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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The Breast Cancer Susceptibility gene 1 (BRCA1) encodes an 1863-amino acid protein that plays a central role in the pathogenesis of hereditary breast cancer. The BRCA1 protein contains an N-terminal RING finger, a central region spanning residues 452-1079 that binds to four-way junction DNA, and two C-terminal BRCT domains, which are critical for BRCA1-mediated tumor suppression and are targets for cancer-causing mutations. This project focuses on the elucidation of the structural determinants of the BRCA1-DNA interaction, an essential step towards a deep understanding of the molecular mechanisms of BRCA1 function in DNA repair during cellular physiology and breast cancer pathogenesis. Specifically, the objective of this proposal is to determine the atomic structure of the central BRCA1 protein region in complex with four-way junction DNA, using X-ray crystallography. Numerous attempts to produce large amounts of soluble BRCA1(452-1079) protein in bacteria were unsuccessful. We therefore identified shorter BRCA1 protein fragments that overlap with BRCA1(452-1079), retain the ability to interact with four-way junction DNA, and can be produced in bacterial cells in a soluble form. These protein fragments are suitable for crystallization experiments with four-way junction DNA and these studies are currently in progress.

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INTRODUCTION

The Breast Cancer Susceptibility gene 1 (BRCA1) encodes an 1863-amino acid protein that plays a central role in the pathogenesis of hereditary breast cancer (1-3). Numerous studies have established that BRCA1 contributes significantly in the maintenance of the genomic stability through its involvement in homologous recombination and in transcription-coupled and double-stranded break DNA repair (1-3). To date, two domains of the BRCA1 protein have been studied extensively: the N-terminal RING finger-encompassing region (residues 1-103) and the C-terminal BRCT domains (residues 1650-1863) (1-3) (Figure 1A). A heterodimer consisting of the RING-encompassing region of BRCA1 and the RING domain of the BRCA1-associated protein BARD1 functions as a ubiquitin ligase *in vitro* (1-3). The BRCT domains interact with a number of cellular proteins involved in transcriptional regulation, DNA repair, and cell-cycle control. Importantly, the BRCA1 region spanning amino acids 452-1079 binds to branched DNA (4). This project focuses on the elucidation of the structural determinants of the BRCA1-DNA interaction, an essential step towards a deep understanding of the molecular mechanisms of BRCA1 function in DNA repair and transcription during cellular physiology and breast cancer pathogenesis. Specifically, the objective of this proposal is to determine the atomic structure of the BRCA1(452-1079) protein region both in the unbound state and in complex with branched DNA, using X-ray crystallography.

BODY

During the one-year award period we proceeded with the experiments proposed in the Statement of Work. A description of our progress in these studies follows below.

Task 1. To crystallize and determine the crystal structure of the BRCA1(452-1079) protein region. To crystallize the BRCA1 region that contains the DNA-binding domain (DBD) it is necessary to produce large amounts (multi-milligram quantities) of this BRCA1 region in bacterial cells as a soluble protein and purified it to homogeneity. The original report that identified the DNA-binding property of BRCA1 mapped grossly this function in the 452-1079 region (4). This means that this protein fragment encompasses the DNA-binding domain but the exact borders of this domain remain unknown. Having only this piece of information, we initiated experiments for the expression, purification, and crystallization of this protein region. Toward this goal, the DNA fragment encoding the human BRCA1 region 452-1079 was amplified by the polymerase chain reaction (PCR) method using the human BRCA1 cDNA as a template and cloned it following standard protocols (5) into pET-6H2, a modified pET-16b prokaryotic vector (Novagen) that allows the expression of recombinant hexahistidine-tagged protein in *Escherichia coli* cells, as we described previously (6). Numerous attempts to produce large amounts of soluble BRCA1(452-1079) protein in many *E. coli* strains using this vector in a wide range of temperatures and in different culture media were unsuccessful. Therefore, we also cloned the DNA fragment coding for BRCA1(452-1079) into the following prokaryotic expression vectors: a) pGEX-2 (Pharmacia) to produce it as a fusion with glutathione S-transferase (GST); b) pET-43a (Novagen) to produce it as a fusion with NusA; c) pMAL-c2 (New England BioLabs) to produce it as a fusion with maltose-binding protein (MBP); and d) pTYB12 (NEB) to produce it as an intein fusion. The resulting constructs were verified by DNA

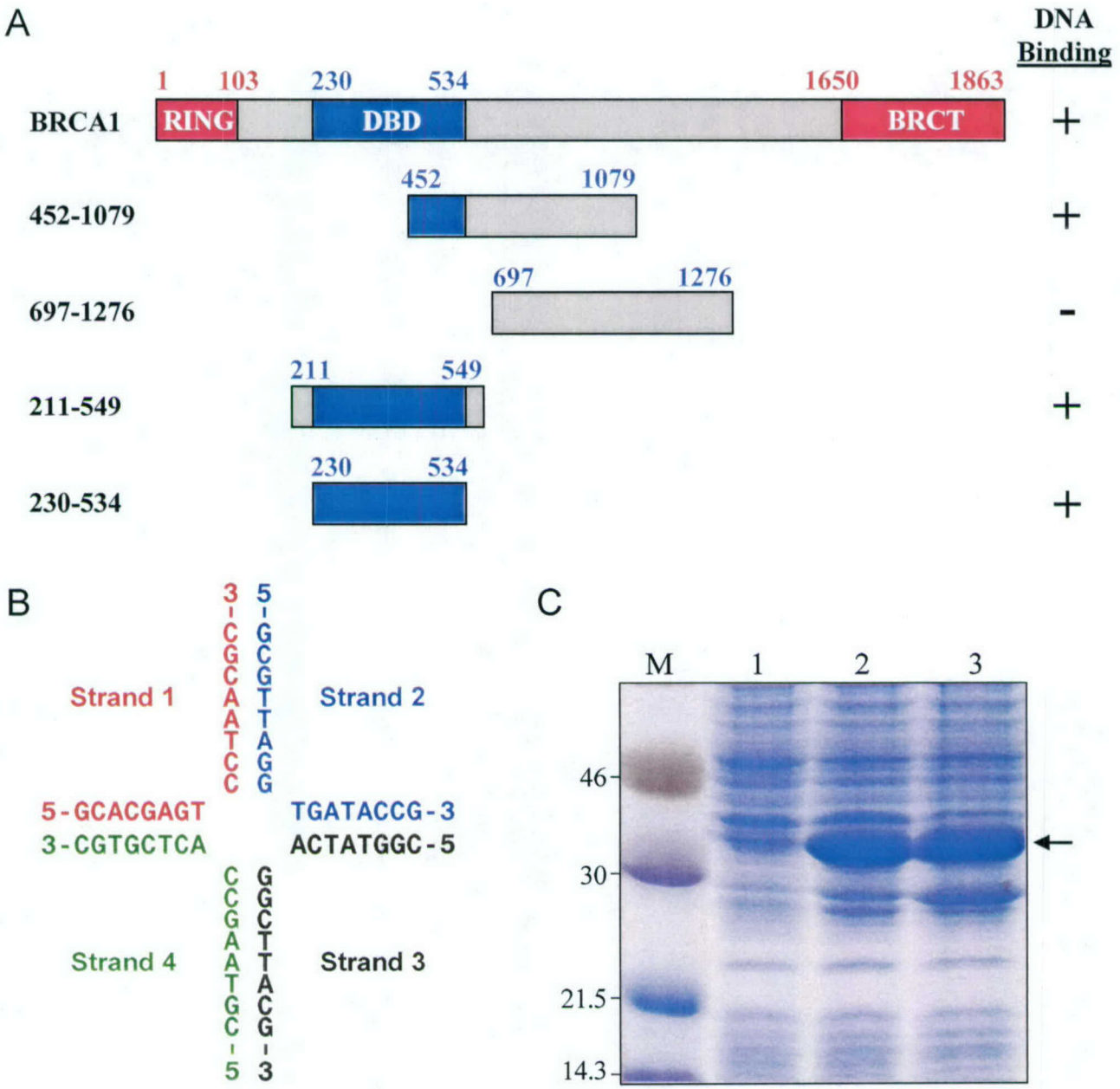

Figure 1. (A) Schematic representation of BRCA1 domains and DNA-binding properties of BRCA1 protein fragments based on our studies and refs. 4 and 7. The BRCA1 region spanning residues 230-534 contains the smaller known protein fragment of BRCA1 that can bind to four-way junction DNA and can be produced in bacterial cells as a soluble protein (7). DBD: DNA-binding domain. **(B)** The four-way junction DNA probe designed for binding and crystallization studies with the BRCA1(211-549) protein. The indicated four oligonucleotide strands are annealed to generate the four-way junction DNA. **(C)** Expression of the BRCA1(211-249) domain having a hexahistidine tag in *E. coli* BL21(DE3) cells. *Lane M*: protein markers in kDa; *lanes 1 and 2*: uninduced and induced whole-cell extracts, respectively, of *E. coli* cells expressing BRCA1(211-549) protein; *lane 3:* induced soluble proteins. The arrow indicates the position of the soluble BRCA1(211-549) protein.

sequencing and used to transform *E. coli* cells. Extensive tests with all these constructs failed to produce large amounts of BRCA1(452-1079) protein in a soluble form necessary for the crystallization experiments. Therefore, we reasoned that a better mapping of the BRCA1 region involved in binding to four-way junction DNA was essential and it might generate a soluble protein fragment suitable for crystallographic analysis. It is noteworthy that since the crude mapping of the original BRCA1 DBD in the region spanning residues 452-1079 (4), no other studies describing a more precise localization of the DBD were reported. The encountered difficulties to produce large amounts of soluble BRCA1(452-1079) protein delayed the successful completion of the proposed tasks within the one-year award period.

Deletion mutagenesis to discover shorter protein fragments that are soluble and retain the ability to bind branched DNA showed that BRCA1(211-549) can bind to four-way junction DNA (Figure 1A and 1B). These results are consistent with a report that was published earlier this month in the *Journal of Molecular Biology* showing that the BRCA1(230-534) protein fragment can be made in bacteria as a soluble protein and binds four-way DNA (7). Therefore, we have for the first time two BRCA1 protein fragments that bind to four-way junction DNA and can be expressed in *E. coli* cells in soluble form. We are now in the process of producing these proteins in order to proceed with the crystallization experiments. The BRCA1(211-549) and BRCA1(230-534) protein fragments will be purified using a combination of affinity, ion exchange and size exclusion chromatography. Crystallization experiments will be performed with the pure protein and nucleoprotein complexes using sparse matrix screenings and vapor diffusion crystallization methods. Because BRCA1(211-549) and BRCA1(230-534) protein fragments bind with high affinity to four-way junction DNA molecules (7, our studies), we will assemble complexes of these proteins with four-way junction DNA molecules generated by annealing synthetic oligonucleotides, similar to those described in Figure 1B. We will take special care to purify monodisperse nucleoprotein complexes for crystallization trials using size exclusion chromatography. For structure determination, crystals of selenomethionine-containing recombinant BRCA1(211-549) and BRCA1(230-534) proteins will be used to collect multiwavelength anomalous diffraction (MAD) diffraction data using synchrotron radiation. The crystal structures will be determined with MAD methods. I would like to emphasize that although the award period is finished, we will continue to pursue very persistently the crystallographic analysis of the BRCA1 DBD bound to four-way junction DNA, as outlined in the original proposal. The one-year Concept Award has provided critical support to initiate this research project and the successful completion of these studies will be reported to DOD in the future.

KEY RESEARCH ACCOMPLISHMENTS

1. Cloning of the BRCA1(452-1079) into prokaryotic expression vectors pET-6H2, pGEX-2, pET-43a, pMAL-c2, and pTYB12 for production in *E. coli* cells (*Task 1*).
2. Induction of expression and performance of solubility tests of the bacterially produced BRCA1(452-1079) protein (*Task 1*).
3. Deletion mutagenesis to map the borders of the BRCA1 DBD and identify smaller BRCA1 protein fragments that can be made in bacteria in soluble form and bind four-way DNA (*Task 1*).

4. Identification of the BRCA1 region spanning residues 211-549 that bind four-way junction DNA and can be produced in *E. coli* cells as a soluble protein (*Task 1*).
5. Initiation of large-scale production of BRCA1(211-549) protein in *E. coli* cells for crystallization with four-way junction DNA (*Tasks 1 and 2*).

REPORTABLE OUTCOMES

The experiments of the proposal are still in progress and no publications have resulted so far.

CONCLUSIONS

During the award period we made numerous attempts to find conditions that would allow the production of large amounts of the BRCA1(452-1079) protein in *E. coli* cells, using the prokaryotic expression vectors pET-6H2, pGEX-2, pET-43a, pMAL-c2, and pTYB12. Because these experiments showed that this protein fragment cannot be readily made in *E. coli* cells, we proceeded with the identification of an overlapping BRCA1 protein fragment spanning residues 211-549 that binds to four-way junction DNA and can be expressed in bacterial cells in a soluble form. We are currently in the process of producing and purifying this recombinant protein fragment in order to crystallize it in complex with four-way junction DNA. The information obtained from these experiments will elucidate the mechanisms underlying the function of BRCA1 in DNA repair and will reveal the molecular changes induced by cancer-causing mutations in BRCA1 that abrogate the activity of this protein and contribute to the pathogenesis of breast cancer.

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