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14. ABSTRACT: We demonstrated that the BRCA2-Rad51 interaction is crucial for HR repair and multiple regions of BRCA2 protein are involved in regulating HR repair. Using biochemical approaches, we found that BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C DNA repair proteins involving a strong interaction between BRCA2 and Rad51, and between Rad51B and Rad51C. We successfully expressed three BRC fragments using baculovirus expression system. However, the purification of these proteins was found to be difficult because these proteins were extremely unstable and tended to be degraded during the purification process. The investigation regarding whether the BRC1-4, BRC5-8 or BRC1-8 proteins affects the Rad51 activities are underway.
We found that, upon replication stresses, DNA-PKcs is phosphorylated and phosphorylated DNA-PKcs co-localizes with Brca1. The possible interaction between DNA-PKcs and Brca1 was confirmed in the co-immunoprecipitation (co-IP) analysis showing that DNA-PKcs could be co-precipitated with the α-Brca1 antibody. Furthermore, GST-Brca1 fusion proteins covering different region of Brca1 were mixed with HeLa nuclear extract followed by co-IP with α-DNA-PKcs antibody and western against α-GST antibody. The result showed that Brca1 fragment #5 may potentially interact directly with DNA-PKcs.

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INTRODUCTION

The BRCA2 gene is associated with hereditary tendency to breast cancer. Exactly how defects in BRCA2 causes predisposition to breast cancer is not yet understood. Recent evidence indicates that the BRCA2 protein has a critical function in DNA repair through homologous recombination (HR). It is very likely that defective HR repair causes the accumulation of unrepaired DNA in genome and results in cancers. We propose to investigate how BRCA2 functions in DNA HR repair using both cellular and biochemical approaches. The BRCA2 gene encodes a large protein of 3418 amino acids with a molecular weight of 384-kDa. The BRCA2 protein physically interacts with Rad51, the key protein in DNA HR repair via two Rad51-binding domains, eight BRC repeats and a extreme C-terminal region (amino acids 3196-3232). These eight conserved BRC repeats (designated as BRC1 to BRC8), located in the central portion of the protein and cover nearly a third of the protein. These two Rad51-binding domains of BRCA2 have been shown to be essential for normal sensitivity to DNA-damaging agents, indicating they are functionally significant. Therefore, we focus our efforts on investigation of the effects of these Rad51-interacting regions of BRCA2 in HR repair. The domain constructs used in our study are diagrammed in Fig. 1. **Our specific aims and final research results are summarized as the following:**

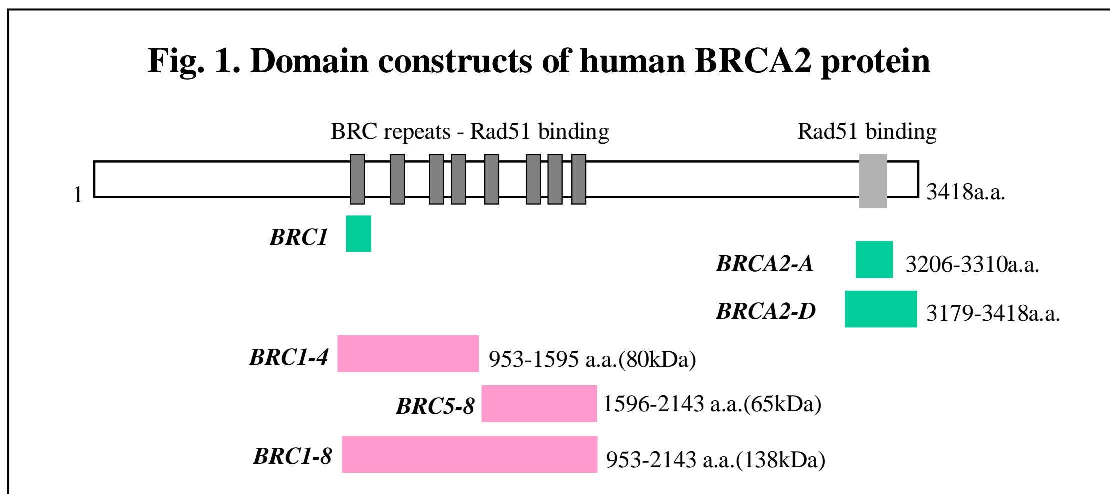


Fig. 1. Domain constructs of human BRCA2 protein

BODY

Specific aim 1. To test the hypothesis that disruption of the BRCA2-Rad51 interaction affects homologous recombination (HR) in human HT1080 cells. We propose that the human BRCA2 participates directly to homologous recombination through the interaction with Rad51 and regulates this process in some critical way, such that disruption of the BRCA2-Rad51 interaction results in a reduction in the capacity of repairing chromosome breaks by HR. We established a cellular approach to assay HR frequency *in vivo*. To measure HR, an artificial reporter locus was installed into a chromosome of human HT1080 cells and clones of cells that integrated the reporter were isolated. A defined DNA double-strand break can be introduced at the integrated reporter locus, by transient expression of a highly site-specific endonuclease I-SceI of *S. cerevisiae*. This reporter locus can detect repair of a specifically I-SceI induced chromosomal double-strand break by HR. The HT1080 cells harboring the reporter were therefore used for the HR assay.

Results:

Using this HR assay system, we investigated the effects of three BRCA2 regions, including a BRC1 motif and two C-terminal fragments (BRCA2-A and BRCA2-D). Fragment BRCA2-A consists of amino acids 3206-33310 and fragment BRCA2-D consists of amino acids 3179-3418. We found that transient expression of the BRC1 motif of BRCA2 during double-strand break induction in HT1080 cells caused a great suppression (~22-fold) as compared with the control (vector alone) in the frequency of HR (Fig. 2.). The result suggests that the BRCA2-Rad51 interaction through BRC1 is important for regulating HR repair, such that disruption of the normal protein-protein interaction between BRCA2 and Rad51 by overexpression of the small BRCA2 domain causes impaired HR. In addition, expression of fragment BRCA2-A or BRCA2-D in HT1080 cells resulted in a smaller but significant reduction in the HR frequency (Fig. 3.), indicating that the C-terminal region of BRCA2 also plays a role in the function of BRCA2 in HRR. Our results provide direct cellular evidence that the BRCA2-Rad51 interaction is crucial for HR repair and that multiple regions of BRCA2 protein are involved in regulating HR repair.

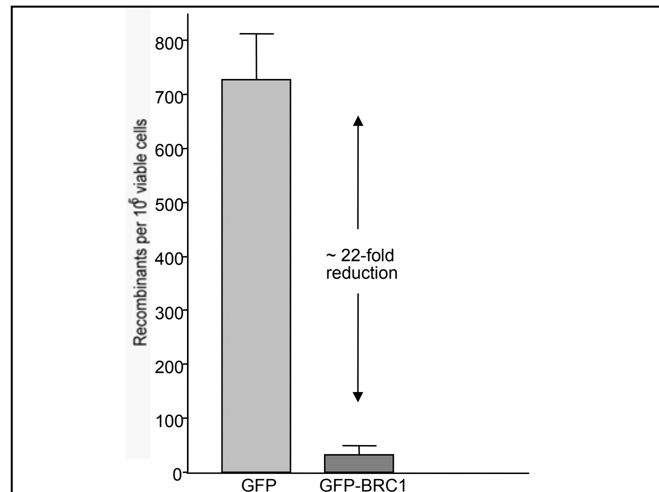


Fig. 2. Expression of BRC1 motif of BRCA2 greatly inhibits repair of a chromosome break by homologous recombination

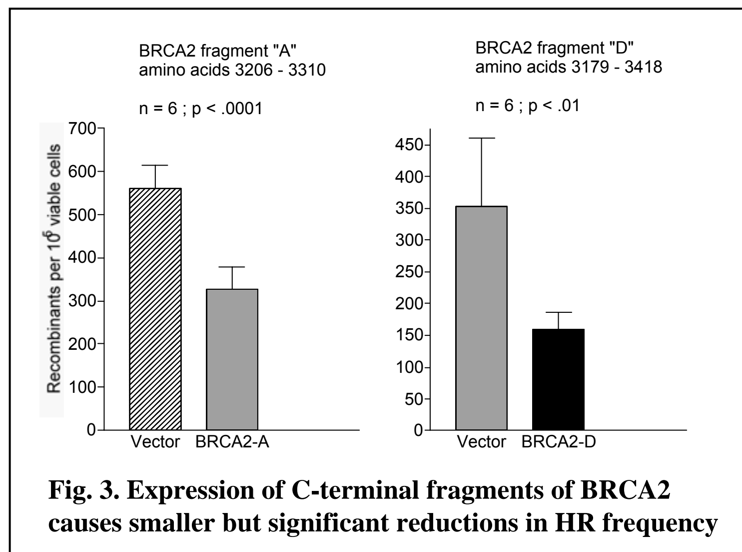


Fig. 3. Expression of C-terminal fragments of BRCA2 causes smaller but significant reductions in HR frequency

Specific aim 2. To test the hypothesis that the BRCA2 protein functions to mediate the biochemical activities of Rad51. It is not clear how the Rad51-BRCA2 interaction regulates DNA homologous recombinational repair. We hypothesize that BRCA2 directly mediates the biochemical activities of Rad51 and subsequently affects homologous recombination. To investigate the effects of BRCA2 protein on the Rad51 activities, we proposed to purify the BRCA2 protein and examine its effects on Rad51 using three biochemical assays, including DNA binding, ATPase and DNA strand exchange. Because the very large size of BRCA2 might hamper attempts to obtain the full-length protein, we decided to purify the functional domain of BRCA2, the BRC repeats. We proposed to express and purify three BRC fragments, including BRC1-4, BRC5-8 and BRC1-8. The purification of the human Rad51 protein is required for the assays as well.

Results on protein purification:

We have expressed the human Rad51 protein in insect cells Sf9 using the baculovirus system. The Rad51 protein was subsequently purified to homogeneity using spermidine precipitation and

sequential column chromatography with hydroxyapatite, Q-Sepharose, heparin affinity and MonoQ column (Fig. 4.). We have been able to obtain about 10 mg of the purified Rad51 protein during our study and this amount of the protein is sufficient for the biochemical assays. To obtain the BRCA2 protein, we constructed the BRC1-4, BRC5-8 and BRC1-8 fragments of BRCA2 (as diagrammed in Fig. 1.) into a 6xHis-tagged baculoviral vector. Employing the baculovirus strategies, we successfully expressed the three BRC fragments of BRCA2 in Sf9 cells. The BRC1-4, BRC5-8, and BRC1-8 domains encode the proteins of 80-kDa, 65-kDa, and 138-kDa, respectively. The identity of these proteins was confirmed by Western Blotting using either α-BRC4 or α-BRC5 antibody as well as α-His antibody (Fig. 5.). When these proteins were subjected to purification, we discovered that these BRC proteins were extremely unstable and tended to be degraded during the purification process. We have been working on several approaches to solve the problem. We tested several conditions to stabilize the proteins, including use of different salts, different concentration of salts, different expression temperature, and co-expression of each protein with Rad51. Although some of the methods improved the stability of the protein, none of them can ultimately produce a protein, which is stable enough for biochemical assays.

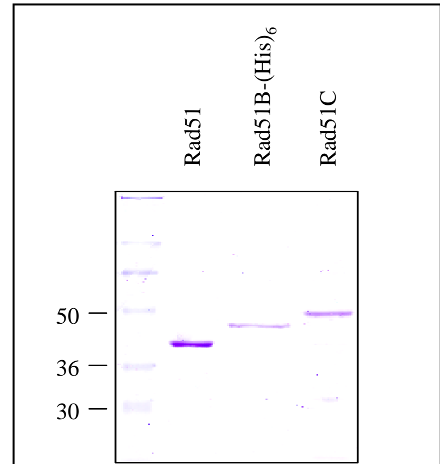


Fig. 4. Purified human Rad51, Rad51B and Rad51C proteins

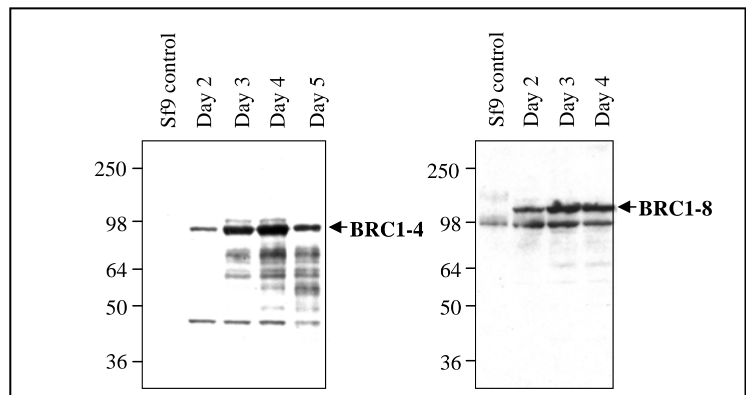


Fig. 5. Baculovirus expression of BRC1-4 and BRC1-8 fragments. Cells were harvested on days 2, 3, 4, 5 post-infection of baculoviruses

Results on activity assays:

We established three *in vitro* assays for Rad51 activity, including DNA binding, ATPase and DNA strand exchange. Using these biochemical assays, we examined the activity for Rad51 and Rad51 paralogs, Rad51B and Rad51C. We also examined the effects of small BRCA2 domains on the activity of Rad51.

1) DNA binding assay. We established a gel shift assay to determine the DNA binding activity using [^{32}P]-labeled oligonucleotides, including single-stranded DNA (ssDNA) 63mer, double-stranded DNA (dsDNA) 63/63mer, and 3'-tailed dsDNA 63/32mer. Using this assay, we have examined the DNA binding activity of Rad51 and two Rad51 paralogs, Rad51B and Rad51C. We demonstrated that Rad51B and Rad51C bind both ssDNA and dsDNA, and show preference for tailed dsDNA. We also compared the DNA binding capabilities of Rad51B and Rad51C with Rad51 using the same three DNA substrates: 63mer, 63/63mer and 63/32mer. We found that Rad51C possesses a higher affinity for all of three DNAs than does Rad51. The relative affinity of these three proteins for DNA was found to be: Rad51C > Rad51 > Rad51B.

2) ATPase assay. We established an *in vitro* ATPase assay using [γ-^{32}P]ATP as the substrates and have determined the ATPase activity of the Rad51, Rad51B and Rad51C proteins. We also

examined whether the BRC1 domain of BRCA2 has effects on the ATPase activity of Rad51. As shown in Fig. 6., various ratios of BRC1 and Rad51 were tested in the assay and an inhibitory effect on the ATPase activity of Rad51 was observed. We found that the ATPase activity of Rad51 was inhibited at approximately 4.0 fold with the molar ratio of BRC1 and Rad51 is equal to 2 and that the inhibitory effect was slightly increased as the BRC1/Rad51 ratio increases. The inhibitory effect of the BRC1 domain on the ATPase activity of Rad51 indicates that the BRC1 domain of BRCA2 is important for regulating the ATP binding and/or hydrolysis of Rad51.

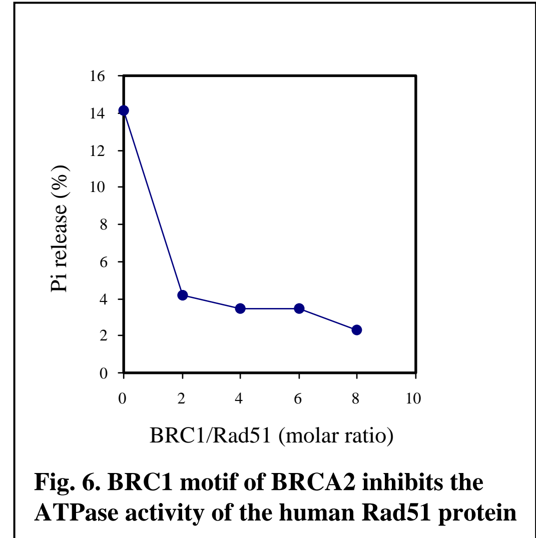


Fig. 6. BRC1 motif of BRCA2 inhibits the ATPase activity of the human Rad51 protein

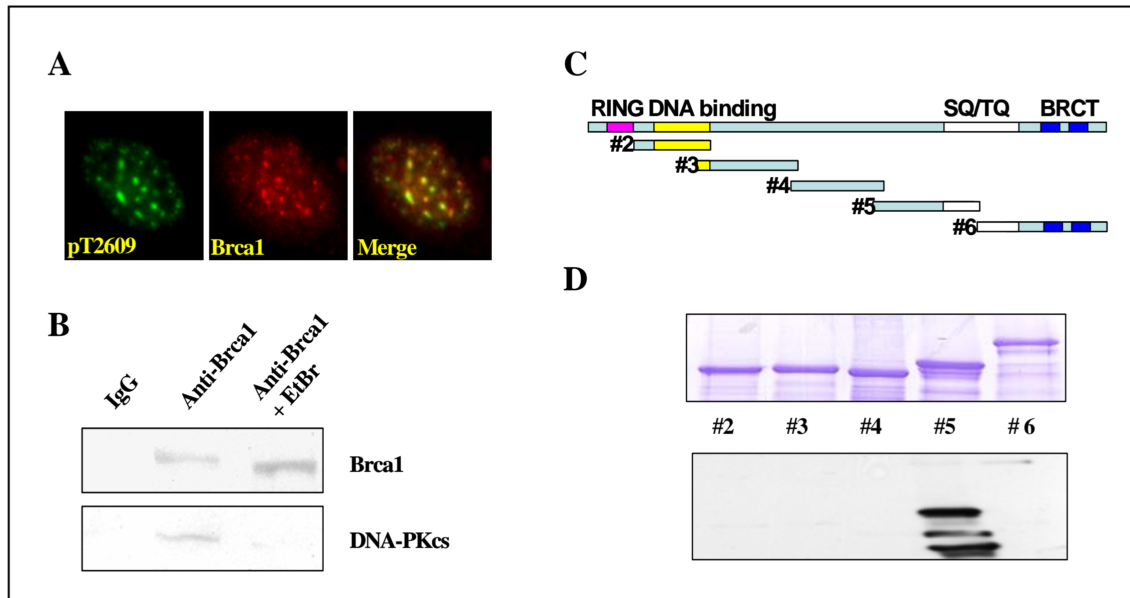
3) DNA strand exchange assay. The key biochemical activity of Rad51 in the recombinational DNA repair process is to promote strand pairing and exchange between two homologous DNA strands. It is important to investigate whether BRCA2 acts to mediate the DNA strand exchange activity of Rad51 and leads to effects on homologous recombination. We established a DNA strand transfer assay using ssDNA 63mers and [^{32}P]-labeled dsDNA 32/32mers as the substrates. The DNA strand transfer activity of Rad51 was determined as a control. The strand transfer products (3'-tailed 63/32mer) were observed and the amount of product formation is dependent on the concentration of Rad51. We also demonstrated that Rad51C displays apparent DNA strand transfer in an ATP-independent manner, while Rad51B shows no such activity.

New finding regards interaction of Brca1 and DNA-PKcs:

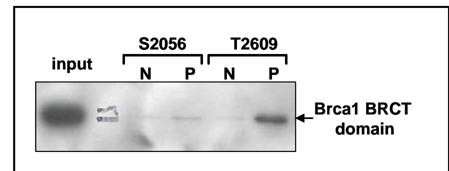
1) Interaction between Brca1 and DNA-PKcs

We have shown previously that, upon replication stresses, DNA-PKcs is phosphorylated at both T2609 and S2056, and phosphorylated DNA-PKcs co-localizes with the newly synthesized DNA (Chen et al., 2005). Additionally, we have found that phosphorylated DNA-PKcs also co-localizes with Brca1 under the same condition (Figure 7A). We hypothesize that DNA-PKcs may indeed interact with Brca1 upon replication stresses. The possible interaction between DNA-PKcs and Brac1 was confirmed in the co-immunoprecipitation (co-IP) analysis showing that DNA-PKcs could be co-precipitated with the α-Brca1 antibody (Figure 7B). To further analyze the interaction between DNA-PKcs and Brac1, GST-Brca1 fusion proteins covering different region of Brca1 (Figure 7C) were mixed with HeLa nuclear extract followed by co-IP with anti-DNA-PKcs antibody and western against anti-GST antibody. The result showed that Brca1 fragment #5 may potentially interact directly with DNA-PKcs (Figure 7D).

Fig. 7 Interaction between Brca1 and DNA-PKcs. (A) Co-localization of DNA-PKcs pT2609 foci and Brca1 upon replication stress. Camptothecin (Topo I inhibitor) induced pT2609 foci co-localize with Brca1 foci. **(B)** Co-IP of DNA-PKcs with anti-Brca1 monoclonal antibody (Ab1, Oncogene). **(C)** GST-Brca1 fusion constructs. **(D)** Purified GST-Brca1 fragments (top panel) and GST-Brca1 fragments co-precipitated with anti-DNA-PKcs antibody (25-4, NeoMarker).



2) BRCT domain expression and binding with DNA-PKcs S/TQ peptides. In the preliminary test, we have found that Brca1 BRCT domain is able to interact specifically with phospho-T2609 peptide column but not non-phosphoT2609 or phospho-S2056 peptide columns. We will test the binding of Brca1 BRCT domain to phospho-peptide columns covering other S/TQ motif within the T2609 phosphorylation cluster region. In addition, we will construct and express BRCT domains form various BRCT-containing molecules including MDC1, 53BP1, XRCC1, Rad9, and etc. We will analyze each individual BRCT domains and examine if they interact specifically with DNA-PKcs S/TQ phospho-peptides. Alternatively, we will collaborate with EMB core and determine the affinity of these BRCT domains to individual S/TQ phospho-peptides via BiaCore analysis.



KEY RESEARCH ACCOMPLISHMENTS

1. The BRC1 motif and two C-terminal fragments (amino acids 3206-3310 and 3179-3418) of the BRCA2 protein was shown to function in DNA repair via homologous recombinational (HR). Our results provide direct cellular evidence that BRCA2-Rad51 interaction is crucial for HR repair and that the multiple regions of BRCA2 protein are involved in regulating HR repair.
2. We found that BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C through a strong interaction between BRCA2 and Rad51 and a weak interaction between Rad51 and Rad51C. No direct interaction between the BRC repeats of BRCA2 and Rad51B (or Rad51C) was found.
3. The human Rad51 protein was expressed in insect cells and purified to homogeneity using spermidine precipitation and sequential column chromatography. The BRC1-4 and BRC1-8

fragments of BRCA2 were expressed. We discovered that these BRC proteins were extremely unstable and tended to be degraded during the purification process.

4. We showed that the BRC1 domain of BRCA2 suppresses the ATPase activity of human Rad51. The result indicates that the BRC1 domain of BRCA2 functions to modulate the ATP binding/hydrolysis of Rad51.

5. We found that, upon replication stresses, DNA-PKcs is phosphorylated and phosphorylated DNA-PKcs co-localizes with the newly synthesized DNA and also co-localizes with Brca1 under the same condition.

6. We hypothesize that DNA-PKcs may indeed interact with Brca1 upon replication stresses. The possible interaction between DNA-PKcs and Brac1 was confirmed in the co-immunoprecipitation (co-IP) analysis showing that DNA-PKcs could be co-precipitated with the α-Brca1 antibody.

7. To further analyze the interaction between DNA-PKcs and Brac1, GST-Brca1 fusion proteins covering different region of Brca1 were mixed with HeLa nuclear extract followed by co-IP with anti-DNA-PKcs antibody and western against anti-GST antibody. The result showed that Brca1 fragment #5 may potentially interact directly with DNA-PKcs.

8. In the preliminary test, we have found that Brca1 BRCT domain is able to interact specifically with phospho-T2609 peptide column but not non-phosphoT2609 or phospho-S2056 peptide columns.

CONCLUSIONS

Using an HR assay system, we found that individual expression of several small BRCA2 regions in human HT1080 cells causes a reduced frequency in homologous recombination. These results indicate that disruption of the normal BRCA2-Rad51 interaction by introducing the small BRCA2 fragments impairs homologous recombination. Our data provide the direct cellular evidence that the BRCA2-Rad51 interaction is crucial for HR repair and multiple regions of BRCA2 protein are involved in regulating HR repair. Using the baculovirus co-expression and Ni-NTA pull-down strategies, we demonstrated that BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C DNA repair proteins involving a strong interaction between BRCA2 and Rad51, and between Rad51B and Rad51C. A weak interaction between Rad51 and Rad51C was observed as well. We also found that the BRC repeats of BRCA2 do not directly interact with Rad51B or Rad51C. In addition, we aim to purify the Rad51-binding domain (BRC repeats) of BRCA2 and investigate its effects on Rad51 activities. We have successfully expressed three BRC fragments using baculovirus expression system, including BRC1-4, BRC5-8 and BRC1-8. These protein expressions were confirmed by Western analysis using specific antibodies. The purification of these proteins was found to be difficult because these proteins were extremely unstable and tended to be degraded during the purification process. We have tested several conditions to stabilize the proteins, including use of different salts, different concentration of salts, different expression temperature, and co-expression of the proteins with Rad51. We have established three biochemical assays for Rad51 activities, including DNA binding, ATPase and DNA strand exchange. We found that the BRC1 domain of BRCA2 inhibits the ATPase activity of Rad51, indicating a role for the BRC1 domain in modulating the ATP binding and/or hydrolysis activity of Rad51. The investigation regarding whether the BRC1-4, BRC5-8 or BRC1-8 proteins affects the Rad51 activities are underway.

We have shown previously that, upon replication stresses, DNA-PKcs is phosphorylated at both T2609 and S2056, and phosphorylated DNA-PKcs co-localizes with the newly synthesized DNA. Additionally, we have found that phosphorylated DNA-PKcs also co-localizes with Brca1 under the same condition. We hypothesize that DNA-PKcs may indeed interact with Brca1 upon replication stresses. The possible interaction between DNA-PKcs and Brac1 was confirmed in the co-immunoprecipitation (co-IP) analysis showing that DNA-PKcs could be co-precipitated with the α-Brca1 antibody. To further analyze the interaction between DNA-PKcs and Brac1, GST-Brca1 fusion proteins covering different region of Brca1 were mixed with HeLa nuclear extract followed by co-IP with anti-DNA-PKcs antibody and western against anti-GST antibody. The result showed that Brca1 fragment #5 may potentially interact directly with DNA-PKcs.

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