

Recombination Mediator and Rad51 Targeting Activities of a Human BRCA2 Polypeptide*

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BRCA2 likely exerts its tumor suppressor function by enhancing the efficiency of the homology-directed repair of injured chromosomes. To help define the DNA repair role of BRCA2, we expressed and purified a polypeptide, BRC3/4-DBD, that harbors its BRC3 and BRC4 repeats and DNA binding domain. BRC3/4-DBD interacted with hRad51 and bound DNA with a distinct preference for single-stranded (ss) DNA. Importantly we demonstrated by biochemical means and electron microscopy that BRC3/4-DBD nucleates hRad51 onto ssDNA and acts as a recombination mediator in enabling hRad51 to utilize replication protein A-coated ssDNA as recombination substrate. These functions of BRC3/4-DBD required both the BRC repeats and the BRCA2 DNA binding domain. The results thus clarify the role of BRCA2 in Rad51-dependent DNA recombination and repair, and the experimental strategies described herein should be valuable for systematically deciphering this BRCA2 function.

Homologous recombination (HR)³ helps eliminate DNA breaks, cross-links, and other deleterious lesions from chromosomes. A failure in HR leads to cancer formation (1, 2), which aptly underscores the importance of delineating the mechanism of the HR machinery. HR is best understood in the context of DNA double strand break repair wherein the break is processed nucleolytically to yield ssDNA, which serves as the nucleation site for the Rad51 recombinase. Polymerization of Rad51 onto the ssDNA results in a helical protein filament called the presynaptic filament. The presynaptic filament harbors a binding site for duplex DNA and provides the catalytic center for the search of homology in the duplex and the formation of a joint between the ssDNA and duplex substrates. Assembly of the presynaptic filament is slow, rendering it prone to interference by the single strand binding factor RPA. In addition, the timely delivery of Rad51 to the single-stranded recombination substrate is complicated by a high affinity of this recom-

binase for duplex DNA. In *Saccharomyces cerevisiae*, several HR factors, termed recombination mediators, act to promote the assembly of the Rad51 presynaptic filament (3, 4).

Mutations in *BRCA2* account for a significant portion of familial breast and ovarian cancers (5) and can lead to the cancer-prone syndrome Fanconi anemia (2). *BRCA2* mutant cells exhibit hypersensitivity to genotoxic agents and HR deficiency (1, 6, 7). *BRCA2* harbors eight copies of a Rad51 binding motif called the BRC repeat (8–10). In addition, the carboxyl terminus of *BRCA2* contains a distinct Rad51 binding domain, and phosphorylation of this domain by cyclin-dependent kinases appears to modulate the interaction with Rad51 (11). Crystallographic and biochemical characterization of mouse *Brca2* has revealed a DNA binding domain that comprises three oligonucleotide/oligosaccharide binding (OB) folds (12).

Several lines of evidence suggest that *BRCA2* functions as a recombination mediator. First, as noted above, *BRCA2* interacts with Rad51 (10) and possesses ssDNA binding activity (12), characteristics of a recombination mediator. Second, *BRCA2*-deficient cells fail to assemble DNA damage-induced nuclear Rad51 foci, reflective of a failure to deliver Rad51 to recombination substrates (13–15). Third, the *Ustilago maydis* Brh2 protein, which harbors a single Rad51-binding BRC repeat and a DNA binding activity, enables Rad51 to utilize RPA-coated ssDNA for recombination (16).

Due to its enormous size (3,418 amino acid residues), it has not been possible to obtain full-length human *BRCA2* for testing its physical and functional interactions with Rad51. To circumvent this limitation, we constructed a polypeptide that harbors the *BRCA2* DNA binding domain and two of the BRC repeats, BRC3 and BRC4. This *BRCA2*-derived polypeptide, BRC3/4-DBD, bound hRad51 and ssDNA and, importantly, was capable of delivering hRad51 to ssDNA and allowing hRad51 to utilize hRPA-coated ssDNA for the catalysis of recombination. Thus, these results provide direct evidence that *BRCA2* targets hRad51 to recombination substrates and possesses recombination mediator activity.

MATERIALS AND METHODS

Plasmids

BRC3/4-DBD harbors the human *BRCA2* BRC repeats 3 and 4 (residues 1409–1596) and the DNA binding domain (DBD) (residues 2477–3194). To facilitate the purification of this *BRCA2* polypeptide, an amino-terminal S tag (17) and a carboxyl-terminal His₆ tag were attached to it. The tagged BRC3/4-DBD polypeptide was introduced into the pET-32b vector (Novagen) that has been deleted for the amino-terminal His₆ tag. The *Escherichia coli* GST-BRC3/4 (residues 1409–1596) expression plasmid was a kind gift from Dr. Wen-Hwa Lee (10). Construction of the plasmid that expresses the amino-terminally His₆-tagged DBD (residues 2449–3224) will be described elsewhere.

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³ The abbreviations used are: HR, homologous recombination; ss, single-stranded; RPA, replication protein A; hRPA, human RPA; hRad51, human Rad51; OB, oligonucleotide/oligosaccharide binding; DBD, DNA binding domain; GST, glutathione *S*-transferase; BSA, bovine serum albumin; ds, double-stranded; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; EM, electron microscopy; SSB, single-stranded DNA-binding protein.

Cell Growth

The plasmids that express BRC3/4-DBD, GST-BRC3/4, and the DBD were introduced into the *E. coli* strain Rosetta (DE3). Following transformation, single colonies were picked and grown for 16 h in 300 ml of Luria broth. The starter culture was diluted 50 times with fresh Luria broth and incubated at 37 °C. When the A_{600} of the cultures reached 0.6–0.8, isopropyl 1-thio- β -D-galactopyranoside was added to 0.1 mM, and the induction continued at 16 °C for 18 h. Plasmid p11d-tRPA was introduced into *E. coli* strain BL21 (DE3), and the induction of hRPA was carried out as described previously (18, 19). Cells were harvested by centrifugation and stored frozen at –80 °C.

Protein Purification

Purification of BRCA2-derived Polypeptides—All the purification steps were carried out at 0–4 °C. For the purification of BRC3/4-DBD, *E. coli* cell paste (120 g from 48 liters of culture) was suspended in 600 ml of cell breakage buffer (50 mM Tris-HCl, pH 7.5, 500 mM KCl, 2 mM dithiothreitol, 10% sucrose, and the following protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A at 3 μ g/ml each, and 1 mM phenylmethylsulfonyl fluoride) and then passed through a French press once at 20,000 p.s.i. The BRC3/4-DBD polypeptide is mostly soluble, and after centrifugation (at 100,000 \times *g* for 90 min), the clarified lysate containing this polypeptide was gently mixed with 8 ml of nickel-nitrilotriacetic acid-agarose beads for 5 h. The nickel beads were collected into a glass column with an internal diameter of 1.5 cm and washed with 150 ml of buffer K (20 mM KH_2PO_4 , pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) containing 1 M KCl and 0.01% Igepal CA-630 (Sigma) followed by a 50-ml wash with buffer K containing 100 mM KCl and 15 mM imidazole. The nickel affinity column was then developed with an 80-ml gradient of 30–300 mM imidazole in K buffer containing 100 mM KCl with BRC3/4-DBD eluting at \sim 150 mM imidazole. Fractions (4 ml each) containing the peak of BRC3/4-DBD were pooled (total of 20 ml) and loaded onto a 1-ml Macro hydroxyapatite (Bio-Rad) column, which was eluted with 20 ml of 50–300 mM KH_2PO_4 in K buffer, collecting 0.5-ml fractions. BRC3/4-DBD eluted from the hydroxyapatite column at \sim 180 mM KH_2PO_4 , and the pool of BRC3/4-DBD (16 ml) was precipitated with ammonium sulfate at 0.21 g/ml. The protein precipitate was collected by centrifugation, dissolved in 0.5 ml of K buffer containing 100 mM KCl, and then mixed gently with 0.5 ml of anti-S-agarose beads (Novagen) to bind BRC3/4-DBD. The anti-S affinity matrix was poured into a glass column with an internal diameter of 0.5 cm and washed with 10 ml of buffer E (50 mM Tris-HCl, pH 7.4, 20% glycerol, 1 mM EDTA, and 0.5 mM dithiothreitol) followed by 10 ml of buffer K containing 1 M KCl. BRC3/4-DBD was released from the affinity matrix by three 10-min elutions at 23 °C with 1 ml of buffer K containing 3 M MgCl_2 and 0.5 M KCl each time. The eluate, containing a total of 100 μ g of BRC3/4-DBD, was dialyzed against 2 liters of buffer K containing 500 mM KCl, concentrated in a Centri-con-30 device (Millipore) to 0.5 mg/ml, and stored in small portions at –80 °C.

For the purification of GST-BRC3/4, extract was made from 10 g of *E. coli* cell paste as described above except that the cell breakage buffer (50 ml) contained 200 mM KCl. The lysate obtained after ultracentrifugation was passed through a 20-ml Q fast flow column and then mixed with 1 ml of glutathione-Sepharose 4 beads for 2 h. The beads were poured into a glass column with an internal diameter of 1 cm, washed with 20 ml of buffer K containing 500 mM KCl, and treated with 10 ml of 20 mM glutathione (Amersham Biosciences), collecting 1-ml fractions. The fractions containing the peak of GST-BRC3/4 (4 ml total) were loaded onto a 1-ml Macro hydroxyapatite column, which was developed

with a 20-ml gradient of 50–320 mM KH_2PO_4 in buffer K. GST-BRC3/4 eluted from the hydroxyapatite column at 85–175 mM KH_2PO_4 , and the peak of GST-BRC3/4 (4.5 ml total containing 1.5 mg of GST-BRC3/4) was concentrated in an Amicon Ultra microconcentrator (Millipore) to 3 mg/ml and stored in small portions at –80 °C. The purification of the DBD will be described elsewhere.⁴

Purification of hRad51, hRad51 K133R, and hRPA—The hRad51 protein was expressed in the *E. coli* RecA-deficient strain BLR (DE3) and purified to near homogeneity using our previously described procedure (18). The hRad51 K133R mutant was expressed and purified to near homogeneity in the same manner. hRPA was purified from *E. coli* BL21 (DE3) cells harboring the plasmid p11d-tRPA that co-expresses the three subunits of this protein (19) as described previously (18).

Other Proteins—*E. coli* RecA and SSB were a kind gift from Michael Cox.

Affinity Pull-downs

For the experiment in Fig. 3A, bovine serum albumin (BSA) (4 μ g), hRad51 (4 μ g), hRad51 K133R (4 μ g), and RecA (10 μ g) with or without BRC3/4-DBD (7 μ g) in 30 μ l of buffer B (20 mM KH_2PO_4 at pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, 0.01% Igepal CA-630, 100 mM KCl, and 10% glycerol) were mixed with 20 μ l of anti-S-agarose beads (Novagen/EMD Biosciences) at 4 °C for 60 min. After washing the beads twice with 100 μ l of the same buffer, bound proteins were eluted with 30 μ l of SDS-PAGE loading buffer. The experiment in Fig. 3C was done in the same manner with GST (15 μ g), GST-BRC3/4 (5 μ g), hRad51 (4 μ g), and RecA (4 μ g) using glutathione-Sepharose beads as the affinity matrix for protein pull-down (Amersham Biosciences). The supernatant, wash, and SDS eluate, 10 μ l each, were analyzed by 10% SDS-PAGE and Coomassie Blue staining.

For the experiment in Fig. 3B, we coupled hRad51 and BSA to Affi-Gel 15 beads (Bio-Rad) to create matrices containing 3 mg/ml Rad51 and 12 mg/ml BSA. To examine complex formation, GST-BRC3/4, DBD, and BRC3/4-DBD (8 μ g each) were mixed with 6 μ l of Affi beads conjugated to Rad51 or Affi beads conjugated to BSA beads in 30 μ l of buffer B at 4 °C for 60 min. The beads were washed twice with 100 μ l of the same buffer before eluting the bound proteins with 30 μ l of SDS-PAGE loading buffer. The supernatant, wash, and SDS eluate, 10 μ l each, were analyzed as above.

DNA Substrates

The oligonucleotides used are listed in Table 1. For DNA binding assays, the 80-mer Oligo 1 was labeled at the 5'-end with [α -³²P]ATP and T4 polynucleotide kinase. To make the 80-bp duplex, the radiolabeled Oligo 1 was hybridized to its exact complement, Oligo 2. For the homologous DNA pairing reactions, 150-mer Oligo 3 was used as ss substrate, and the duplex substrate was prepared by hybridizing complementary 40-mer Oligo 4 (³²P-labeled at the 5'-end) and Oligo 5. To prepare magnetic beads containing ssDNA, biotinylated (dT)₈₃ (7.5 μ g) was mixed with streptavidin-coated magnetic beads (Roche Applied Science) in 200 μ l of buffer C (10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 1 mM EDTA) for 4 h at 25 °C. The beads were washed twice with 400 μ l of buffer C containing 1 M NaCl and stored in buffer C at 4 °C. The 60-bp dsDNA used as Rad51 trap in the magnetic bead pull-down assay (Fig. 5) consisted of Oligo 6 hybridized to Oligo 7. DNA hybridization was carried out by heating pairs of complementary oligonucleotides at 75 °C for 10 min in buffer H (50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , and 100 mM NaCl) followed by slow cooling to 25 °C. All the DNA

⁴ L. Krejci, J. Etchin, J. San Filippo, M. G. Sehorn and P. Sung, manuscript in preparation.

TABLE 1

Oligonucleotides used in this study

Oligo 1	5'-TTATATCCTTTACTTTGAATTCTATGTTTAACCTTTACTTATTTTGTATTAGCCGGATCCTTATTTCAATTATGTTTCAT-3'
Oligo 2	5'-ATGAACATAAATGAAATAAGGATCCGGCTAATACAAAATAAGTAAAAGTTAAACATAGAAATCAAGTAAAGGATATAA-3'
Oligo 3	5'-TCTATTTATGTCCTTTTATTTTCATTTCCTATATTTATTCCTATTATGTTTTATTCACTTACTTATTTCTTTATGTTTCATTTT TTATATCCTTTACTTTTATTTTCTCTGTTTATTCATTACTTATTTTGTATTATTCCTTATCTTATTTA-3'
Oligo 4	5'-TAATACAAAATAAGTAAATGAATAAACAGAGAAAATAAAG-3'
Oligo 5	5'-CTTTATTTTCTCTGTTTATTCATTACTTATTTTGTATTA-3'
Oligo 6	5'-TGCTCTTTTATTTTCATTTTCGTTTATTCACATATATTTTTTATTTCTATTTTACTTATTTT-3'
Oligo 7	5'-AAATTAAGTAAAATAGAAAATAAAAATATAGTGAATAAACGAAATGAAATAAGAGACA-3'

substrates were purified from 10% polyacrylamide gels run in TAE buffer (40 mM Tris acetate, pH 7.4, with 0.5 mM EDTA) and eluted from gel slices by overnight diffusion at 4 °C into the same buffer.

DNA Binding Assay

BRC3/4-DBD (20–500 nM) was incubated with 30 nM ssDNA or dsDNA or the combination of the two substrates in 10 μ l of buffer R (50 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM dithiothreitol, and 100 μ g/ml BSA) containing 60 mM KCl at 37 °C for 5 min. The reaction mixtures were resolved in 10% non-denaturing polyacrylamide gels in TAE buffer at 4 °C. The gels were dried and then subjected to phosphorimaging analysis for data quantification.

Targeting of hRad51 to Magnetic Bead-bound Biotinylated ssDNA by BRC3/4-DBD

hRad51 (2.7 μ M) was incubated with the indicated amounts of GST-BRC3/4, DBD, or BRC3/4-DBD in 18 μ l of buffer R with 0.1 mM ATP for 10 min at 25 °C and then mixed with (dT)₈₃-containing magnetic beads (12 μ M in nucleotides) and 60-mer dsDNA trap (72 μ M base pairs) for 10 min at 37 °C; the final volume of the reaction mixtures was 20 μ l. The beads were captured with the Magnetic Particle Separator (Roche Applied Science), and the supernatants were set aside for further analysis. After washing the beads twice with 20 μ l of buffer T (25 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5 mM EDTA, and 0.5 mM dithiothreitol) containing 0.01% Igepal CA-630, 0.1 mM ATP, and 1 mM MgCl₂, bound proteins were eluted from half of the beads with 10 μ l of 2% SDS. The various supernatants and SDS eluates (5 μ l each) were analyzed by SDS-PAGE and Coomassie Blue staining to determine their content of proteins and also by polyacrylamide gel electrophoresis followed by ethidium bromide staining to quantify the amount of dsDNA.

ATPase Assay

For the experiment in Fig. 5C, the remaining half of the beads (Fig. 5B) were resuspended in 6 μ l of buffer R containing 60 mM KCl and 0.1 mM [γ -³²P]ATP (1 μ Ci) followed by incubation at 37 °C. For the experiment in Fig. 5D, hRad51 (2.7 μ M) was incubated with the indicated amounts of BRC3/4-DBD in 10 μ l of buffer R that contained 0.1 mM [γ -³²P]ATP (1 μ Ci) and (dT)₈₃ (12 μ M nucleotides) bound on magnetic beads at 37 °C. At the indicated times, 1 μ l of the reaction mixtures was withdrawn and added to 1 μ l of 500 mM EDTA to stop the reaction. The level of ATP hydrolysis was determined by thin layer chromatography in polyethyleneimine sheets (J. T. Baker Inc.).

Homologous DNA Pairing Assay

The reaction mixtures were assembled in buffer R that contained 1 mM ATP and had a final volume of 12.5 μ l. Unless stated otherwise, all the reaction steps were carried out at 37 °C. In the standard homologous DNA pairing reaction, the 150-mer Oligo 3 (6 μ M nucleotides) was incubated with hRad51 (2 μ M) with or without the indicated concentration of BRC3/4-DBD for 5 min followed by the addition of ³²P-labeled homologous dsDNA (6 μ M base pairs) and 4 mM spermidine hydrochloride.

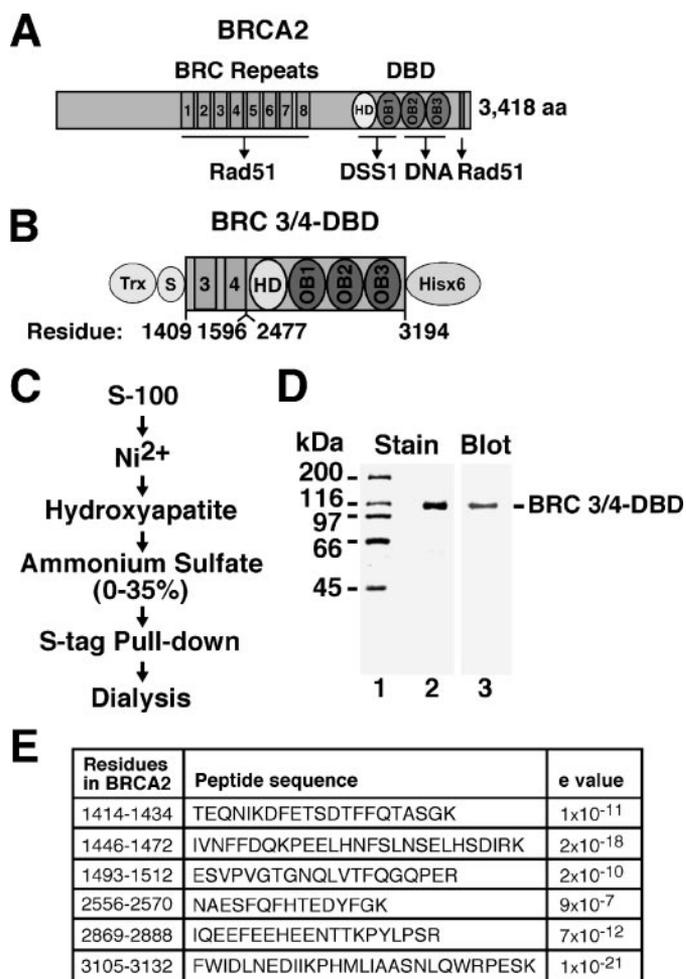


FIGURE 1. Purification of BRC3/4-DBD. **A**, schematic outlining the functional domains in BRCA2. The DBD consists of a helical domain (HD) and three oligonucleotide binding (OB1, OB2, and OB3) folds that confer the ability to interact with DSS1 and ssDNA. In addition to the BRC repeats, the carboxyl terminus possesses a distinct Rad51 binding domain. **B**, the human BRCA2-derived polypeptide BRC3/4-DBD harbors two of the BRC repeats (BRC3 and BRC4 within residues 1409–1596) linked to the DBD (residues 2477–3194). BRC3/4-DBD contains thioredoxin (Trx) and S tags at its amino terminus and a His₆ tag at its carboxyl terminus. **C**, schematic of the chromatographic procedure devised for BRC3/4-DBD purification. **D**, purified BRC3/4-DBD was analyzed by SDS-PAGE and Coomassie Blue staining (2 μ g in lane 2) or immunoblotting with anti-histidine antibodies (200 ng in lane 3). **E**, results from MALDI-TOF analysis of purified BRC3/4-DBD. Six representative fragments and their corresponding e values are shown. aa, amino acids.

ride. After 30 min of incubation, the reactions were mixed with an equal volume of 1% SDS containing 1 mg/ml proteinase K. Following a 10-min incubation, samples were run in 10% non-denaturing polyacrylamide gels in TAE buffer at 4 °C. The gels were dried and analyzed in a PhosphorImager. For evaluating the recombination mediator function of the various BRCA2-derived polypeptides, the ssDNA was preincubated with hRPA (600 nM) or SSB (4 μ M) for 5 min; all the subsequent steps were as described above. The reactions with RecA were done in buffer R

BRCA2-Rad51 Interactions

that contained 5 mM MgCl₂ but were otherwise assembled and processed in the same manner.

Electron Microscopy

Reaction mixtures were assembled with combinations of hRad51 K133R (2 μM), hRPA (600 nM), BRC3/4-DBD (200 nM), and the 150-mer ssDNA (6 μM nucleotides) as described for the homologous DNA pairing reactions except that BSA was omitted from the reaction buffer. After a 10-min incubation, the reactions were diluted 6 times with the reaction buffer, and a 3-μl portion was applied to 400-mesh grids coated with fresh carbon film that had been glow-discharged in air. Samples were stained with 2% uranyl acetate for 15 s and examined with a Tecnai 12 transmission electron microscope equipped with a LaB6 filament and operated at 120 keV in conjunction with a GATAN 794 charge-coupled device camera at a nominal magnification of ×15,000.

RESULTS

Expression and Purification of hBRCA2-derived Polypeptides—Human BRCA2 harbors eight BRC repeats in its middle portion and, as deduced from studies with mouse Brca2, a DBD toward the carboxyl terminus (Fig. 1A) (12). Due to its uncommonly large size, we have not

yet been able to purify full-length BRCA2. However, because several BRCA2-like molecules from organisms such as *U. maydis* and *Caenorhabditis elegans* are much smaller in size and contain only a single BRC (20), we reasoned that a BRCA2 polypeptide harboring the DBD and some of the BRC repeats may be capable of physical and functional interactions with hRad51. We therefore constructed a BRCA2 polypeptide, BRC3/4-DBD (Fig. 1B), that comprises BRC3 and BRC4, both of which bind hRad51 very strongly (9, 10), and the DBD. We added S and His₆ tags to BRC3/4-DBD to allow the use of anti-S antibody-agarose and nickel-nitrilotriacetic acid-agarose as affinity steps during protein purification.

BRC3/4-DBD was expressed in *E. coli*, and a procedure was devised (Fig. 1C) for its purification to near homogeneity (Fig. 1D). The identity of purified BRC3/4-DBD was established by immunoblot analysis with antibodies specific for the His₆ affinity tag on the polypeptide (Fig. 1D) and by MALDI-TOF (Fig. 1E). Four independent preparations of BRC3/4-DBD gave very similar results in all the assays performed. For the functional characterization of BRC3/4-DBD, we obtained *E. coli* RecA and SSB from Michael Cox and also purified to near homogeneity hRad51, the hRad51 K133R variant, and the heterotrimeric ssDNA binding factor hRPA using our published procedures (18). As controls in the biochemical analyses, we also expressed and purified to near homogeneity a GST fusion protein that contains the BRC3 and BRC4 repeats (GST-BRC3/4) and also a His₆-tagged form of the DBD. The SDS-PAGE analysis of the aforementioned proteins can be found in Fig. 2.

Association of BRC3/4-DBD with Rad51—We used *in vitro* pull-down assays to test whether BRC3/4-DBD binds hRad51. First we incubated purified hRad51, hRad51 K133R, or RecA with BRC3/4-DBD and then captured the resulting protein complexes on anti-S antibody-agarose beads through the S tag on BRC3/4-DBD. Fig. 3A shows that BRC3/4-DBD bound both hRad51 and hRad51 K133R but was unable to associate with RecA. We next used Affi-Gel beads containing cross-linked hRad51 to confirm protein complex formation and to ascertain the role

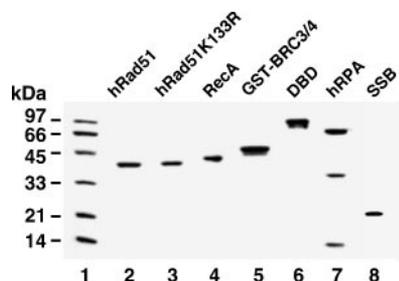


FIGURE 2. Analysis of proteins by SDS-PAGE and Coomassie Blue staining. Lane 2, hRad51; lane 3, hRad51 K133R; lane 4, RecA; lane 5, GST-BRC3/4; lane 6, BRC3/4-DBD; lane 7, hRPA (RPA70, RPA34, and RPA14 subunits); lane 8, SSB.

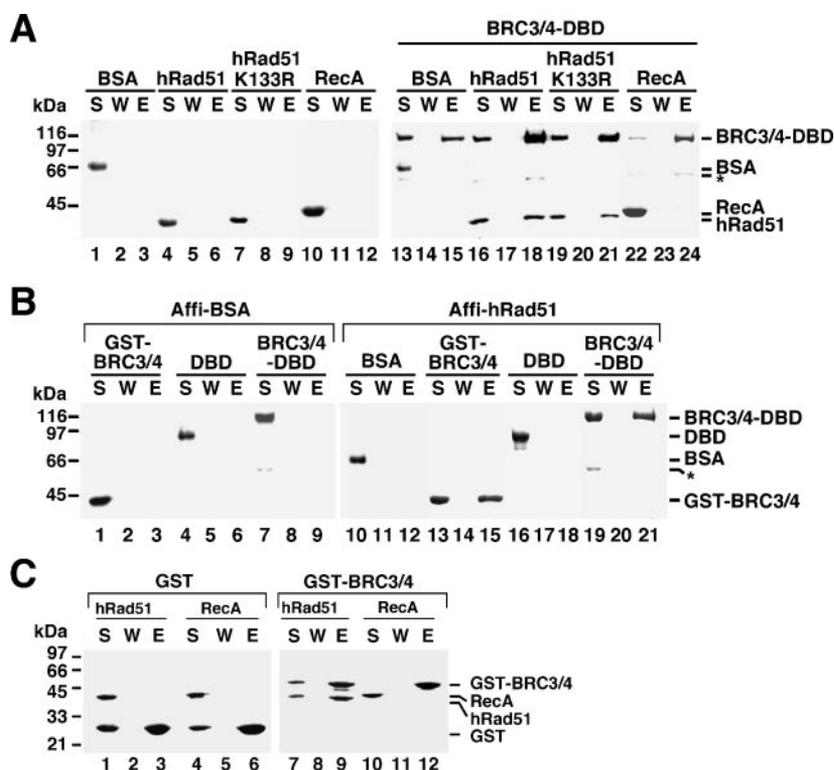


FIGURE 3. BRC3/4-DBD binds hRad51. A, BSA, hRad51, hRad51 K133R, and RecA were mixed with anti-S protein-agarose beads in the absence (lanes 1–12) or presence of BRC3/4-DBD (lanes 13–24) and then subjected to affinity pull-down with anti-S-agarose beads. B, GST-BRC3/4, DBD, and BRC3/4-DBD were subjected to affinity pull-down with Affi beads conjugated to BSA (Affi-BSA; lanes 1–9) or hRad51 (Affi-hRad51; lanes 10–21). C, pull-down assays using GST or GST-BRC3/4 on glutathione-Sepharose confirmed that BRC3/4 binds Rad51 but not RecA. The supernatant (S), wash (W), and SDS eluate (E) from the above reactions were analyzed by SDS-PAGE and Coomassie Blue staining. A proteolytic product of BRC3/4-DBD is marked by the asterisk.

of the BRC repeats. As shown in Fig. 3B, although BRC3/4-DBD and GST-BRC3/4 were retained on the Affi-hRad51 beads as anticipated, DBD was not. In control experiments (Fig. 3B), none of the BRCA2-derived polypeptides were retained on Affi beads containing BSA. When we performed pull-down through the GST tag on GST-BRC3/4 using glutathione-Sepharose, we saw complex formation with hRad51 but not with RecA (Fig. 3C). From these results, we conclude that BRC3/

4-DBD associates with hRad51 and hRad51 K133R in a specific manner through the BRC repeats.

DNA Binding by BRC3/4-DBD—To determine whether BRC3/4-DBD has DNA binding capability, increasing amounts of the purified polypeptide were incubated with either 32 P-labeled 80-mer ssDNA or 80-bp duplex. The reaction mixtures were resolved in polyacrylamide gels followed by phosphorimaging analysis to reveal shifting of the DNA species by BRC3/4-DBD. As shown in Fig. 4, A and B, BRC3/4-DBD bound both DNA forms but with a distinct preference for the ssDNA species. Consistent with these results, when we incubated BRC3/4-DBD with a mixture of the ssDNA and dsDNA, it first shifted the ssDNA substrate before shifting the dsDNA (Fig. 4C). Half-maximal binding of ssDNA occurred at ~ 60 nM BRC3/4-DBD, but even at 500 nM BRC3/4-DBD, only a relatively small fraction (~ 30 – 40%) of the dsDNA was shifted. We have verified that 1) DBD alone also binds DNA with a strong preference for the ssDNA species and a similar affinity (data not shown) and 2) GST-BRC3/4 has no DNA binding activity (data not shown). Taken together, the results clearly show that BRC3/4-DBD and by inference full-length BRCA2 harbor a DNA binding function that is highly specific for ssDNA.

Targeting of Rad51 to ssDNA by BRC3/4-DBD—Although Rad51 forms filaments on both ssDNA and dsDNA, only the Rad51-ssDNA filament species is active in homologous DNA pairing (21). In fact, assembly of the Rad51-ssDNA nucleoprotein filament can be hampered by sequestration of Rad51 on dsDNA. Given that BRC3/4-DBD physically interacts with hRad51 and binds ssDNA avidly, we wished to test whether BRC3/4-DBD could target hRad51 to ssDNA. To address this,

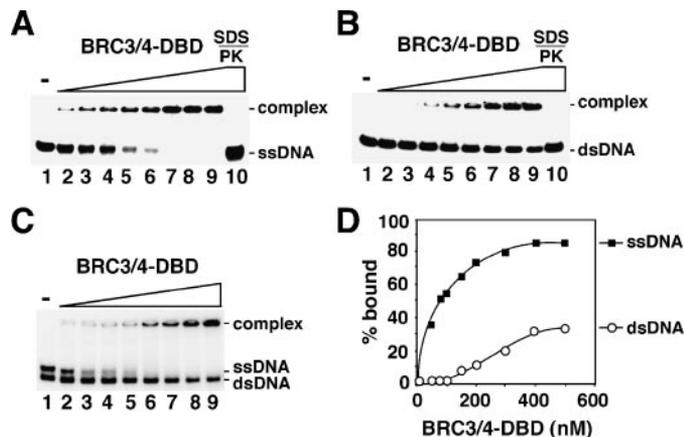


FIGURE 4. BRC3/4-DBD has high affinity for ssDNA. A and B, increasing amounts of purified BRC3/4-DBD (20–500 nM, lanes 2–9) were incubated with 30 nM 32 P-labeled ssDNA or dsDNA and then analyzed. Treatment of the nucleoprotein with SDS and proteinase K (SDS/PK) released the DNA substrate (lane 10). C, BRC3/4-DBD (50–500 nM) was incubated with the mixture of 32 P-labeled ssDNA and dsDNA and then analyzed. D, the results from the experiment in C are plotted.

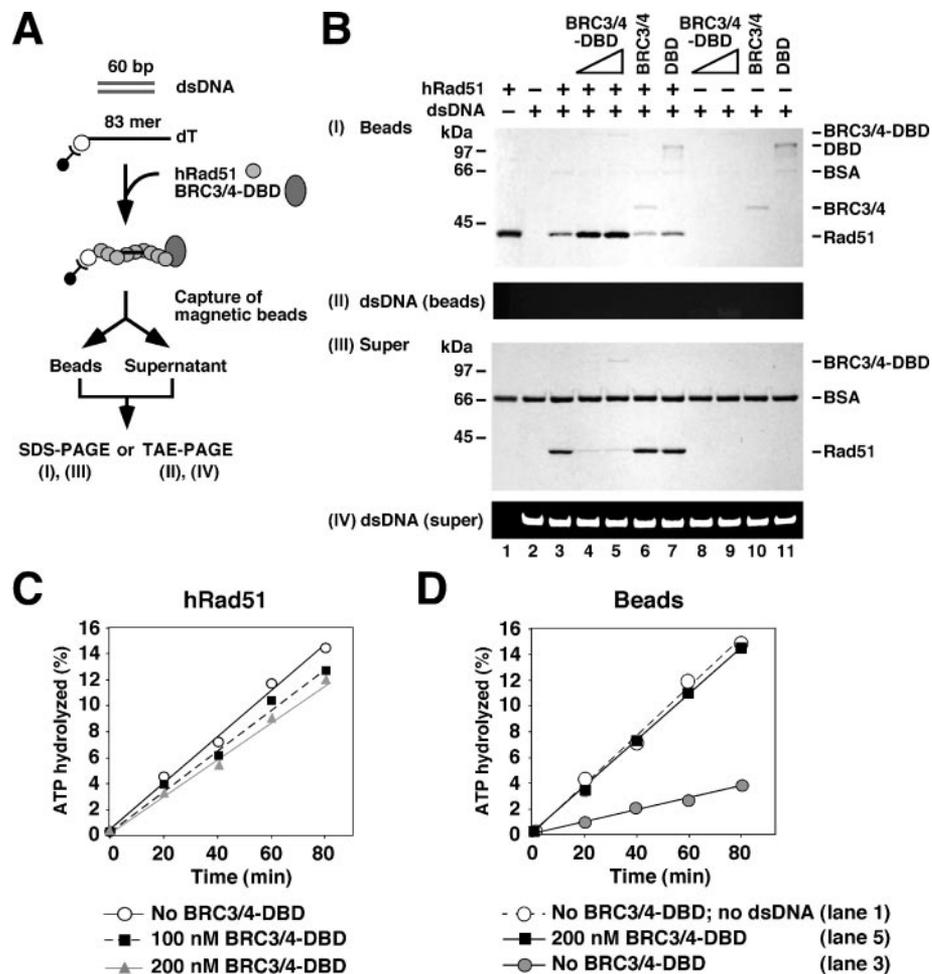


FIGURE 5. BRC3/4-DBD targets hRad51 to ssDNA. A, schematic of the assay. Magnetic bead-bound oligo(dT) was incubated with hRad51, BSA, and BRCA2-derived polypeptides, without or with an excess of dsDNA, as indicated. Proteins bound to the oligo(dT) were captured with a magnet and then eluted with SDS. B, the supernatants (super) and SDS eluates (beads) were analyzed for their protein and DNA contents. Although the majority of hRad51 was trapped on the dsDNA as expected (lane 3), BRC3/4-DBD efficiently targeted hRad51 to the ssDNA (lanes 4 and 5). GST-BRC3/4 (BRC3/4) or DBD was ineffective in this regard (lanes 6 and 7, respectively). C, the bead-bound fractions (from lanes 1, 3, and 5 in B) were examined for hRad51-mediated ATP hydrolysis. D, Rad51-mediated ATP hydrolysis was assayed in the presence of ssDNA and with or without BRC3/4-DBD.

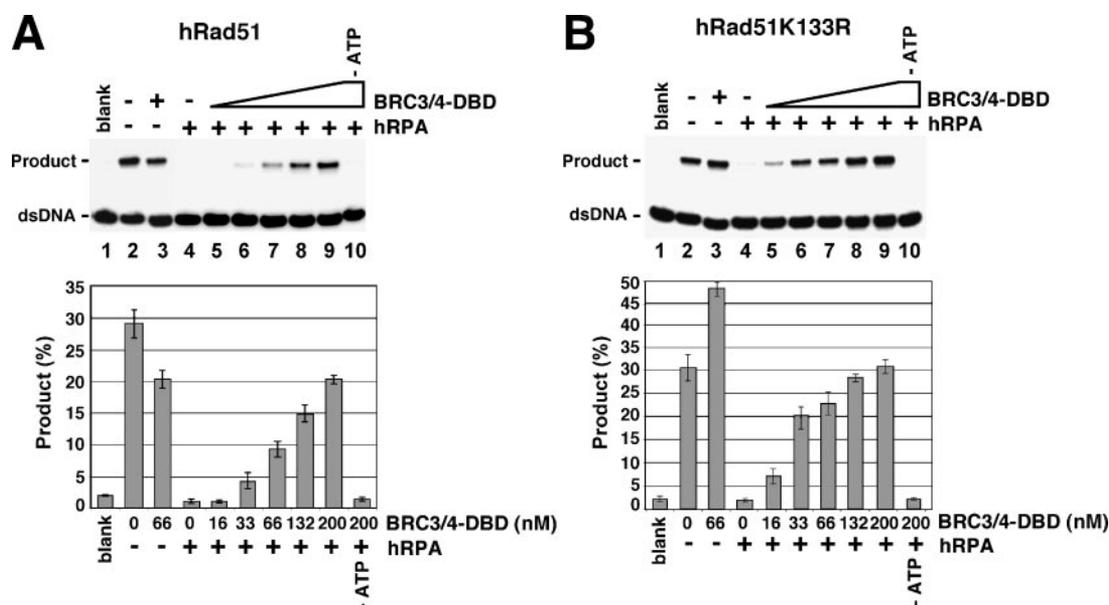


FIGURE 6. **Recombination mediator activity of BRC3/4-DBD.** A and B, homologous DNA pairing reactions containing hRad51 or hRad51 K133R and varying amounts of BRC3/4-DBD were carried out with ssDNA or hRPA-coated ssDNA as indicated. The averaged values of results from three independent experiments are presented in the histograms. The no protein control (lane 1) is marked as "blank," and ATP was omitted from the reaction in lane 10.

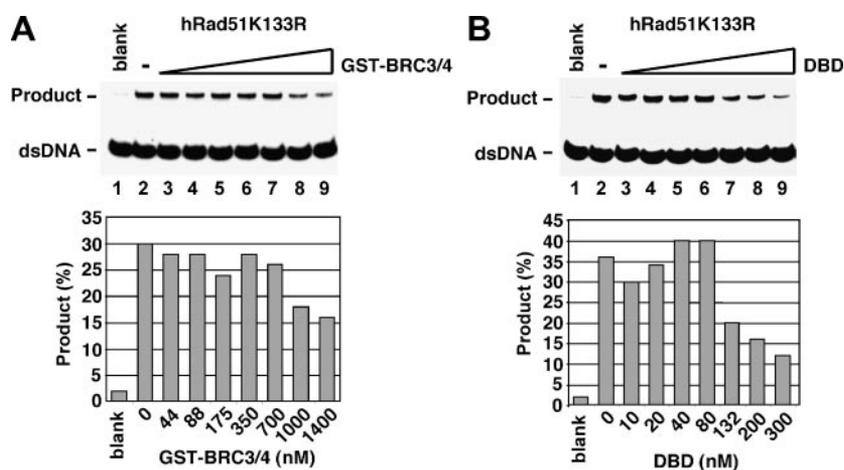


FIGURE 7. **Effects of GST-BRC3/4 (A) and DBD (B) on homologous DNA pairing by hRad51 K133R.** The reactions were carried out exactly as described for Fig. 6 but without hRPA.

we examined the partition of hRad51 between a 60-mer duplex molecule and biotinylated oligo(dT) immobilized on streptavidin magnetic beads with or without BRC3/4-DBD. The amount of hRad51 associated with the oligo(dT) and duplex was determined by SDS-PAGE. Because Rad51 has an ATPase activity (22–24) but BRC3/4-DBD does not hydrolyze ATP or stimulate the hRad51 ATPase activity (Fig. 5C), we could also independently determine the level of hRad51 on the magnetic bead-bound oligo(dT) by measuring ATPase activity. Results from SDS-PAGE and ATPase analyses indicated that amounts (100 and 200 nM) of BRC3/4-DBD substoichiometric to that of hRad51 (2.7 μ M) enhanced the fraction of oligo(dT)-bound hRad51 by 4–5-fold (Fig. 5B, compare lanes 4 and 5 with lane 3) and correlated to an equivalent amount of ATPase activity (Fig. 5D). In contrast, neither GST-BRC3/4 (400 nM) nor the DBD (400 nM) (Fig. 5B, lanes 6 and 7) was effective in enhancing the association of hRad51 with the ssDNA. Likewise no enhanced ssDNA targeting of hRad51 was seen with the combination of GST-BRC3/4 and DBD (data not shown). Taken together, the results provide support to the notions that 1) BRC3/4-DBD targets hRad51 to ssDNA and 2) both the BRC repeats and the DBD are indispensable for this hRad51 targeting activity.

Recombination Mediator Activity of BRC3/4-DBD—As a result of competition for binding sites, RPA can exclude Rad51 from ssDNA and cause marked suppression of the Rad51 recombinase activity (3, 4, 25). We used an oligonucleotide-based homologous DNA pairing assay to assess whether BRC3/4-DBD can help overcome the suppressive effect of hRPA on the hRad51 recombinase function. We included in the analyses both the wild type hRad51 protein and a variant, hRad51 K133R, which harbors the change of the conserved lysine (Lys-133) residue in the Walker type A ATP-binding pocket to arginine. The hRad51 K133R protein has little ATPase activity but forms a highly stable presynaptic filament and retains recombinase activity (Ref. 26 and see below). In the absence of RPA, BRC3/4-DBD (40–160 nM) slightly attenuated the homologous DNA pairing activity of wild type hRad51 but exerted a significant stimulatory effect on hRad51 K133R (Fig. 6). These effects of BRC3/4-DBD on the hRad51 and hRad51 K133R proteins were highly reproducible. GST-BRC3/4, up to 700 nM, did not affect the homologous DNA pairing reaction, but inhibition of the reaction was seen at concentrations of 1 μ M and above of this polypeptide (Fig. 7A). The homologous DNA pairing reaction by either hRad51 or hRad51 K133R was not affected by up to an 80 nM concentration of the isolated DBD,

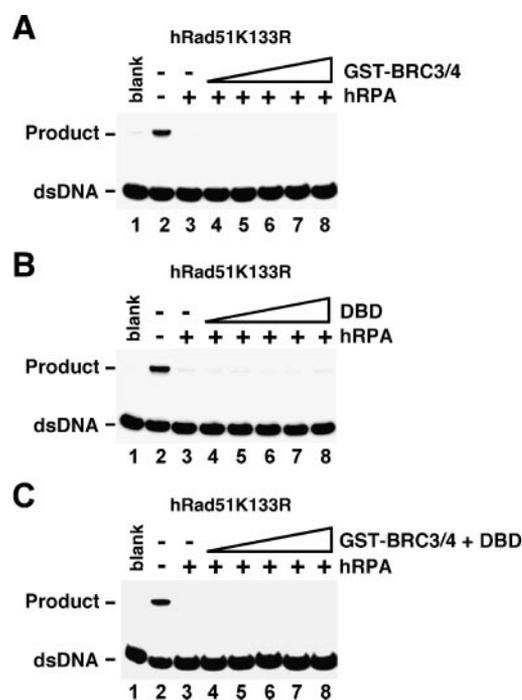


FIGURE 8. Experiments showing that neither GST-BRC3/4 (A), DBD (B), nor a mixture of the two polypeptides (C) is capable of overcoming the inhibitory effect of hRPA on homologous DNA pairing by hRad51 K133R. The concentrations of the BRCA2 polypeptides were 25, 50, 100, 150, and 200 nM.

but higher levels of it had a significant inhibitory effect (Fig. 7B). The effects of these BRCA2-derived species were also highly reproducible.

We next investigated whether BRC3/4-DBD has recombination mediator activity. Before testing the effect of BRC3/4-DBD, we predetermined the concentration of RPA (600 nM) sufficient to ablate homologous DNA pairing catalyzed by either hRad51 or hRad51 K133R (2 μ M each) with the amount of ssDNA template (40 nM template or 6 μ M nucleotides) used. Importantly with both hRad51 and hRad51 K133R, restoration of homologous DNA pairing was seen upon addition of an amount of BRC3/4-DBD (33 nM) similar to the concentration of the ssDNA template. Full restoration of homologous DNA pairing in reactions containing either hRad51 or hRad51 K133R required 200 nM BRC3/4-DBD. We note that even the highest concentration of BRC3/4-DBD used in these experiments was still 10 times lower than that of hRad51 or hRad51 K133R, indicative of a catalytic mode of action in BRC3/4-DBD. Control experiments showed that BRC3/4-DBD alone is devoid of homologous DNA pairing activity (data not shown) and that the restored homologous DNA pairing reaction, as expected, was strictly ATP-dependent (Fig. 6) (4, 24). Significantly neither DBD, GST-BRC3/4, nor the combination of both of these polypeptides, in the same concentration range of BRC3/4-DBD used, was able to overcome the suppressive effect of RPA (Fig. 8).

Examination of Recombination Mediator Function by Electron Microscopy—We used electron microscopy (EM) to further examine the recombination mediator activity of BRC3/4-DBD. We used hRad51 K133R because it forms a more stable presynaptic filament than the wild type protein (26). In the EM analyses, hRad51 K133R-ssDNA filaments exhibited the characteristic striations noted in previous studies (Fig. 9A), whereas the nucleoprotein complexes of RPA with ssDNA had a nondescript appearance (Fig. 9B). The resolution of the EM images was such that we could reliably quantify the abundance of the hRad51 K133R-ssDNA filaments in reaction mixtures. From previous work (4, 27), we know that each helical repeat of the Rad51 K133R presynaptic

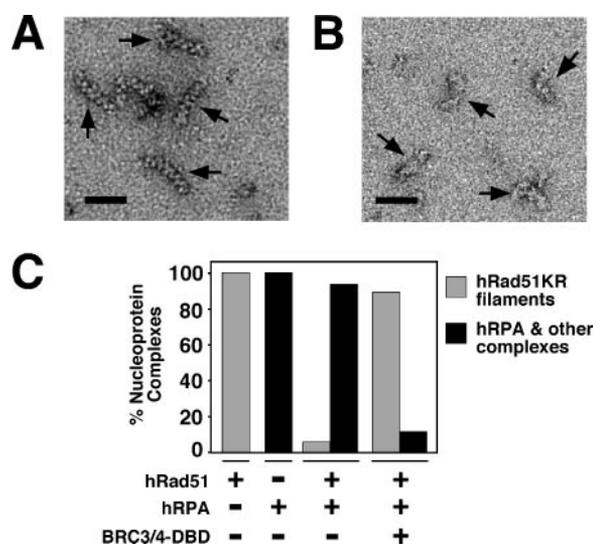


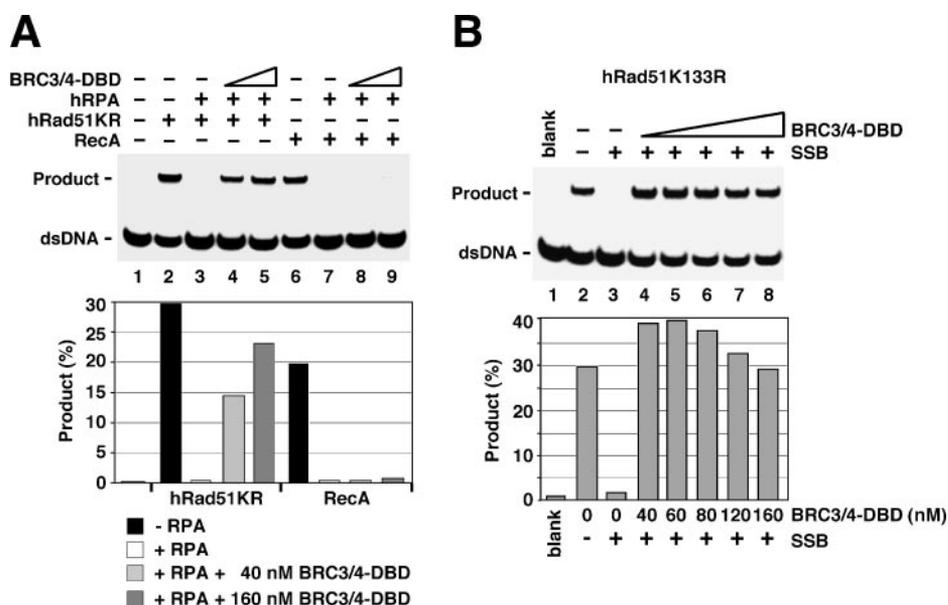
FIGURE 9. Examination of recombination mediator activity by electron microscopy. Examples of hRad51 K133R-ssDNA nucleoprotein filaments (A) and hRPA-ssDNA complexes (B) are shown (arrows). Control experiments confirmed the requirement for ssDNA in the formation of the hRad51 K133R filaments and hRPA-containing complexes. The bar in black denotes a length of 50 nm. C, data quantification of reaction mixtures that contained either hRad51 K133R and hRPA or hRad51 K133R, hRPA, and BRC3/4-DBD. Over 1,500 nucleoprotein complexes were counted to determine the relative abundance of the hRad51 K133R-ssDNA filaments.

filament should consist of \sim 18–19 nucleotides of ssDNA and six protein molecules. In the experiments with the BRC3/4-DBD, we used the same concentrations of proteins and ssDNA as in the homologous pairing assay to allow a direct comparison of the EM results with those from the biochemical experiments. The majority of the hRad51 K133R presynaptic filaments made with the 150-mer ssDNA substrate consisted of seven to eight helical repeats just as anticipated from the known biophysical attributes of these filaments. In congruence with the biochemical data, when the ssDNA was first coated with hRPA before hRad51 K133R was added, we saw an abundance of hRPA-ssDNA complexes but very few presynaptic filaments (Fig. 9C). Importantly the inclusion of 200 nM BRC3/4-DBD, sufficient to restore homologous pairing with hRPA-coated ssDNA template to near the uninhibited level (Fig. 6B), led to robust presynaptic filament formation (Fig. 9C). Thus, the results from the EM analyses are also clearly indicative of a recombination mediator function in BRC3/4-DBD.

Specificity and Versatility of the Recombination Mediator Activity—As shown earlier in the pull-down assay, neither BRC3/4-DBD nor GST-BRC3/4 (Fig. 3) has any affinity for *E. coli* RecA protein. Because results from our biochemical analysis showed that the BRC repeats are indispensable for recombination mediator activity, we wished to test whether RecA-mediated homologous DNA pairing is responsive to BRC3/4-DBD. As shown in Fig. 10A (lane 7), the same amount of RPA (600 nM) that was sufficient to ablate hRad51-mediated homologous DNA pairing was similarly effective in suppressing the RecA recombinase activity. Importantly little or no restoration of the RecA-mediated homologous DNA pairing reaction was seen upon addition of BRC3/4-DBD (Fig. 10A).

We also asked whether BRC3/4-DBD could overcome the inhibitory effect of *E. coli* SSB on hRad51-catalyzed homologous DNA pairing. We found that preincubating the ssDNA with 1 μ M SSB tetramer led to near ablation of the hRad51-mediated reaction (Fig. 10B). Importantly the addition of as little as 40 nM BRC3/4-DBD was sufficient to completely reverse the inhibitory effect of SSB, whereas, as demonstrated earlier, a significantly higher level (200 nM) of the BRCA2 polypeptide was necessary to restore hRad51-mediated homologous DNA pairing to near

FIGURE 10. Specificity and versatility of the BRC3/4-DBD recombination mediator activity. A, BRC3/4-DBD is unable to overcome the suppressive effect of hRPA on RecA-mediated homologous DNA pairing (lanes 6–9). hRad51 K133R (hRad51KR) was included in this analysis as control (lanes 2–5). B, *E. coli* SSB attenuates homologous DNA pairing by hRad51 K133R, and this suppressive effect is overcome by BRC3/4-DBD (lanes 4–9). The no protein control (lane 1) is marked as "blank." The results are presented in the histograms.



the uninhibited level. These results revealed an unanticipated versatility of BRC3/4-DBD in nucleating hRad51 onto ssDNA occupied by ssDNA-binding proteins.

DISCUSSION

We used a modular approach, by making polypeptides that harbor functional domains, to begin dissecting the HR function of the human tumor suppressor BRCA2. Our studies with the BRCA2-derived polypeptide BRC3/4-DBD unveiled two distinct roles in its interactions with the Rad51 recombinase: a ssDNA targeting role and also a recombination mediator role. We have provided strong evidence that both the BRC repeats and the DNA binding activity in BRC3/4-DBD are needed for functional interactions with Rad51. The activity of BRC3/4-DBD is specific for Rad51 as no restoration of homologous DNA pairing was seen with *E. coli* RecA. Interestingly BRC3/4-DBD could efficiently nucleate Rad51 onto SSB-covered ssDNA, in fact more so than when RPA-coated ssDNA was used. Based on these biochemical findings, we suggest that in homologous recombination reactions BRCA2 fulfills the two aforementioned functions documented for BRC3/4-DBD. This suggestion is congruent with 1) earlier cytological data indicating that BRCA2 is needed for the assembly of DNA damage-induced nuclear Rad51 foci believed to correspond to nucleoprotein filaments of Rad51 on the recombination (ssDNA) substrate (15) and 2) the recent demonstration of a recombination mediator activity in the *U. maydis* Brh2 protein (16), a BRCA2-like molecule. However, it remains to be established whether Brh2 also serves to target Rad51 to ssDNA as we have shown for BRC3/4-DBD herein.

Certain aspects of our results warrant further comments and analysis. First, we note that an amount of BRC3/4-DBD substantially below that of Rad51 is sufficient for ssDNA targeting of the latter and also recombination mediator activity, indicative of a catalytic mode of action. Second, over the same concentration range of BRC3/4-DBD used in the biochemical experiments, the fusion of BRC3 and BRC4 neither stimulates nor suppresses the Rad51 recombinase, thus eliminating the possibility that it serves a negative regulatory function as previously suggested by Davies *et al.* (13). Interestingly we found that BRC3/4-DBD enhances the basal homologous DNA pairing activity of hRad51 K133R, which binds but does not hydrolyze ATP (26, 28), while slightly attenuating the hRad51 activity. Because ATP hydrolysis promotes the turn-

over of hRad51 from ssDNA, the presynaptic filament made with hRad51 K133R is much more stable than that consisting of the wild type protein. Because complexes of the Rad51 paralogs may serve to stabilize the presynaptic filament (3, 29), it seems possible these complexes could work in conjunction with BRCA2 to synergistically enhance the assembly and stability of the presynaptic filament.

Several important issues concerning the HR function of BRCA2 remain. BRCA2 contains eight BRC repeats, and future studies will determine whether these repeats in isolation can shepherd Rad51 to the ssDNA substrate and whether they do so more efficiently when present in tandem. In addition to the BRC repeat, the carboxyl-terminal region of BRCA2 (residues 3215–3418) also harbors a Rad51 interaction domain (8, 30). Recently it was shown that phosphorylation of serine 3291 in this Rad51 interaction domain reduces its ability to associate with Rad51 (11). The function of this carboxyl-terminal domain remains to be defined. BRCA2 associates with a 70-residue acidic polypeptide called DSS1, and the interaction site lies within the DNA binding domain of BRCA2 (12, 31). DSS1 appears to be critical for the stability of BRCA2 (32), but whether it also affects the functional properties of BRCA2 has not yet been determined. The assays and experimental strategies described herein should be valuable for addressing these outstanding issues.

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