LOCATING THE PROTEIN BINDING REGION OF P53 AND THE INTRINSICALLY DISORDERED REGION OF BRCA1

By

Anna Norman

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LOCATING THE PROTEIN BINDING REGION OF P53 AND THE INTRINSICALLY DISORDERED REGION OF BRCA1

Project Approved:

Supervising Professor: Mikaela Stewart, Ph.D.

Department of Biology

Laura Luque, Ph.D.

Department of Biology

Carol Thompson, Ph.D.

Department of Sociology

ABSTRACT

BRCA1 and p53 have been shown to interact in tumor suppressor pathways that protect against hereditary breast and ovarian cancer. Finding the physical binding location associated with this interplay is important in assessing cancer-risk and determining molecular details of the interaction. This project aimed to identify the protein binding region of p53 with the intrinsically disordered region of BRCA1. We cloned select regions of human BRCA1 and p53 protein into *E. coli* bacteria, then harvested and purified the proteins. A pull-down assay was performed to test binding affinity between a segment of p53 and two different length BRCA1 constructs. The assay showed that neither the construct that contained BRCA1 amino acids between 772-1126 nor the construct with amino acids between 896-1190 interacted with p53. This indicates that these amino acids alone are not sufficient for binding of p53 and BRCA1. Our results could also indicate that a third-party binding mediator is required *in vivo*. This information expands upon our knowledge of the p53 and BRCA1 binding interaction and can be used in a clinical setting to evaluate risk associated with mutations in the experimental regions.

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TABLE OF CONTENTS

INTRODUCTION 6

METHODS....8

Protein expression....8

Material and reagents....9

Affinity purification....9

SDS-PAGE....10

Dialysis and Concentration....10

Pull-down assay....10

RESULTS....12

DISCUSSION....14

REFERENCES 17

Introduction

In 2017, there were 252,710 new cases of breast cancer and 40,610 deaths due to breast cancer in the United States, making breast cancer the most diagnosed form and the second leading cause of cancer death in women.[1] In 2018, there were an estimated 22,240 new cases of ovarian cancer and 14,070 ovarian cancer deaths in the US.[2] The prevalence of breast and ovarian cancer diagnosis and death makes identifying risk factors for cancer development a topic of interest in biological research. Mutations in tumor suppressor proteins and oncoproteins are known predisposing factors for cancer. The aim of this project is to explore these proteins and pinpoint areas within the proteins that are critical for proper function where mutations would be detrimental.

BRCA1 is a tumor suppressor protein involved in DNA double-strand break repair, transcriptional regulation, induction of apoptosis, homologous recombination, cell-cycle checkpoint control, and growth signaling.[3] Studies have shown that if BRCA1 exhibits a germline mutation in humans, one outcome is hereditary breast and ovarian cancer (HBOC) syndrome. This syndrome often results in early onset breast cancer but is also associated with other cancers, including ovarian. HBOC syndrome accounts for 5-7% of all cases of breast cancer and those with the syndrome have a 50-80% chance of developing breast cancer and a 30-50% chance of developing ovarian cancer.[3]

The BRCA1 protein contains a central disordered region that is ~1500 amino acids long as determined by NMR spectroscopy and CD spectroscopy.[4] Most of the disordered region does not have independently folded globular domains, but electrophoretic mobility shift assays and intrinsic tryptophan fluorescence experiments suggest that this region is responsible for interaction with p53, another tumor suppressor protein.[4] These studies suggest that the disordered

region acts as a flexible scaffold for molecular interactions with many different molecules critical for the DNA damage response pathway, including p53.[4] BRCA1 has been implicated in the proper functioning of p53, though the exact mechanism of action is not known. It is known, however, that BRCA1 is involved in p53 phosphorylation through cell cycle regulation and that p53 stability and abundance was decreased in BRCA1 mutant mice. The two proteins are thought to function together to contribute to overall genome stability, though a concrete direct physical interaction between the proteins has not been confirmed *in vitro*.[3]

p53 has also been found to regulate BRCA1 nuclear export after DNA damage as shown by immunoprecipitation, western blotting, and immunofluorescence assays. This export is linked to initiation of apoptosis in damaged cells. p53 dysfunction leads to lack of BRCA1 shuttling, meaning that there is not enough cytoplasmic BRCA1 to regulate cytotoxic damage, indicating the importance of their interaction in tumor suppression.[5]

An *in vitro* study found that the region between amino acid residues 224-500 of BRCA1 and the region between residues 311-393 of p53 were sufficient for binding of the two proteins.[6] However, a conflicting study using *in vivo* immunoprecipitation and western blotting showed that BRCA1 and p53 interaction was consistent with BRCA1 amino acids between residue 772 and 1292, but that p53 interaction with a shorter BRCA1 constructed of amino acids 772-1036 was negligible. This suggests that amino acids 1036 to 1292 of BRCA1 contain the binding region for BRCA1 and p53.[7] Interaction between the two proteins was stronger in cells after exposure to UV light or Adriamycin treatment, both of which lead to DNA damage. This indicates that BRCA1 and p53 do work together in response to DNA damage and may suppress tumor formation.[7] Though this study suggests a physical binding of these two proteins, the results are not conclusive, and our study attempts to confirm this binding.

To locate the binding region of BRCA1 and p53, our project aimed to test the claim that a physical binding region exists within amino acids 772-1292 on BRCA1 and amino acids 311-393 on p53. To accomplish this, we transformed *E. coli* cells using plasmids to express BRCA1 and p53 constructs of differing lengths (Table 1), then harvest and purify the proteins to use in a pull down assay to investigate the binding patterns of these tumor-suppressor proteins.

Construct Name	Protein Region	Molecular Weight
p53	p53 amino acids 355 - 393	31 kDa
B3	BRCA1 amino acids 772-1126	49 kDa
B6	BRCA1 amino acids 896 - 1190	46 kDa

Table 1. Description of BRCA1 and p53 constructs

Methods

Protein Expression

A fresh transformation of *E. coli* cells was performed to produce the proteins of interest for our assays. Plasmids contain the regions coding for minimized constructs of p53 and BRCA1 were transformed into BL21 (DE3) *E. coli* cells, then grown on plates containing kanamycin for BRCA1 or plates containing ampicillin for p53 overnight at 37 °C. These cells were then harvested and grown in 1 L LB broth cultures to an optical density of 0.5-0.6 before inducing protein expression with Isopropyl ß-D-1-thiogalactopyranoside (IPTG))to a final concentration of 0.3 mM. BRCA1 constructs were allowed to induce overnight at 16°C and p53 constructs were induced for 5 hours at 25 °C with shaking. After induction, cultures were centrifuged for 10 minutes at 4°C and resuspended in 24 mL of GST Buffer A for p53 or 24 mL of Nickel Buffer A for BRCA1 (Table 2). The constructs were then stored at - 80 °C if

Buffer Name	Composition
GST Buffer A	140 mM NaCl, 2.7 mM KCl, 10 mM $NaH_2PO_4 \cdot H_20$, 1.8 mM Na_2HPO_4 (pH = 7.4)
GST Buffer B	10 mM glutathione, 50 mM Tris, 1mM DTT (pH = 7.4)
Nickel (Ni) Buffer A	10 mM imidazole, 300 mM NaCl, 50 mM Tris (pH 7.4)
Nickel (Ni) Buffer B	1 M imidazole, 300 mM NaCl, 50 mM Tris (pH =7.4)
Coomassie Blue	.25 g/mL Coomassie, 10% acetic acid, 50% methanol, 40% double deionized water
BME Load Dye	5% 2-mercaptoethanol, 2.5% SDS, 62.5 mM Tris-HCl, 0.002% bromophenol blue, 10% glycerol
Destaining Buffer	10% glacial acetic acid, 50% methanol, 40% double deionized water

Table 2. Description of buffers and reagents used throughout experiment

purification could not be performed directly after growth and expression. If frozen, the samples were thawed by placing the closed container containing the solubilized cells into water prior to affinity purification. DNAse, lysozyme, protease inhibitor cocktail, and PMSF in ethanol were added to the thawed samples to protect the protein during cell lysis. The samples were sonicated on ice for 30 minutes total with 15 second pulses and 30 second rest periods at 80% amplitude. Then, the cells were centrifuged to remove insoluble cell debris at 1300 rpm for 25 minutes at 4 °C. The desired protein regions are listed below with their given names and molecular weights.

Affinity Purification

Affinity purification was conducted with the AKTA liquid chromatography system to isolate the BRCA1 and p53 proteins from other cellular proteins. A 5 mL GSTtrap affinity column was used for p53 constructs, and a 5 mL HiTrap TALON cobalt column was used for BRCA1 constructs. The samples were applied to the columns which were initially equilibrated with water, ethanol, and the corresponding buffer A. Unbound proteins were washed with the correct buffer A and disposed of in a waste container. Bound proteins were eluted with GST buffer B for p53 and Ni Buffer B for BRCA1 proteins. Eluted fractions were analyzed for

absorbance at 280 nm to determine which of the fractions contained the proteins of interest. These fractions were analyzed with SDS PAGE to confirm the presence of proteins then stored at -80 °C before dialysis and concentration.

SDS PAGE

Sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis was used to confirm the identity of the proteins present post induction as well as post purification. SDS was also used to analyze binding after the pull-down assay described below. Each sample was mixed in a 1:1 ratio with BME load dye then inserted into wells of a 15% polyacrylamide gel and run for 45 minutes at 195 V. Coomassie blue was then added to stain the gel before microwaving for 15 seconds and staining for 15 minutes. Then, the stain was poured off and a destaining solution was added to visualize results on the polyacrylamide gel.

Dialysis and Concentration

After all of the proteins were purified, the fractions were thawed and dialyzed in SnakeSkin cellulose tubing in 4L of ITC running buffer overnight at 4°C to remove glutathione and imidazole, which can interfere with pull-down assays. Then, protein concentrating centrifugal devices were used three times each for 20 minutes at 3000 rpm and 4 °C to increase the concentration of both proteins to approximately 600 mM. The concentrated proteins were aliquoted into 0.5 mL fractions and stored at -80 °C until it was time to perform the pull-down assay.

Pull-Down Assay

A pull-down assay was performed to check for binding between p53 and the BRCA1 constructs. Frozen protein samples were thawed on ice, and the absorbance of each protein sample at 280nm (A280) was measured with Thermo Scientific Nanodrop 100. The extinction

coefficient was calculated from the primary sequence using ExPaSy ProtParam tool, and the final concentration in mM was calculated using the following formula.

$$Final\ Concentration = \frac{A_{280}}{Extinction\ Coefficient\ x\ 10^{3}}$$

Using this formula, we completed appropriate dilutions of the protein samples with buffer A to reach a final concentration of 0.6 mM for each protein. For both BRCA1 proteins, this was a dilution of around 20 µL of protein to 50 µL of buffer A. For p53, this was about 15 µL of protein to 55 µL of buffer. 50 µL of Pierce Glutathione Agarose in Bioscience spin columns was equilibrated with 100 µL of GST buffer A. Then, 100 µL of p53 was incubated with the resin for 30 minutes to ensure binding to the resin. Next, the columns were washed twice with more GST buffer A to remove everything except for the p53 protein which was bound to the glutathione

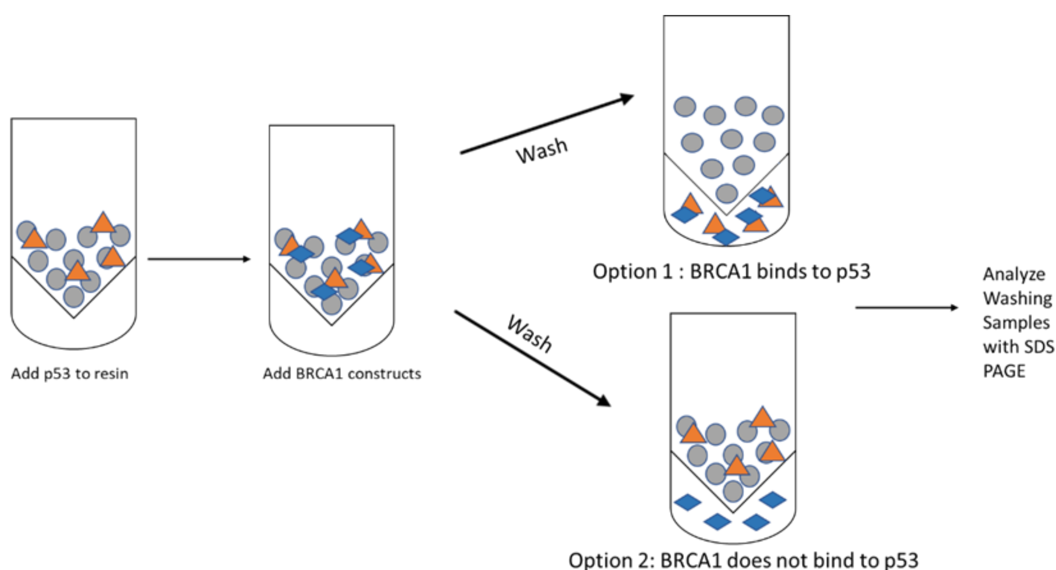

Figure 1. Methods for pull-down assay.

agarose resin. Then, two spin columns were incubated with either 100 µL B3 or B6 BRCA1

constructs for 30 minutes to encourage BRCA1 binding to the already bound p53. Unbound BRCA1 proteins were washed off of the resin using two 40 μL aliquots of GST buffer A. The remaining bound proteins were eluted with GST buffer B to see if both BRCA1 and p53 are present. Samples from the GST buffer A washings and elution were analyzed using the SDS PAGE procedure detailed above. Figure 1 depicts this process.

Results

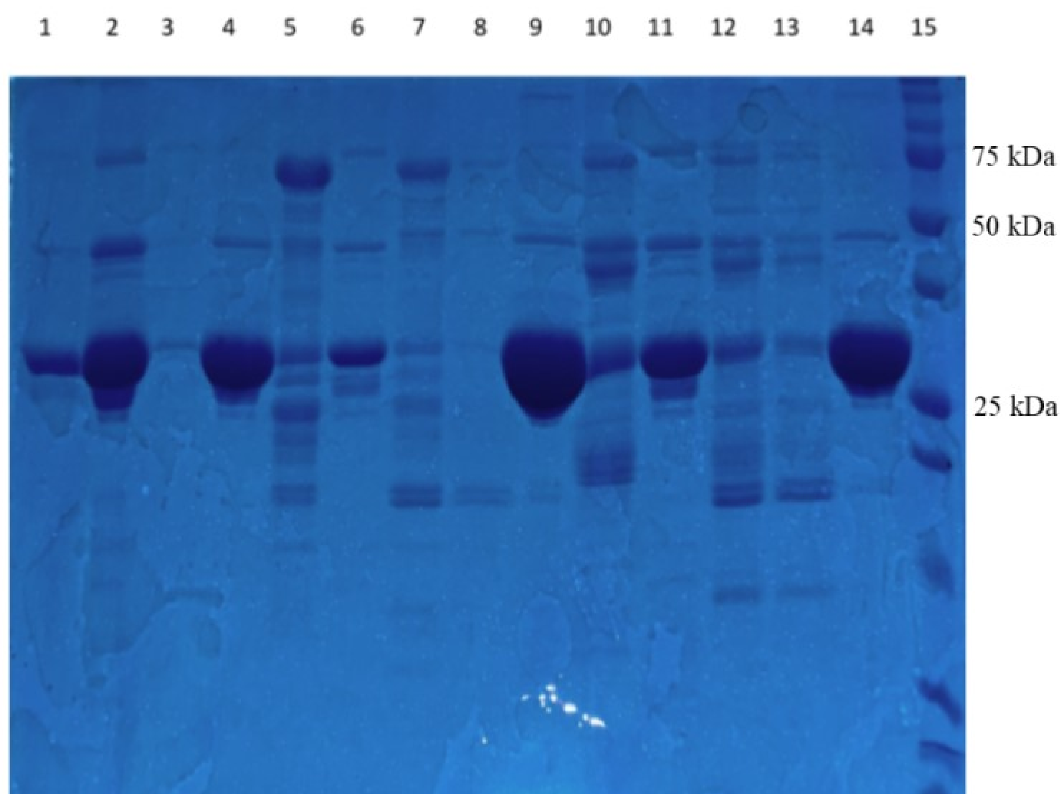

Figure 2. Pull down assay showing no binding between BRCA1 constructs and p53. Samples were taken after each step of the pull-down assay and analyzed via SDS PAGE gel. Results are shown for the p53 control and B3 and B6 experiment. Lane 1 is purified p53, lane 5 is purified B3, and lane 10 is purified B6. Washing lanes 7 and 8 show all B3 being washed away without any p53 binding. Washing lanes 12 and 13 show he same result for B6. Lanes 9 and 14 show the p53 being removed from both experimental runs in the elution independent from any BRCA1 construct.

The presence of the desired proteins was confirmed after each affinity purification by running gel electrophoresis on the collected fractions and comparing the proteins present to the expected sizes of our proteins as calculated by the ExPaSy ProtParam tool and using a standard protein molecular weight ruler for reference (Figure 2, lane 15). Lanes 1, 5 and 10 of Figure 2 show that we were able to isolate a protein corresponding to the molecular weight of each of our desired constructs. B3 stained at around 74 kDa, which is 14 kDa larger than we would expect from the protein segment that we tried to isolate. Plasmid sequencing to confirm the identity of both protein constructs failed, so we were not able to positively establish the identity of B3. However, some studies of Sumoylated proteins have reported that the SUMO tag that was included in our proteins can add up to 20 kDa to the apparent weight of a protein as represented on SDS PAGE.[8] We did not predict that SUMO would add this much weight to the B3 construct, so it is possible that is why the protein is staining at 74 kDa. B6 stained at around 50 kDa, as expected, so we feel most confident in that experimental trial. However, future studies should use plasmid sequencing to confirm the identity of all constructs involved. After the identities of our proteins were mostly confirmed, we performed a pull-down assay to assess their interaction.

After the purified human proteins were tested using a pull-down assay, samples were taken after washing and elution steps for both BRCA1 constructs, then tested using gel electrophoresis to visualize protein sizes. As seen in Figure 2, the SDS PAGE gel indicated that p53 (355 - 393) did not bind to either truncated BRCA1 construct. Our p53 protein contained a tag which would allow it to stick to the glutathione agarose resin until an elution buffer (GST buffer B) was used to remove all proteins. Therefore, any proteins that bind to p53 would remain bound to the resin via p53 until the elution. We used this assumption to test the binding affinity of B3 and B6 to p53. The gel showed a stain consistent with our construct of p53 at a molecular

weight of around 35 kDa in the elution samples for the control and experimental runs (lanes 4, 9, and 14) This indicates that p53 was correctly bound to the resin in all trials and was washed off with elution buffer, as expected. B3 and B6 both stained at around the expected molecular weight in their respective experimental washings (lanes 7, 8, 12, and 13), but neither appeared on the gel in the elution sample with p53. This indicates that the simple wash buffer was able to dislodge BRCA1 from the spin column before the elution buffer was added. If p53 and either of the two BRCA1 constructs were bound, we would expect to see BRCA1 present in the experimental elution lanes 9 and 14, because the wash buffer would not be strong enough to interrupt the binding between p53 and BRCA1, and the BRCA1 would stay present in the column until the elution. The absence of BRCA1 in the final elution shows that both BRCA1 constructs washed off early and remained unbound from p53 throughout the pull-down assay. We can conclude that these protein regions of p53 and BRCA1 are not sufficient to support protein binding.

Discussion

Our study analyzed the binding interaction between p53 and BRCA1, which is necessary for proper tumor suppression in many breast and ovarian cancers. Past studies have shown the importance of amino acids 311-392 in p53 and amino acids 1036-1292 in BRCA1 for interaction with p53, and we aimed to better understand the specifics of this relationship by studying the interaction by investigating minimized constructs *in vitro*. Identifying the site of binding between these two proteins is important in the expansion of our knowledge about tumor suppression cascades in breast and ovarian cancer and assessing risk for cancer development in humans. We also aimed to narrow down the possible binding site to a small enough section of amino acids that the molecular details of the binding interaction could be identified. A pull-down assay indicated that neither minimized BRCA1 construct interacted with p53 (355-393). While this

result does not reveal the exact protein binding location of these two tumor suppressor proteins as intended, it does provide useful information about this pair of proteins.

We can conclude that the experimental regions of p53 and BRCA1 we used in our pulldown assay are not sufficient for the binding of these two tumor suppressor proteins. The assay showed a lack of binding between the two proteins. Additionally, our results could be explained by the need for a separate third-party binding partner in this interaction that would engage with p53 and BRCA1 to facilitate binding. The absence of this binding intermediate in our assay would also result in a lack of binding as shown by the pull-down assay. This knowledge allows us to utilize the process of elimination when continuing to examine this interacting region, because we can conclusively say these regions do not bind on their own. We can use this information in a clinical context to hypothesize that in humans, mutations in the region that we tested will not result in significant tumor suppression deficiency, as this region does not seem to be important for this interaction. Additionally, this study continues to expand our understanding of the intrinsically disordered region of BRCA1, which interacts with many molecular counterparts to carry out its tumor suppressor function. In the future, studies on this topic should continue to test unique constructs of BRCA1 and p53 within the area of interest to conclusively locate the amino acid binding region. Future experiments should also test binding under different experimental conditions to expand upon our conclusions.

While this experiment did yield significant results, the process was plagued by obstacles. We originally planned to test six unique BRCA1 constructs and two unique p53 constructs, but after growing these human proteins in bacteria, they sustained significant proteolysis during purification. We altered many steps in the procedure to favor the stability of the proteins, but after multiple unsuccessful attempts at harvesting the unreported constructs, we eventually

moved forward with the two most stable BRCA1 constructs and shorter p53 construct. This proteolysis was likely due to the intrinsically disordered nature of our proteins of interest and in the future, the genes related to these proteins should be cloned into plasmids and placed into *E. coli* to harvest the proteins directly from inclusion bodies which would prevent proteolysis. The additional data that could have been gathered from our lost constructs would have provided a more thorough understanding of this region, and these protein regions could be useful in future research which investigates this protein interplay.

Considering the limitations of our experiment, there is much work to be done in conclusively determining the protein binding location of BRCA1 and p53. These results offer a promising starting point for future research that can answer the still unanswered questions about this important cancer-related oligomerization event.

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