The Cancer-predisposing Mutation C61G Disrupts Homodimer Formation in the NH2-terminal BRCA1 RING Finger Domain*

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The breast and ovarian cancer tumor suppressor gene, BRCA1, encodes for a Zn2+-binding RING finger motif located near the protein NH2 terminus. The RING finger motif is characterized by eight conserved Cys and His residues which form two Zn2+-binding sites termed Site I and Site II. We used limited proteolysis in conjunction with matrix-assisted laser desorption ionization time-of-flight mass spectroscopy to investigate the metal binding properties and to probe the solution structures of wild-type and mutant BRCA1 constructs that include the RING finger. Our results show that the RING finger motif is part of a larger proteolysis-resistant structural domain which encompasses the first 110 residues of BRCA1. Analytical gel-filtration chromatography and chemical cross-linking experiments demonstrate that the BRCA1 NH2-terminal domain readily homodimerizes in solution. The cancer-predisposing C61G mutation, which alters a conserved Zn2+-binding residue, abolishes metal binding to Site II of the RING finger motif, while Site I remains intact and functional. The C61G mutation also results in increased proteolytic susceptibility of the COOH-terminal portion of the NH2-terminal domain and perturbs the oligomerization properties of BRCA1.

The BRCA1 tumor suppressor gene encodes for a large protein of 1863 amino acid residues that bears little homology to previously described proteins (1). The NH2-terminal region of BRCA1, however, contains a conserved pattern of cysteine and histidine residues associated with the RING finger family. The RING finger is a Zn2+-binding motif found in a wide variety of proteins that are diverse both in function and origin (2–4). Although generally thought to mediate macromolecular interactions, a specific function for RING finger domains has yet to be defined. Nonetheless, the RING finger domain is clearly important for BRCA1 function. The first 100 residues, which include the RING finger motif (residues 24–64), comprise the most highly conserved region among known BRCA1 genes (5, 6). Cancer-predisposing missense mutations linked to the development of breast and ovarian cancers have been identified within the RING finger domain of BRCA1 (7, 8). In addition, another RING finger-containing protein, BARD1 (BRCA1-associated RING domain 1), has been identified that specifically interacts with the NH2-terminal region of BRCA1 (9).

Structures of RING finger domains have been determined for three proteins; the promyelocytic leukemia proto-oncogene PML (10), immediate-early EHV-1 protein from equine herpesvirus (IEEHV) (11, 12), and the VDJ recombination-activating protein (RAG1) (13). The structures share common folding topologies for the core of the domain (Fig. 1), which include at least two antiparallel β-strands and tetrahedral coordination of two Zn2+ atoms by the eight conserved residues that define the RING finger motif. The two Zn2+ sites are formed by alternating pairs of coordinating ligands with the first and third pairs forming Site I and the second and fourth pairs forming Site II. The PML and IEEHV solution structures show that the RING finger domain alone can behave as an autonomous Zn2+ dependent folding motif, but the RING finger of RAG1, in combination with a second RAG1 Zn2+-binding module, forms a larger structural unit that specifically homodimerizes in solution. Thus, there are significant differences among RING finger structures that are likely of functional significance. To understand properties of BRCA1 that may contribute to its function, we have undertaken a detailed analysis of the NH2-terminal region of BRCA1 that includes the RING finger motif.

EXPERIMENTAL PROCEDURES

Materials—Sequencing grade endoproteinase Lys-C (Pierce) and V8 (Worthington) were used. Bovine serum albumin, ovalbumin, a-chymotrypsinogen, ribonuclease A, and glutaraldehyde (Sigma) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and 2,2′,6′,2″-terpyridine (Teryp) (Aldrich) were used without further purification.

Expression and Purification of BRCA1 Constructs—Constructs containing the first 76, 112, and 172 amino acid residues of BRCA1 were prepared by PCR amplification of the desired regions using human lymphoblast cDNA as a template. Primers were synthesized to obtain BRCA1 PCR products incorporating NheI and BamHI endonuclease restriction sites in the 5′ primer and 3′ primer, respectively. PCR products and pET11a vector (Novagen) were digested with NheI and BamHI, and the DNA insert was cloned into the vector. The ligated construct was used to transform Escherichia coli BL21 cells for protein expression. Cloned inserts were sequenced using a Perkin-Elmer ABI prism 377 DNA sequencer. Due to the restriction sites used, each construct contains two extra amino acids (Ala and Ser), at the amino terminus. Construct and fragment designations refer to the number and position of BRCA1 residues.

Expression and Purification of BRCA1 NH2-terminal Constructs—Starter cultures grown from single colonies were inoculated into 500-ml Luria broth/2-liter flasks containing 100 μg/liter ampicillin to an initial A600 nm of 0.1 and grown at 37 °C with shaking. When the A600 nm
reached ~0.5–0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5–1.0 mM to induce expression. Cells were allowed to grow for 4 h after induction and harvested by centrifugation.

Cell pellets were resuspended in ~30 ml of lysis buffer (50 mM Tris, 0.1 mM KCl, 5 mM EDTA, 10 mM dithiothreitol, 0.1% Triton X-100, pH 7.6), phenylmethylsulfonyl fluoride (4 mM) added, and cells were lysed with a French press. The homogenate was incubated with DNase, RNase, and MgCl2 (10 mM) at 4 °C for 30 min prior to centrifugation at 20,000 × g for 30 min at 4 °C. Each BRCA1 construct separated into the pellet fraction, which was washed with lysis buffer, solubilized in 6 M guanidine HCl in the presence of Zn2+, and dialyzed overnight at 4 °C against 0.1% HOAc. The resulting supernatant was lyophilized, reconstituted in 0.1% HOAc, and further purified by reverse-phase high performance liquid chromatography using a Waters Delta Pak C4 column with a water/ACN gradient containing 0.1% trifluoroacetic acid. Protein was applied at 30% ACN and eluted with a linear 30–50% ACN gradient varying at a rate of 0.5%/min. Protein-containing fractions were lyophilized, resuspended in 2 M guanidine HCl in the presence of Zn2+, and purified by gel-filtration chromatography (see below). Protein purity (typically ≥99%) and amino acid composition were determined by SDS-PAGE and MALDI-TOF mass spectrometry. Protein concentrations were determined by UV spectroscopy. Approximate extinction coefficients at 276 nm, calculated on the basis of amino acid composition, were 4.06 and 5.51 mM−1 cm−1 for wt BC-76 and BC-112, respectively.

UV-visible Spectroscopy—Co2+ binding to BC-76 peptides was monitored by acquiring UV-visible spectra over a range of 290–800 nm using an AVIV model 14DS UV-visible spectrophotometer. Buffer A was sparged with N2 to reduce oxidation of cysteine residues. Peptide concentrations were approximately 50 μM. The concentration of Co2+ stock solutions were determined spectrophotometrically using Terpy (14).

Limited Proteolysis and MALDI-TOF Spectroscopy—Fractions purified by gel-filtration chromatography in Buffer A were quantitated and used directly for limited proteolysis experiments. Protease, either endoprotease Lys-C or V8, was added to a final protein:protease ratio of 250–1000:1 (w/w). Reaction aliquots (5–μl) were removed at 0, 3, 10, 25, 60, and 120 min after the addition of protease and quenched by dilution into an equal volume of 0.5% trifluoroacetic acid. Proteolysis of metal-free protein samples was performed both in the absence and presence of EDTA.

A saturated 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix solution was prepared in 30% ACN solution and 0.1% trifluoroacetic acid. MALDI samples were prepared using the dried droplet method (15), and mass spectra were acquired with a Perseptive Voyager Elite MALDI mass spectrometer equipped with delayed ion extraction.

RESULTS

Oligomerization State of BRCA1 RING Finger Constructs—Gel-filtration chromatography was used to assess the oligomerization properties of the NH2-terminal region of BRCA1. We engineered three constructs of BRCA1 (termed BC-76, BC-112, and BC-172 based upon the number of BRCA1 residues), all of which start at the NH2 terminus of BRCA1 and include the RING finger motif. Fig. 2A shows gel-filtration elution profiles for the BC-76 and BC-112 constructs. Both wt BC-76 and
BC-112 elute primarily as sharp peaks with peak widths nearly identical to those of standard proteins used for column calibration (profiles of standards not shown). This indicates that each protein elutes predominantly as a single species. Relative to the standard proteins, the apparent molecular weight for each construct is slightly more than twice that predicted from their monomeric molecular weights. These findings suggest that the BRCA1 RING finger constructs specifically oligomerize in solution.

To verify the self-association inferred from the gel-filtration experiments and to determine oligomer stoichiometry, wt BC-76 and BC-112 were subjected to glutaeraldehyde cross-linking (Fig. 2B). The results show that the predominant species captured by the cross-linking reaction is a dimer. Some higher order oligomers are observed at higher glutaeraldehyde concentrations and longer incubation times. Taken together, the gel-filtration and cross-linking experiments indicate that the NH$_2$-terminal BRCA1 constructs form homodimers in solution.

The C61G cancer-predisposing mutation alters one of the conserved Site II Zn$^{2+}$-binding ligands in the RING finger motif (Fig. 1). As shown in Fig. 2A, both the BC-76 and BC-112 C61G mutant constructs elute from the gel-filtration column as a broad peak with a large apparent molecular weight. This behavior is indicative of proteins involved in weak, nonspecific aggregation. Wild-type BRCA1 RING finger constructs in the absence of Zn$^{2+}$ show similar elution profiles (data not shown).

**Metal Binding Properties of the BRCA1 RING Finger**—Metal binding to both the wild-type and C61G mutant RING fingers was investigated using Co$^{2+}$ as a spectroscopic probe. Previous studies have shown that addition of Co$^{2+}$ to the RING finger domains of PML and BRCA1 yields characteristic spectral bands in the 600–800 nm region of the visible spectrum that are consistent with tetrahedral coordination of the Co$^{2+}$ at both Site I and Site II by the conserved RING finger ligands (Fig. 1; Refs. 10 and 16). The absorbance at 695 nm increases linearly with concentration up to approximately 1 equivalent of Co$^{2+}$ (Fig. 3). Saturation occurs at slightly more than 2 equivalents of Co$^{2+}$/BC-76 peptide. These results indicate that Co$^{2+}$ binding occurs in two discrete stages, consistent with the findings of Roehm and Berg (16), who assign the first binding step to coordination of Co$^{2+}$ by the four cysteine ligands of Site I. Titration of C61G BC-76 with Co$^{2+}$ shows that stoichiometric binding to the higher affinity Site I is retained. The initial phase of the titration is nearly superimposable on the wild-type curve. However, binding saturates at ~1 equivalent of Co$^{2+}$. Thus, in the mutant RING finger metal binding to Site I is retained, while binding to Site II is either abolished or substantially reduced in affinity.

**Effects of Zn$^{2+}$ on the Structure and Stability of the BRCA1 NH$_2$-terminal Region**—Proteolytic susceptibility can be used to identify both stable structural regions and flexible segments within proteins (17–19). We used limited proteolysis in conjunction with MALDI-TOF mass spectrometry to probe structural changes that result from Zn$^{2+}$ binding to the wild-type and C61G BC-76 constructs. MALDI-TOF mass spectra were collected on reaction samples quenched at various times after the addition of the endoprotease (Endo) Lys-C (Fig. 4). Prior to the addition of protease, only a single species corresponding to intact wt BC-76 is observed in the mass spectrum. Since Endo Lys-C cleaves COOH-terminal to lysine residues, there are nine potential cleavage sites in BC-76 (Fig. 4). In the absence of Zn$^{2+}$, considerable cleavage is observed throughout the protein only 3 min after the addition of protease (Fig. 4A), indicating that nearly every site is accessible to protease. After 60 min, proteolytic degradation is complete. Similar results were obtained with V8, which primarily targets glutamate residues (data not shown). These data show that in the absence of Zn$^{2+}$, BC-76 is fairly flexible, with little or no protease-resistant structure.

In contrast, wt BC-76 in the presence of Zn$^{2+}$ is quite resistant to proteolysis (Fig. 4B). Cleavage is restricted to two sites, resulting in fragments 1–70 and 1–55. Lys-70, the primary cleavage site after 3 min, is outside the minimal RING finger motif. Lys-55 is located between the third and fourth pairs of Zn$^{2+}$-binding residues in the RING finger motif. Both fragments persist after 60 min of digestion. Thus, Zn$^{2+}$ binding to BC-76 yields two protease-resistant fragments: one that contains both Zn$^{2+}$-binding sites and one that contains Site I only.

Consistent with the Co$^{2+}$ binding studies that indicated C61G BC-76 still binds one metal ion, the BC-76 mutant is clearly less susceptible to proteolysis than the apo-peptide. Instead, the proteolysis profile of C61G shows similarities to the wt Zn$^{2+}$-bound BC-76. At early time points, the same two fragments (1–55 and 1–70) as are seen in the wt BC-76 digest are observed for C61G. Inspection of Fig. 4C reveals that a fragment (1–65) not observed during proteolysis of wt BC-76 accumulates in the C61G reaction mixture after 3 min. Lys-65 is immediately adjacent to the last conserved cysteine residue of the RING finger motif and is rendered susceptible to proteolysis as a consequence of the C61G mutation. These results show that the C61G mutant is capable of binding Zn$^{2+}$ at Site I, but the mutation significantly alters the metal binding properties at Site II, which, in turn, affects the COOH-terminal structure of BC-76 rendering it more susceptible to proteolytic degradation.

**Determination of the Minimal NH$_2$-terminal Domain of BRCA1 That Contains the RING Finger Motif**—To determine whether the RING finger motif itself represents a complete and autonomous folding domain or is instead part of a larger structural domain, we performed limited proteolysis on two longer constructs, BC-112 and BC-172. Addition of Endo Lys-C results in rapid cleavage at residue 109 of BC-112, three residues from the COOH terminus (Fig. 5A). The remaining fragment is relatively stable against further proteolytic degradation. As observed for BC-76, some cleavage occurs at Lys-55 within the RING finger motif, generating fragments 1–55 and 56–109 and showing that this region of the RING finger domain is still accessible to protease despite the presence of the extra COOH-terminal residues. The stability of BC-112 is strongly Zn$^{2+}$-dependent, as rapid degradation of the entire construct occurs upon addition of excess EDTA (data not shown). Identical ex-
periments with the larger BC-172 construct also show rapid cleavage at residue 109 by Endo Lys-C (Fig. 5B). As with BC-112, residues 1–109 are moderately protected from further proteolysis, while the COOH-terminal fragment is rapidly degraded. Similar profiles were obtained for cleavage of BC-112 and BC-172 with V8 protease (data not shown). We therefore conclude that the first 110 residues of BRCA1 form a unique structural domain.

Limited proteolysis of the C61G BC-112 mutant yields a proteolytic profile very different from that of wt BC-112 (Fig. 5C). The mutant is degraded much faster than the wild-type construct. Furthermore, there is very little accumulation of the 1–109 fragment, while rapid cleavage at both Lys-55 and Lys-65 is readily apparent. Fragments corresponding to either residues 56–109 or 66–109 are not observed, indicating that the COOH-terminal region of C61G BC-112 is rapidly degraded. The appearance and persistence of the fragment 1–55 indicates that the mutant is still capable of binding Zn$^{2+}$ at Site I, similar to the shorter C61G BC-76 construct. Therefore, loss or reduction of Zn$^{2+}$ binding at Site II has a profound effect on proteolytic susceptibility throughout the COOH-terminal portion of the molecule.

**DISCUSSION**

Although the importance of BRCA1 in tumor suppression and cell growth regulation is widely recognized, little is known about properties of the protein that are pertinent to its function. To begin to address this question, we characterized a number of solution properties of the NH$_2$-terminal portion of both wild-type and mutant BRCA1. Our results show that the first 110 residues of BRCA1, which include the RING finger motif, form a structural domain that readily homodimerizes in solution. As will be detailed elsewhere, the BRCA1 NH$_2$-terminal domain identified by limited proteolysis of the homodimer is the same as that required for specific heterodimer formation with BARD1 (9).

The cancer-predisposing C61G mutation affects several important properties of the BRCA1 NH$_2$-terminal domain. Constructs possessing this mutation bind a single metal ion (either Zn$^{2+}$ or Co$^{2+}$) at Site I, while Site II is nonfunctional. The inability to bind Zn$^{2+}$ at Site II has two clear ramifications for the solution properties of the NH$_2$-terminal domain. First, gel-filtration experiments reveal that the mutant constructs behave as aggregates that are larger than the well-behaved dimers formed by wild-type constructs. Second, the C61G mutation causes increased susceptibility to proteolysis not only at a site directly following Zn$^{2+}$ (i.e. Lys-65), but throughout the COOH-terminal region of the domain. The increased flexibility (and possible unfolding) of the COOH-terminal portion of the domain may contribute to its tendency to aggregate.

Recent biophysical characterization of the RING finger do-
The asterisk denotes the presence of known impurities in BC-172 samples. The arrow in A highlights fragment 56–109, which persists in the wild-type proteolysis, but is absent from the mutant cleavage reaction. The arrow in C indicates cleavage at Lys-65, immediately adjacent to Site II, which is fully protected in the wild-type protein. The asterisk denotes the presence of known impurities in BC-172 samples.

The protein concentrations used in our studies are undoubtedly lower than the concentrations required for BRCA1 homodimerization in vitro. It will therefore be important to determine an accurate K_d for BRCA1 homodimerization to assess the functional significance of these observations.

The identification of a BRCA1 NH2-terminal domain permits characterization of specific protein-protein interactions and investigation of possible regulatory events that mediate these functions. Determination of the boundaries of the BRCA1 NH2-terminal structural domain also affords a starting point for detailed structural investigations of BRCA1. Although BRCA1 exhibits some traits in common with the RAG1 homodimer (13), there is no evidence for the existence for another BRCA1 Zn2+-binding motif in proximity to the RING finger motif, and the sequence homology between RAG1 and BRCA1 in the regions surrounding the RING finger motif is low. Thus, the structure of the BRCA1 dimerization interface is likely to be unique.

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