Nonhomologous End-Joining Activity in Cell-free Extracts from Brca1-null Fibroblasts[1]

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ABSTRACT

BRCA1 ensures genomic stability, at least in part, through a functional role in DNA damage repair. BRCA1 interacts with the Rad50/Mre11/Nbs1 complex that occupies a central role in DNA double-strand break repair mediated by homologous recombination and nonhomologous end joining (NHEJ). NHEJ can be catalyzed by mammalian whole cell extract in a reaction dependent upon DNA ligase IV, Xrcc4, Ku70, Ku80, and DNA-PKcs. Here, we show that under identical cell-free reaction conditions, the addition of antibodies specific for BRCA1 and Rad 50 but not Rad51, inhibits end-joining activity. Cell extracts derived from Brca1-deficient mouse embryonic fibroblasts exhibit reduced end-joining activity independent of the endogenous protein amounts of DNA ligase IV, Ku80, and Ku70. The Brca1-dependent NHEJ activity predominates at the lower concentrations of Mg2+ (0.5 mM); elevated Mg2+ or Mn2+ concentrations (10 mM) dramatically increase overall end-joining activity and abrogates the requirement for Brca1, Xrcc4, and Ku70. The addition of partially purified BRCA1, in association with Rad50/Mre11/Nbs1 complex, complements the NHEJ deficiency of Brca1-null fibroblast extracts. These results suggest a role for Brca1 in NHEJ and in the maintenance of genome integrity.

INTRODUCTION

Inactivation of the hereditary breast cancer susceptibility gene, *BRCA1*,[3] leads to genomic instability (1–3). Extensive chromosomal abnormalities have been observed in Brca1-deficient murine fibroblasts (4), as well as the BRCA1-mutant human breast cancer cell line HCC1937 (5). The function of BRCA1 in genome stability is attributable to its central role in the cellular response to DNA damage response, and emerging evidence supports a role for BRCA1 in DNA damage repair. For example, Brca1-deficient murine and human cells are sensitive to DNA-damaging agents, including IR (6–8). Furthermore, HCC1937 cells expressing mutant BRCA1 protein exhibit a reduction in both the rate and extent of DSB repair after IR when compared with cells expressing wild-type BRCA1 protein (9). Finally, BRCA1 physically interacts with the Rad50/Mre11/Nbs1 DSB repair complex and colocalizes to nuclear foci along with this complex after treatment of cells with IR (8).

In eukaryotic cells, DSBs are repaired through two distinct pathways: homologous recombination and NHEJ. BRCA1 has been implicated in homology-based repair because cells expressing a Brca1 exon-11 deletion mutant exhibit defects in gene targeting, single-strand annealing, and gene conversion (10). BRCA1 may also influence NHEJ by virtue of its interaction with the Rad50/Mre11/Nbs1 complex. The orthologous complex in *Saccharomyces cerevisiae* Rad50/Mre11/Xrs2 is critical for NHEJ, sister chromatid recombination, and telomere maintenance. Yeast strains deficient in any of the components of the Rad50/Mre11/Xrs2 complex are 10–100-fold less efficient in nonhomologous joining of DNA ends (11, 12). The Rad50/Mre11/Nbs1 complex is characterized by 3′ to 5′ exonucleolytic activity on double-stranded DNA and endonucleolytic activity on single-stranded DNA and hairpin structures. Furthermore, in the presence of a DNA ligase, Mre11 can facilitate DNA end joining using microhomologies at or near DNA termini (13, 14). Recently, the yeast Rad50/Mre11/Xrs2 complex was found to exhibit DNA end-binding activity and end-bridging activity (15). Thus, the Rad50/Mre11/Nbs1 complex may fulfill a functionally conserved role in NHEJ.

In mammalian cells, a NHEJ pathway has been identified that comprises the heterodimeric DNA end-binding activity Ku70/Ku80 and the DNA-PKcs (reviewed in Ref. 12). Recently, Baumann *et al.* (16) developed a cell-free system that faithfully reflects the genetic requirements for this NHEJ pathway. In this system, accurate intermolecular ligation of DNA ends was found to be dependent on DNA ligase IV/Xrcc4 and requires Ku70, Ku80, and DNA-PKcs. However, the role of Rad50/Mre11/Nbs1 in this NHEJ assay has not been addressed.

We report here the use of this cell-free assay to investigate the role of BRCA1 in DNA end joining. We observed that antibodies specific for BRCA1, Rad50, and Ku70, but not Rad51, inhibit the end-joining activity present in extracts prepared from a human lymphoblastoid cell line. Comparison of extracts derived from Brca1-null MEFs with that from isogenic Brca1-proficient MEFs for their respective abilities to catalyze end joining *in vitro* revealed that Brca1-deficient MEF extracts exhibit a significantly reduced end-joining activity. This deficiency can be complemented by partially purified BRCA1 in association with the Rad50/Mre11/Nbs1 complex. Finally, we found the BRCA1-dependent NHEJ activity in mammalian WCE to be sensitive to the reaction concentration of divalent cations. Elevated concentrations of Mg2+ or Mn2+ (to 10mM) stimulated the overall level of DNA end-joining activity and masked a BRCA1, Ku, and Xrcc4 requirement. These results provide evidence that BRCA1 promote NHEJ in a Mg2+ concentration-dependent manner.

MATERIALS AND METHODS

MEFs and Lymphoblastoid Cell Line. The *Brca1*$^{-/-}$:*p53*$^{-/-}$ and p53$^{-/-}$ MEFs were derived from 9.5-day old embryos of a cross between *Brca1*$^{+/-}$ and *p53*$^{+/-}$ mice as described (17) and cultured in DMEM plus 10% FCS. Human lymphoblastoid cell line, LEM, was immortalized by Epstein-Barr virus and cultured in DMEM plus 10% FCS.

Cell-free NHEJ Assay. Cell extracts were prepared and *in vitro* reactions were performed according to previously described procedure (16). WCEs were normalized for their respective total protein levels using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Reactions (16 μl) were carried out in 50 mM triethanolamine-HCl (pH 7.5), 0.5 mM $Mg(OAc)_2$, 80 mM potassium acetate, 2 mM ATP, 1 mM DTT, and 100 μg/ml BSA. Cell-free extracts were incubated for 5 min at 37°C before the addition of 5 fmol ^{32}P-labeled DNA. pBSK(+) duplex plasmid DNA (2.96 kb; Stratagene, La Jolla, CA) was linearized with *Eco*RI, dephosphorylated using calf intestinal phosphatase, and was 5′ ^{32}P-end-labeled using polynucleotide kinase. In each reaction, 5 fmol of labeled DNA was used. After incubation at 37°C for 1 h, ^{32}P-labeled DNA products were deproteinized by proteinase K (500 μg/ml) and 1% SDS at 37°C for 20 min and analyzed by electrophoresis through 0.7% agarose gels, followed by autoradiography. Quantitation of DNA end-joining efficiency was carried out

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[3] The abbreviations used are: *BRCA1*, human breast cancer 1 gene; BRCA1, protein product of *BRCA1;* *Brca1*, mouse breast cancer 1 gene; Brca1, protein product of *Brca1*; IR, ionizing radiation; NHEJ, nonhomologous end joining; DSB, double-strand break; MEF, mouse embryonic fibroblast; WCE, whole cell extract; mAb, monoclonal antibody; RPB1, RNA polymerase II.

by densitometry. For antibody inhibition experiments, cell extracts were preincubated with specific antibodies on ice for 30 min before shifting to 37°C for 5 min, followed by the addition of ^{32}P-labeled DNA.

Antibodies and Antisera. A recombinant protein containing glutathione *S*-transferase fused with mouse Brca1 of amino acids 788-1135 in frame was used as an antigen for the production of antimurine Brca1 mouse polyclonal antisera. Purified goat IgG specific for XRCC4, Ku70, and Ku80 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified mouse monoclonal antibody specific for BRCA1 (Ab-1) was purchased from Oncogene Research Products (San Diego, CA). Other antibodies specifically against BRCA1, Rad50, and Rad51 have been described (8).

The amounts of the following antibodies were used in NHEJ inhibition experiments: 10 μg of antimouse IgG; 1 μg of purified goat anti-Ku70 IgG; 1 μg of anti-BRCA1 mAb Ab-1; 2 μg of anti-BRCA1 mAb 17F8; 5 μg of anti-Rad50 mAb 13B3; 1 μg of purified rabbit anti-Rad51 IgG; and 1 μg of purified rabbit anti-Xrcc4. Only mAbs and commercial available purified antisera were used in antibody inhibition assays. Most of the polyclonal antisera contain high levels of nuclease activity and cannot be used in the cell-free end-joining assay.

Protein Purification. HeLa cell nuclear extract was subjected to successive phosphocellulose P-11, DEAE-Sepharose, and Superose 6 gel filtration chromatography as described previously (18). Individual fractions derived from Superose 6 chromatography corresponding to the peaks of the Rad50, Mre11, and NBS1 proteins were pooled and designated as fraction C1. The fraction C1 was concentrated on phosphocellulose P-11 by elution with 0.5 M KCl D buffer [20 mM HEPES (pH 7.9), 0.2 mM EDTA, and 20% glycerol], followed by dialysis with 0.1 M KCl in D buffer.

RESULTS

To explore the function of BRCA1 in DNA repair, we used an *in vitro* DNA end-joining assay that has been described previously (16). In this system, NHEJ catalyzed by human WCE was observed by rejoining ^{32}P-labeled linear duplex DNA in a reaction that is dependent upon all of the mammalian factors thus far genetically implicated in NHEJ, including DNA ligase IV, Xrcc4, Ku70, Ku80, and DNA-PKcs. We initially tested WCE from a human lymphoblastoid cell line, LEM, for its ability to catalyze NHEJ and observed that 25–35% of the input DNA molecules were rejoined during a 1-h incubation with 20 μg of this cell extract. Under identical reaction conditions, BRCA1-specific antibodies Ab-1 and 17F8, preincubated with LEM WCE before the addition of ^{32}P-end-labeled linear DNA into the reaction, dramatically inhibited end joining (Fig. 1). Antibodies specific for Rad50 also inhibited the end-joining activity present in WCE. Consistent with previous observations, antibodies specific for Ku70 inhibited end-joining activity *in vitro* (16). However, antibodies specific for Rad51 or normal murine IgG did not inhibit end-joining activity. These results indicate that BRCA1 and Rad50, but not Rad51, may be involved in NHEJ in this cell-free system.

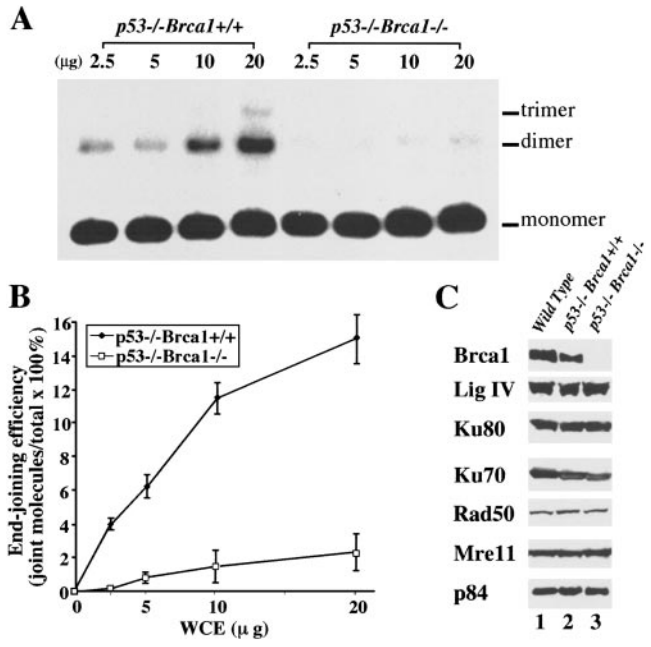

Fig. 2. Deficient end-joining activity in Brca1 mutant MEFs. *A*, end-joining activity of MEF extracts. Increasing amounts of WCE from each of the indicated MEF cell lines were incubated with ^{32}P-end-labeled linear DNA and assayed for end-joining activity. *B*, quantitative analysis by phosphorimaging. End-joining efficiency was calculated as: intensity of end-joining products/total substrate × 100%; *error bars* indicate the experimental deviation. *C*, immunoblot analysis demonstrating equivalent levels of representative NHEJ proteins and an unrelated nuclear matrix protein, p84, in the MEF WCEs assayed for end-joining activity.

To further substantiate the requirement for BRCA1 in NHEJ, we directly compared WCEs derived from *Brca1*$^{-/-}$*p53*$^{-/-}$ MEFs with extracts from both *p53*$^{-/-}$ and wild-type MEFs for their respective NHEJ activities *in vitro*. WCEs from wild-type or *p53*$^{-/-}$ MEFs could rejoin 35–50% of input DNA. Significantly, WCE from *Brca1*$^{-/-}$*p53*$^{-/-}$ MEFs was reduced 3–10-fold consistently, relative to WCE from either *p53*$^{-/-}$ or wild-type MEFs, for end-joining activity (Fig. 2, *A* and *B*). For quantitative standardization, WCEs

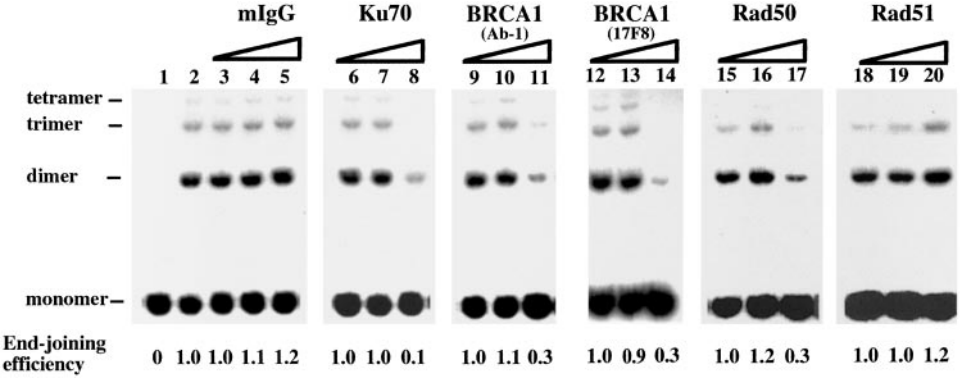

Fig. 1. Antibodies specific for BRCA1, Rad50, and Ku70 inhibit the end-joining activity present in human cell extract. Human lymphoblastoid LEM WCE was preincubated with BRCA1-specific antibodies Ab-1 (*Lanes 9–11*) and 17F8 (*Lanes 12–14*), Ku70-specific antibody (*Lanes 6–8*), Rad50-specific antibody (*Lanes 15–17*), Rad51-specific antibody (*Lanes 18–20*), or control antibody murine IgG (*Lanes 3–5*) before the addition of ^{32}P-end-labeled linear DNA (5 fmol/reaction) and subsequent incubation. Antibodies or antisera were added at 1:6 serial dilutions as follows (the actual amounts were described in the "Materials and Methods" section): straight, *Lanes 5, 8, 11, 14, 17*, and *20*; diluted 1/6, *Lanes 4, 7, 10, 13, 16*, and *19*; diluted 1/36, *Lanes 3, 6, 9, 12, 15*, and *18*. *Lane 1*: no extract. *Lane 2*: LEM extract alone. Note that BRCA1-, Rad50-, and Ku-70-specific, but not control, antibodies inhibited end joining. The end-joining efficiency was calculated as the end-joining activity [intensity of multimers/(multimers + monomer)] in each reaction expressed relative to the end-joining activity in WCEs without antibodies as determined by densitometric analysis.

were first normalized for total protein levels and subsequently analyzed by immunoblot analyses for their respective expression levels of Ku70, Ku80, DNA ligase IV, Rad50, Mre11, or the nuclear matrix protein p84 (19). No significant difference in protein levels could be observed among these WCEs (Fig. 2*C*), excluding the possibility that the reduced end-joining activity in Brca1-null cell extract is because of variations in the expression levels of these NHEJ proteins.

It is known that divalent cations such as Mg2+ and Mn2+ affect NHEJ catalyzed by mammalian WCE *in vitro* (20). Our NHEJ reactions included 0.5 mM Mg2+ and were performed identically to those described initially by Baumann *et al.* (16). To test whether the Brca1-dependent NHEJ activity present in WCEs of MEFs is affected by the concentration of divalent ions, we compared extracts derived from Brca1-deficient and wild-type MEFs for their respective NHEJ activities in the presence of increasing concentrations of Mg2+ or Mn2+. As shown in Fig. 3, augmentation of the Mg2+ or Mn2+ concentration dramatically increased the level of DNA end joining catalyzed by both Brca1-null and wild-type cell extracts and concomitantly abrogated the requirement for Brca1 regardless the amount of WCE used in the reactions. Thus, at reaction concentrations of divalent ions exceeding 1.5 mM Mg2+ or 0.5 mM Mn2+ plus 0.5 mM Mg2+, the difference in NHEJ catalyzed by Brca1-null and wild-type MEF extracts was indistinguishable, possibly indicating the involvement of a Brca1-independent pathway for NHEJ under these conditions.

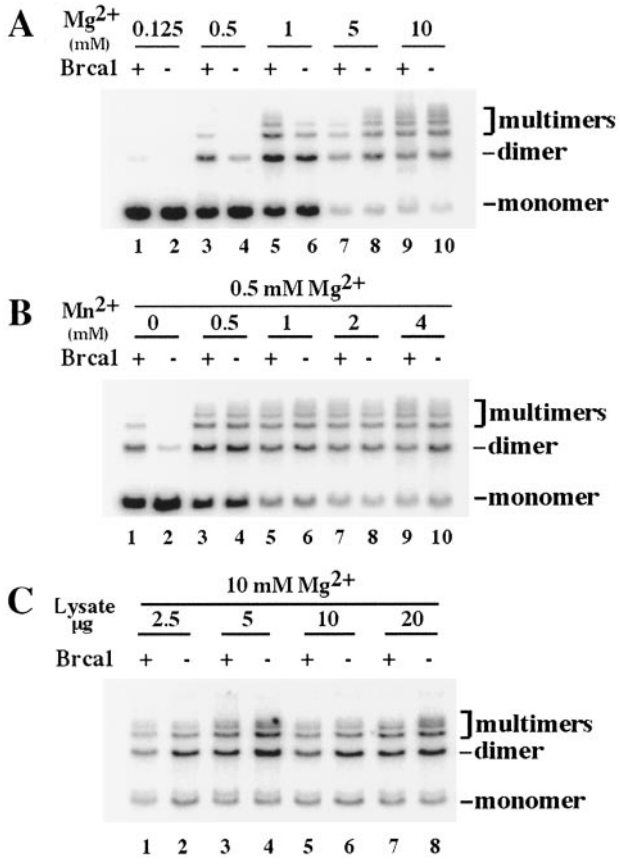

Fig. 3. DNA end-joining activity in cell extracts is sensitive to Mg2+ and Mn2+. *A*, determination of optimal concentration of Mg2+. Cell extracts (10 μg) derived from Brca1-proficient (*odd-numbered lanes*) or Brca1-deficient (*even-numbered lanes*) MEFs were incubated with different concentration of Mg2+ as follows: *Lanes 1–2*, 0.125 mM; *Lanes 3–4*, 0.5 mM; *Lanes 5–6*, 1 mM; *Lanes 7–8*, 5 mM; and *Lanes 9–10*, 10 mM. *B*, determination of the effect of Mn2+. Extract (10 μg) derived from Brca1-proficient (*odd-numbered lanes*) or Brca1-deficient (*even-numbered lanes*) MEFs were incubated at 0.5 mM Mg2+ with different concentration of Mn2+ as follows: *Lanes 1–2*, 0 mM; *Lanes 3–4*, 0.5 mM; *Lanes 5–6*, 1 mM; *Lanes 7–8*, 2 mM; and *Lanes 9–10*, 4 mM. *C*, titration of the amount of cell extracts. In the presence of 10 mM Mg2+, cell extracts (2.5, 5, 10, and 20 μg) derived from Brca1-proficient (*odd-numbered lanes*) or Brca1-deficient (*even-numbered lanes*) MEFs were assayed for DNA end-joining activity.

To explore the relationship between the Brca1-dependent NHEJ activity and known components of the NHEJ pathway, including Xrcc4 and Ku70, at different divalent ion concentrations, we performed reactions with either 0.5 mM or 10 mM Mg2+ (Fig. 4). Addition of Brca1-specific antibody reduced end-joining activity in Brca1 proficient MEFs to the level of Brca1 mutant cells at 0.5 mM Mg2+. Similarly, the addition of antibodies against Ku70 and Xrcc4 completely eliminated end-joining activity of Brca1 deficient or proficient cells. These observations suggest that BRCA1 may function along with Xrcc4 and Ku70 in NHEJ. However, at 10 mM Mg2+, the additions of antibodies against Ku70, Xrcc4, and BRCA1 have no apparent inhibitory function against the robust end-joining activities in both Brca1-proficient and -deficient MEFs (Fig. 4), indicating an existence of an alternative pathway.

To determine whether a cellular fraction containing BRCA1 can complement the diminished NHEJ activity in Brca1-deficient cells, we fractionated human HeLa cell nuclear extract according to the scheme outlined in Fig. 5*A*. The bulk of BRCA1 protein present in a soluble HeLa nuclear extract bound to phosphocellulose PC-11 and eluted predominantly and approximately equally between 0.1–0.3 and 0.3–0.5 M KCl step fractions (fractions B and C, respectively, Fig. 5*A*). The bulk of cellular Rad50 protein was also recovered in the PC-11 B and C fractions, although more eluted in the B fraction than in the C fraction (Fig. 5*A*). Most of the BRCA1 protein present in the PC-11 C fraction bound to, and eluted from, a DEAE-Sepharose anion exchange resin in a 0.1–0.25 M KCl step fraction (fraction CB). After Superose 6 gel filtration chromatography of the CB fraction, BRCA1 was eluted in fractions corresponding to peaks of the Rad50, Mre11, and Nbs1 proteins, indicating cofractionation of BRCA1 with the Rad50/Mre11/Nbs1 protein complex (Fig. 5*A*). Western blot analysis of individual Superose 6 column fractions with antibodies specific for BRCA1 and the large subunit of RPB1 also revealed that the bulk of BRCA1 eluted ahead of RPB1 in a number of high molecular weight fractions (Fig. 5*A*). Reciprocal coimmunoprecipitation of BRCA1 and Rad50 from peak Superose 6 column fractions demonstrated that BRCA1, Rad50, and Nbs1 all reside in a single high molecular weight complex of M_r ~1,000,000 (Fig. 5*B*).

Individual Superose 6 column fractions corresponding to the peak of the BRCA1/Rad50/Mre11/Nbs1 complex were pooled and concentrated on phosphocellulose PC-11 to yield a partially purified protein fraction termed C1. Fraction C1 was tested for its ability to complement WCE of Brca1-deficient MEF for end-joining activity *in vitro*. Although it catalyzed no end-joining activity on its own, the addition of fraction C1 to WCE of Brca1-deficient MEFs increased its end-joining activity about 2.5-fold (Fig. 5, *C* and *D*) to 60% of the end-joining activity catalyzed by WCE derived from Brca1-proficient MEFs. The addition of fraction C1 to WCE of Brca1-proficient MEFs has no effect on end-joining activity (data not shown). These results suggest that the partially purified BRCA1 complex facilitated the BRCA1-dependent NHEJ process.

DISCUSSION

BRCA1 plays an important role in maintaining genomic stability through its participation in DNA repair and cell cycle checkpoint control. For DNA DSB repair, BRCA1 has been shown to be critical for homologous recombination (10). However, it is not known whether BRCA1 has a role in NHEJ. In this study, we showed that under identical cell-free reaction conditions described by Baumann *et al.* (16), the addition of antibodies specific for BRCA1 and Rad50, but not Rad51, inhibits end-joining activity. Cell extracts derived from Brca1-deficient MEFs exhibit reduced end-joining activity independent of the endogenous protein amounts of DNA ligase IV, Ku80, and Ku70. The BRCA1-dependent NHEJ activity predominates at the lower concentra-

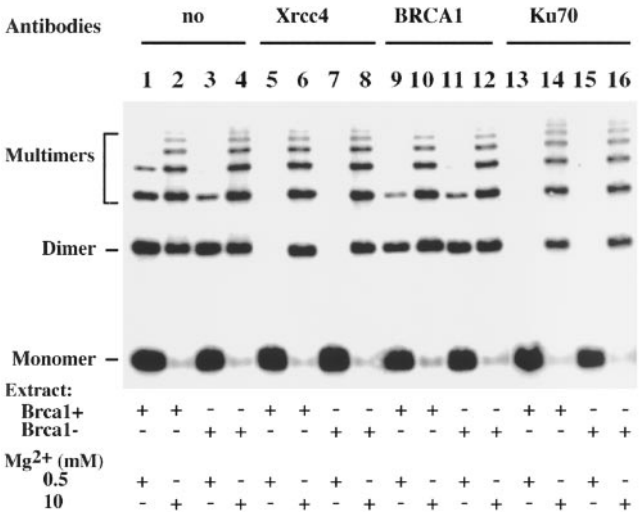

Fig. 4. Antibodies specific against BRCA1, Ku, and Xrcc4 inhibit NHEJ at 0.5 mM, but not at 10 mM, Mg2+. At 0.5 mM Mg2+ (*odd lanes*), antibodies against BRCA1 (*Lanes 9–12*) reduced the end-joining activity in Brca1-proficient MEF extracts (*Lanes 1* and *9*) to the level of Brca1-deficient cells (*Lanes 3* and *11*). Antibodies against Xrcc4 (*Lane 5–8*) or Ku70 (*Lanes 13–16*) completely inhibited the end-joining activity in Brca1-proficient cells (*Lanes 5* and *13*) and the residual activity in Brca1-deficient cells (*lanes 7* and 15). However, at 10 mM Mg2+ (*even lanes*), robust end-joining activity in both Brca1-proficient and Brca1-deficient MEFs is resistant to antibodies against Ku70 (*Lanes 6* and *8*), Xrcc4 (*Lanes 14* and *16*), and BRCA1 (*Lanes 10* and *12*), indicating an existence of an alternative pathway.

tions of 0.5 mM Mg2+; elevated reaction concentrations of Mg2+ or Mn2+ to 10 mM dramatically increases overall end-joining activity and abrogates the requirement for Brca1, Xrcc4, and Ku70. The addition of partially purified BRCA1 in association with Rad50/Mre11/Nbs1 complements the NHEJ deficiency of Brca1-deficient MEF extracts. These results support a role for BRCA1 in NHEJ.

BRCA1 is likely to participate in NHEJ by virtue of its interaction with the Rad50/Mre11/Nbs1 complex. Previous work has demonstrated that BRCA1 interacts physically with the Rad50/Mre11/Nbs1 complex *in vivo* and *in vitro* (8). Furthermore, BRCA1 can be isolated from cells in a high molecular complex with Rad50/Mre11/Nbs1. These observations suggest that the function of BRCA1 in DSB repair may be mediated, at least in part, through its association with the Rad50/Mre11/Nbs1 complex. It is recently shown that the yeast counterpart Rad50/Mre11/Xrs2 has DNA end-binding and bridging activities. Addition of the Ku homologous protein HdfA and B enhances the DNA end-bridging activity of the Rad50/Mre11/Xrs2 complex. The Rad50/Mre11/Xrs2 complex then directly recruits Dnl4/Lif1 (equivalent to mammalian DNA ligase IV and Xrcc4) to complete DNA end ligation (15). Similarly, mammalian Rad50/Mre11NBS1 has also been shown to exhibit DNA end-tethering activity (21, 22). Therefore, NHEJ *in vitro* can be carried out by components, including Rad50/Mre11/Nbs1, Ku homologues, and DNA ligase IV/Xrcc4. Although the precise role of BRCA1 in NHEJ remains unclear, the ability of BRCA1 to form a tight complex with Rad50/Mre11/Nbs1 suggests that BRCA1 may facilitate the NHEJ function of this complex. Recently, a purified recombinant BRCA1 was shown to have a direct DNA-binding activity (23). Whether this DNA-binding activity has a direct contribution to the NHEJ warrants additional investigation.

The cell-free system for NHEJ that we used in this study has been reported to be dependent on a DNA-PK-mediated pathway (16). Nonetheless, substantial evidence accumulated from *in vitro* studies indicates that eukaryotic cells rely on more than one DNA end-joining pathway (24–27). For example, extracts derived from the DNA-PK mutant cell line MO59J have been reported to exhibit wild-type end-joining activity, suggesting the involvement of a DNA-PK-independent end-joining pathway in these cells (27). The relative contribution of a particular pathway to the overall end-joining activity observed in mammalian WCEs likely

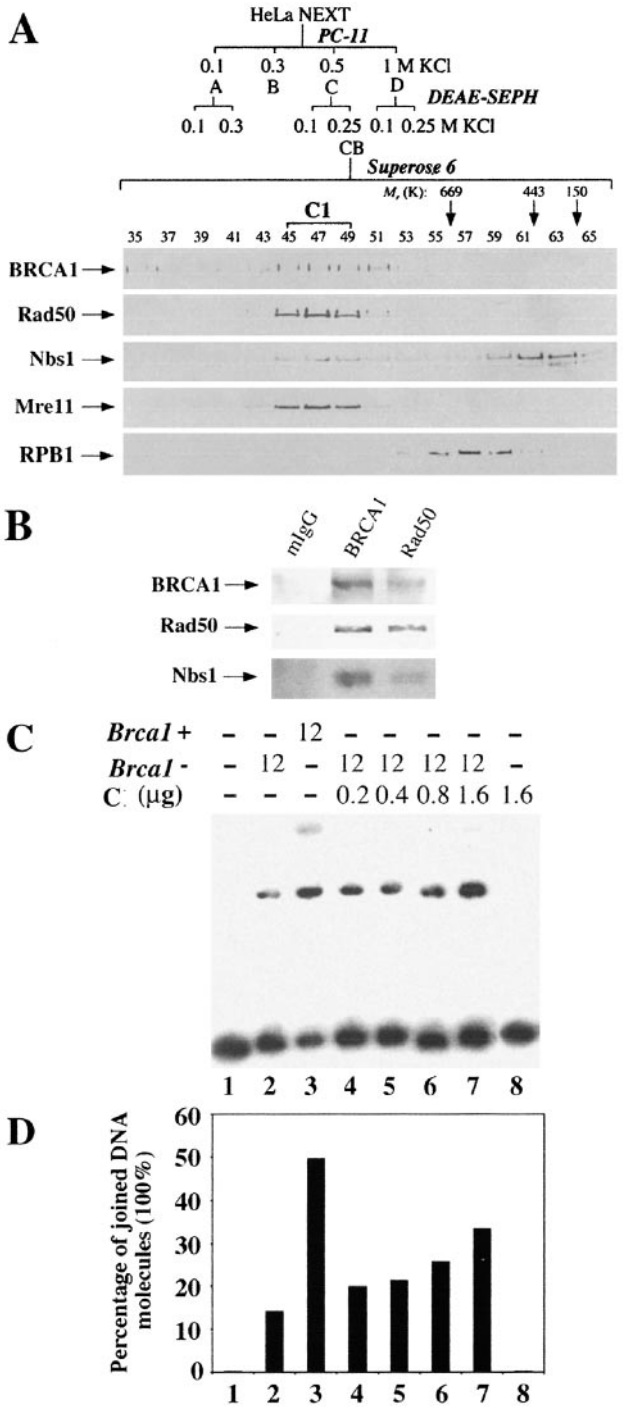

Fig. 5. Complementation of NHEJ activity in BRCA1-deficient cell extracts by exogenous BRCA1. *A*, schematic diagram of purification of a BRCA1/Rad50/Mre11/NBS1-containing complex. HeLa cell nuclear extract (NEXT) was subjected to successive phosphocellulose PC-11, DEAE-Sepharose, and Superose 6 gel filtration chromatography. CB protein fraction (4 mg/ml) was subjected to Superose 6 gel filtration analysis. Individual fractions were resolved by 10% SDS-PAGE and subjected to immunoblot analysis using antibodies specific for the proteins indicated to the *left* of the blot (RPB1: large subunit of RNA polymerase II). *Vertical arrows* above the immunoblot panels indicate marker protein peaks. *B*, coimmunoprecipitation of BRCA1, Rad50, and NBS1 from peak Superose 6 column fractions. Column fractions 45–49 (fraction C1) from the experiment in *A* were pooled, and a portion subjected to immunoprecipitation with the antibodies indicated above the immunoblot (mIgG = murine IgG). Immunoprecipitates were resolved by 10% SDS-PAGE and subjected to immunoblot analysis with antibodies specific for the proteins indicated to the left of the blot. *C*, complementation of Brca1-deficient MEF extracts for end-joining activity by a BRCA1/Rad50/Mre11/Nbs1 complex. Fraction C1 was added as indicated to Brca1-deficient WCE and incubated on ice for 30 min before assay of end-joining activity. Fraction C1 alone (*Lane 8*) does not catalyze end-joining activity. *D*, end-joining activity in *C* was measured as [intensity of multimers/(multimers + monomer) × 100%] by phosphoimage analysis and converted to percentage of joined DNA molecules.

reflects the *in vitro* reaction conditions used. One factor that could alter the relative influence of a particular end-joining pathway in the reaction is the concentration of divalent cations, particularly Mg2+ and Mn2+. In this regard, we observed that the BRCA1-dependent NHEJ activity present in mammalian WCE is sensitive to the reaction concentration of Mg2+ and Mn2+. Elevated concentrations of these divalent cations stimulates overall end-joining activity and masks the requirement for BRCA1, suggesting the involvement of a BRCA1-independent pathway to achieve end joining. A similar phenomenon has recently been reported for DNA ligase IV using ligase IV mutant 180BR cell (20). Elevated reaction concentrations of Mg2+ (10 mM) stimulated DNA end joining through an apparent DNA ligase IV-independent pathway, whereas reduced concentrations of Mg2+ (0.5 mM) revealed a DNA ligase IV dependency for low levels of end-joining activity. Interestingly, we observed that under similar conditions, antibodies specific for Ku70, Xrcc4, and BRCA1 efficiently suppressed DNA end-joining activity at reduced concentrations of Mg2+ (Fig. 4). These observations raise the possibility that BRCA1 works along with Rad50, Ku, and Xrcc4 in NHEJ at a low concentration of Mg 2+. Interestingly, mammalian cell extracts deficient in Fanconi anemia proteins had a 3–9-fold reduction in DNA end-joining activity at high Mg2+ concentration (10 mM) in a pathway independent of DNA-PK/Ku (28). Therefore, it is very likely that multiple NHEJ pathways may exist in mammalian cells.

Previously, BRCA1 has been implicated in homology-based repairs of DNA breaks because cells expressing a Brca1 exon 11-deletion mutant exhibit defects in gene targeting, single-strand annealing, and gene conversion (10). Interestingly, no defects in plasmid integration and nonhomologous repair processes were observed in these Brca1 mutant cells. However, it is possible that the NHEJ activity observed in this previous study described by Moynahan *et al*. (10), reflects the contribution of a BRCA1-independent NHEJ pathway. Alternatively, genetic differences between independently derived Brca1 mutant cell lines may contribute to different conclusions regarding the importance of Brca1 in NHEJ. Our Brca1 mutant allele carries a reverse-oriented neomycin cassette inserted into the 5′ end of Brca1 exon 11, which will lead to premature termination of translation (29). No stable Brca1 protein derivative can be detected in our Brca1$^{-/-}$ MEFs. The embryonic stem (ES) cells previously characterized for defects in homologous and NHEJ repair (10), express a Brca1 exon 11 splice variant (30) and the homozygote embryos derived from these ES cells exhibit a less severe phenotype than our homozygotes (29, 30). Using our Brcal$^{-/-}$ MEFs, we have demonstrated a 50–100-fold reduced efficiency in microhomology-mediated end-joining activity of a defined chromosomal DNA DSB introduced by a rare-cutting endonuclease I-SceI (31). These results further support a role of BRCA1 in NHEJ.

The findings presented here suggest that BRCA1 functions in the repair of DSBs through a novel role in NHEJ, consistent with the recent report that a single mutated BRCA1 allele leads to impaired fidelity of DSB end joining (32). Inefficient or error-prone DNA repair resulting directly from inactivation of BRCA1 could lead to global genomic instability and a concomitant accrual of functionally inactivating mutations at genetic loci involved in breast tumorigenesis. Additional mechanistic studies concerning the role of BRCA1 in DNA damage response and repair should expedite the design and implementation of strategies to delay, and ultimately to prevent, breast cancer formation.

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