Mapping the Functional Domains of BRCA1

INTERACTION OF THE RING FINGER DOMAINS OF BRCA1 AND BARD1*

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Breast cancer 1 (BRCA1) and BRCA1-associated RING domain 1 (BARD1) are multidomain proteins that interact in vivo via their N-terminal RING finger motif regions. To characterize functional aspects of the BRCA1/BARD1 interaction, we have defined the structural domains required for the interaction, as well as their oligomerization state, relative stability, and possible nucleic acid binding activity. We have found that the RING finger motifs do not themselves constitute stable structural domains but are instead part of larger domains comprising residues 1–109 of BRCA1 and residues 26–119 of BARD1. These domains exist as homodimers and preferentially form a stable heterodimer. Shorter BRCA1 RING finger constructs do not interact with BARD1 or with longer BRCA1 constructs, indicating that the heterodimeric and homodimer interactions are mediated by regions outside the canonical RING finger motif. Nucleic acid binding is a generally proposed function of RING finger domains. We show that neither the homodimers nor the heterodimer displays affinity for nucleic acids, indicating that the proposed roles of BRCA1 and BARD1 in DNA repair and/or transcriptional activation must be mediated either by other regions of the proteins or by additional cofactors.

Unraveling the cellular basis for BRCA1-mediated tumor suppression is a challenging goal that is complicated, in part, by the multifunctional nature of the protein. In large multifunctional proteins, such as BRCA1, different functions are often dispersed among discrete structural regions or domains. In such cases, functional studies can be facilitated by characterization of the properties of individual domains or subunits. Unfortunately, in the case of BRCA1, a large protein of 1863 amino acids, clues regarding the location or extent of independent functional domains are not readily available on the basis of sequence homologies. A noteworthy exception is the RING finger consensus motif located at the N-terminal end of the protein (1). The RING finger is a zinc-binding motif defined by a conserved pattern of cysteine and histidine residues that is found in a wide variety of proteins of diverse origin and function (2). Although more than 80 RING finger-containing proteins have been identified to date, a specific cellular function has not yet been associated with the motif (3). Nevertheless, the importance of the RING finger to BRCA1 function is supported by the following observations: 1) the first 100 residues, including the RING finger motif (residues 24–64), are the most highly conserved regions among known BRCA1 genes (4, 5); 2) several cancer-predisposing mutations have been identified within this region of BRCA1 (6, 7); and 3) yeast two-hybrid studies have identified two novel proteins that interact with this region of BRCA1 (8, 9).

One of the proteins found to associate with the BRCA1 N-terminal region, BARD1 (8), also contains an N-terminal RING finger motif (residues 50–86). Yeast two-hybrid studies showed that the N-terminal RING finger regions of both BRCA1 and BARD1 are required for specific heterocomplex formation between the proteins. Subsequent cellular localization studies demonstrated that BRCA1 and BARD1 localize to discrete nuclear foci (dots) during S phase and disperse during other phases of the cell cycle (10). In addition, complexes containing BRCA1 and BARD1 have been detected on damaged, replicating DNA structures (10). Taken together, these observations suggest that a multiprotein complex involving BRCA1 is responsive both to the cell cycle and to DNA damage and identify an important cellular interaction among the proteins. Despite identification of heterologous protein interactions, the cellular basis of BRCA1-mediated tumor suppression remains elusive. When initially identified, it was suggested that the BRCA1 RING finger may play a role in specific DNA binding (1). Indeed, there has been considerable speculation regarding a general function for RING finger proteins in nucleic-acid interactions. To date, published data on the DNA binding properties of RING fingers are conflicting and equivocal: there are reports that particular RING finger proteins or domains bind synthetic oligonucleotides or DNA cellulose, whereas other reports provide evidence to the contrary (11–14).

In no case, however, has specific nucleic acid binding been demonstrated. Nonetheless, BRCA1 has been reported associated with certain chromatin structures in vivo and linked to the RNA polymerase II holoenzyme complex (15, 16). These findings raise the possibility that BRCA1 might associate directly with nucleic acids either alone or upon formation of a complex with BARD1.

In order to characterize the functional significance of the BRCA1/BARD1 interaction further, we have sought to define the extent of the structural region encompassing the RING finger consensus motifs for both proteins. We find that the RING finger motifs do not themselves constitute stable struc-
tural units but are instead part of larger structural domains. Interactions and properties that may contribute to the function of these proteins, including their oligomerization states, relative stabilities, and nucleic acid binding activities are described herein.

EXPERIMENTAL PROCEDURES

Materials—3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and 2,2’,6,2’-terpyridine were obtained from Aldrich and used without further purification. Limited proteolysis studies used sequencing grade endoproteinase Lys-C (Pierce) and V8 endo proteinase (Worthington). Bovine serum albumin, ovalbumin, α-chymotrypsinogen, and ribonuclease A, and glutaraldehyde were purchased from Sigma.

Expression and Cloning of BRCA1 and BARD1 Constructs—BC-76, BC-112, and BC-172 constructs were prepared as described previously (17). BARD1 constructs were prepared in a similar fashion using primers to obtain the desired BARD1 polymerase chain reaction products from human lymphoblast cDNA. The primers were synthesized with NheI and BamHI endonuclease restriction sites in the 5’- and 3’-ends, respectively. The polymerase chain reaction products and pET11a vector (Novagen) were then digested with NheI and BamHI, ligated, and transformed into Escherichia coli BL21 cells, and clones were sequenced as described previously for BRCA1 constructs (17). Because of the restriction sites used, each construct contains two extra amino acids (Ala and Ser) at the N terminus. BC-112 also contains two additional residues at its C terminus (Gly and Lys) as a result of the cloning procedure. Construct and fragment designations refer to the number and position of BRCA1 or BARD1 residues.

Expression and Purification of BRCA1 and BARD1 N-terminal Constructs—BRCA1 constructs were expressed and purified as described previously (17). Purification of BARD1 constructs was identical to that of BRCA1 constructs with one minor modification. The BARD1-containing insoluble fraction was re-solubilized into 6 M guanidine-HCl as before (17), diluted to 2 M guanidine-HCl by dropwise addition of Tris buffer (50 mM Tris, 10 mM dithiothreitol, pH 7.6) and purified to homogeneity by sequential chromatography. Protein purity and concentration was determined as described previously for the BRCA1 constructs (17). Approximate extinction coefficients at 280 nm for BARD1 constructs, calculated on the basis of amino acid composition, are 19.55, 13.86, and 13.86 M\(^{-1}\) cm\(^{-1}\) for BD 26–152, BD 26–140, and BD 26–131, respectively.

Fluorescence Spectroscopy—Fluorescence spectra were measured on a Spex Fluorolog fluorometer using a 1 M 0.5-cm cuvette at 25°C. BRCA1 homodimer and BRCA1/BARD1 heterodimer sample (1.0 ml) was eluted with a 50–350 mM NaCl gradient (5 column volumes), and the intermediate elution volume relative to the two individual constructs elutes as a single sharp peak with a width comparable to those of standard proteins used for column calibration. The intermediate elution volume relative to the two individual species is indicative of a heterodimeric complex between BC-172 and BD 26–152. Similar results were obtained with BC-112 and BD 26–152 (data not shown).

Glutaraldehyde cross-linking of peak fractions collected from gel-filtration columns, followed by SDS-PAGE, confirmed the oligomerization state of each species. The elution profile of BC-112/BD 26–152 is blue-shifted an equimolar mixture of BC-112 and BD 26–152 (data not shown).

Limited proteolysis were performed on the BARD1 homodimer and BRCA1/BARD1 heterodimer sample (1.0 ml) were digested with sequencing grade trypsin (Promega) and analyzed by MALDI-TOF mass spectrometry experiments were performed as described previously (17).

Nucleic Acid Binding—BRCA1, BARD1, and the ADR1 zinc finger domain constructs were purified by gel-filtration chromatography in 25 mM Tris, 50 mM NaCl, pH 7.6 (Buffer 1). Peak fractions (approximately 10 μM by absorbance) were loaded onto double- and single-stranded DNA cellulose (calf thymus DNA, Sigma) and synthetic RNA (poly(A), poly(U), poly(G), and poly(C) agarose (Sigma) columns at a flow rate of 0.2 ml/min in Buffer 1. Columns were washed with 5 column volumes of Buffer 1 to remove nonspecifically bound material. The bound material was eluted with a 50–350 mM NaCl gradient (5 column volumes), followed by a 350–600 mM NaCl gradient (2 column volumes), and a final wash with 2 M NaCl to remove tightly bound protein. Each column (Perseptive Biosystems) was packed with approximately 1 ml of nucleic acid resin. The identity of eluted material was verified by analysis of peak fractions on 14% SDS-PAGE.

RESULTS

The BRCA1 RING Finger Structural Domain Extends beyond the RING Finger Homology Region—Three different sized BRCA1 RING finger constructs (termed BC-76, BC-112, and BC-172; Table I) were tested for their ability to interact with a BARD1 RING finger construct. The BARD1 construct (BD 26–152) encompasses residues 26–152 and was chosen on the basis of reported two-hybrid studies (8). We used gel-filtration chromatography to assess the oligomeric states of the constructs. Fig. 1A illustrates gel-filtration elution profiles for BC-172, BD 26–152, and a 1:1 mixture of the two constructs. Individually, each construct elutes primarily as a single peak at an elution volume that corresponds to a molecular weight roughly twice the expected value, suggesting that both BC-172 and BD 26–152 homodimerize in solution. An equimolar mixture of the two constructs elutes as a single sharp peak with a width comparable to those of standard proteins used for column calibration. The intermediate elution volume relative to the two individual species is indicative of a heterodimeric complex between BC-172 and BD 26–152. Similar results were obtained with BC-112 and BD 26–152 (data not shown).

| Construct name | Protein residues | Calculated monomer molecular mass Da | MALDI-TOF molecular mass Da |
|----------------|------------------|------------------------------------|---------------------------|
| BC-76          | BRCA: 1–76       | 8,839                              | 8,840 ± 2                 |
| BC-112         | BRCA1: 1–110     | 12,960                             | 12,961 ± 2                |
| BC-172         | BRCA1: 1–172     | 19,877                             | 19,884 ± 8                |
| BD 26–131      | BARD1: 26–131    | 12,133                             | 12,129 ± 3                |
| BD 26–140      | BARD1: 26–140    | 13,121                             | 13,126 ± 4                |
| BD 26–152      | BARD1: 26–152    | 14,584                             | 14,589 ± 5                |

2 BARD1 contains two potential initiator methionines (residues Met1 and Met3). The yeast two-hybrid studies showed that the first 25 residues of BARD1 are not required for the BRCA1/BARD1 interaction.
trum of the heterodimer isolated by gel-filtration is virtually identical to the spectrum obtained by direct mixing of the proteins (data not shown).

We have previously demonstrated that the short BRCA1 construct, BC-76, forms a homodimer (17). However, using the approaches described above, we do not detect heterodimer formation with BC-76 and BD 26–152 (data not shown). Furthermore, mixtures of BC-76 and BC-172 elute as two separate
peaks from the gel-filtration column at volumes corresponding to the expected molecular weights of BC-76 and BC-172 homodimers (Fig. 1C). Similar results were obtained with mixtures of BC-76 and BC-112 (data not shown). Thus, the BC-76 RING finger construct does not dimerize with either BC-112, BC-172, or BD 26–152, indicating that specific and high affinity BRCA1 homodimerization and heterodimerization with BARD1 requires BRCA1 residues C-terminal to position 76.

**Determination of the Structural BRCA1 and BARD1 Domains Required for Heterodimerization**—To identify the structural domains of BRCA1 and BARD1 required for heterodimerization, we used limited proteolysis in conjunction with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The premise behind this experimental approach is that regions of proteins that are structured are less susceptible to proteolysis than regions of proteins that are flexible or disordered. This approach has also been used to map regions of proteins involved in mediating interactions with other molecules (18).

The BC-112/BD 26–152 heterodimer was probed with endo-protease Endo Lys C, which cleaves on the C-terminal side of lysine residues. Fig. 3 shows MALDI-TOF mass spectra that were collected at various times after the addition of protease. Although there are potential cleavage sites throughout the primary sequence of both BC-112 and BD 26–152 (see Fig. 6), cleavage only occurs in a limited number of sites. Twenty-five minutes after the addition of protease, BC-112 is cleaved mainly at Lys20, generating fragment BC 1–109 (Fig. 3B). BC 1–109 is quite resistant to further proteolysis, persisting even 60 min into the reaction (Fig. 3C). Cleavage also occurs at Lys55 of BC-112, generating fragments 1–55 and 56–109, which are also resistant to further proteolysis. Our previous studies showed that this same site is also accessible in the BC-112 homodimer (17). BD 26–152 quickly loses 22 residues from its C terminus, generating a BD 26–130 fragment (Fig. 3B), which is very stable: no additional cleavage occurs within the fragment even after 60 min of proteolysis (Fig. 3C).

Limited proteolysis was also performed with V8, which cleaves on the C-terminal side of glutamate residues (see Fig. 6 for sequences). Sixty minutes after the addition of protease, BC-112 remained intact, whereas BARD1 was cleaved at residue 119, generating a stable BD 26–119 fragment (see Fig. 5B).
dimerizing with BD 26–140.

To further probe differences in the N-terminal region of BRCA1, limited proteolysis was performed with V8. The mass spectra shown in Fig. 5 illustrate a pronounced difference in the proteolytic susceptibility between the BRCA1 homodimer and heterodimer to V8. The enzyme readily cleaved the N-terminal region of the homodimer, generating fragments BC 11–112, BC 11–100, and BC 11–75 (Fig. 5A). In contrast, BC-112 remained completely intact in the heterodimer (Fig. 5B). Taken together, the proteolysis results, summarized in Fig. 6, clearly illustrate that in each case the homodimers are more susceptible to proteolysis than the heterodimer. Similar results were observed with the slightly shorter BD 26–131 construct (data not shown).

Neither Homodimeric nor the Heterodimeric Complex Binds Nucleic Acids—We tested the ability of our RING finger constructs to bind nucleic acids using double- and single-stranded DNA cellulose and single-stranded RNA agarose (poly(A), poly(C), poly(G), and poly(U)) columns. Homodimeric (BC-112, BD 26–131, and BD 26–140) and heterodimeric (BC-112/BD 26–131 and BC-112/BD 26–140) complexes were applied to each nucleic acid column and eluted with a NaCl gradient. The zinc finger domain from ADR1 (a known DNA-binding domain from a yeast transcription factor) was used as a positive control (19). Fig. 7 shows the elution profiles for BC-112, BD 26–140, BC-112/BD 26–131, and the ADR1 zinc finger domain from a double-stranded DNA column. Whereas the ADR1 zinc finger was retained on the column, eluting at ~400 mM NaCl, the RING finger complexes showed no affinity for double-stranded DNA, eluting in the void volume (50 mM NaCl). A general property of nucleic acid-binding proteins is that they exhibit detectable affinity to nonspecific nucleic acids. The sequence-specific DNA-binding ADR1 zinc finger domain displays this property. ADR1 bound to every column assayed, eluting from single-stranded DNA, poly(A), poly(C), poly(G), and poly(U) RNA columns at NaCl concentrations of ~350, ~325, ~200, ~400, and ~150 mM, respectively. In direct contrast, neither homodimer nor the heterodimer displayed any affinity for the columns tested, with the protein eluting in the 50 mM NaCl wash in every case (data not shown). Similar results are observed with the BD 26–131 construct (data not shown).

DISCUSSION

Although BRCA1 has been implicated in the maintenance of genome integrity (10, 15) and in transcriptional activation (16, 20), little is known about its specific function in either of these processes. In an approach aimed at characterizing functional properties of BRCA1, we have focused on its interactions with BARD1, a protein shown to interact with BRCA1 in vivo (8). Our results demonstrate that, individually, BRCA1 and BARD1 RING finger constructs exist as homodimers and that they preferentially form heterodimers. Limited proteolysis analysis defines the RING finger structural domains required for heterodimerization as comprising residues 1–109 of BRCA1 and residues 26–119 of BARD1. The heterodimer is remark-
ably stable, existing at concentrations well below $10^{-2}\text{M}$. The RING finger motifs correspond to residues 24–64 and residues 50–86 in BRCA1 and BARD1, respectively. Thus, our results demonstrate that these motifs are part of a larger structural domain, rather than representing stable autonomous domains themselves. A similar situation exists for the recombination-activating gene 1 (RAG1) protein, the RING finger motif of which is also contained within a larger domain that exists as a homodimer in solution (21). The three domains share little homology aside from the consensus RING finger motif residues, and RAG1 does not share additional sequence homology with either BRCA1 or BARD1.

There are several reports of studies in which constructs that consist of a minimal RING finger motif sequence have been characterized, including a metal binding study of the RING finger (residues 22–77) of BRCA1 (22) and solution structure studies of the RING fingers from promyelocytic leukemia proto-oncogene and immediate-early equine herpesvirus-1 protein (11, 23). In contrast to BRCA1, BARD1, and RAG1, the short RING finger constructs used in the structure determinations were reported to be monomeric. In each case, however, the authors observed low solubility and a tendency of the constructs to aggregate. This is consistent with our experience with shorter RING finger constructs of BRCA1. Although results derived from studies involving minimal RING finger constructs may provide interesting information, the use of suboptimal domains may not reflect properties of the native proteins. In contrast, longer constructs of BRCA1 and BARD1 have good solubility properties and are well behaved in solution, which are general properties of stable, folded structural domains.

Our studies and others (8, 9) demonstrate that the N-terminal domain of BRCA1 is capable of high affinity protein-protein interactions with heterologous partners. There has been much speculation in the literature, however, concerning the role of RING finger domains as potential DNA-binding modules (3). The conflicting reports on the DNA binding ability of RING finger domains, along with studies that implicate BRCA1 and BARD1 in DNA repair and transcriptional activation, led us to test the nucleic acid binding properties of our well behaved and well characterized RING finger constructs. Despite the fact that all the constructs are positively charged at neutral pH, with calculated pI values ranging from 7.7 to 8.4, neither homodimer nor the heterodimer exhibited any nucleic acid binding activity. Consequently, if BRCA1 and BARD1 play a role in DNA repair and/or transcriptional activation, interactions with DNA must be mediated either by other regions of the proteins or by additional cofactors.

Proteolysis mapping and fluorescence experiments provide general structural insights as a preliminary step toward building a picture of the BRCA1/BARD1 heterodimer. The strong protection from proteolysis of regions outside the RING finger motif afforded in the heterodimer suggests that these regions may play a direct role in the heterodimer interface. In contrast,

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3 Serial dilution experiments failed to detect dissociation of the dimers by either fluorescence or gel-filtration to the lowest concentrations at which a signal could be measured (J. Meza, P. Brzovic, and R. Klevit, unpublished observation).

4 J. Meza, P. Brzovic, and R. Klevit, unpublished observations.
studies reported here are a first step in our goal to identify and characterize can greatly aid in biochemical and functional classification of structural domains and binding partners. Such a involvement. An important early step in such efforts is the identification of the functions of the proteins involved. A major challenge in the study of breast cancer and other diseases is the determination of the functions of the proteins involved. An important early step in such efforts is the identification of structural domains and binding partners. Such a characterization can greatly aid in biochemical and functional studies of large multidomain, multifunctional proteins. The studies reported here are a first step in our goal to identify and understand the structure and function of the N-terminal regions of BRCA1 and BARD1.

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