A region of human BRCA2 containing multiple BRC repeats promotes RAD51-mediated strand exchange

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ABSTRACT

Human BRCA2, a breast and ovarian cancer suppressor, binds to the DNA recombinase RAD51 through eight conserved BRC repeats, motifs of ~30 residues, dispersed across a large region of the protein. BRCA2 is essential for homologous recombination in vivo, but isolated BRC repeat peptides can prevent the assembly of RAD51 into active nucleoprotein filaments in vitro, suggesting a model in which BRCA2 sequesters RAD51 in undamaged cells, and promotes recombinase function after DNA damage. How BRCA2 might fulfill these dual functions is unclear. We have purified a fragment of human BRCA2 (BRCA2BRC1–8) with 1127 residues spanning all 8 BRC repeats but excluding the C-terminal DNA-binding domain (BRCA2 CTD). BRCA2BRC1–8 binds RAD51 nucleoprotein filaments in a ternary complex, indicating it may organize RAD51 on DNA. Human RAD51 is relatively inefficient in vitro at strand exchange between homologous DNA molecules unless non-physiological ions like NH₄⁺ are present. In an ionic milieu more typical of the mammalian nucleus, BRCA2BRC1–8 stimulates RAD51-mediated strand exchange, suggesting it may be an essential co-factor in vivo. Thus, the human BRC repeats, embedded within their surrounding sequences as an eight-repeat unit, mediate homologous recombination independent of the BRCA2 CTD through a previously unrecognized role in control of RAD51 activity.

INTRODUCTION

BRCA2, a tumour suppressor whose inactivation is associated with hereditary breast and ovarian cancer predisposition, is essential for DNA repair in mammalian cells (1–3). BRCA2-deficient cells are defective in the repair of DNA double-strand breaks by error-free homologous recombination (4), allowing error-prone repair processes to create gross chromosomal re-arrangements that may promote carcinogenesis (2). The role of BRCA2 in homologous recombination has been linked to its functions in the regulation of RAD51, a RecA-related recombinase that forms the nucleoprotein filaments on damaged DNA that are crucial to recombinational repair (5,6). BRCA2 binds directly to RAD51 through 6 of the 8 BRC repeats, ~30 amino acid motifs encoded within the central exon 11 region of all known mammalian BRCA2 genes (7–9). This feature distinguishes mammalian BRCA2 molecules from simpler eukaryal homologues such as Brh2 in Ustilago maydis, which contains a single BRC repeat (10).

Available data concerning the nature and consequences of the interaction between human BRCA2 and RAD51 mediated by the BRC repeats raise an important but unresolved question. On the one hand, peptides encoding isolated BRC repeats such as BRC3 when present in excess can prevent the assembly of RAD51 into nucleoprotein filaments on DNA substrates, and can dissolve pre-formed RAD51 complexes (11). A structural basis for this apparently inhibitory activity is suggested by the fact that BRC repeats can bind to RAD51 by mimicking its mode of self-association; thus preventing multimerization and dispersing existing multimers (12). Conversely, however, there is evidence that isolated BRC repeat peptides under certain conditions can bind to the RAD51 nucleoprotein filament, rather than disrupting it...
Thus, despite the apparently inhibitory effects of isolated BRC repeats, we show here that purified BRCA2 BRC1–8 can promote recombination (15). Studies on the dynamics of functional GFP–RAD51 fusion proteins in the nuclei of living cells before and after DNA damage offer support for this view (16), although the lack of direct biochemical evidence that human BRCA2 is a recombination mediator has been noted as an important hiatus in current understanding (17).

How can BRCA2–RAD51 complexes delivered to sites of DNA damage operate to promote repair by homologous recombination? Whilst the function of the C-terminal domain of BRCA2 in recombination can be rationalized by its ability to bind DNA and stimulate RAD51-mediated strand exchange in vitro through the displacement of the single-strand DNA-binding protein RPA (18), the role of the BRC repeat-containing region is less clear, given the ability of isolated BRC repeat peptides to both disrupt filaments (11,12) and bind to them (13), depending on the experimental conditions. These studies—and indeed, most previous work on the biochemical function of the BRC repeats—have utilized isolated BRC repeat peptides. However, all mammalian BRCA2 molecules contain eight conserved BRC repeats in the exon 11 region (Figure 1A).

Several features of the organization of the exon 11 region, shared across mammalian evolution, point to the fact that isolated BRC repeat peptides may not accurately reflect the biological activities of the eight BRC repeats embedded within the context of surrounding sequences in exon 11. First, although the exon 11 sequence that flanks the BRC repeats is poorly conserved even in close mammalian species, the spacing that it provides between consecutive BRC repeats has been well conserved in evolution (9). Second, there is a considerably higher degree of sequence conservation between the same BRC repeat of different mammalian species than between different BRC repeats of one species (9,19). This raises the possibility that during evolution, diverse selective pressures have been placed on the BRC repeats at different sites in exon 11, with each BRC repeat adopting an idiosyncratic function (5,19). This is supported by evidence of differences in the functional characteristics of BRC3/BRC4 and BRC7 in vitro (11,13). However, the practical difficulties of producing either full-length mammalian BRCA2 (>3000 amino acids) or the BRC repeat-containing exon 11 region (>1000 amino acids) in a form suitable for experimentation, have meant that experiments have thus far been devoted to studying the function of isolated BRC repeats rather than the more physiologically relevant collective function of the eight BRC repeats within the context of the exon 11 region.

To address this, we have purified a 1127 amino acid fragment of human BRCA2 that contains all 8 BRC repeats (BRCA2BRC1–8). We show here that purified BRCA2BRC1–8 forms a ternary complex with RAD51 and DNA, and is capable of promoting RAD51-mediated strand exchange in vitro. Thus, despite the apparently inhibitory effects of isolated BRC repeats, within the framework provided by the exon 11 region, BRC repeats function to stimulate DNA recombination. This novel activity provides direct biochemical evidence that BRCA2 functions as a mediator of homologous recombination in mammalian cells.

**MATERIALS AND METHODS**

**Protein expression, purification and peptide synthesis**

A region of human BRCA2 corresponding to a 1127 amino acids fragment between residues 987 and 2113 of the encoded protein was cloned into a PET28a vector (Novagen) and expressed in Rosetta (DE3) competent cells (Novagen). Cells were lysed in 20 mM Tris–HCl, pH 8.0, 300 mM NaCl and 5 mM DTT containing protease inhibitors (Roche); the insoluble pellet was clarified and washed with 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 1% Triton X-100 and 5 mM DTT containing protease inhibitors (Roche). The insoluble material was solubilized in 50 mM Tris–HCl, pH 8.0, 8 M urea, 5 mM DTT and purified under denaturing conditions by anion exchange using Q Sepharose HP resin (Amersham) with elution over a NaCl gradient. The pH of the eluted sample was reduced by dialysis in 50 mM sodium acetate, pH 5.0, 8 M urea and 5 mM DTT and purification was completed by cation exchange using SP Sepharose HP resin (Amersham) with elution over a NaCl gradient. Urea used in the purification was freshly deionized by passage over AG 501–X8 (D) resin (Bio-Rad) in order to prevent protein carbamylation. The BRCA2 fragment was renatured by dialysis in 50 mM Tris–HCl, pH 8.0, 500 mM l-arginine and 10 mM DTT, and was subsequently transferred in to 20 mM Tris–HCl, 10% glycerol and 5 mM DTT by thorough dialysis. The sample was concentrated using an Amicon Ultra-4 centrifugal filter device and stored at −80°C.

Full-length human RAD51 was cloned into a PET11d vector (Novagen), expressed in recA cells BLR (DE3) competent cells (Novagen), and purified by a method described previously (20). Recombinant human RPA protein was made using the expressing vector p11d-rRPA (gift from Mark Wold) and purified in Dr Richard Wood’s lab (Cancer Research UK Clare Hall Labs) as described previously (21).

Protein concentrations were estimated by UV absorbance at 280 nm (Varian Cary 50 spectrophotometer) with extinction coefficients estimated by ProtParam (22).

The BRC4 repeat peptide (KEPTLLGFHTASGKKVKTA-KESLDKVKNLFDEKEQ) was synthesized at the Cancer Research UK Peptide Synthesis Unit, London. Stock solutions of the peptides dissolved in dH2O and stored at −20°C were diluted appropriately for strand exchange assays.

**Co-immunoprecipitation**

Protein A–Sepharose 6 M (50 µl) (Sigma) was incubated with 20 µg rabbit polyclonal anti-RAD51 IgG (Santa Cruz Biotech) or with 20 µg non-immune rabbit serum IgG for 3 h at 4°C in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1% Triton X-100. The resulting complexes were then incubated
with the purified BRCA2 fragment (0.9 μM) and/or purified human RAD51 (0.9 μM) in the presence of 10 mg/ml BSA (Sigma) in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 at 4°C for 3 h. The complexes were thoroughly washed in buffer containing 500 mM NaCl and subsequent pellets were suspended in loading buffer for analysis by SDS–PAGE (Invitrogen).

Co-immunoprecipitation reactions were also performed using anti-BRCA2 antibodies (Ab-1; Calbiochem) through the method described above but using 2 μg antibodies and with 10 nM BRCA2_{BRC1–8} and 1 nm, 10 or 100 nM RAD51. Further reactions were performed using 2 μg anti-BRCA2 antibodies with 0.1 μM BRCA2_{BRC1–8} and 0.05, 0.1, 0.5 or 1.0 μM RAD51. These reactions were visualized by western blot; samples were run on an SDS–PAGE gel (Invitrogen) and transferred on to nitrocellulose membrane (Invitrogen) at 25 mV for 90 min using transfer buffer 20 mM Tris, 150 mM glycine, 1% SDS and 20% methanol. The membrane was blocked in 50 ml blocking buffer [phosphate-buffered saline (PBS), 0.05% Tween and 10% milk powder] for 1 h at room temperature. It was then incubated with 2 μg mouse monoclonal anti-BRCA2 antibodies or 2 μg rabbit polyclonal anti-RAD51 IgG (Santa Cruz Biotech) in 20 ml blocking buffer for 1 h, before being washed three times in PBS and 0.05% Tween, each for 10 min. The membrane was then incubated with 2 μg alkaline phosphatase conjugated...
Circular dichroism spectroscopy

The BRCA2<sub>BRC1–8</sub> sample was prepared for CD analysis by dialysis into 10 mM phosphate buffer (pH 7.5) and concentration was confirmed by absorbance at 280 nm, measured on a Varian Cary 50 spectrophotometer. CD spectra were obtained using a JASCO J-810 CD spectrometer. A native far UV CD spectrum was recorded over the range 185–260 nm using a 0.16 mg/ml sample in a cuvette of path length 0.05 cm. A denatured far UV CD spectrum was also recorded of a 0.16 mg/ml sample in the presence of 6 M guanidine–HCl (Fluka). CD data were converted from theta machine units to mean residue ellipticity and were deconvoluted using the CDSSTR algorithm of the DichroWeb server (23,24).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (13) either by pre-incubating RAD51 and BRCA2<sub>BRC1–8</sub> before incubation with radiolabelled DNA, or by adding BRCA2<sub>BRC1–8</sub> to pre-formed RAD51-DNA complexes. In the first instance, RAD51 (5 μM) was pre-incubated in the absence or presence of varying concentrations of BRCA2<sub>BRC1–8</sub> for 15 min at 37°C in Buffer A (see Strand Exchange Assay) containing KCl to a final concentration of 200 mM and 1 mM AMP-PNP. ApaL1 linearized<sup>32</sup>P-labelled dsX174 DNA (10 μM) was added, and the reaction was incubated at 37°C for a further 60 min. In the second instance, RAD51 (5 μM) was first incubated at 37°C for 5 min, before 10 μM of ApaL1 linearized<sup>32</sup>P-labelled dsX174 DNA was added, and the protein–DNA complex allowed to form for 10 min at 37°C before the addition of varying concentrations of BRCA2<sub>BRC1–8</sub>. Reactions were incubated at 37°C for 60 min. Control reactions with DNA alone, or DNA plus BRCA2<sub>BRC1–8</sub> were incubated for 75 min at 37°C. The protein–DNA complexes were resolved by 0.5% agarose gel electrophoresis in TAE buffer. The dried gels were exposed to Phosphoimager screens and data processed on the Fuji FLA-5000 scanner.

RESULTS

The expression and purification of BRCA2<sub>BRC1–8</sub>

A 1127 amino acid fragment of human BRCA2 corresponding to amino acid residues 987–2113 and spanning all 8 BRC repeats (BRCA2<sub>BRC1–8</sub>; Figure 1A) was expressed in Escherichia coli using a pET28a vector (Figure 1B). The 126 kDa protein was found to be highly over-expressed in the form of inclusion bodies. The BRCA2<sub>BRC1–8</sub> polypeptide was recovered from the insoluble fraction and purified under denaturing conditions through anionic and cationic exchange chromatography (Figure 1B). Once purified, the denatured sample was renatured in the presence of the refolding additive L-arginine; thereafter the BRCA2<sub>BRC1–8</sub> protein was found to be soluble and stable in standard protein buffers, thus allowing its use in biochemical investigations. The sequence of the purified BRCA2<sub>BRC1–8</sub> protein was confirmed and shown by mass spectrometry to be unaffected by possible chemical alteration due to the high concentrations of denaturant (data not shown). The protein was found to be functional in RAD51 binding through the co-immunoprecipitation of a RAD51–BRCA2<sub>BRC1–8</sub> complex using either anti-RAD51 (Figure 1C) or anti-BRCA2 (Figure 1D) antibodies. The RAD51-binding capacity of BRCA2<sub>BRC1–8</sub> was tested through RAD51–BRCA2<sub>BRC1–8</sub> co-immunoprecipitation using anti-BRCA2 antibodies over molar ratios from 0.5:1 up to 10:1 (Figure 1E). An increasing amount of RAD51 was co-immunoprecipitated between 0.5:1 and 5:1 molar.
ratios, with binding saturation observed between 5:1 and 10:1 molar ratios. This indicates the presence of multiple RAD51-binding sites, with each BRCA2_BRC1–8 molecule binding several RAD51 molecules.

Biophysical features of BRCA2_BRC1–8

In order to investigate the conformation of BRCA2_BRC1–8, the structure of the purified protein was probed by circular dichroism spectroscopy (Figure 2A). Far UV spectra comparing renatured and denatured samples of BRCA2_BRC1–8 revealed that the renatured sample does contain secondary structural elements including both α-helices and β-sheets (Figure 2B). Indeed, the secondary structure makeup of BRCA2_BRC1–8 was further defined through the deconvolution of a far UV spectrum over the range 185–240 nm. The sample was found to consist of 9% α-helix, 33% β-sheet, 24% turns and 34% random coil (data were fitted with normalized r.m.s.d. of 0.031). The finding that the BRCA2_BRC1–8 protein consists of 42% secondary structural elements and 58% turns or random coil suggests that whilst it contains some structured regions that are potentially of functional significance, it is also intrinsically flexible with much unordered sequence. It is important to note that the sequence of the exon 11 region displays in general only a moderate degree of conservation amongst closely related BRCA2 orthologues. Whilst the BRC repeats are themselves well conserved, the intervening sequences between consecutive repeats are remarkably poorly conserved. However, the length of the spacing they provide between BRC repeats is preserved. This is consistent with a portion of the BRCA2 exon 11 sequence constituting intrinsically unstructured flexible linkers rather than structured regions in their own right. The structural features of the renatured BRCA2_BRC1–8 protein are thus in keeping with the predicted nature of this region of human BRCA2 (5).

BRCA2_BRC1–8 forms a ternary complex with RAD51 and DNA

Isolated BRC repeats can either prevent RAD51 nucleoprotein filaments (11) or form a ternary complex with RAD51 on DNA (13). We tested the ability of BRCA2_BRC1–8–RAD51 complexes to bind DNA, or the effect of BRCA2_BRC1–8 on RAD51–DNA complexes, by EMSA. As expected, radiolabelled linear φX174 dsDNA (Figure 3, lane 1) was retarded in its mobility by addition of RAD51, marking formation of a nucleoprotein filament (lane 2). Interestingly, pre-formed BRCA2_BRC1–8–RAD51 complexes induce a further, concentration-dependent retardation in the mobility of the radiolabelled dsDNA (lanes 3–9). An apparent plateau in retardation occurs at >2.8 μM of BRCA2_BRC1–8 that may reflect saturation of binding. Addition of increasing amounts of BRCA2_BRC1–8 to pre-formed RAD51–DNA complexes (lanes 10–16) reveals a similar retardation in the mobility of the radiolabelled dsDNA. Retardation peaks at 1.7–3.3 μM BRCA2_BRC1–8, with a slight decrease in retardation at 3.9 μM, whose significance could not readily be ascertained, because the BRCA2_BRC1–8 fragment could not be purified at higher concentrations. We speculate, however, that this biochemical feature could reflect the proposal that BRCA2 may not only deliver RAD51 for filament formation at sites of recombinational repair, but also help to dissolve RAD51 filaments following the completion of repair (16).

Further evidence for ternary complex formation comes from affinity chromatography (Figure 4). A short, 73 bp
biotinylated-ssDNA oligonucleotide was annealed to a homologous circular 5386 bp φX174 ssDNA molecule in order to create a biotinylated substrate, which predominantly represents a ssDNA circle (Figure 4A). RAD51 and BRCA2BRC1–8 (either alone or together, as indicated) were then incubated with the DNA substrate. Proteins bound to the DNA-joint molecule (labelled ‘b’ in the figure) were isolated by affinity chromatography on magnetic-streptavidin beads, resolved by SDS–PAGE, and revealed by protein staining. Unbound proteins left behind in the supernatant (labelled ‘s’ in the figure) were compared with proteins bound to the DNA-joint molecules.

As expected, RAD51 alone binds efficiently to DNA-joint molecules (Figure 4B, lanes 1 and 2), marking the formation of a nucleoprotein filament. In the absence of RAD51, the majority of BRCA2BRC1–8 remains in the supernatant.
(Figure 4B, lane 3), whilst a small amount of BRCA2_{BRC1–8} binds to the DNA substrate (Figure 4B, lane 4). Since similar levels of BRCA2_{BRC1–8} bind to the streptavidin beads alone (data not shown), BRCA2_{BRC1–8} pull-down in this case likely reflects a non-specific interaction with the beads rather than DNA binding. Importantly, when RAD51 is present, the majority of BRCA2_{BRC1–8} is brought down with the DNA-joint molecule (Figure 4B, compare lanes 3 and 4 with 5 and 6). Ternary complex formation between BRCA2_{BRC1–8} and RAD51 and DNA was observed over a range of nucleotide/protein concentration ratios, including 1:1 (data not shown), 2:1 and 3:1 (Figure 4C), at which RAD51 has been shown previously to perform strand exchange in vitro (26).

This conclusion is further substantiated by the concentration-dependent binding of BRCA2_{BRC1–8} to a fixed amount (5 µM) of the RAD51–DNA-joint molecule complex formed at a nucleotide/protein concentration ratio of 1:1 (Figure 4D). At 1 µM concentration, BRCA2_{BRC1–8} is entirely bound to the RAD51–DNA complex (compare lanes 2 and 6), further confirming the results in Figure 4B. Increasing amounts of BRCA2_{BRC1–8} are pulled down with the RAD51–DNA complex, reaching saturation at ~3 µM (compare lanes 3 and 7 versus 4 and 8). This is similar to the results of EMSA shown in Figure 3B, where saturation of binding occurs at 2.8–3.9 µM. Each BRCA2_{BRC1–8} molecule contains multiple BRC repeats potentially capable of binding RAD51 with varying affinities (8,12). Thus, the saturation we observe as the concentration of BRCA2_{BRC1–8} is increased could reflect intermolecular competition for binding to limiting amounts of RAD51, with only those BRC repeats in each BRCA2_{BRC1–8} molecule that have the highest affinity for RAD51 able to bind when the BRCA2_{BRC1–8}/RAD51 molar ratio approaches 1:1.

Taken together, the findings we have presented in Figures 3 and 4 provide direct evidence that the multiple BRC repeat region of BRCA2 can form a ternary complex with RAD51 on DNA rather than disrupting it. This is in contrast to the apparently inhibitory effect of individual BRC repeats on RAD51 nucleoprotein filament formation ([11] and data not shown), suggesting that function of BRC repeats is modified and directed when present as an eight-repeat unit within the context of the exon 11 region.

BRCA2_{BRC1–8} promotes RAD51-mediated strand exchange

The recombination function of RAD51 may be demonstrated through its ability to perform strand exchange between circular ssDNA and linearized dsDNA (radiolabelled and non-radiolabelled) in the presence of ssDNA-binding protein, RPA (25–27) (Figure 5A). The strand exchange assay is now well established as a method of demonstrating recombination in vitro, and has been used to study the recombination functions of all RAD51 recombinases. Interestingly, whilst E.coli RecA and yeast RAD51 are both highly active in recombination, mammalian RAD51 is a relatively poor recombinase when isolated in vitro (28–32). Clearly, co-factors present in mammalian cell nuclei are required to promote RAD51-mediated recombination that are not necessary for the recombinase function of yeast and bacterial RAD51 homologues.

In order to study RAD51-mediated strand exchange outside its normal cellular environment, buffer systems have been developed that optimally stimulate RAD51 recombinase activity in vitro (25,33,34). Importantly, the addition of different ionic species stimulates the recombinase activity of mammalian RAD51. It has been reported that the inclusion of either (NH₄)₂SO₄ or Ca²⁺ in the reaction buffer enhances RAD51-mediated strand exchange in vitro (25,33,34). This has provided a useful tool for investigating the recombinase activity of mammalian RAD51, which would otherwise be difficult to study. Consistent with these previous reports, we find that in the presence of ammonium sulphate, RAD51 mediates strand exchange in a concentration-dependent manner with respect to DNA (Figure 5B and C) that is absolutely dependent on RPA as has been reported previously (25).
Similarly, RAD51 is effective at strand exchange in the presence of 0.3–0.6 mM Ca$^{2+}$, also as reported previously (data not shown).

However, the conditions used in these in vitro assays for RAD51-mediated strand exchange do not conform to the ionic milieu found within the mammalian cell nucleus, in which K$^+$ and Mg$^{2+}$ are the predominant ionic species (35–38). Indeed, there is structural evidence that K$^+$ may modulate the ATPase activity of an archaeal homologue of RAD51 (39). Accordingly, we performed experiments under approximately physiological conditions (200 mM K$^+$ and 1 mM Mg$^{2+}$), in the absence of ammonium or calcium ions, and in the presence of an ATP regenerating system (34). The concentration of RPA in the reaction was also adjusted so that the need for the BRCA2 CTD, which is required to displace RPA, was obviated (18,40).

At ssDNA/RAD51 ratio of 3:1, RAD51 was found to weakly stimulate strand exchange, as has been reported previously (Figure 6A, lane 1) (34). However, upon the addition of BRCA2$_{BRC1-8}$, RAD51-mediated strand exchange was robustly stimulated in a dose-dependent manner, with the formation of joint molecule and complete recombination products in the form of nicked circles (Figure 6A, lanes 2–4). BRCA2$_{BRC1-8}$ stimulated strand exchange when present at one-fifth of the concentration of RAD51 (Figure 6A, lane 2). Efficient formation of a ternary complex with RAD51 and DNA is observed at a non-saturating concentration of BRCA2$_{BRC1-8}$ (Figure 4D, lane 6) which would suggest that the binding of RAD51 is thus likely to involve multiple BRC repeats of each BRCA2$_{BRC1-8}$ molecule. Stimulation of strand exchange by BRCA2$_{BRC1-8}$ is also clearly dependent upon the presence of Mg$^{2+}$ and ATP (Figure 6B).

We observe a stimulatory effect of $\sim$3 $\mu$M BRCA2$_{BRC1-8}$ on RAD51-mediated strand exchange, equating to $\sim$24 $\mu$M of single BRC repeats. However, under the same conditions, an equivalent concentration of isolated BRC4 peptide inhibits, rather than promotes, strand exchange (Figure 6C), consistent with its ability to inhibit RAD51 nucleoprotein formation [(11) and data not shown]. This suggests that the eight BRC repeats, when organized in the context of the BRCA2$_{BRC1-8}$ fragment, may possess an activity not shared with isolated BRC peptides. However, given the differences in sequence between the different BRC repeats in human BRCA2, which are poorly characterized in terms of function, further investigation is warranted.

Another ion found in the mammalian nucleus is Ca$^{2+}$, which is reportedly present at concentrations of $\sim$0.1 $\mu$M free Ca$^{2+}$ (41), although higher local concentrations might be achieved during biological signalling. Although Ca$^{2+}$ alone at 0.3–0.6 mM can stimulate RAD51-mediated strand exchange in vitro [(33) and data not shown], we find that when physiological concentrations of K$^+$ and Mg$^{2+}$ are also present, RAD51 activity is relatively low (Figure 6D, compare lane 1 with lanes 2–5). However, under these conditions, a sub-stoichiometric amount of BRCA2$_{BRC1-8}$ provided a substantial stimulus to the strand exchange reaction (Figure 6D, compare lane 6 with lanes 7–10). Thus, in an ionic milieu approaching the physiological conditions of the mammalian nucleus, we find that whilst recombinase activity of RAD51 diminishes, the level of stimulation achieved by BRCA2$_{BRC1-8}$ greatly increases.

We have demonstrated that, at the 3:1 DNA/RAD51 ratio used in the strand exchange assays, BRCA2$_{BRC1-8}$ forms a ternary complex involving RAD51 and ssDNA (Figures 3 and 4), which plausibly represents the species active in stimulating strand exchange. However, to rule out the possibility that BRCA2$_{BRC1-8}$ promotes strand exchange by sequestering RAD51 away from the linear dsDNA used in these reactions, we examined the ability of RAD51 to promote strand exchange—in the absence or presence of an increasing concentration of BRCA2$_{BRC1-8}$—at different DNA/RAD51 ratios ranging from 1:1 to 5:1 (Figure 6E). If BRCA2$_{BRC1-8}$ were simply serving as a sink for free RAD51, this experiment should reveal an increase in strand exchange by RAD51 alone as the DNA/RAD51 ratio increases. This does not occur. Instead, we find that BRCA2$_{BRC1-8}$ stimulates strand exchange best when the DNA/RAD51 ratio is between 1:1 and 3:1, the same optimum reported with RAD51 alone, but under different ionic conditions in which species such as NH$_4^+$ (25) and Ca$^{2+}$ (33) substitute for BRCA2$_{BRC1-8}$.

Collectively, our findings have two important implications. First, they highlight an interesting difference between the high-RAD51 activity under the relatively non-physiological conditions so far used in vitro and its markedly diminished capacity to mediate strand exchange in ionic conditions more typical of the mammalian nucleus. We infer from our work that in vivo, additional co-factors such as BRCA2 are essential for efficient homologous recombination mediated by RAD51. This is entirely consistent with the cellular effects of BRCA2 inactivation on DNA repair or chromosomal integrity (1–3), and on homology-mediated gene conversion (4,42).

Moreover, the experiments we report here demonstrate a role for the multi-BRC repeat region conserved in all mammalian BRCA2 molecules, which is independent of the BRCA2 CTD. The DNA-binding binding BRCA2 CTD has been shown to stimulate RAD51-mediated strand exchange in vitro; its role is believed to involve a displacement of RPA from single-stranded DNA in order to allow RAD51 to bind (18,40,43). However, we find that when RPA concentrations are lowered to obviate the need for its displacement by the BRCA2 CTD, a previously unrecognized activity of BRCA2$_{BRC1-8}$ in directly promoting RAD51-mediated strand exchange is revealed. Thus, our findings also establish a biological function for the evolutionarily conserved region of BRCA2 containing the eight BRC repeats.

**DISCUSSION**

In this paper we describe a significant advance in understanding the role of mammalian BRCA2 in homologous recombination. We have expressed and purified a 1127 amino acid region of human BRCA2 containing all 8 BRC repeats (BRCA2$_{BRC1-8}$), and have demonstrated that as a collective unit, the multiple BRC repeats can associate in a ternary complex with RAD51 on ssDNA and dsDNA. Ternary complex formation and the stimulation of RAD51-mediated strand exchange occur at sub-stoichiometric concentrations of BRCA2$_{BRC1-8}$ relative to RAD51, indicating that each BRCA2$_{BRC1-8}$ molecule probably binds several molecules of RAD51.
Figure 6. Stimulation of RAD51-mediated strand exchange by BRCA2 BRC1–8. (A) Stimulation of RAD51-mediated strand exchange by BRCA2 BRC1–8 in the presence of KCl and Mg²⁺. Reactions were carried out using ssDNA and RAD51 at a ratio of 3:1. RAD51 was pre-incubated without (lane 1) or with increasing concentrations of BRCA2 BRC1–8 (lanes 2–4) for 5 min before adding ssDNA and RPA (0.26 μM). After a further 5 min incubation, radiolabelled linear dsDNA (10 μM) was added and incubated for a further 60 min. The reactions steps were incubated at 37°C. The strand exchange products were analysed by gel electrophoresis and phosphoimager. (B) Stimulation of RAD51-mediated strand exchange by BRCA2 BRC1–8 is dependent on Mg²⁺ and ATP. Reactions were carried out as described in (A) either in the absence of both co-factors Mg²⁺ and ATP (lanes 1–4), in the absence of ATP but presence of Mg²⁺ (lanes 5–8) or in the presence of both co-factors (lanes 9–12). BRCA2 BRC1–8 was present as indicated. (C) Effect of BRCA2 BRC1–8 (lane 2) or isolated BRC4 peptide (lane 3) on RAD51-mediated strand exchange when BRC repeats are present at equivalent molarities. Reactions were carried out as in (A). The first lane shows a reaction without added proteins. (D) The effect of BRCA2 BRC1–8 on RAD51-mediated strand exchange is not dependent on physiologically relevant Ca²⁺ concentrations. Reactions are as described in (B) except that RAD51, either in the absence (lanes 1–5) or presence of BRCA2 BRC1–8 (2 μM; lanes 6–10), was incubated with buffer containing no Ca²⁺ ions (lanes 1 and 6) or an increasing concentration of Ca²⁺ (lanes 2–5 and 7–10). (E) BRCA2 BRC1–8 stimulates RAD51-mediated strand exchange at different DNA/RAD51 ratios. Reactions are as described in (A).
We have further shown that BRCA2 BRC1–8 stimulates RAD51-dependent strand exchange under physiologically relevant conditions in which human RAD51 shows only poor recombinase activity. Crucially, our description of a novel function for the BRCA repeat region of mammalian BRCA2, does not depend on the BRCA2 CTD. Previous work had shown stimulation of strand exchange by the Mus musculus BRCA2 CTD, the U.maydis BRCA2 homologue Brh2 containing a CTD and single BRC repeat and the human BRCA3–4 fused to a CTD (18,40,43). Our results establish BRCA2 BRC1–8 as a co-factor necessary to promote RAD51-dependent homologous recombination under physiological conditions resembling those present in the nuclei of living cells, consistent with the known importance of BRCA2 to homologous recombination in vivo.

Our data are consistent with a model (11,16) inferred from the observations that BRCA2 is complexed to RAD51 even before DNA damage (44) and is required to target RAD51 to sites of DNA damage (2,14,16) protected by RPA (45) in preparation for homologous recombination (Figure 7). Thus, BRCA2 is expected to function both in sequestering RAD51 to prevent promiscuous filament formation in undamaged cells, and in targeting and promoting RAD51’s recombinase function at sites of DNA damage, perhaps as a result of activation by post-translational modifications (15,16). At sites of damage, BRCA2 is likely to perform multiple functions in promoting RAD51-mediated homologous recombination (Figure 7). The displacement of RPA by the BRCA2 CTD may arrange the exon 11-BRC repeat-bound RAD51 molecules in a manner conducive to their loading on to damaged DNA for nucleoprotein filament formation (Figure 7). Indeed, whilst the in vitro strand exchange reaction presented here does not depend on the DNA-binding BRCA2 CTD, it is likely that in vivo, the CTD helps, not only to target the active BRCA2–RAD51 complex to junctions between ssDNA and dsDNA (18) but also to orientate the complex with respect to the polarity of the resected double-strand break.

Our proposal for the function of the multiple BRC repeats within the exon 11 region is supported by the finding that under specific conditions, BRCA3 and BRCA4 can associate with a nucleoprotein filament without causing its disruption (13). Given the known antagonism between the BRC repeat–RAD51 interaction and RAD51 oligomerization, this might conceivably be explained by BRC repeats possessing two modes of RAD51 binding, one that is inhibitory to filament formation (12) and another that is compatible with filament formation (13), reflecting the duality of the function of BRCA2 in regulating RAD51 activity during homologous recombination (11,15). RAD51 repeats may thus interact with a RAD51 filament as a ternary complex if present in sub-stoichiometric amounts, but then disrupt the filament through their inhibitory binding mode if present in excess (11,13,40). The large exon 11 sequence that houses the eight BRCA repeats would thus be expected to control the binding mode of the BRCA repeats, allowing them to form a ternary complex with RAD51 and DNA, as described in this study.

However, it is also possible that the inhibition and the stimulation of RAD51 activity by BRCA2 reflect only a single mode of BRC repeat–RAD51 binding, and not two distinct modes. One major difficulty with the hypothesis of a second mode of binding of the BRC repeat to RAD51 (13) lies in the observation that the amino acid conservation within BRC repeats is already accounted for by the known inhibitory interaction between BRC repeats and RAD51 through mimicry of self-association (12,19,46). It would therefore be surprising for a second RAD51-binding mode to involve a different mode of binding. The large exon 11 sequence that houses the eight BRCA repeats would thus be expected to control the binding mode of the BRCA repeats, allowing them to form a ternary complex with RAD51 and DNA, as described in this study.

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to have emerged for BRC repeats with a similar pattern of sequence conservation as that of the first binding mode.

We therefore favour an alternative explanation for the stimulation of RAD51-mediated strand exchange by BRCA2, which does not invoke a second binding mode. We envision that the BRC repeats of BRCA2 BRC1–8 bind RAD51 in only one mode, inhibitory of RAD51 oligomerization, with each BRC repeat capping one self-association surface of RAD51, but permitting filament growth from its opposite surface (Figure 7 circled inset). The resulting nucleoprotein filament would thus consist of multiple filaments separated by inter-leaved BRC repeats. Indeed, the long exon 11 sequence between adjacent BRC repeats would allow for considerable growth of each capped filament. It is important to note, however, that our results are consistent with either of these structural possibilities explaining the novel activity of BRCA2 BRC1–8 in promoting RAD51-mediated strand exchange.

In summary, we have established a unique tool for research into mammalian homologous recombination through the expression and purification of BRCA2 BRC1–8, a fragment of BRCA2 containing all eight BRC repeats. We report that within the context of the wider sequence provided by BRCA2 BRC1–8, the eight BRC repeats exert a previously unrecognized, collective function in promoting RAD51-mediated strand exchange through ternary complex formation, independent of the BRCA2 C-terminal domain. Thus, our work provides direct biochemical evidence that human BRCA2 can function as a recombination mediator—a critical but unresolved issue (17). The identification of a mechanism for the stimulation of RAD51-mediated recombination by the multiple BRC repeat-containing region of human BRCA2 provides an important insight into the process of recombinational DNA repair in mammalian cells.

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