RAD51 Interacts with the Evolutionarily Conserved BRC Motifs in the Human Breast Cancer Susceptibility Gene brea2*

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Recent work has shown that the murine BRCA2 tumor suppressor protein interacts with the murine RAD51 protein. This interaction suggests that BRCA2 participates in DNA repair. Residues 3196–3232 of the murine BRCA2 protein were shown to be involved in this interaction. Here, we report the detailed mapping of additional domains that are involved in interactions between the human homologs of these two proteins. Through yeast two-hybrid and biochemical assays, we demonstrate that the RAD51 protein interacts specifically with the eight evolutionarily conserved BRC motifs encoded in exon 11 of brea2 and with a similar motif found in a Caenorhabditis elegans hypothetical protein. Deletion analysis demonstrates that residues 98–339 of human RAD51 interact with the 59-residue minimal region that is conserved in all BRC motifs. These data suggest that the BRC repeats function to bind RAD51.

Germline mutations in the brea1 and brea2 tumor suppressor genes account for approximately 5–10% of all breast cancer cases (1–4). In addition, deleterious alleles of brea1 or brea2 are responsible for almost all familial ovarian cancer, and deleterious alleles of brea2 are involved in hereditary male breast cancer (1–4). Currently the mechanism of action of these two genes remains largely undefined. The brea1 and brea2 genes encode large proteins, 1863 and 3418 amino acids, respectively (1, 3). A search of the public sequence data bases has revealed little sequence homology to previously identified proteins. However, analysis of the protein sequence has revealed several statistically significant repeated motifs in these genes (5, 6). For example, eight internal repeats, known as BRC motifs, are found clustered in exon 11 of the human BRCA2 protein. These motifs are not found in BRCA1 but are conserved in all mammalian BRCA2 proteins that have been sequenced. A similar motif is also present in the hypothetical protein encoded by the Caenorhabditis elegans gene T07E3.5 (5, 7).

A number of studies have been conducted in the past year to elucidate the biological roles of the brea1 and brea2 genes.

Knock-outs of the mouse homologs of these genes indicate that they are essential during development. The ablation of either brea1 or brea2 results in an embryonic lethal phenotype characterized by failure of proliferation during approximately days 6–8 of gestation (8, 9). Levels of RNA expression from brea1 and brea2 are coordinately regulated during proliferation and differentiation in mammary epithelial cells (11, 12). Furthermore, their expression varies at different stages of the cell cycle, with RNA levels peaking at the G1/S boundary (13, 14). These findings provide preliminary evidence that these breast cancer genes participate in a common functional pathway. Three independent studies have now established that the RAD51 DNA repair protein is linked to the BRCA1 and BRCA2 pathways (9, 10, 15). Immunoprecipitation experiments reveal that RAD51 forms a complex with BRCA1, and immunofluorescence analysis shows that both proteins are co-localized to the synaptonemal complex of mouse meiotic chromosomes (15). The direct interaction of murine RAD51 with murine BRCA2 has been demonstrated in a yeast two-hybrid assay (9, 10). The loss of interaction of these two proteins probably accounts for the hypersensitivity to γ irradiation that is observed in mouse embryos deficient in BRCA2 (9). rad51, a homolog of the Escherichia coli recA gene, is known to function in recombination and DNA repair (16, 17). It is currently unclear how the interaction of RAD51 with either BRCA1 or BRCA2 affects its function. Alteration of normal DNA repair function may lead to genomic instabilities that eventually contribute to tumorigenesis.

We have conducted yeast two-hybrid searches using various segments of the human brea2 gene as “baits” and identified a number of interacting proteins, one of which is RAD51. To further understand the structural and functional relationships between human BRCA2 and RAD51, we map the minimal regions of the human BRCA2 protein that mediate binding to RAD51. Specifically we show that RAD51 interacts with the eight BRC motifs of the human BRCA2 protein. These sites of interactions are distinct from those that have been previously reported for the murine BRCA2 and RAD51 proteins.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—Overlapping fragments of BRCA2 cDNA were ligated into the Gal4p DNA-binding domain vector pGBT.C and the Gal4p activation domain vector pGAD.C (18) (see Table I). Homologous recombination in yeast was also used to create a number of fusion constructs (19). BRCA2-Gal4p DNA-binding domain fusions were co-transformed into the yeast strain J692 with activation domain libraries from B-cell, liver, testis, and breast (20, 21). The yeast transformants were plated on yeast minimal media lacking tryptophan, leucine, and histidine and containing 25 μM 3-amino-1,2,4-triazole. After incubation for approximately 8 days at 30 °C, β-galactosidase activity was determined by a filter assay (22).

Templates for the eight BRC motifs of the human BRCA2 protein and the BRC motif encoded by C. elegans T07E3.5 were generated by PCR amplification using the following primer pairs: motif 1F, GGGAATTC-CCAGAAAAAAATAATGTTACATGAAAC; motif 1R, GGTTCGACCT-GTAAATTTTGCAGATACAGTTAATTTG; motif 2F, GGGAATTCCT-GTTGAAAATAATGTTACATGAAAC; motif 2R, GGTTCGACCATTA-TTTGGTTATATCAGTTGGCATTTATTA; motif 3F, GGGAATTCTCAA-GAGAAAATATACTGCTACTAA; motif 3R, GGGTCGACTTGATCTTCAGAAAAATAATGATTACATGAACAA; motif 4F, GGTTCGACCT-GTAAATTTTGCAGATACAGTTAATTTG; motif 4R, GGTTCGACCATTA-TTTGGTTATATCAGTTGGCATTTATTA; motif 5F, GGTTCGACCTGTCGATACACGATACATGAAAC; motif 5R, GGTTCGACCATTA-TTTGGTTATATCAGTTGGCATTTATTA; motif 6F, GGTTCGACCTGTCGATACACGATACATGAAAC; motif 6R, GGTTCGACCATTA-TTTGGTTATATCAGTTGGCATTTATTA; motif 7F, GGTTCGACCTGTCGATACACGATACATGAAAC; motif 7R, GGTTCGACCATTA-TTTGGTTATATCAGTTGGCATTTATTA; motif 8F, GGTTCGACCTGTCGATACACGATACATGAAAC; motif 8R, GGTTCGACCATTA-TTTGGTTATATCAGTTGGCATTTATTA.

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RAD51 Interacts with BRC Motifs

were washed extensively and released by heating to 90 °C in an equal
gels, were used in each assay. The protein complexes bound to the beads
proteins, as determined by Coomassie Blue staining of aliquots run on
Sande and Privalsky (25). Equal amounts of GST and GST fusion
with the radiolabeled protein was performed according to the protocol of
saline with 0.1% Triton X-100. The binding assay of GST fusion protein was
in vitro (Stratagene). The transformants were induced by isopropylthiogalacto-
binding domain fusion were co-expressed in yeast cells. The
in vitro -galactosidase filter assays are summarized in Ta-
In Vitro Protein-Protein Interaction Assay—Template for making radiolabeled proteins were either from PCR-generated DNA fragments or recombinant plasmids. The primers T7-forward (TAATACGAC-
engineered as activation domain fusions and RAD51 was engineered as a DNA-binding domain fusion. After co-transforma-
were cloned into both pGBT.C and pGAD.C using standard techniques (23). Similarly, various truncated segments of RAD51 were created by PCR for subcloning. The sequence of all fusion constructs were verified by sequencing. In
In Vitro Protein-Protein Interaction between BRCA2 and RAD51—In vitro biochemical assays provide an alternative means to investigate protein-protein interaction. Different segments of RAD51 protein, residues 1–44, 1–95, and 98–339, cloned as glutathione S-transferase fusions, were examined for in vitro association with radiolabeled BRCA2. The BRCA2 segment encompassing residues 1912–2126 was first used to optimize the in vitro assay. It was found that this region interacts with GST-RAD98–339 but not with nonrecombinant GST, GST-
and Adh-act-reverse (CCCTACATCATCATGCAGTATCTACGATTCA-
TAGATCTCTG) specific for the pGAD.C vector were used to produce PCR templates of the BRC motifs and all other inserts that were fused to the Gal4p activation domain (24). The T7-forward primer contains the T7 RNA polymerase promoter. The PCR DNA templates were transcribed in vitro by T7 RNA polymerase using the Ribomax Kit (Promega) and translated in vitro in the presence of [35S]methionine using rabbit reticulocyte lysate (Promega). In some experiments, the Single Tube Protein System 2 from Novagen was used to synthesize radiolabeled proteins.

GST fusion protein constructs were made by cloning the PCR prod-
ucts containing amino acid residues 1–44, 1–95, or 98–339 of human RAD51 and residues 1912–2126 of BRCA2 into the vector pGEX (Pharmacia Biotech Inc.). All GST and GST fusion con-
structs were transformed into E. coli BL21(DE3)pLysS competent cells
(Stratagene). The transformants were induced by isopropylthiogalacto-
pyranoside, harvested, and lysed by sonication in phosphate-buffered saline with 0.1% Triton X-100. The binding assay of GST fusion protein with the radiolabeled protein was performed according to the protocol of Sande and Privalsky (25). Equal amounts of GST and GST fusion proteins, as determined by Coomassie Blue staining of aliquots run on gels, were used in each assay. The protein complexes bound to the beads were washed extensively and released by heating to 90 °C in an equal volume of 2 × SDS-PAGE loading buffer. The radiolabeled proteins were resolved by SDS-PAGE and visualized using the Molecular Dynamics Storm PhosphorImager System (Molecular Dynamics).

RESULTS

Interaction of the Internal Region of BRCA2 with RAD51—The yeast two-hybrid system provides a powerful approach for identifying protein-protein interactions in vivo. In our initial experiments, we cloned the DNA segment of brca2 encoding amino acid residues 1912–2126 as a Gal4p DNA-binding do-
main fusion (Table I). This clone was used as a "bait" to search for sites for RAD51 interaction. To test this hypothesis, each

| Binding domain plasmid (pGST.C) | Activation domain plasmid (pGAD.C) | β-Galactosidase activity |
|---------------------------------|-----------------------------------|-------------------------|
| aa 196–386                      | RAD51                              | White                   |
| aa 272–379                      | RAD51                              | White                   |
| aa 361–667                      | RAD51                              | White                   |
| aa 444–479                      | RAD51                              | White                   |
| aa 570–715                      | RAD51                              | White                   |
| aa 736–1253                     | RAD51                              | Blue                    |
| aa 1253–1708                    | RAD51                              | Dark Blue               |
| aa 1708–2099                    | RAD51                              | Dark Blue               |
| aa 1912–2126                    | RAD51                              | Blue                    |
| aa 2683–2870                    | RAD51                              | White                   |
| aa 2706–3014                    | RAD51                              | White                   |
| aa 2758–2996                    | RAD51                              | White                   |
| aa 736–1253                     | No Insert                          | White                   |
| aa 1253–1708                    | No Insert                          | Light Blue              |
| aa 1708–2099                    | No Insert                          | Light Blue              |
| aa 1912–2126                    | No Insert                          | White                   |
BRC motifs and RAD51 were cloned into either pGBT.C or pGAD.C. The numbers refer to amino acid residue positions in the BRCA2 protein or the C. elegans gene T07E3.5 (accession number U13643). Blue and white colors indicate a positive and negative interaction, respectively. The diagram indicates the position of each BRC motif, 1–8, in the BRCA2 protein, which is 3418 amino acids in length. The exon 11 boundaries are at the indicated amino acid positions 638 and 2280.

**Fig. 1. In vitro binding of BRC motifs to RAD51.** Segments of RAD51 (amino acid residues 1–44, 1–95, and 98–339) were fused to GST. Similar amounts of GST and GST fusion proteins immobilized on glutathione-Sepharose beads (100 μl) were incubated with 15 μl of in vitro translated 35S proteins. A, BRC2 fragment, residues 1912–2126. B, C. elegans motif, residues 8–113. C, BRC motif 3, residues 1404–1502. D, RAD51, residues 1501–1589. After washing four times with HEMO buffer (25), the beads were resuspended in 30 μl of 0.1% SDS-PAGE loading dye, boiled, and spun. 20 μl of the supernatant was resolved by SDS-PAGE. The input radiolabeled protein in each gel represents the control sample before incubation with GST or GST fusions.

**Table II**

| Binding domain (pGBT.C) | Activation domain plasmid (pGAD.C) | β-Galactosidase activity |
|------------------------|-----------------------------------|-------------------------|
| BRC 1 (987–1069)       | RAD51                             | White                    |
| BRC 2 (1198–1293)      | RAD51                             | White                    |
| BRC 3 (1407–1498)      | RAD51                             | Blue                     |
| BRC 4 (1501–1589)      | RAD51                             | Blue                     |
| BRC 5 (1649–1735)      | RAD51                             | White                    |
| BRC 6 (1822–1914)      | RAD51                             | White                    |
| BRC 7 (1955–2035)      | RAD51                             | Blue                     |
| BRC 8 (2006–2112)      | RAD51                             | Blue                     |
| C. elegans (8–113)     | RAD51                             | Blue                     |
| BRC 1, 2, 3, 4, 5, 6, 7, or 8 | No Insert | White |
| C. elegans             | No Insert                          | White                    |
| RAD51                  | BRC5                              | White                    |
| RAD51                  | BRC6                              | White                    |

**Table III**

Mapping of the minimal binding domain in BRC motif 3 and RAD51

| Binding domain plasmid (pGBT.C) | Activation domain plasmid (pGAD.C) | β-Galactosidase activity |
|---------------------------------|-----------------------------------|-------------------------|
| BRC 3 (1404–1502)              | RAD51 (98–339)                    | Blue                    |
| BRC 3                            | RAD51 (120–339)                  | White                   |
| BRC 3                            | RAD51 (150–339)                  | White                   |
| BRC 3                            | RAD51 (200–339)                  | White                   |
| BRC 3                            | RAD51 (250–339)                  | White                   |
| BRC 3                            | RAD51 (98–200)                   | White                   |
| BRC 3                            | RAD51 (98–150)                   | White                   |
| BRC 3                            | RAD51 (98–1432)                  | White                   |
| BRC 3                            | RAD51 (98–1443)                  | White                   |
| BRC 3                            | RAD51 (98–1453)                  | Blue                    |
| BRC 3                            | RAD51 (98–1483)                  | Blue                    |
| BRC 3                            | RAD51 (98–1502)                  | White                   |
| BRC 3                            | RAD51 (98–1542)                  | Blue                    |
| BRC 3                            | RAD51 (98–1562)                  | White                   |
| BRC 3                            | RAD51 (98–1572)                  | White                   |
| BRC 3                            | RAD51 (98–1592)                  | White                   |

**DISCUSSION**

The identification of components in the BRCA2 functional pathway should further our understanding of BRCA2 function and its role in tumorigenesis. Through yeast two-hybrid screens of human cDNA libraries, we identified RAD51 as a BRCA2-binding protein. Sharan et al. (9) previously demonstrated that the C-terminal region of murine BRCA2 interacts with the N-terminal portion of murine RAD51. We identified eight sites of interaction between these proteins. Specifically, the eight BRC motifs encoded in exon 11 of the human brca2 gene interact with RAD51. In addition, a similar motif found in a C. elegans hypothetical protein also interacts with RAD51. These data suggest that the BRC motifs function to bind RAD51.

Sequence comparison of the BRCA2 proteins from six mammalian species have revealed sequences conserved among the eight BRC motifs, which strongly implies functional significance (5, 7). Our findings that RAD51 binds to different protein sequences encoded by the 5-kilobase exon 11 of BRCA2 led us to examine whether the BRC internal repeats are the actual sites of interactions with RAD51. Because the amino acid sequences of these motifs are not identical, we analyzed each BRC motif individually to determine if they could associate with RAD51. All eight human BRC motif proteins showed interaction in biochemical assays, but only six of eight BRC motifs bound specifically to RAD51 in the yeast two-hybrid assays. This discrepancy in binding specificity might be caused by the different protein context of Gal4p and GST fusions. Subtle changes in the amino acid sequence of BRC motif 5 and 6 could have affected the proper folding of the predicted globular domain in these motifs in yeast.

Deletion analyses of the BRC motif 3 and RAD51 identified the minimal regions in both proteins that are required for binding. Our results indicate that the minimal region defined for the BRC motif 3 encompasses the entire consensus sequence that is highly conserved among mammalian species (7). Similarly, the minimal region of human RAD51, amino acid
residues 98–339, that interacts with human BRCA2 is conserved among prokaryotic and eukaryotic species. The homologous region in the E. coli recA protein has been demonstrated to contain ATPase activity and is involved in oligomer formation and recombination (26). The role of multiple RAD51-binding sites in BRCA2 is open to interpretation. It seems reasonable that BRCA2 might bind several molecules of RAD51 simultaneously and therefore might serve as a scaffold for RAD51 assembly during DNA repair and recombination. Alternatively, the multiple binding sites might play a role in the sequestration of active RAD51 or the facilitation of functional interactions of RAD51 with itself or other proteins. Because a similar BRC motif in a hypothetical protein from C. elegans is capable of binding to human RAD51, this same structural and functional relationship may hold in other eukaryotic species.

Using biochemical assays, we have confirmed that the association between RAD51 and BRCA2 is direct (10). In contrast, the binding of RAD51 to BRCA1 may be mediated through another protein in a complex (15). We have searched extensively for possible interactions between RAD51 and BRCA1 using the yeast two-hybrid assay, but so far without success (data not shown). It is interesting to note that the tumor suppressor gene p53 also associates with RAD51 (27). Together these data strongly suggest that RAD51 represents a crucial functional junction linking recombination and repair to cell cycle checkpoint signaling.

Our protein-protein interaction data, coupled with the previous observations that 1) brca2-null mouse embryos are hypersensitive to γ irradiation, 2) brca1 and brca2-null mouse embryos drastically overexpress p21 and suffer a failure of embryonic cell proliferation, and 3) there is a direct protein-protein interaction between RAD51 and p53, raise a critical question about the function of BRCA1 and BRCA2 (28–31). Is the primary deficit in brca1-null and/or brca2-null tumors a failure of DNA repair, or is it a failure of signal transduction associated with the need for DNA repair? The answer to this question could have bearings on the rationale for use of brca1 and brca2 as anti-tumor gene therapy agents in patients. The restoration of BRCA1 and BRCA2 function might stabilize genome integrity in the tumor without reversing the damages that have already occurred. In contrast, restoration of signal transduction might render the tumors more sensitive to apoptosis, a process that can be triggered by p53 or possibly chemotherapeutic agents. Further research is needed to address these possibilities.

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