BRCA1 RING Domain Cancer-predisposing Mutations
STRUCTURAL CONSEQUENCES AND EFFECTS ON PROTEIN-PROTEIN INTERACTIONS*

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Peter S. Brzovic‡, Jose E. Meza§, Mary-Claire King§, and Rachel E. Klevit‡‡
From the ‡Departments of Genetics and Medical Genetics and §Department of Biochemistry and Biomolecular Structure
Center, University of Washington, Seattle Washington 98195-7742

Cancer-predisposing missense mutations in the RING domain of BRCA1 primarily target Zn$^{2+}$-liganding residues. Here we report on the structural consequences of such mutations introduced into the second Zn$^{2+}$ site (Site II) of the BRCA1 RING domain and their effect on the interaction with the BARD1 RING domain. Each of the BRCA1 Site II mutants still interact and form a stable heterodimer with BARD1. Limited proteolysis of BRCA1/BARD1 complexes, monitored by matrix-assisted laser desorption ionization time-of-flight spectrometry, show that the mutations cause a local structural perturbation that is primarily confined to the second Zn$^{2+}$ binding loop of the BRCA1 subunit. These findings are consistent with the structure of the BRCA1/BARD1 heterodimer, which shows this region is well removed from the helices required for dimerization with BARD1. Instead, the mutations alter a region of BRCA1 that appears to be required for interaction with ubiquitin-conjugating enzymes.

In the reducing environment of the cell where conditions disfavor disulfide bonds, small modular protein domains often require metal ions to confer structural stability and integrity. Zn$^{2+}$ is commonly used in this role and is found at the core of a number of domains including Zinc fingers, GATA domains, hormone receptors, PHD and Lim domains, and RING domains (1). These domains generally utilize a characteristic combination of cysteine and histidine residues as metal ligands. Because of the central importance of Zn$^{2+}$ as a structural component, missense mutations that target Zn$^{2+}$-liganding residues can adversely affect protein structure with a concomitant impairment or loss of protein function. Often Zn$^{2+}$-dependent domains are found as individual components of larger multi-domain proteins. The breast and ovarian cancer susceptibility gene BRCA1 encodes an 1863-residue protein that contains a Zn$^{2+}$-dependent RING motif (2). The functional importance of the RING domain is underscored by its high degree of sequence conservation among BRCA1 species and the existence of cancer-predisposing missense mutations that target Zn$^{2+}$-liganding residues.

The RING motif is characterized by a conserved pattern of seven cysteine and one histidine residues arranged in an inter-leaved fashion forming two distinct Zn$^{2+}$-binding sites (termed Site I and Site II, Fig. 1) (3). The key to structural characterization was the determination that the BRCA1 RING motif, which encompasses residues 24–64, is part of a larger domain (residues 1–109) that includes residues both N- and C-terminal to the core RING motif (4). This domain of BRCA1 preferentially forms a stable heterodimer complex with the RING domain of BARD1 (5, 6), a protein that also binds to mRNA polyadenylation factor CtsF-50 to inhibit 3' end processing of mRNA precursors (7). BRCA1 and BARD1 interact via an extensive anti-parallel 4-helix bundle interface formed by helices that flank the central RING motifs (Fig. 1). Although this arrangement positions the two central RING motifs of BRCA1 and BARD1 adjacent to each other, there are few direct interactions between them (8).

BRCA1 and BARD1 have been shown to co-localize in vivo in a cell cycle-dependent manner and in response to DNA damage (9). This association has received increased attention because recent studies have shown that the BRCA1/BARD1 heterodimer plays a functional role in facilitating ubiquitin transfer reactions (10). A growing number of RING domains has been demonstrated to function as RING ubiquitin ligases that work in concert with ubiquitin-conjugating enzymes to facilitate the transfer of ubiquitin to target proteins (11). By itself, the BRCA1 RING domain exhibits some activity as a RING E3 ubiquitin ligase (12). This activity increases dramatically upon formation of a BRCA1/BARD1 RING domain heterodimer (10). Cancer-predisposing mutations that target Zn$^{2+}$-liganding residues abolish this activity.

Undoubtedly, Zn$^{2+}$ plays a critical role in stabilizing the BRCA1 RING domain, but the structural effects caused by mutation of Zn$^{2+}$ ligands is not known. To investigate the consequences of cancer-predisposing mutations, we constructed a series of single-site mutations that alter Zn$^{2+}$-liganding residues in Site II of the BRCA1 RING domain (Fig. 1). We found that Site II mutants not only fold and bind Zn$^{2+}$ at Site I but also retain the ability to bind Zn$^{2+}$ at Site II, albeit with reduced affinity. Furthermore, each of the BRCA1 mutants retain the ability to spontaneously form heterodimers with the RING domain of BARD1. Using limited proteolysis in conjunction with MALDI-TOF mass spectrometry, we demonstrate that the structural effects of these BRCA1 Site II mutations are primarily localized to the second Zn$^{2+}$ binding loop.

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§ Supported by a Research Supplement for Underrepresented Minorities, National Institutes of Health Grant ROI CA27632-18S1. Present address: Dept. of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143.
‡ To whom correspondence should be addressed: University of Washington, Dept. of Biochemistry and Biomolecular Structure Center, Box 357742, Seattle, WA 98195-7742. Tel.: 206-543-5891; Fax: 206-543-8394; E-mail: klevit@u.washington.edu.

¶ The abbreviations used are: E3, ubiquitin ligase; E2, ubiquitin-conjugating enzyme; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; TOF, time-of-flight; wt, wild type; Endo, endoproteinase.
within the central RING motif of BRCA1, a region not involved in the BRCA1-BARD1 dimerization interface. Thus, BRCA1 Site II mutations cause a local structural perturbation and do not abolish one function of the BRCA1 RING domain, the ability to form a specific complex with BARD1. Instead, Site II cancer-predisposing mutations may alter a binding surface required for BRCA1 to interact with a ubiquitin-conjugating enzyme.

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). Bovine serum albumin, ovalbumin, α-chymotrypsinogen, ribonuclease A, apo-equine myoglobin, and bovine insulin (as standards) were purchased from Sigma. The QuikChange site-directed mutagenesis Kit (Stratagene) and verified by DNA sequencing. Expression, purification, and quantitation of wild-type BRCA1 and BARD1 N-terminal domain constructs were prepared as previously described (4, 5). Single-site mutagenesis of BRCA1 was performed using the QuikChange site-directed mutagenesis Kit was purchased from Stratagene.

Cloning and Purification of BRCA1 and BARD1 N-terminal Domain Constructs—Wild-type BRCA1 and BARD1 N-terminal domain constructs were prepared as previously described (4, 5). Single-site mutations were introduced into the wild-type BRCA1 plasmid, ovulation, α-chymotrypsinogen, ribonuclease A, apo-equine myoglobin, and bovine insulin (as standards) were purchased from Sigma. The QuikChange site-directed mutagenesis Kit (Stratagene) and verified by DNA sequencing. Expression, purification, and quantitation of wild-type and mutant BRCA1 and BARD1 constructs were as previously described (4, 5).

Characterization of Heterodimer Formation—Gel filtration chromatography, fluorescence spectroscopy, and glutaraldehyde cross-linking experiments were performed as previously described (4, 5).

Limited Proteolysis and Collection of MALDI-TOF Mass Spectrometry Data—Heterodimer samples purified by gel filtration chromatography in 25 mM Tris, 0.2 mM NaCl, pH 7.6, were quantitated and used directly in limited proteolysis experiments. Proteolysis was performed under conditions designed to limit multiple cleavage events within a single polypeptide chain. Endoproteinase Lys-C was added to 8 μM heterodimer samples at a protein:protease ratio of either 4000:1 or 8000:1. These ratios were optimal for following the degradation and accumulation of protein fragments. Reaction aliquots (15 μl) were removed at 0, 3, 7, 15, 25, 45, 60, 90, and 130 min after the addition of protease, and the reaction was quenched by addition of 5 μl 3% trichloroacetic acid solution.

A saturated matrix solution was prepared by dissolving 35 mg of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid. Matrix was mixed with sample in a ratio of 7:3 and spotted immediately on a MALDI sample plate in duplicate. MALDI-TOF mass spectra were acquired and analyzed using a Bruker Daltonics Biflex-III MALDI-TOF mass spectrometer equipped with delayed ion extraction.

Identification of peptides resulting from proteolysis with Endo Lys-C was accomplished using the mass spectrometry analysis program PAWS (www.proteometrics.com). A cubic-spline baseline correction was applied to each spectra before the determination of peak intensities for all observable peptides. Proteolytic time courses were generated from the MALDI-TOF data by normalizing observed peak intensities. Peptide fragments were divided into two groups, either N-terminal or C-terminal peptides as shown in Table I. Normalized peak intensities were calculated by dividing the observed peak intensity for a peptide by the sum of all observed intensities for peaks within a group. Differentiating between N- and C-terminal peptides prevents a single cleavage event from being counted twice. Full-length BC-112 is considered to be either an N- or C-terminal peptide. Fragments ending at residue 109 were placed in the same group as the corresponding fragment terminating at residue 112.

Nanoflow Electrospray Ionization (ESI)-TOF Mass Spectrometry—Wild-type and mutant heterodimer samples were prepared in 50 mM ammonium acetate, pH 7.0, by gel filtration chromatography. All samples were centrifuged before analysis. Nanospray ionization time-of-flight (ESI-TOF) spectra were acquired using a Mariner Biospectrometry work station (PerSeptive Biosystems) equipped with a syringe pump. Sample flow rate was between 0.5 and 1.0 μl/min. Spectra shown are the average of 25–100 scans/sample. Spectra were deconvoluted using the data explorer software provided with the system.

RESULTS

Heterodimerization of BRCA1 Site II Mutants with BARD1—Some of the most frequently reported cancer-predisposing mis-
sense mutations in BRCA1 target the Zn\textsuperscript{2+}-liganding residues Cys-61 and Cys-64 within the second Zn\textsuperscript{2+}-binding site (Site II) of the RING domain. To understand the structural consequences of these mutations in BRCA1, we constructed and purified BRCA1 Site II mutants in which each Zn\textsuperscript{2+}-liganding residue (Cys-39, His-41, Cys-61, and Cys-64) was individually altered (Table II). As reported previously (4, 5), both wild-type BRCA1 and BARD1 proteins can homodimerize but preferentially form heterodimers when mixed. This behavior was demonstrated using gel filtration chromatography, fluorescence spectroscopy, and glutaraldehyde cross-linking techniques. We tested the ability of each BRCA1 Site II mutant to form heterodimers with BARD1 using the same experimental approaches. The elution profiles for all mutant BRCA1/BARD1 heterodimers constructs on an analytical gel-filtration column were essentially identical to the wild-type heterodimer complex (data not shown). Since gel filtration chromatography separates molecules based on their relative size, these results indicate that both wild-type and mutant species form well-defined heterodimers with BARD1 with little predilection of the mutant complexes to form higher order aggregates at concentrations (μM) used in this work.

Fluorescence provides a useful means to follow BRCA1/BARD1 heterodimer formation. The fluorescence spectrum of BD-115 (which contains two Trp residues, whereas BC-112 has none) undergoes a significant shift in emission wavelength and increase in fluorescence intensity upon heterodimer complex formation with BC-112 (5). Identical spectral shifts are observed for the complex with each of the BRCA1 Site II mutants and BARD1 (data not shown). The observation that the fluorescence spectrum of BARD1 is the same for each heterodimer complex suggests that the structure of BD-115 in the heterodimer, at least in the regions surrounding the tryptophan residues, is unaffected by mutations in the BRCA1 subunit of the heterodimer. Heterodimer formation between BRCA1 Site II mutants and BARD1 was also confirmed by glutaraldehyde cross-linking experiments (data not shown).

Limited Proteolysis of BRCA1 Site II Mutant/BARD1 Heterodimer Complexes—Introduction of BRCA1 Site II mutations has hampered detailed structural investigations as the concentrations required (~μM) are difficult to obtain. Therefore, we used limited proteolysis in conjunction with MALDI-TOF mass spectrometry to investigate structural changes caused by BRCA1 Site II mutations. MALDI-TOF is particularly amenable to these studies given the ability of this technique to resolve and analyze mixtures of proteins and peptides from aqueous buffer solutions (14). For these studies, we relied primarily on the protease endoproteinase Lys-C based on the favorable distribution of lysine residues throughout the primary sequence of BRCA1 (Fig. 2E), although similar results are obtained using other proteases.

Mass spectra collected on reaction samples quenched at various stages during limited proteolysis of wild-type and mutant BRCA1/BARD1 heterodimers are presented in Fig. 2. As shown for the wild-type complex, prior to addition of protease only full-length BRCA1 (BC-112) and BARD1 (BD-115) RING domain constructs are present (Fig. 2A). Figs. 2, B–D, compare the proteolytic patterns observed for wild-type and mutant heterodimers 130 min after the addition of Endo Lys-C. The wild-type complex (Fig. 2B) is cleaved primarily at the extreme C-terminal ends of both the BRCA1 and BARD1 constructs to yield fragments of 109 (wt BC 1–109) and 105 (BD 1–105) residues in length, respectively. Although some minor cleavage is observed at residue 55 within BRCA1 to generate BC 1–55 and BC 56–109 fragments (right panel), BC 1–109 and BD 1–105 are relatively resistant to further proteolytic degradation and represent the extent of the BRCA1 and BARD1 N-terminal structural domains (5).

The different BRCA1 Site II mutant heterodimers exhibit a variable degree of proteolytic susceptibility. Limited proteoly-
The point represents the average intensity obtained from analysis of at least protein:protease ratios of either 8000:1 (1) or 4000:1 (2) and the accumulation of BC-112 are shown for reactions utilizing – heterodimer complex. Time courses for the degradation of BC-112 and the accumulation of BC 1–109 are shown for reactions utilizing protein:protease ratios of either 8000:1 (1×) or 4000:1 (2×). Each time point represents the average intensity obtained from analysis of at least five different spectra. The error bars indicate maximum deviation observed during a time course and are similar for all subsequent experiments.

The time courses collected during proteolysis of the wild-type BC-112 heterodimer complex. The ratio of BD-115/BC-112 peak intensity remains constant across the different concentrations of heterodimers. B, effect of protease concentration on time courses derived from MALDI-TOF mass spectra. Heterodimer samples were prepared by serial dilution from a concentrated stock solution. The ratio of BD-115/BC-112 peak intensity remains constant across the different concentrations of heterodimers. B, effect of protease concentration on time courses derived from MALDI-TOF mass spectrometry data. Time courses for N-terminal fragments observed during proteolysis of wild-type BC-112/BD-115 (A), C39A BC-112/BD-115 (B), and C64A BC-112/BD-115 (C) heterodimer complexes. The time courses derived from proteolysis of the H41A BC-112/BD-115 heterodimer complex (not shown) were nearly identical to those shown for the wild-type heterodimer. Similarly, time courses observed for the C61A heterodimer complex were identical to the C64A heterodimer. The distribution of potential Lys cleavage sites within the primary sequence of BRCA1 is depicted at the bottom. Observed cleavage sites are highlighted by arrows and labeled with residue numbers.

In conclusion, the H41A heterodimer gives a proteolytic profile that is essentially indistinguishable from that of the wild-type complex (data not shown), whereas the C39A, C61G, C61A, and C64A complexes display distinctly different proteolytic patterns (in our experiments the C61G, C61A, and C64A complexes yielded nearly identical patterns of proteolysis with Endo Lys-C; therefore, only the results observed with C64A are presented). The signal arising from the BC 1–55 fragment is substantially greater during proteolysis of the C39A, C61G, C61A, and C64A mutants relative to the wild-type complex. Secondly, alteration of individual cysteine residues in Site II unmask cleavage sites at residues 65 and 70 in BRCA1 that are protected in the wild-type protein. These differences correspond to changes in proteolytic susceptibility within a 15-residue segment of BRCA1, a region within the second Zn2+ binding loop that includes the last pair of Zn2+–liganding residues that form Site II (see Fig. 1). An additional fragment corresponding to cleavage at residue 38 is observed during proteolysis of the C39A mutant complex. Importantly, as is also observed for the wild-type heterodimer, no internal cleavage fragments of BARD1 are observed during proteolysis of any of the mutant heterodimer complexes. Thus, the BD 1–105 fragment rapidly accumulates in solution and remains resistant to further proteolytic degradation despite proteolysis within the BRCA1 subunit of the heterodimer.

Although proteolysis of both the C39A and C64A (and C61G and C61A) heterodimers generates peptide fragments corresponding to cleavage at residues 55, 65, and 70 in the BRCA1 subunit, comparison of Figs. 2, C and D, reveals that the intensity of signals arising from these fragments differs substantially. This reproducible pattern occurs despite the similarity of the mutations in which a Site II Cys Zn2+ ligand is replaced with Ala. Therefore, we sought to determine whether we could extend our analysis of limited proteolysis data to generate time courses from MALDI spectra and follow the accumulation and disappearance of species during the course of a reaction.

MALDI-TOF mass spectrometry is not generally considered a quantitative technique. However, the reproducibility of our results and the similarity of all the proteins used in this study indicated that direct comparisons might be feasible (14). It was first necessary to establish how well the mass spectrometry data relate to well defined concentrations of reactants in solution. Fig. 3A shows a plot of concentration versus intensity for a serial dilution of undigested wild-type heterodimer. Although the concentrations of BRCA1 and BARD1 in the heterodimer are equal, under our experimental conditions, the intensity of the peaks that correspond to the two polypeptides differ. However, the observed ratio of BARD1 to BRCA1 remains constant. Secondly, we reasoned that varying the concentration of protease should be reflected in the observed accumulation and degradation of species in the reaction mixture. This is demonstrated in the time courses for the conversion of BC-112 to BC
1–109 obtained for the wild-type heterodimer (Fig. 3B). Increasing the concentration of Endo Lys-C in the reaction mixture increases the observed rates for the disappearance of BC-112 and accumulation of BC-109. Similar responsiveness to protease concentration was observed for the disappearance and accumulation of all species monitored during proteolysis of both wild-type and mutant heterodimers (data not shown). Thus, by these criteria the technique is responsive to the relative concentration of peptides and reflects differences in the rates of formation and decay of species in solution.

Time courses for N-terminal fragments observed during proteolysis of the wild-type and mutant BRCA1/BARD1 heterodimer complexes with Endo Lys-C are shown in Fig. 4. As described above, wt BC-112 in the wild-type heterodimer is susceptible to proteolytic cleavage almost exclusively at the C-terminal end of the construct (Fig. 4A). As a result, wt BC 1–109 appears concomitantly with the disappearance of wt BC-112. The peptide wt BC 1–55 appears relatively late in the time course and with weak intensity. The reaction does not go to 100% completion probably due to loss of protease activity during the course of the reaction. Time courses generated from limited proteolysis of the H41A heterodimer (data not shown).

Fig. 4B shows time courses for fragments observed during the proteolytic digestion of the C39A heterodimer. In contrast to the wild-type reaction, loss of C39A BC-112 is accompanied by an accumulation of both C39A BC 1–109 and C39A BC 1–55 (generation of the BC 1–55 fragment can arise by cleavage of either BC 1–112 or BC 1–109). These data show that the BRCA1 subunit is rendered more susceptible to cleavage at residue 55 as a result of the C39A mutation. A fragment corresponding to residues BC 1–38 also accumulates in the reaction mixture (Figs. 2C, 4B). The slow, steady accumulation of C39A BC 1–38 relative to C39A BC 1–55 (Fig. 4B) suggests this fragment represents a secondary cleavage event within the C39A BC 1–55 fragment rather than a primary cleavage event within either C39A BC 1–109 or BC 1–112. This is consistent with the absence of accumulation of a C39A BC 39–109 or 39–112 fragment in solution. BC 1–38 is only observed during proteolysis of the C39A mutant heterodimer, and the increased susceptibility at residue 38 is likely due to the mutation in the adjacent residue. Thus, the primary effect of the C39A mutation is increased proteolytic susceptibility at residue 55, relative to the wild-type complex.

Inspection of Figs. 2, C and D, show that both the C39A and C64A heterodimers undergo proteolytic cleavage at residues 55, 65, and 70. However, examination of time courses for BC 1–55 and BC 71–112 (Figs. 5A and B) reveal large differences in proteolytic susceptibility within this limited region of BRCA1. As shown in Fig. 5A, proteolysis at residue 55, which
is only weakly susceptible in the wild-type heterodimer, readily occurs in both the C39A and C64A heterodimers, as shown by the rapid accumulation of the BC 1–55 fragment. Only modest differences are observed between the C64A and C39A BC 1–55 time courses. In marked contrast, the time course for C64A BC 71–112 C-terminal fragment reveals this species rapidly accumulates in solution immediately after the addition of protease, whereas there is a pronounced lag time of several minutes before detection of this species during proteolysis of the C39A heterodimer. In each case, the amount of the BC 71–112 fragment slowly declines as it is converted to BC 71–109, a limit peptide that cannot be further degraded. Close examination of Fig. 4C shows that fragments C64A BC 1–65 and C64A BC 1–70 also rapidly appear in solution after the addition of protease. These N-terminal peptides probably do not accumulate to significant levels relative to other species as they can be quickly degraded to C64A BC 1–55. These data show that the Cys-39 and Cys-64 mutations, both, result in increased proteolytic susceptibility at residue 55, whereas the C64A mutation (and C61A and C61G mutations, data not shown) results in increased susceptibility throughout the region of BRCA1 that includes residues 55, 65, and 70. 

Zn$^{2+}$ Binding to Heterodimer Complexes Examined by ESI-TOF Mass Spectrometry—Although the C39A, C61A, and C64A mutations each remove a cysteine ligand from Site II of BRCA1, the differences observed in the proteolytic profiles for these mutant complexes (Figs. 2, 4, and 5) prompted us to examine whether any of the mutant heterodimer species are still capable of binding Zn$^{2+}$ at Site II. Procedures for collecting MALDI data, which include sample preparation in acidic matrix solutions and the process of laser desorption, disrupt most non-covalent and ionic interactions. Thus, MALDI spectra show only the individual peptide components. However, ESI of aqueous solutions at neutral pH may allow observation of non-covalent and ionic interactions, permitting characterization of protein tertiary and sometimes quaternary associations. This
ESI-TOF spectra for BRCA1/BARD1 heterodimers at neutral pH are shown in Fig. 6. Although each of the heterodimers is stable in solution under the experimental conditions (50 mM ammonium acetate, pH 7.0) as judged by gel filtration chromatography (data not shown), the primary species observed is not the intact heterodimer complex but the separate BRCA1 and BARD1 subunits. However, as shown in Fig. 6A, the observed masses for the wild-type proteins correspond to the binding of two Zn\(^{2+}\) atoms per subunit, as expected for RING domains. With a mass of 65 a.m.u., the absence or presence of Zn\(^{2+}\) is easily resolved. The presence of bound Zn\(^{2+}\) was verified by acidification of the sample with acetic acid (final solution pH < 4); lowering the pH denatures both the BRCA1 and BARD1 subunits with concomitant loss of bound Zn\(^{2+}\). This effect is illustrated in Fig. 6F for the C64A heterodimer complex. Under these solution conditions, the observed masses correspond to the proteins in the absence of Zn\(^{2+}\). Thus, information about protein-metal interactions are preserved in the ESI-TOF mass spectrometry experiment.

The results for the mutant complexes are shown in Fig. 6, B-F. The H41A subunit is found exclusively in the two-Zn\(^{2+}\) form (data not shown), consistent with its similarity to the wild-type subunit in limited proteolysis experiments (see “Results”). Unexpectedly, a two-Zn\(^{2+}\) species is also observed for both the C39A and C64A (and C61A) subunits. C39A (Fig. 6B) is almost exclusively in the two-Zn\(^{2+}\) form, whereas the C64A (Fig. 6C) and C61A (data not shown) subunits exist as a mixture of one-Zn\(^{2+}\) and two-Zn\(^{2+}\) species. All of the BRCA1 mutants bind at least one Zn\(^{2+}\) atom, almost certainly in Site I. This interpretation is consistent with our limited proteolysis studies (see “Results”) and reports of sequential metal binding to the BRCA1 RING domain with Site I of higher affinity (16). In all cases the BARD1 subunit contains two bound Zn\(^{2+}\) atoms.

It is possible that the two-Zn\(^{2+}\) form observed for C64A represents a nonspecific protein-Zn\(^{2+}\) interaction. To test this, excess Zn\(^{2+}\) was added to each heterodimer sample. We did not observe Zn\(^{2+}\)-protein complexes with a stoichiometry greater than two Zn\(^{2+}\) atoms/subunit, although large excess concentrations of Zn\(^{2+}\) eventually cause precipitation of the heterodimers. Interestingly, the addition of 40 \(\mu\)M Zn\(^{2+}\) (0.5 eq/all Zn\(^{2+}\) sites) to the C64A heterodimer shifts C64A from a mixture of one-Zn\(^{2+}\) and two-Zn\(^{2+}\) species (Fig. 6C) to predominantly the two-Zn\(^{2+}\) form (Fig. 6D). Alternatively, the addition of EDTA, a metal chelating agent, yields primarily the one-Zn\(^{2+}\) species with nearly complete loss of the two-Zn\(^{2+}\) form (Fig. 6E). A small amount of the C64A subunit in the zero-Zn\(^{2+}\) form is also observed. The BARD1 subunit remains relatively unaffected by the addition of excess Zn\(^{2+}\) or EDTA. This is also true for both subunits of the wild-type heterodimer complex (data not shown). Thus, these experiments show that despite the loss of a metal ligand, the mutant heterodimers are still capable of binding Zn\(^{2+}\) at Site II of the BRCA1 subunit.

**DISCUSSION**

The three-dimensional structure of the BRCA1/BARD1 heterodimer complex (8) provides a framework for understanding the results of the limited proteolysis experiments (Fig. 7). BRCA1 and BARD1 interact by formation of an extensive 4-helix bundle formed by helices N- and C-terminal to the central RING motifs of both subunits. This arrangement juxtaposes the two central RING motifs next to each other, but there are few direct interactions between them. Residues that comprise Site II of BRCA1 are well removed from the dimerization interface. Thus, mutation of Site II Zn\(^{2+}\)-liganding residues (i.e. Cys-39, Cys-61, or Cys-64) has a predominantly local structural effect that is reflected in the increased proteolytic susceptibility of the second Zn\(^{2+}\) binding loop of BRCA1. Other regions of the heterodimer are relatively unperturbed. Even excision of a 15-residue segment from the middle of the RING domain of BRCA1 (residues 56–70 in the C61A or C64A mutants) does not lead to perceptible dissociation of the heterodimer and further proteolytic degradation expected for the individual BRCA1 or BARD1 subunits (5). These results are indicative of the overall stability of the BARD1/BRCA1 heterodimer complex.

The limited proteolysis studies revealed inherent differences between the various Site II mutants. Although H41A yields a protein that is indistinguishable from wild-type by our criteria, loss of a Site II cysteine ligand has a more pronounced effect that varies depending on the site of the mutation. The proteolytic susceptibility of the second Zn\(^{2+}\) loop is much more pronounced for the Cys-61 and Cys-64 mutants than for the C39A mutant (Figs. 2, 4, and 5). This difference is also reflected in the ESI-TOF data (Figs. 6, B and C). As the intrinsic binding affinity of individual thiolate ligands must be nearly identical, the overall protein structure must superimpose an important contextual effect. One clear distinction among the three Cys residues of Site II is the sequence context in which they are found. The ligands that comprise RING metal sites are arranged in pairs within the primary sequence (Fig. 1). Cys-39 is part of a Cys-X-His motif that constitutes the first pair of Site II ligands, and Cys-61 and Cys-64 are part of a Cys-X-Cys motif that makes up the other half of the tetrahedral Zn\(^{2+}\) binding site. A prominent feature of the Cys-X-Cys motif commonly observed in a number of Zn\(^{2+}\)-binding proteins including the crystal structure of the RAG1 RING domain (17) is the presence of a hydrogen bond between the SY of the first Cys residue (i) and the backbone amide proton of the i+2 residue when the ligands are coordinated to Zn\(^{2+}\). NMR data suggest that this H-bonding pattern is present in the first and second Zn\(^{2+}\) binding loops of both BRCA1 and BARD1.\(^2\) Thus, a Cys-X-Cys motif may contribute more to the Zn\(^{2+}\) binding energy

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\(^2\) P. S. Brzovic and R. E. Klekit, unpublished results.
than a Cys-X-His due to enhanced structural stability afforded by its H-bonding capacity. Mutation of either Cys-61 or Cys-64 would not only alter the ability of the Cys-X-Cys motif to bind Zn$^{2+}$ (Fig. 6C) but also disrupt this H-bonding interaction.

It has been generally assumed that the cancer-predisposing mutations in Site II of BRCA1 yield a protein that is either misfolded or unfolded due to loss of Zn$^{2+}$ binding at that site. This would surely disrupt the ability of the BRCA1 RING domain to form complexes with interacting proteins. We find instead that the Site II mutations cause local structural perturbations and do not abrogate the ability of the BRCA1 RING domain to interact with BARD1. This presents an apparent conflict with in vivo assays, which found that BRCA1 constructs encompassing the first 304 residues lost the ability to interact with BARD1, as judged by both two-hybrid and co-immunoprecipitation assays (6). It is possible that the protein concentrations used for in vitro characterization (µM) mask biologically relevant alterations in the BRCA1/BARD1 dissociation constant (likely in the nM region) (5). Alternatively, the ability of Cys-61 or Cys-64 BRCA1 mutants to properly fold and bind BARD1 may be influenced by protein fusions required for two-hybrid analysis, the presence of regions outside the BRCA1 RING domain of unknown structural integrity, or aberrant interactions with other proteins present in the cellular milieu, all of which could lead to an apparent loss of function. In any case, the in vitro data demonstrate that the BRCA1 Site II mutants retain the requisite structural information for this domain to properly fold and dimerize with BARD1.

If Site II mutations do not necessarily disrupt the interaction between BRCA1 and BARD1, are there other functional ramifications of mutations at Cys-39, Cys-61, and Cys-64 that predispose individuals to breast cancer? Recent studies show that the BRCA1 RING domain exhibits ubiquitin ligase (E3) activity, facilitating the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme (UbcH5) to protein targets (10, 12, 18). The BRCA1 RING domain to properly fold and dimerize with BARD1.

This would surely disrupt the ability of the BRCA1 RING domain to form complexes with interacting proteins. We find instead that the Site II mutations cause local structural perturbations and do not abrogate the ability of the BRCA1 RING domain to interact with BARD1. This presents an apparent conflict with in vivo assays, which found that BRCA1 constructs encompassing the first 304 residues lost the ability to interact with BARD1, as judged by both two-hybrid and co-immunoprecipitation assays (6). It is possible that the protein concentrations used for in vitro characterization (µM) mask biologically relevant alterations in the BRCA1/BARD1 dissociation constant (likely in the nM region) (5). Alternatively, the ability of Cys-61 or Cys-64 BRCA1 mutants to properly fold and bind BARD1 may be influenced by protein fusions required for two-hybrid analysis, the presence of regions outside the BRCA1 RING domain of unknown structural integrity, or aberrant interactions with other proteins present in the cellular milieu, all of which could lead to an apparent loss of function. In any case, the in vitro data demonstrate that the BRCA1 Site II mutants retain the requisite structural information for this domain to properly fold and dimerize with BARD1.

If Site II mutations do not necessarily disrupt the interaction between BRCA1 and BARD1, are there other functional ramifications of mutations at Cys-39, Cys-61, and Cys-64 that predispose individuals to breast cancer? Recent studies show that the BRCA1 RING domain exhibits ubiquitin ligase (E3) activity, facilitating the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme (UbcH5) to protein targets (10, 12, 18). Formation of a BRCA1/BARD1 heterodimer greatly enhances this activity (10). Although the original report utilized larger constructs of BRCA1 and BARD1, the wild-type heterodimer used in our studies behaves similarly in ubiquitination activity assays. Of particular importance is the finding that mutations at Cys-39, Cys-61, and Cys-64 within Site II abolish the E3 activity of BRCA1 (10, 18). It is likely that these mutations directly affect the BRCA1 RING(E3)-E2 binding interface. This postulate is based on the structure of another RING domain protein, cCbl, in complex with the E2 ubiquitin-conjugating enzyme UbcH7 (19). cCbl is a RING family E3 that promotes the ubiquitination of receptor tyrosine kinases. This activity is mediated via a direct interaction between the cCbl RING domain and an E2 ubiquitin-conjugating enzyme. Whereas the interface between BRCA1 and BARD1 is formed by helices that flank the central RING motif, the cCbl binding interface for UbcH7 is formed predominantly by the first Zn$^{2+}$ loop, the central helix, and the second Zn$^{2+}$ loop, regions that reside within the core RING motif of cCbl. The corresponding regions of BRCA1 are labeled in Fig. 7. It is the second Zn$^{2+}$ loop, which forms a critical part of the RING(E3)-E2 interface of cCbl, that is perturbed by mutations within Site II of BRCA1.

In addition to BARD1 and UbcH5c, the BRCA1 RING domain also interacts with BAP1, a de-ubiquitinating enzyme (13). It remains to be determined how BAP1 recognizes and binds to BRCA1, whether it interacts in a manner similar to BARD1 (8) or that proposed for UbcH5. The interplay of these proteins is consistent with important functions in protein regulation via ubiquitination. Accordingly, it has been suggested that missense mutations in the BRCA1 RING domain such as those described in Site II predispose to cancer by interfering with the ubiquitin-ligase activity of BRCA1 (10, 18). The data presented herein show that cancer-predisposing missense mutations at Cys-61 and Cys-64 do affect a potential binding interface for at least one partner of the BRCA1 RING domain.

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