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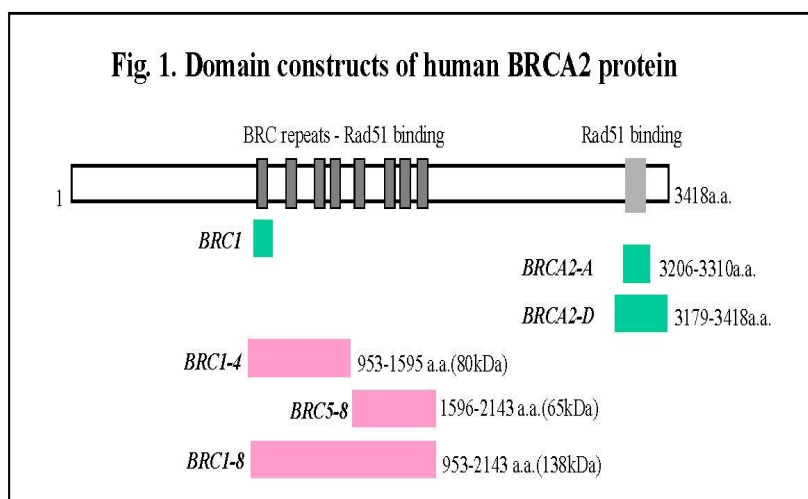
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14. ABSTRACT During this no cost extend funding period we concluded that BRCA2-Rad51 interaction is crucial for HR repair and multiple regions of BRCA2 protein are involved in regulating HR repair. A manuscript has been prepared for submission. In addition, 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. We plan to explore this direction in the future.					
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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.

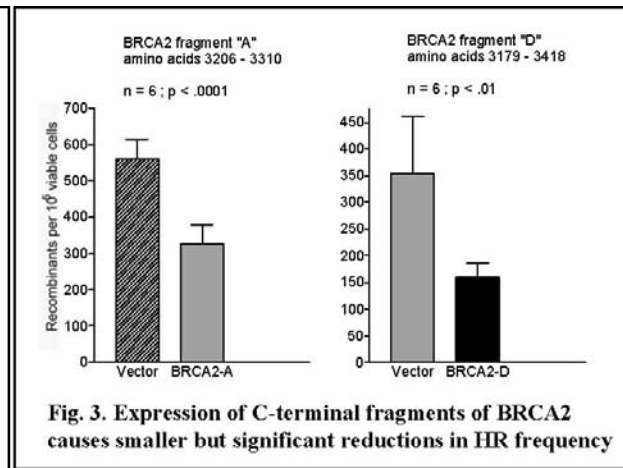
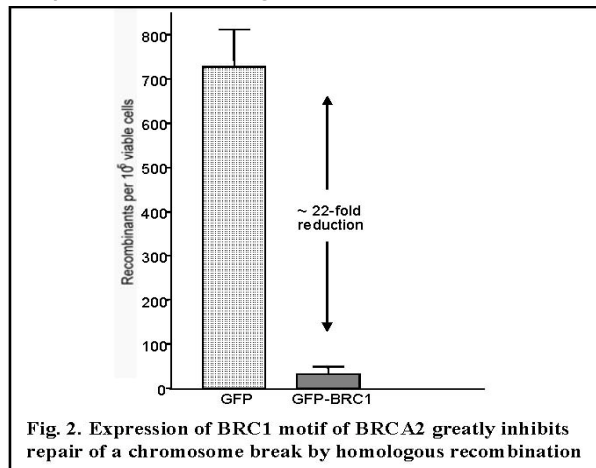


Body

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.

2. Interaction of BRCA1 and DNA-PK

Results: 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 4A). 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 4B). To further analyze the interaction between DNA-PKcs and Brac1, GST-Brca1 fusion proteins covering different region of Brca1 (Figure 4C) 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 4D).

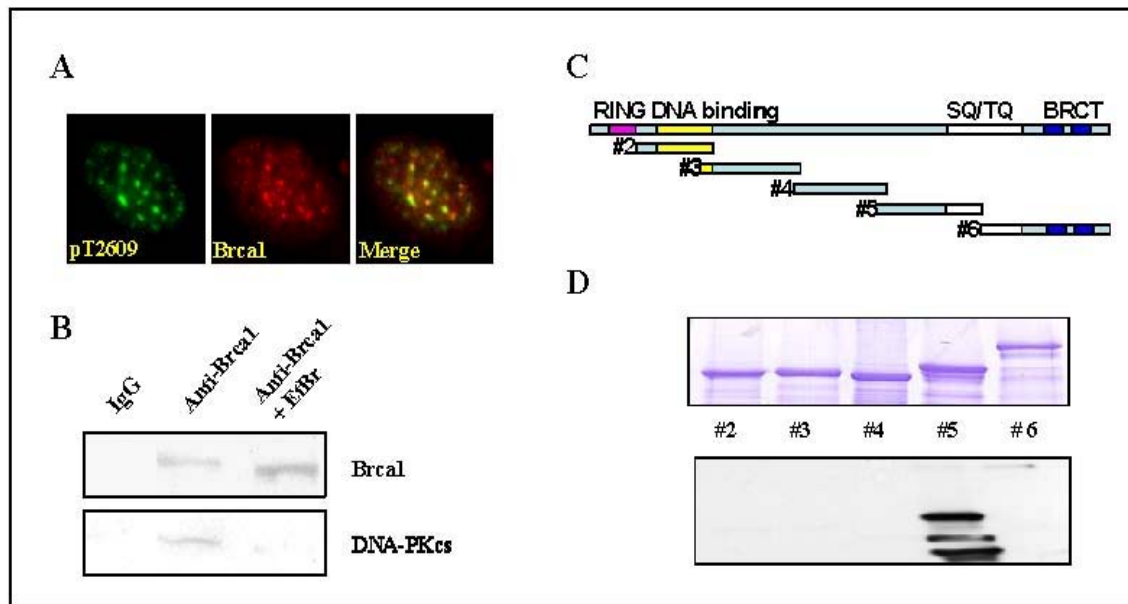
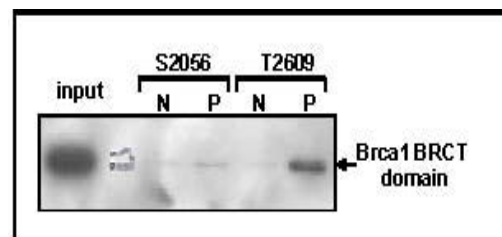


Fig. 4 Interaction between Brca1 and DNA-PKcs. (A) Co-localization of DNA-PKcs pT2609 foci and Brca1 upon replication stress. Camptothecin (Topo I inhibitor) induced pT2609 foci co-localize with Brca1 foci. (B) Co-IP of DNA-PKcs with anti-Brca1 monoclonal antibody (Ab1, Oncogene). (C) GST-Brca1 fusion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

3) 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 from 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 Achievement and Conclusion

During the course of the funding period of this project, 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.

During the last funding period, 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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