DETERMINING BIOCHEMICAL AND BIOPHYSICAL METHODS TO EVALUATE THE INTERACTION BETWEEN BRCA1 AND ESTROGEN RECEPTOR ALPHA

by

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ABSTRACT

BRCA1 is a gene whose protein (also named BRCA1) is found throughout all human cells and engages in DNA repair, cell cycle regulation, gene transcription regulation, and apoptosis. However, mutations in BRCA1 typically confer a higher risk of cancer in estrogen-responsive tissues, including breast epithelial tissue. This increase in incidence of tissue-specific cancers is thought to be in part due to the role of BRCA1 in the estrogen-response pathway and interaction with the estrogen receptor alpha (ERα). Previous studies identified possible regions of each protein involved in the binding interface between BRCA1 and ERα. Using these regions (amino acids 177-240 in BRCA1 and the ligand binding domain of ERα) as our constructs, our studies further analyzed the molecular details of this direct interaction and determined methods conducive to studying the BRCA1-ERα interaction. A pull-down assay qualitatively confirmed binding between the constructs of BRCA1 and ERα. Data collected from NMR spectroscopy reaffirmed the direct interaction between BRCA1 and ERα first seen in the pull-down assay and provided evidence demonstrating that the presence of estrogen in the samples increased binding affinity. Finally, fluorescence spectroscopy of quenching experiments confirmed the previous results – that a direct interaction occurs between the constructs of BRCA1 and ERα used – and allowed us to describe the binding curve of the system being studied. The molecular details confirmed here provide further avenues of study, such as documenting variants of unknown significance or studying the role estrogen plays in the function of the BRCA1-ERα complex, which could lead to novel findings that expand our understanding of the role either protein plays in cancer development.

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INTRODUCTION

Breast cancer is the second most common cancer worldwide and the most common cancer diagnosis found among women[1]. Although both genetic and environmental factors play a part in the development of breast cancer in an individual, the proteins BRCA1 and BRCA2 – named after their connection to breast cancer development – play a large role in both inherited and non-inherited breast cancers[2]. Half of all non-inherited breast cancers show decreased expression of BRCA1, and mutations in BRCA1 and BRCA2 contribute to 9 out of every 10 cases of inherited breast cancers[2]. Early detection and monitoring is an essential part to developing breast cancer therapy; although imaging techniques such as mammography and ultrasounds are standard practices for detecting tumor growth in breast tissue, current research is also interested in the use of biochemical markers for breast cancer detection and monitoring[1]. One of these biochemical markers is estrogen receptor alpha (ERα) because of its involvement in breast epithelial tissue growth and regulation[1]. BRCA1 has both direct and indirect interaction with ERα in breast tissue[2-7].

BRCA1 is essential for many functions within all human cells, including DNA repair, cell cycle regulation, apoptosis, ubiquitination, and regulation of gene transcription[2-3, 6]. Although BRCA1 holds critical functions in all cell types, inherited mutations of BRCA1 lead to the greatest increase in risk for cancer in estrogen-responsive tissues, like breast and ovarian tissue[3].

ERα comes from a family of nuclear receptors which bind estrogen-response elements on DNA and induce transcription of estrogen-responsive genes relating to cell proliferation within epithelial breast tissue[2, 4]. The increased risk of developing tissue-specific cancers through

inherited mutations in BRCA1 is theorized to be in part because of its interaction with ERα in the estrogen-response pathway[5].

The estrogen-response pathway utilizes two regions within ERα: the DNA-binding domain and the ligand binding domain[4-6]. The ligand binding domain (LBD), in the absence of bound estrogen, suppresses the activity of the DNA-binding domain[4]. Once estrogen binds, the LBD no longer suppresses the DNA-binding domain, and ERα undergoes a conformational change that allows both dimerization of the protein and for the DNA-binding domain to bind estrogen-responsive elements on DNA and induce transcription of its target genes[4-5]. However, further regulation of this pathway occurs via the ubiquitin ligase activity of BRCA1[5]. The monoubiquitylation of ERα prevents increased activity of the estrogen signaling pathway and regulates the growth of mammary epithelial cells[4-5]. In other words, ubiquitination of ERα decreases its transcriptional activity.

Although domains of BRCA1 found in both the amino- and carboxyl-termini are required for proper inhibition of ERα, the first 302 amino acids of BRCA1 are most important for this interaction[7]. Previous research demonstrated that the capacity for ERα-LBD ubiquitination by BRCA1 becomes limited when BRCA1 is missing amino acids 177-240, indicating the importance of these amino acids for binding and ubiquitination[5]. BRCA1 also shows specificity for the LBD over the DNA-binding domain, meaning that although the function of the DNA-binding domain is regulated, BRCA1 binds at the LBD[5].

The binding profile between ERα and BRCA1 predicts that amino acids 177-240 of BRCA1 and part of the LBD ERα are directly involved in binding, but these regions are intrinsically disordered[2, 8-9]. Intrinsically disordered regions (IDRs) of a protein have multiple conformations instead of a single state or form of the protein[10]. IDRs, when bound to another

protein or molecule in a system, can favor a single conformation or continue to vary in conformation from moment to moment[10]. Studies of other compounds binding to the ERα-LBD indicate a single conformation of this region when the ligand is bound[10], but the effect of BRCA1 binding to this same region is not known[2]. Methods such as nuclear magnetic resonance (NMR), circular dichroism (CD), and fluorescence would help document the molecular details of the interaction between BRCA1 and ERα and the kinetics of this system[10-11].

Little is understood regarding the kinetics or molecular details of the binding between ERα and BRCA1 beyond the acknowledgement that the interaction between these two proteins is complex[7]. Co-factors, such as p300 and cyclin D1, influence the association and function of BRCA1 with ERα, and the multi-faceted functions of BRCA1 – including an indirect interaction with this system – also play a part in its capacity to inhibit ERα[7]. Documenting the molecular details of the ERα-BRCA1 complex will further our understanding of this system and provide a basis for further research, such as the investigation of genetic variants of unknown significance, within these regions of BRCA1 and ERα[12].

Our goal – to document the molecular details of the estrogen receptor binding region of BRCA1 – was accomplished through *in vitro* methods of nuclear magnetic resonance (NMR) spectrometry and fluorescence spectroscopy after expression and purification of each protein and a qualitative assay to confirm binding of our protein constructs. NMR tracks chemical shifts of each nitrogen in the backbone of an isotopically-labelled protein (in our case, BRCA1) that occur after the addition of a compound to the sample (ERα)[10]. Analyzing these chemical shifts allowed us to determine the presence of binding between ERα and BRCA1. Fluorescent spectroscopy measurements of a receptor (ERα) with and without its ligand (BRCA1) can lead to a change in emission measured by a cuvette-based fluorimeter[11]. Measuring the difference in intensity based

off the concentration of BRCA1 in solution creates a binding curve to help understand the kinetics of the system being studied[11]. Additionally, we determined biochemical and biophysical methods conducive to studying this interaction to facilitate future research of this system.

METHODS

BRCA1 Estrogen Receptor Regions (ERR) Expression and Purification

BRCA1 estrogen receptor binding regions (amino acids 177-240 and 177-258) were expressed using a pETSUMO plasmid in *E. coli* BL21(DE3) cells. Transformations were completed using 30 seconds of heat shocking at 42 ºC. The cells were plated on LB agar plates containing 50 μg/mL kanamycin and grown overnight (12-16 hours). These colonies were then transferred into 1 L of LB broth containing 50 μg/mL kanamycin and grown at 37 ºC shaking at 250 RPM until an optical density (OD_{600}) of 0.6 was reached. Temperature was reduced to 16 ºC, and the cultures were left to equilibrate at the new temperature. After an hour, protein expression was induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG). After 16 hours, the cells were harvested by centrifugation at 4 ºC for twenty minutes at 3500 RPM. Cells were resuspended in a buffer containing 0.5 M NaCl, 20 mM TRIS, 5 mM imidazole, at pH 7.4.

Protein purification began by adding 0.1 mg/mL egg lysozyme, bovine DNase I (Goldbio), and protease inhibitor cocktail tablets (Roche cOmplete protease inhibitor cocktail tablets, EDTA-free) to the resuspended cells. The suspension was transferred to a 50 mL glass beaker on ice for lysis through the Vibra Cell sonicator, using pulses of 15 seconds on, 30 seconds off at 80% amplitude for a total of 10 minutes of sonication. Phenylmethylsulfonyl fluoride (PMSF) was added to the beaker in three 60 μL segments: once before, once during, and once after sonication. The lysate was clarified through centrifugation at 4 ºC for 25 minutes at 14000 RPM to remove any insoluble cellular debris. The supernatant of the clarified lysate was

transferred to a 50 mL conical tube and loaded into the Äkta Start GE system to purify the BRCA1 proteins using a cobalt affinity column following the manufacture protocol (His-Trap TALON column from GE Healthcare). The presence of a His tag on SUMO (a string of six histidines found before the BRCA1 protein) allowed the protein to bind to the column with high affinity, and the rest of the proteins found in the clarified lysate flowed through the column without binding. Increasing the concentration of imidazole, which contains a higher affinity to the cobalt column than the His tag, allowed for the protein to elute and be collected in fractions that were identified through absorbance readings at 280 nm, measured by the UV bulbs found on the Äkta machine. Fractions containing the protein of interest were combined and dialyzed overnight with an H3C protease buffer containing 25 mM $NaPO_4$, 150 mM NaCl, and 1 mM dithiothreitol (DTT) at a pH of 7.

After dialysis, the fractions were incubated for one hour with H3C protease and allowed to cleave the His tag from the protein. To separate the His tag from the protein in solution, the solution was run through a GST and Nickel affinity column. The GST column contains an affinity for the H3C protease, and the Nickel column contains an affinity for the His-tag-containing SUMO protein. Therefore, the protease and His-tag stick to the column, and the BRCA1 protein flows through both columns without any affinity. The flow-through is then concentrated with a 3 kDa cutoff PALL concentrator using 20 minute cycles of centrifugation at 3,000 RPM until reaching a volume less than 2 mL. The 2 mL of protein was loaded onto NGC Quest10 Plus Bio-Rad Chromatography System and ran through a gel filtration column to separate the contents in solution by size. The fractions containing purified BRCA1 were identified through absorbance readings at 280, 215, and 255 nm, measured by UV bulbs found within the size exclusion chromatography system. These fractions are concentrated using a 3 kDa

concentrator with 20 minute cycles of centrifugation at 3,000 RPM until the protein is at a concentration between 0.2 and 0.4 mM. The remaining protein is aliquoted in microcentrifuge tubes and placed in the -80 ºC freezer for storage.

ERα LBD Expression and Purification

ERα LBD expression and purification followed closely with the expression and purification detailed above. However, the ERα LBD was expressed using a pET15B plasmid in *E. coli* BL21(DE3) cells. Transformations were still completed using 30 seconds of heat shocking at 42 ºC, but the cells were plated on LB agar plates containing 50 μg/mL ampicillin and allowed to grow overnight (12-16 hours). These colonies were transferred into 100 mL of LB broth containing the same concentration of ampicillin and grown overnight before being aliquoted into 6 L of LB broth and grown in the same conditions detailed above. Protein expression was induced using IPTG at a 0.3 mM concentration. The resuspension buffer also differed, containing 50 mM HEPES, 0.5 M NaCl, 1 mM TCEP, 20 mM imidazole, and 10% glycerol at pH 8. While the resuspended BRCA1 cell pellets could be placed in the -80 ºC freezer for an extended period of time before purifying, ERα LBD required immediate purification after expression to prevent aggregates of the protein.

Protein purification utilized a total of 5 minutes of sonication, with 10 second pulses on and off to lyse the bacterial cells. Dialysis, proteolysis, and the GST/Nickel affinity columns are not necessary for ERα, because the pET15B plasmid does not contain the same His-tag on SUMO as the BRCA1 constructs that needed to be cleaved from the protein. ERα contains a His-tag allowing it to bind to the cobalt affinity column found on the Äkta Start GE system, but cleavage of the His-tag was not performed. Gel filtration with the NGC Quest10 Plus Bio-Rad Chromatography System separated the insoluble ERα aggregates from the usable protein by size,

and concentration of the purified protein was performed using a 10 kDa PALL concentrator to reach a concentration between 0.2 and 0.3 mM.

Pull-Down Assay

In order to confirm qualitatively the presence of an interaction between ERα LBD and the short BRCA1 construct (amino acids 177-240) in vitro, a pull-down assay was performed. Two microcentrifuge tubes were equilibrated with 150 μL of resin through a series of washings with a buffer containing 50 mM HEPES, 0.5 M NaCl, 1 mM TCEP, 20 mM imidazole, and 10% glycerol at pH 8. Because of its affinity to the resin, ERα at a concentration of 0.238 mM was added to one tube and allowed to incubate. The resin is washed with the same buffer three times, and 60 μM of the BRCA1 construct was added to the microcentrifuge tube and incubated for 30 minutes. After incubation, the resin is washed again three times with the same buffer, and gel samples are taken after each wash. To completely remove (elute) all the protein contained in the resin and confirm the presence of both proteins, buffer containing a higher concentration of imidazole (50 mM HEPES, 1 M NaCl, 1 mM TCEP, and 500 mM Imidazole at pH 8) was added to the tube, and another gel sample was taken. The other tube containing resin follows the same steps but leaves out the addition of ERα to the resin to act as a control. Polyacrylamide gel electrophoresis (PAGE) was run with the gel samples collected for both the control and experimental assays and analyzed for results.

NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was utilized to measure the change in chemical environment of ^{15}N isotopically labeled BRCA1 short construct (amino acids 177-240) in the presence and absence of ERα LBD and in the presence and absence of 6 mM estrogen in order to study the effect of ERα binding to BRCA1. Four samples were prepared – 120 μM

BRCA1; 120 μM BRCA1 + 0.35 mM estradiol (E_2); 120 μM BRCA1 + 120 μM ERα; and 120 μM BRCA1 + 120 μM ERα + 6 mM E_2, all in 8% D_2O – and data were collected on a 600 mHz spectrometer by Dr. Lisa Tuttle at the University of Washington. Expression and purification of isotopically labeled ^{15}N BRCA1 followed the same procedure detailed above with the exception of a growth period in 1L minimal media containing an isotope of nitrogen (^{15}N ammonium chloride) transferred from 4L of LB broth.

Fluorescence

In order to investigate the kinetics of the ERα - BRCA1 interaction, a quenching experiment using titration fluorimetry was performed. Using a BMG Labtech FLUOstar Omega microplate reader, eight points of the curve were analyzed with a constant concentration of ERα at 2.4 μM and varying concentrations of the ligand (BRCA1): 0 μM, 1.5 μM, 3 μM, 4 μM, 5 μM, 6 μM, 8 μM, and 12 μM. Samples were prepared in triplicate with and without 6 μM estrogen present, and each 100 μL sample was placed into a 96-well microplate and analyzed through a TRP fluorescence program on the microplate reader. Fluorescence at 294 nm excitation and 340 nm emission was measured with a gain of 1095. Any points that deviated any more than two standard deviations away from the average standard deviation were removed before analysis of the data.

Fluorescence was also measured, using the parameters detailed above, at each of these titration points after 300 mM KI was added to the samples. The KI quenches, or decreases the fluorescence of, ERα, and the addition of BRCA1 binds to ERα and displaces the surrounding KI, leading to a reappearance of fluorescence. This change in fluorescence between the samples with and without 300 mM KI was used to perform statistical analyses to determine the fraction of

ERα bound to BRCA1, assuming the highest concentration of ligand used corresponded to ERα being 100% bound.

RESULTS

BRCA1 and ERα minimal binding domains interact *in vitro*

After expression and purification of both constructs, the pull-down assay was performed in order to confirm qualitatively an *in vitro* interaction between the two constructs. This assay is the first time that the construct and purification protocol of ERα LBD was used in combination with this minimized BRCA1 (amino acids 177-240) construct and purification protocol. In theory, these constructs contain amino acids essential to the *in vitro* interaction between ERα and BRCA1 seen in previous studies, and this assay confirmed this interaction[5].

In this pull-down assay, ERα LBD has affinity for the resin used, but BRCA1 does not. When the resin was washed, any BRCA1 not bound to ERα was removed from the resin. When the resin was eluted, all protein, bound or unbound to the resin, was removed. ERα LBD is present in the resin in the experimental resin, but absent in the control resin. The control assay demonstrated that without ERα LBD present in the resin, most of the BRCA1 washed out of the resin after the second wash (lane 11 of Figure 1). The first wash experienced little protein elution due to displacement of the buffer instead of any movement of protein out of the resin. In comparison, when ERα LBD was present in the resin, the greatest concentration of BRCA1 flowed through in the elution (lane 8 of Figure 1), once the affinity of ERα LBD to the resin was disrupted, allowing it to flow through the resin and pull BRCA1 down with it.

Because BRCA1 demonstrated low affinity to the resin in the control assay but eluted as if it had an affinity in the presence of ERα LBD, we can conclude that the minimized construct of BRCA1 interacts with the LBD of ERα.

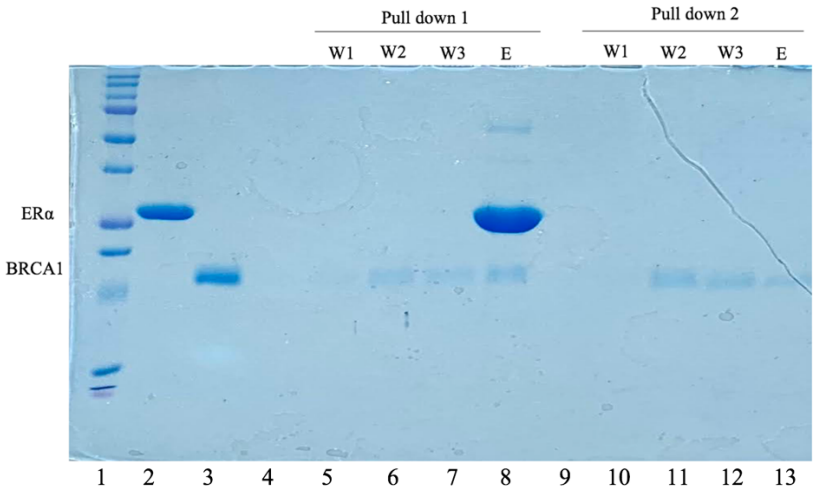

Figure 1. Pull-down assay confirming binding between BRCA1 construct (amino acids 177-240) and ERα LBD. Samples were taken after each step of the pull-down assay and ran on an SDS-PAGE gel to analyze the results of the experiment. Lane 2 is purified ERα LBD and lane 3 is purified BRCA1 (177-240) to help guide analysis of the results. A higher volume of BRCA1 (177-240) eluted (E) after the last wash (W3) when ERα LBD was present in the resin (lanes 5-8) compared to the control resin without ERα (lanes 10-13), where the highest volume of BRCA1 (177-240) eluted after the second wash (W2).

Estrogen improves the BRCA1-ERα binding interaction

To gain molecular details regarding the interaction, effects of binding were studied further through NMR analysis. Four samples were prepared of ^{15}N BRCA1 (amino acids 177-240) in varying conditions – ^{15}N BRCA1; ^{15}N BRCA1 + estrogen (E_2); ^{15}N BRCA1 + ERα; and ^{15}N BRCA1 + ERα + E_2 – and their NMR spectra were compared against each other. The peaks of this BRCA1 construct are clustered closely together within a small range of the hydrogen axis, reaffirming that this region of BRCA1 is highly disordered (Figure 2, blue spectrum). The addition of ERα or E_2 does not drastically alter the clustering of the peaks (Figure 2, gold spectrum), indicating that BRCA1 continues to be intrinsically disordered, but the data provides some interesting findings regarding the BRCA1-ERα interaction. As part A of Figure 2 demonstrates, the addition of ERα corresponds with the appearance of at least six additional

peaks. Each peak represents one N-H bond on the backbone of the polypeptide chain, and while a total of 65 peaks are possible for this protein construct, about 16 N-H bonds are missing, indicating the hydrogen atom is exchanging with the solution, preventing measurement with NMR. The appearance of six new peaks might indicate that six backbone atoms previously exchanging with water are now less exposed to water, possibly due to interaction with ERα.

The addition of ERα to BRCA1 in the presence of E_2, however, induced more significant chemical shifts within the NMR spectra. As parts B and C of Figure 2 demonstrate, while the general cluster of peaks still indicates an intrinsically disordered region, the environment in which the peaks are found has shifted and decreased intensities, as evidenced by the decrease in the number of contours found at each peak, which indicates that estrogen addition improves binding of our two protein constructs.

Peak intensity relates to the ability of the molecule being measured to tumble in solution at a certain rate. The smaller the molecule is, the faster it tumbles in solution and the more intense the peak will be – or greater amount of contours will be seen – when measured. Therefore, when the NMR spectra demonstrate a decrease in peak intensity with the addition of ERα in the presence of E_2, one infers slower tumbling due to an increase in the size of the molecule being measured, BRCA1 in our case. All samples used the same constructs of BRCA1, implying that this increase in size comes from ERα directly interacting with BRCA1 in solution. Part A of Figure 3 provides an overlay of the changes that occur to the ^{15}N BRCA1 spectra as ERα and E_2 are added, allowing the chemical shifts and change in intensity between the samples to be observed quantitatively. Part B of Figure 3 quantifies this the average peak intensity. Two-tailed *t* tests ($P < 0.05$) demonstrate a significant decrease upon addition of ERα and E_2. As described above, the average peak intensity of the APO sample (^{15}N BRCA1) did not change

with the addition of ERα alone, indicating estrogen strengthens the interaction between the proteins.

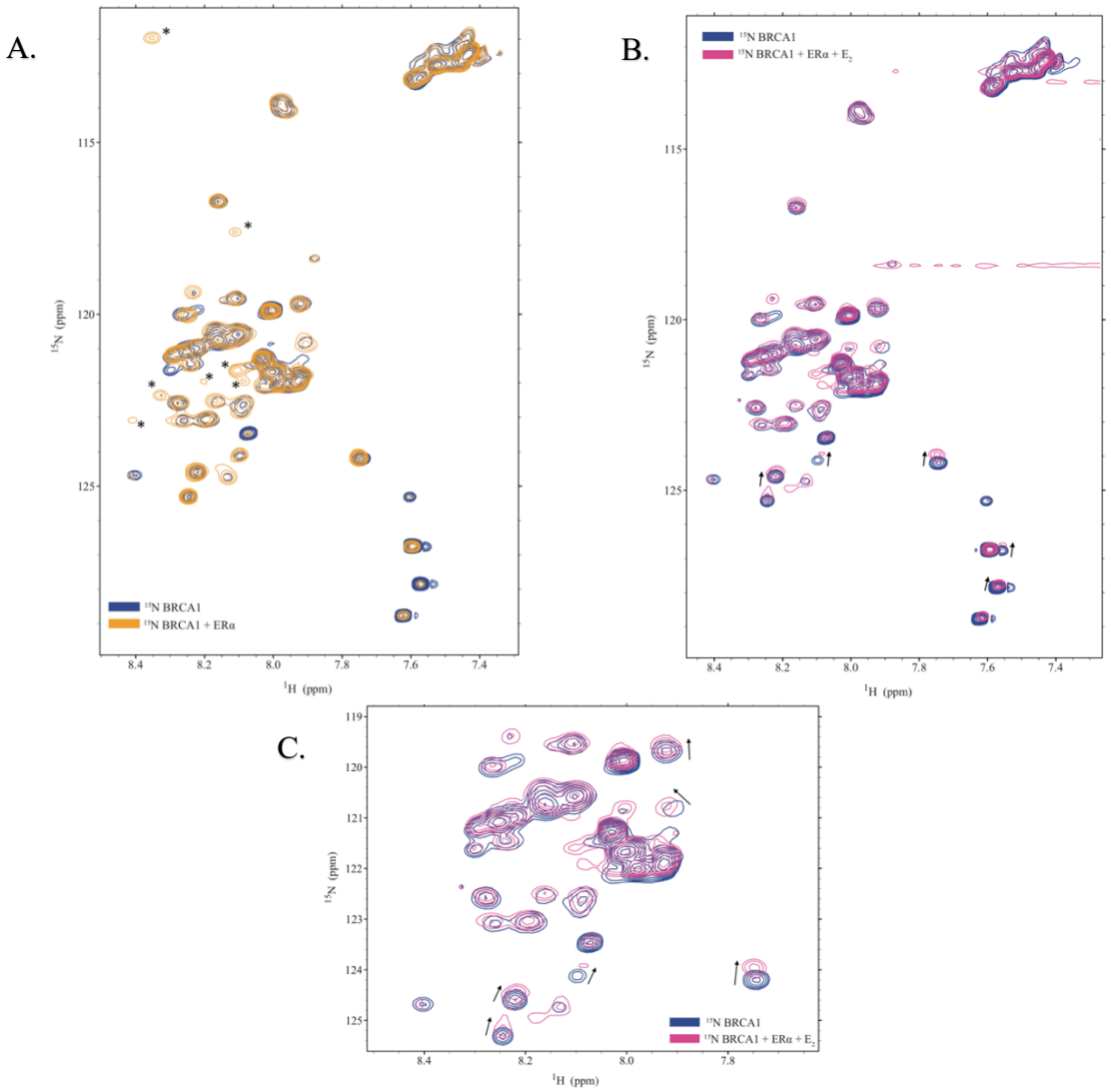

Figure 2. Estrogen improves interaction between BRCA1 and ERα constructs. Nuclear magnetic resonance spectra depicting the chemical environment of ^{15}N and ^{1}H atoms in the backbone of the BRCA1 protein construct. **A.** Clustering of peaks within a short range of the ^{1}H axis confirms the intrinsically disorder (ID) within the BRCA1 region. Comparison between the APO sample (^{15}N BRCA1 in blue) and ^{15}N BRCA1 + ERα (in gold) shows the appearance of at least six new peaks within the NMR spectra (marked with *), indicating an interaction between BRCA1 and ERα constructs. **B.** Comparison between the APO sample and ^{15}N BRCA1 + ERα + E_2 (pink) demonstrates more visible chemical shifts indicating a change in environment and also changes in peak intensity indicating improved BRCA1-ERα interaction. All spectra continue to display clustering indicative of ID of BRCA1 even in the bound state. **C.** Spectra from part **B** zoomed in to better display peak shifts. Chemical shifts are indicated with an arrow (➔).

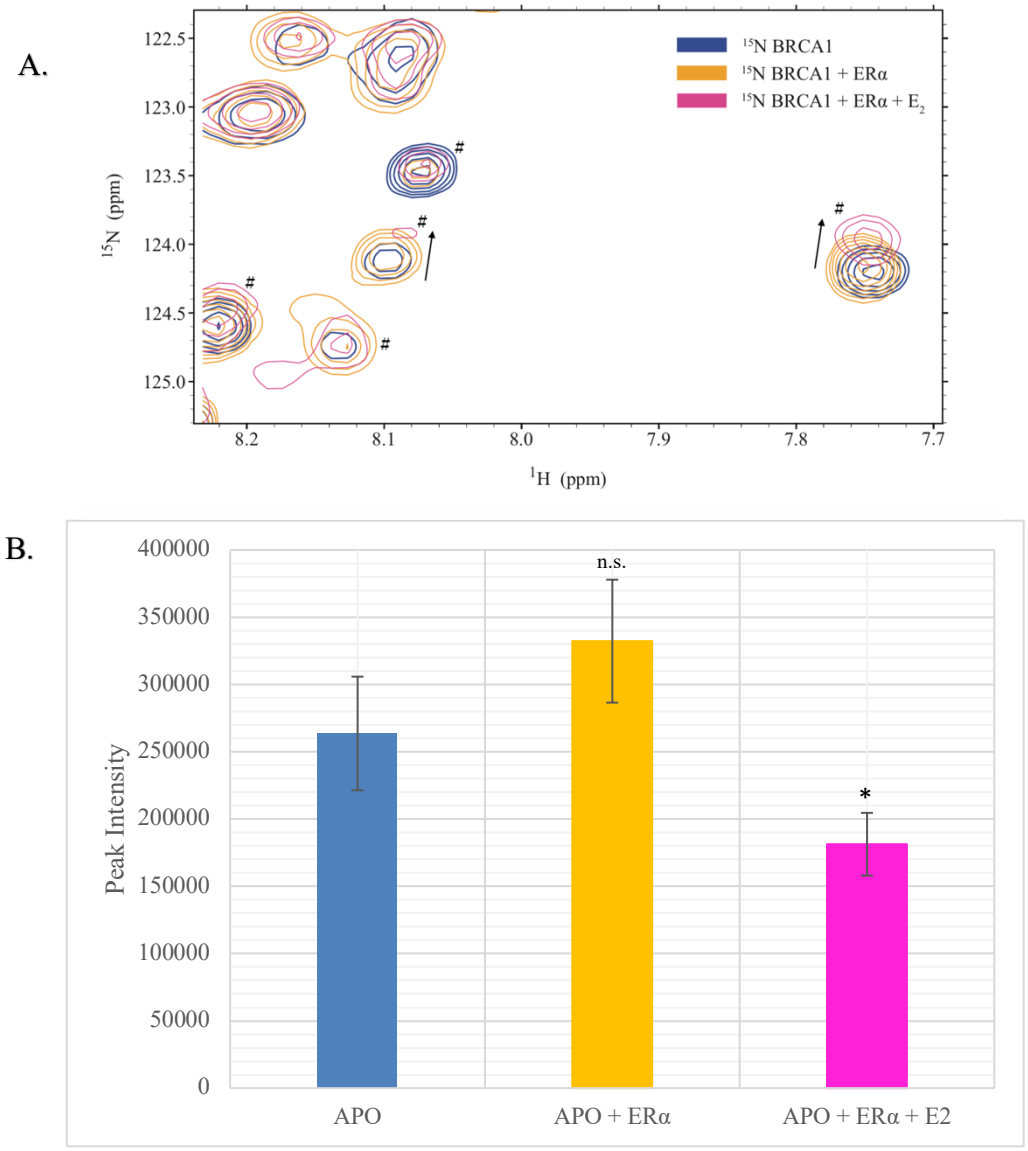

Figure 3. Quantification of peak intensity demonstrates a decrease in intensity of the BRCA1 sample bound to ERα and E_2.

A. Comparison of APO (^{15}N BRCA1) spectra in blue, ^{15}N BRCA1 with ERα in gold, and ^{15}N BRCA1 with ERα and E_2 in pink. Addition of ERα does not significantly alter peak distribution and intensity, while the presence of E_2 leads to more significant chemical shifts and a decrease in peak intensity, indicated by an arrow (➔) and pound sign (#), respectively. **B.** Quantification of the average peak intensity of each sample (APO, ^{15}N BRCA1 + ERα, and ^{15}N BRCA1 + ERα + E_2). An asterisk (*) signifies a change in average peak intensity with a p-value < 0.05 using a two-tailed *t* test compared with the APO sample, and n.s. denotes no significance.

Estrogen does not seem to influence K_d of ERα-BRCA1 system

Fluorescence spectroscopy allowed quantification of the *in vitro* interaction confirmed in the pull-down assay, as well as an analysis on the effect of estrogen (E_2) on binding between these two constructs. A 294 nm wavelength excites tryptophan, an amino acid found in our construct of ERα but not in BRCA1, that emits along a range of wavelengths but peaks around 340 nm. Any increase or decrease in fluorescence of the construct is either the result of an increase or decrease in concentration of ERα, or a change in the ability of tryptophan to emit fluorescence. The second of these two options occurs primarily when there is another molecule, such as KI or E_2, blocking the tryptophan from responding to the wavelength of light. We used this quality of KI to perform quenching experiments and analyze the ability of BRCA1 at variable concentrations to bind to ERα at a single concentration and displace the surrounding KI.

Figure 4 shows the analyzed results of the quenching experiments. Initial results on the cuvette-based fluorimeter showed a promising binding curve, but were unreliable and unable to be repeated because of ERα absorbing onto the glass cuvette. Further studies done in a plastic 96-well microplate proved more consistent. Fluorescence measurements of each sample were taken before and after the addition of 300 mM KI, and the difference in fluorescence between these two measurements was used in analysis. KI addition to samples with smaller concentrations of BRCA1 led to a greater difference in fluorescence, indicating a higher volume of quenching taking place. As the concentration of BRCA1 increased, the fraction of quenched ERα decreased, signifying a higher rate of BRCA1 binding with ERα. Assuming the change in fluorescence at 12 μM BRCA1 indicated 100% bound ERα, we determined the fraction bound for each of the other concentrations of BRCA1. A nonlinear regression curve was fit to the data using the equation: $y = \frac{K_d + P_0 + x - \sqrt{(K_d + P_0 + x)^2 - 4P_0 x}}{2P_0}$, where P_0 = concentration of ERα; x =

concentration of BRCA1; y = fraction bound; and K_d is the dissociation constant of ERα to be determined.[13]

As Figure 4 demonstrates, the binding curve with estrogen present had a K_d of 1.32 μM, and in the absence of estrogen, the K_d was 1.07 μM, with no significant difference between the two values when considering the 95% confidence intervals. This finding conflicts with the data collected through NMR, because it seems to indicate a similar binding curve (and consequently binding affinity) regardless of estrogen content in the sample. However, this does not necessarily mean that estrogen has no effect on binding. The concentration of our protein constructs may alter binding affinity – our NMR samples contained 100 times higher protein concentrations than what is seen in this experiment – or it may be the salt content of our sample interfering with binding. The buffer used to store and dilute samples contains 150 mM NaCl, so an additional 300 mM KI might outcompete and disrupt any binding that is ionic. The lack of data around the steepest part of the curve might imply that this line of best fit is not representing the binding curve as accurately as possible, and the large standard deviation (indicated by the error bars on the graphs in Figure 4) in the data suggests that this experiment should be repeated to try to minimize experimental error.

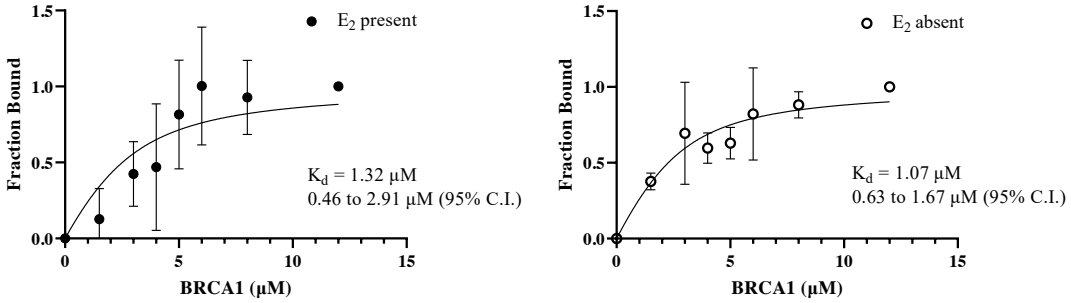

Figure 4. No significant difference found between the K_d of ERα-BRCA1 binding in the presence or absence of E_2. Fraction of ERα bound to BRCA1 at various concentrations in the presence (left) and absence (right) of estrogen (E_2). A nonlinear regression curve defining the K_d of both curves found no significant difference between the ERα–BRCA1 binding affinity in the presence or absence of E_2 in these experimental conditions.

DISCUSSION

BRCA1 is a fundamental regulatory protein found within all human cells and taking part in apoptosis, cell cycle regulation, gene transcription regulation, DNA repair, and ubiquitylation. Yet mutant BRCA1 proteins increase the risk for cancer in breast epithelial, ovarian, and prostate tissues over any other cell types[2-3, 6]. This increase in incidence of tissue-specific cancers is thought to be related to the role BRCA1 takes within the estrogen-response pathway[5]. ERα is known to interact both indirectly and directly with BRCA1[2-7], and previous analysis of the direct interaction between BRCA1 and ERα indicates that amino acids 177-240 of BRCA1 and the ERα LBD are involved in the binding interface of the BRCA1-ERα complex[5]. However, the molecular details of the binding interface of BRCA1 and ERα are not well understood[7]. Here we describe biochemical and biophysical methods useful in studying this interaction and document the molecular details of the estrogen receptor binding region of BRCA1 utilizing these methods.

The main purpose of this research comes from the lack of information we have on the interaction between BRCA1 and ERα. BRCA1 is an extremely large protein, containing 1,863 amino acids in its complete structure, with multifaceted functions in our cells. The regions of each protein theorized to be important in their direct interaction are found within intrinsically disordered regions, meaning the variability of conformations within these regions makes analyzing its molecular details more challenging[5, 10, 14]. We used smaller constructs in an effort to isolate the effect of BRCA1 binding to ERα and determine the molecular details important in this direct interaction, but this is the first time that the simplified construct of BRCA1 we used (consisting of the 86 amino acids found between the 177th and 240th position of the wild type protein) was tested for binding affinity with ERα LBD. Previous studies isolated these amino acids as important and our work confirmed its importance within the binding interface[5].

Furthermore, the protocol used to grow and purify ERα LBD did not require estrogen to be present, allowing us to observe the interaction of BRCA1 and ERα with and without estrogen in the sample to better understand its role within this system.

The growth and purification protocol for ERα proved to be a challenge, and the protein produced tended to have a shorter shelf life than other synthesized proteins. Protein aggregation and absorption onto glass not only limited the amount of protein produced, but also made studies more challenging. Initial fluorescence studies showed varying amounts of success, but we adjusted the experiment by using plastic 96-well microplates and a fluorescence reader in place of a glass cuvette and traditional fluorescence spectrophotometer. This adjustment allowed us to successfully measure fluorescence of ERα at variable BRCA1 concentrations and determine a binding curve and K_d value for the ERα-BRCA1 system with and without estrogen present. For future studies, we recommend avoiding glassware as often as possible and using ERα soon after purification to produce the best results.

Looking toward future studies of the BRCA1-ERα system, this research could lead to many interesting, novel findings. Although binding between these two constructs was confirmed, the exact amino acids within the BRCA1 construct were not identified. Analyzing the NMR spectra further to assign each peak to an amino acid would increase our understanding of the binding profile between these two proteins. Directed mutagenesis of the BRCA1 construct would also allow us to determine in greater detail where BRCA1 interacts with ERα LBD and theorize the effects of not being able to bind to ERα. Further studies with directed mutagenesis might also provide information on certain variants of unknown significance within this region of BRCA1 and its effect on the direct interaction between ERα and BRCA1. Repeating the methods performed from this paper and quantifying the difference between the nonmutated, or wild type,

constructs against the mutant constructs would give insight on the variants of unknown significance. A slightly longer construct of BRCA1 (spanning the amino acids between the 177th and 254th position of the original protein) was also proposed to contain the region essential in the BRCA1-ERα system and could be studied and compared to the construct used here to further analyze the binding interface of the system of proteins. Utilizing even longer constructs of BRCA1 with its ubiquitylation function intact would allow researchers to study the effects of estrogen – and its absence – on ubiquitylation. In short, there are many avenues with which one could take to further our understanding of the BRCA1-ERα system given the findings from this study.

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