Autoubiquitination of the BRCA1·BARD1 RING Ubiquitin Ligase*

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The RING finger of BRCA1 confers ubiquitin ligase activity that is markedly enhanced when complexed with another RING-containing protein, BARD1, and is required for the function of this tumor suppressor protein in protecting genomic integrity. Here, we report that co-expression of BRCA1-(1–639) and BARD1 in bacteria can assemble a potent ubiquitin ligase activity. Purified BRCA1-(1–639)-BARD1 stimulated the Ubc5c-mediated monoubiquitination of histone H2A/H2AX in vitro, suggesting a possible role for BRCA1-BARD1 in modifying chromatin structure. Moreover, the truncated BRCA1-BARD1 complex exhibited efficient autoubiquitination activity in vitro capable of assembling non-lysine 48-linked polyubiquitin chains on both BRCA1-(1–639) and BARD1. When co-expressed in cells by transient transfection, the recombinant BRCA1-(1–300)-BARD1 complex was found to be associated with polyubiquitin chains, suggesting that BRCA1-(1–300)-BARD1 was ubiquitinated in vivo as well. These results raise the possibility that BRCA1-BARD1 acts to assemble non-lysine 48-linked polyubiquitin chains that may serve as part of a signaling platform required for coordinating DNA repair-related events.

When mutated, BRCA1 confers a genetic predisposition to breast and ovarian cancer. Germline mutations can be attributed to tumorigenesis in 45% of families with a history of breast cancer and 90% of families with both breast and ovarian cancer. The penetrance is such that female carriers have an estimated 80% lifetime risk of developing breast cancer (1).

As a tumor suppressor, BRCA1 exerts a pleiotropic effect, playing a role in the maintenance of genomic integrity. To this end, several functions have been ascribed to BRCA1 including double strand DNA break repair, transcription-coupled DNA repair, transcriptional regulation, chromatin remodeling, and ubiquitin ligation (2). Despite implications in a diverse array of cellular pathways, the exact mechanism by which BRCA1 executes its function remains largely unknown.

Within the first one hundred residues of BRCA1, which contains 20% of the biologically relevant mutations (3), lies a RING domain. RING fingers are “cross-brace” structures formed by conserved cysteine and histidine residues that coordinate two zinc ions (4). The RING finger motif has been well established to be able to mediate ubiquitin ligation, an activity intrinsic to a significant subset of the RING protein family. It does so by interacting with E2 ubiquitin-conjugating enzymes (5–7) thus and constitutes one of the two classes of E3 ubiquitin ligases, the other being the HECT domain containing E3 ligases (8). Traditionally, a RING E3 ubiquitin ligase interacts with both an E2, which has previously been charged with ubiquitin activated by an E1 ubiquitin-activating enzyme, and a substrate to catalyze the transfer of ubiquitin from the E2 to the substrate. However, some RING fingers have been operationally defined as having ubiquitin ligase activity without substrates. It is common to test RING fingers by simply assaying for the E1- and E2-dependent formation of ubiquitin polymers. In these assays, there is the potential for heterogeneous reaction products. For example, it has been shown that RING fingers can mediate autoubiquitination (9), E2 ubiquitination (10), and unanchored polyubiquitin chains free from any substrate (11) in vitro. When using ubiquitin as the only means by which to visualize the reaction products, it is difficult to discern among these possibilities. Furthermore, their biological significance is not well understood. There are some examples where the autoubiquitination of a RING finger protein, such as Mdm2, is involved in its turnover (9). Whereas it is plausible that E2 ubiquitination might also be a means of regulating the abundance of the E2, this has not been rigorously investigated. Similarly, although significant amounts of free polyubiquitin chains have been detected in cells (12), their role is unknown. Thus, some RING finger proteins, such as BRCA1, have been described as having ubiquitin ligase activity, although the nature and function of their reaction products remain unclear (5, 13, 14).

The ability of the RING in BRCA1 to confer ubiquitin ligase activity was first demonstrated using a RING-containing truncation of BRCA1. Furthermore, this activity was Zn2+-dependent (5), consistent with the requirement for a properly structured RING finger. Subsequently, it was demonstrated that this activity could be markedly enhanced when complexed with another RING finger protein, BARD1 (13). The association between BRCA1 and BARD1 is the first example of a heterodimeric RING complex, interacting through residues adjacent to the RING structure (3, 15). Interestingly, the mutation of BARD1 is believed to be involved with similar tissue-specific tumors as is the mutation of BRCA1, namely of the breast and ovaries (16). Tumor-derived RING mutations in BRCA1 abolish the ubiquitin ligase activity of the BRCA1-BARD1 heterodimer without significantly affecting the interaction between the two

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1 The abbreviations used are: E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; E1, ubiquitin-activating enzyme; GST, glutathione S-transferase; PK, protein kinase; HA, hemagglutinin.
proteins (3, 13). Moreover, the RING mutations in BRCA1 were found to be incompetent to protect cells containing inactive BRCA1 alleles from ionizing radiation as well as inadequate to restore a G2/M checkpoint, thus correlating the tumor suppression function of BRCA1 with its ubiquitin ligase activity (14).

Additional evidence implicating BRCA1 function within the ubiquitin pathway is its interaction with a deubiquitinating enzyme, BAP1, through its RING domain (17). Also, the monoubiquitination of the Fanconi anemia protein subtype D2, FANCd2, has been connected with BRCA1. FANCd2 co-localizes with BRCA1 after ionizing radiation, and levels of its monoubiquitination are up-regulated in cells containing inactive BRCA1 alleles in the presence of exogenous BRCA1 and ionizing radiation (18). Interestingly, Fanconi anemia, a human autosomal recessive disorder characterized by cancer susceptibility, bears some resemblance to the phenotypes observed with BRCA1 mutations, displaying an increased sensitivity to ionizing radiation (19).

However, important questions remain regarding the activity of the putative BRCA1-BARD1 E3 ligase. The most obvious issue is to identify substrates, because this may help better understand the mechanism by which BRCA1 elicits its function. Once the substrates have been defined, the type of ubiquitination that the substrate undergoes needs to be determined so as to understand the consequence of the modification. For example, the substrate may be polyubiquitinated for purposes of degradation or to serve as a signal, or it may be monoubiquitinated as in the case of FANCd2 and histones. Additionally, other alternatives need to be addressed, such as the actual E2 enzyme that BRCA1-BARD1 coordinates with in vivo as well as other potential small ubiquitin-like modifying molecules that may be employed by it. Here, we characterize the ubiquitin ligase activity of the BRCA1-BARD1 heterodimer. The monoubiquitination of histone H2A/H2AX can be mediated by BRCA1 (−1–639)BARD1 in vitro. The complex displays a robust autoubiquitination activity in vitro where non-lysine 48-linked chains can be polymerized, implying that these chains rather than target for degradation may serve an alternative role.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

**Generation of BRCA1 Constructs**—FLAG-BRCA1 was subcloned into the pBose vector by engineering a NcoI site as well as sequences encoding for six histidines and a FLAG epitope and a BamHI site. The PCR product was then cloned into the pcDNA3.1 TOPO TA vector. The His-FLAG-H2A was then released with Ncol/BamHI digestion and subcloned into pET15b (Novagen), creating pET15b-His-FLAG-H2A.

**His-FLAG-H2AX** was created using the pcDNA3.1-His-FLAG-H2A as a template using the primers 5′-CAATGGGCCATCATCATCATTACAGTTGACGATGATAATCGGCAGCCGAGG-3′ and 5′-GGGGAGCTGCGTGGCAGAATGTTGCTGCTGCTGCCAAAGAAAGGAAAA-3′ followed by cloning of the PCR product into the pcDNA3.1 TOPO TA vector. The His-FLAG-H2A was then released with Ncol/BamHI digestion and subcloned into pET15b (Novagen), creating pET15b-His-FLAG-H2A.

**Generation of Histone H2A/H2AX Constructs**—H2A was then released with Ncol/BamHI digestion and subcloned into pET15b, creating pET15b-His-FLAG-H2A. All constructs were verified by DNA sequencing.

**Protein Expression and Isolation**

**Expression of GST-BRCA1**—GST-BRCA1 was expressed using BL21(DE3) with the pGEX4T3-GST-BRCA1 (1–639) and the pET29b-S-BARD1-FLAG, which contain selectable markers for ampicillin and kanamycin, respectively.

**Transfection, Metabolic Labeling, and Extract Preparation**—293T cells in 100-mm plates were transfected using the standard calcium phosphate precipitation method (up to 20 μg). Where noted, cells were metabolically labeled 48–68 h post-transfection as described previously (6) and harvested. The pellets were resuspended in 0.2 ml/plate buffer B (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.5 mM NADH, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml antipain, 0.2 μg/ml leupeptin, and 0.5 μg/ml leupeptin).

**Ubiquitination Protein Preparation**—Ubc5c was expressed by transforming E. coli transformed with pET3E-His-Ubc5c were grown, induced, and harvested as described above for GST-BRCA1. The harvested cells were resuspended in 0.04 culture volume of lysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 μg/ml antipain, and 0.2 μg/ml leupeptin) followed by sonication. The suspension was sonicated and centrifuged at 17,000 rpm at 4 °C for 30 min.

**Generation of BRCA1 constructs**—FLAG-BRCA1 was subcloned into the pBose vector by engineering a Ncol site flanking BRCA1. This then served as a template for the amplification of a fragment of BRCA1 encoding for amino acids 1–300 that was engineered to contain a 5′-BamHI site and a 3′-Ncol site. The PCR product was then cloned in-frame into a modified pBose vector that contained sequences encoding for a GST tag. A detailed description and map can be given upon request. Of a fragment of BRCA1 encoding for residues 1–639 was amplified from the FLAG-BRCA1 construct using the primers 5′-GACCCTCGAGACATGATTTATCTGCTTTGCTGTT-3′ and 5′-GAAGATCTCATATGATTTATCTGCTTTGCTGTT-3′. The PCR product was cloned into the pcDNA3.1 TOPO TA vector (Invitrogen). BRCA1 (1–639) was released with Ncol digestion and subcloned into pGEX4T3 vector (Amersham Biosciences), creating pGEX4T3-GST-BRCA1 (1–639).

**Generation of BARD1 Constructs**—BARD1 was amplified from a BARD1-m1/pSP6 (15) construct using the primers 5′-CCGCTCGAGATGGCTTTATCTGCTTTGCTGTTGCTGCTGCTGCCAAAGAAAGGAAAA-3′ followed by cloning of the PCR product into the pcDNA3.1 UNI vector, creating pCR3.1-BARD1.

**Generation of Histone H2A/H2AX Constructs**—H2A was amplified from a human mammary epithelial cell line cDNA expression library (20) using the primers 5′-CCATGGGACATCATTACATCATCATGAT TACAAGGATGACGATGATAATCGGCAGCCGAGG-3′ and 5′-GGGGAGCTGCGTGGCAGAATGTTGCTGCTGCTGCCAAAGAAAGGAAAA-3′ followed by cloning of the PCR product into the pcDNA3.1 TOPO TA vector. The His-FLAG-H2A was then released with Ncol/BamHI digestion and subcloned into pET15b (Novagen), creating pET15b-His-FLAG-H2A.
did not exhibit any ubiquitin ligase activity (lanes 7 and 8), demonstrating the requirement of both proteins to achieve any notable levels of ubiquitin polymerization, consistent with previous studies (13).

**BRCA1-BARD1 Stimulates the Ubc5c-mediated Mono-ubiquitination of Histone H2A/H2AX in Vitro**—Knowing that BRCA1 is recruited to sites of DNA damage (2) and that it co-localizes with γ-H2AX (histone H2AX phosphorylated at serine 139) after DNA damage (22), we tested histone H2A or H2AX, which is known to be monoubiquitinated, as potential substrate for the BRCA1-BARD1 ubiquitin ligase. Recombinant FLAG-H2A and FLAG-H2AX were used as substrates, and the reaction products were analyzed by Western blot analysis. In the presence of bacterially assembled BRCA1-(1–639)-BARD1, both FLAG-H2A and FLAG-H2AX were monoubiquitinated (Fig. 2, lanes 1 and 4, denoted with an asterisk and a circle, respectively). Ubiquitination was completely dependent on the presence of BRCA1-(1–639)-BARD1 (lanes 2 and 5) as well as Ubc5c (lanes 3 and 6) and H2A/H2AX substrate proteins (lane 7). Additionally, neither BRCA1-(1–639) nor BARD1 alone activated the monoubiquitination of FLAG-H2A or FLAG-H2AX (lanes 8–11). These results demonstrate that BRCA1-(1–639)-BARD1 stimulated the Ubc5c-mediated monoubiquitination of histone H2A/H2AX in vitro. Importantly, this activity required the presence of both the truncated BRCA1 and BARD1.

**Auto-ubiquitination of BRCA1-BARD1 in Vitro and in Vivo**—We next explored the possibility of autoubiquitination of BRCA1-BARD1. To address this, the bacterially assembled BRCA1-(1–639)-BARD1 was immobilized on an S-agarose matrix and incubated with E1, Ubc5c, and ubiquitin. The reaction products were then analyzed by Western blot. An α-GST immunoblot of the reaction showed high molecular weight GST-BRCA1-(1–639) species being formed that required Ubc5c and BRCA1-(1–639)-BARD1 (Fig. 3A, lanes 1–3). This same blot when probed with α-FLAG demonstrated that BARD1 was also converted into high molecular weight species that were also dependent on the presence of Ubc5c and BRCA1-(1–639)-BARD1 (Fig. 3B, lanes 1–3). To further verify that polyubiquitin chains were being assembled on the BRCA1-(1–639)-BARD1 heterodimer, the same ubiquitination reaction was carried out, but the BRCA1-BARD1-immobilized matrix was washed prior to analysis by SDS-PAGE. Thus, any ubiquitin remaining would presumably be covalently linked to BRCA1-(1–639)-BARD1. As the α-ubiquitin blot demonstrated, this was in fact the case (Fig. 3C). These results demonstrated that both the truncated BRCA1 and BARD1 were ubiquitinated extensively.

A kinetic analysis was carried out to measure the rate with which the autoubiquitination reaction occurred. The BRCA1-(1–639)-BARD1-mediated ubiquitination reaction was terminated at various time points and analyzed by Western blot (Fig. 4). A substantial reduction in unmodified GST-BRCA1-(1–639) was observed by 9 min, concomitant with an accumulation of high molecular weight species (Fig. 4A, lane 3). By 60 min, almost all of BRCA1-(1–639) was polyubiquitinated (lane 5). BARD1 reflected similar kinetics. Within 9 min, a significant amount of BARD1 was reduced, being converted into high molecular weight species (Fig. 4B, lane 3). Thus, the BRCA1-(1–639)-BARD1 heterodimer was capable of rapid and efficient autoubiquitination.

The observation that the BRCA1-(1–639)-BARD1 heterodimer can autoubiquitinate seems counter-intuitive given that Joukov et al. (23) recently reported that the two proteins mutually stabilize each other. We considered the possibility that these chains assembled onto BRCA1-(1–639)-BARD1 may

okadaic acid, 2 mM ATP, 0.6 mM dithiothreitol, 3 μg of [32P]ubiquitin, 0.6 pmol of E1, and Ubc5c (0.15 μg for H2A/H2AX monoubiquitination unless otherwise indicated and 0.5 μg for BRCA1-BARD1 autoubiquitination) or Cdc34 (10 pmol). Ubiquitination reactions monitored by Western blot analysis contained reaction mixtures with unlabeled PK-ubiquitin (or PK-ubiquitin K48R) and without the NaF and okadaic acid. The reactions were incubated at 37 °C for 1 h unless otherwise indicated. The reactions were stopped with 20 μl of 4-fold concentrated Laemmli loading buffer and boiled for 3 min prior to SDS-PAGE analysis followed by autoradiography or Western blot analysis.

**RESULTS**

**BRCA1-(1–639)/BARD1 Exhibits High Levels of Ubiquitination Activity**—In an effort to efficiently generate complexes of BRCA1-BARD1, a truncated form of BRCA1, GST-BRCA1-(1–639), and S-BARD1-FLAG were co-expressed in E. coli. An approximately stoichiometric complex as judged by Coomassie Blue staining, was isolated (Fig. 1A, lane 1), in agreement with the recent solution structure of the BRCA1-BARD1 RING domain complex, which shows that the two proteins form a heterodimeric complex (3). A titration experiment showed that this complex was capable of catalyzing ubiquitin polymerization (Fig. 1B, lanes 1–5) in an E2-dependent manner (lane 6). Note that at the highest level tested (lane 5), there was virtually a complete conversion of the free monomeric ubiquitin substrate to high molecular weight polyubiquitin species. Comparable levels of GST-BRCA1-(1–639) and GST-BARD1 alone
serve alternative functions other than targeting the heterodimer for proteasomal-mediated degradation. Because lysine 48-linked polyubiquitin chains are the principle signal that targets proteins for proteasomal degradation (24), we tested whether BRCA1-(1–639)/BARD1 could assemble non-lysine 48-linked ubiquitin polymers. For this purpose, a ubiquitin mutant containing K48R was used in the autoubiquitination assay. The BRCA1-(1–639)/BARD1 heterodimer indeed formed polyubiquitin chains linked through lysine residues other than lysine 48 in a Ubc5c-dependent manner (Fig. 5A, lanes 3 and 4). In addition, the efficiency with which chains were formed with ubiquitin K48R was comparable to that with the wild-type ubiquitin (compare lane 1 with lane 3). As shown in Fig. 5B, this ubiquitin K48R preparation when used in place of the wild-type ubiquitin was able to inhibit K48-linked polyubiquitin chain formation catalyzed by ROC1/CUL1, Cdc34, and E1 (compare lanes 3–5 with lanes 8–10). This is in keeping with our previous observations that ubiquitin K48R blocks the SCF<sup>HOS</sup>-ROC1-dependent Lys-48-mediated ubiquitination of IκBα (11) as well as substrate-free ubiquitin polymerization (21), thus establishing the feasibility of using this mutant ubiquitin to probe for non-Lys-48-linked ubiquitin chain formation. It has been reported that Ubc5c uses either lysine 48 or lysine 29 chains to synthesize ubiquitin polymers (25, 26). Presumably, when the ubiquitin K48R was used, the majority of the non-lysine 48 chains were linked through lysine 29. These results suggest that the polyubiquitin chains assembled onto the BRCA1-(1–639)/BARD1 complex have the potential to serve as signals other than for degradation.

We then sought to determine whether BRCA1-BARD1 assembled in mammalian cells could also exhibit this autoubiquitination activity. For this purpose, a truncated form of BRCA1, GST-BRCA1-(1–300), and BARD1 were co-expressed in 293T cells followed by metabolic labeling with [35S]methionine and cysteine. Glutathione beads were then used to isolate GST-BRCA1-(1–300) and its associated proteins, and the resulting matrix was incubated with E1, Ubc5c, and HA-ubiquitin. Following the reaction, the beads were washed, and the

**Fig. 2.** BRCA1-(1–639)-BARD stimulates the monoubiquitination of recombinant H2A and H2AX. Bacterial extracts containing GST-BRCA1-(1–639)–S-BARD1-FLAG (0.6 pmol) (lanes 1, 3, 4, 6, and 7), GST-BRCA1-(1–639) (1 pmol) (lanes 8 and 9), and GST-BARD1 (2 pmol) (lanes 10 and 11) were immobilized on S-agarose (lanes 8–11). In the reactions shown in lanes 2 and 6, uncoupled S-agarose was used. The beads were then incubated with a reaction mixture containing 1 μg of either FLAG-H2A (lanes 1–3, 8, and 10) or FLAG-H2AX (lanes 4–7, 9, and 11). No H2A/H2AX was added in the reaction shown in lane 7. Ubc5c was omitted from lanes 3 and 6. Ubiquitination was carried out as described under “Experimental Procedures.” Aliquots of the reactions were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were probed with a α-FLAG antibody. The asterisk denotes monoubiquitinated FLAG-H2A, whereas the closed circle denotes monoubiquitinated FLAG-H2AX. Ub, ubiquitin.

**Fig. 3.** BRCA1-(1–639)-BARD1 undergoes autoubiquitination. A–C, S-agarose beads immobilized with (4 pmol) (lanes 1 and 2) or without (lane 3) GST-BRCA1-(1–639)-S-BARD1-FLAG was incubated with the ubiquitination components. Ubc5c was omitted from lane 2 in A–C. Ubiquitination was carried out as described under “Experimental Procedures.” Aliquots of the reactions were separated by 6% SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were probed with a α-GST antibody (A), a α-FLAG antibody (B), or a α-ubiquitin antibody (C). C, the beads were washed subsequent to the ubiquitination reaction. To visualize products that did not enter the separating gel presumably because of their large size, proteins that remained in the stacking gel were transferred to the nitrocellulose membrane as well.
reaction products were analyzed by Western blot (Fig. 6A) and autoradiography (Fig. 6B). An α-HA immunoblot, recognizing the HA epitope on the recombinant ubiquitin, of the reaction showed that GST-BRCA1-(1–300) precipitates assembled ubiquitin polymers that required Ubc5c and GST-BRCA1-(1–300)+BARD1 (Fig. 6A, lanes 1–3). The same reaction products then were analyzed by autoradiography. An accumulation of 35S-high molecular weight species was evident in the complete reaction (Fig. 6B, lane 1). Concomitantly, the amounts of both the unmodified GST-BRCA1-(1–300) and BARD1 were substantially reduced (compare lane 1 with lane 2). This conversion of both GST-BRCA1-(1–300) and BARD1 to high molecular weight species was dependent on the presence of Ubc5c and GST-BRCA1-(1–300)+BARD1 (lanes 2 and 3). These results confirmed that the BRCA1-(1–300)+BARD1 complex assembled in mammalian cells can autoubiquitinate, excluding the possibility that this activity was specific only to the bacterially assembled complex.

To determine whether or not the observed autoubiquitination is a bona fide activity, GST-BRCA1-(1–300), BARD1, and HA-ubiquitin were co-transfected in 293T cells. A GST pull-down assay of metabolically labeled extracts was performed, and the bound proteins were probed with a α-HA antibody. High molecular weight species, indicative of polyubiquitin chains, were only detected when both GST-BRCA1-(1–300)+BARD1 and HA-ubiquitin were transfected (Fig. 7A). The same reaction products when analyzed by autoradiography demonstrated that similar amounts of GST-BRCA1-(1–300)+BARD1 were being expressed when transfected with HA-ubiquitin and when transfected alone (Fig. 7B, lanes 1 and 2). Furthermore, the co-expression of GST and HA-ubiquitin did not yield any polyubiquitin chains that were associated with GST (data not shown). These results suggest that autoubiquitinated BRCA1-BARD1 can be detected in vivo. We cannot exclude the possibility that polyubiquitin chains associated with BRCA1-(1–300)+BARD1 or a portion of them were conjugated to proteins that are tightly associated with BRCA1-(1–300)+BARD1. Nonetheless, this provides direct evidence that truncated BRCA1-BARD1 is associated with ubiquitination activity in cells.

**FIG. 4. Kinetics of BRCA1-(1–639)-BARD1 autoubiquitination.** GST-BRCA1-(1–639)+S-BARD1-FLAG (4 pmol) was immobilized on S-agarose beads. Ubiquitination was carried out as described under “Experimental Procedures.” The time indicated reflects the amount of incubation time subsequent to the addition of Ubc5c. Aliquots of the reaction products were separated by 6% SDS-PAGE and transferred to a nitrocellulose membrane.

**DISCUSSION**

The discovery of the BRCA1-BARD1 heterodimer possessing ubiquitin ligase activity has opened up a new avenue into the investigation of BRCA1 and how it may function, at least in part, to elicit its tumor suppressor activity. The ubiquitination activity is dependent on critical residues within the RING finger of BRCA1 that are found to be naturally and frequently mutated in breast and ovarian tumors (13, 14). The RING finger is also required to restore responsiveness of the HCC1937 cell line to ionizing radiation (14). BRCA1-BARD1 is the first example of a RING-dependent ubiquitin ligase that depends on heterodimeric RING fingers to exhibit ubiquitination activity. BARD1 markedly increases the intrinsically low activity of BRCA1 (13). BRCA1 can be modeled based on the c-Cbl/UbcH7 structure (7) to interact with Ubc5 in an analogous manner (3). Consistent with this model is the fact that a naturally occurring mutation found in the potential binding surface within the RING of BRCA1, C61G, abolishes ubiquitin ligation (13, 14). A reasonable hypothesis for the mechanism of BARD1 activation may be that the RING of BARD1 helps to stabilize the interaction between the RING of BRCA1 and Ubc5. Whereas some important characteristics of the putative BRCA1-BARD1 E3 enzyme have been delineated, much remains to be defined.

In this study, we describe a new system for co-expressing the BRCA1-(1–639)+BARD1 heterodimer that yields a stoichiometric complex fully capable of supporting ubiquitin ligation (Fig. 1). We also demonstrate that BARD1 complexed with this truncated form of BRCA1 is capable of catalyzing the monoubiquitination H2A/H2AX (Fig. 2) as well as autoubiquitination both in vitro and in vivo (Figs. 3–7). Although BRCA1-(1–639)+BARD1 possesses a potent ubiquitin ligase activity, it lacks a significant portion of the BRCA1 molecule, most notably the C-terminal BRCT domain. However, our repeated attempts in producing sufficient amounts of full-length BRCA1 either in transfected mammalian cells or in bacteria for biochemical studies have been unsuccessful. Because structural studies have revealed that the BRCA1-BARD1 interaction occurs at their respective N termini (3), it is unlikely that the C
terminus of BRCA1 would significantly alter the recruitment of an E2 by the BRCA1-BARD1 RING domain complex. Thus, the ubiquitin ligase activity mediated by the BRCA1-(1–639)/BARD1 complex probably reflects the ubiquitin ligase activity possessed by the full-length protein. It remains to be determined whether the C terminus of BRCA1 significantly influences the ability of BRCA1-BARD1 to stimulate the monoubiquitination of H2A/H2AX and/or to promote autoubiquitination. If this proves to be the case, it suggests that the C terminus of BRCA1 may bind a cellular factor and orient it toward the vicinity of the RING domain for ubiquitination, hence blocking monoubiquitination of H2A/H2AX and/or autoubiquitination. Alternatively, the interactions between the BRCA1-BRCT domain and cellular factors may alter the BRCA1 conformation, leading to activation or inhibition of the BRCA1-BARD1 RING domain complex for monoubiquitination of H2A/H2AX and/or autoubiquitination.

Mono-ubiquitinated histones H2A and H2B, which constitute ~10% total H2A and ~1% total H2B pool in mammals (27), have been associated with transcriptionally active DNA (28–30). It has been postulated that the covalent attachment of a single ubiquitin moiety to histones may possibly alter nucleosome or higher order chromatin structures, thereby opening up the DNA to allow for access of the transcriptional machinery (27). Mono-ubiquitination as a post-translational modification has also recently been shown to be involved in membrane trafficking (31). It might be possible that one response of BRCA1 to DNA damage is to monoubiquitinate histones in

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**Fig. 5.** BRCA1-(1–639):BARD1 can assemble polyubiquitin chains linked through lysine(s) other than Lys-48. 

A. GST-BRCA1-(1–639):S-BARD1-FLAG (4 pmol) was immobilized on S-agarose beads and incubated with an ubiquitination reaction mixture containing wild-type ubiquitin (lanes 1 and 2) or ubiquitin K48R (lanes 3 and 4) with (lanes 1 and 3) or without (lanes 2 and 4) Ubc5c. Ubiquitination was carried out as described under "Experimental Procedures." The beads were washed prior to analysis by SDS-PAGE. Aliquots of the reaction products were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were probed with an α–ubiquitin antibody. To visualize products that did not enter the separating gel presumably because of their large size, proteins that remained in the stacking gel were transferred to the nitrocellulose membrane as well. 

B. Extracts (0.5 mg of protein) from 293T cells co-transfected with pcDNA-HA-ROC1 and pcDNA-CUL1 were immunoprecipitated with α-HA antibodies and incubated with ubiquitination components. Ubiquitination was carried out as described under "Experimental Procedures" with Cdc34 (0.5 μg) as the E2. The time indicated reflects the amount of incubation time subsequent to the addition of Cdc34. Aliquots of the reaction products were separated by 15% SDS-PAGE followed by autoradiography.
conjunction with BARD1, thereby remodeling the chromatin that allows the DNA repair machinery access to the damaged DNA. Preliminary experiments have indicated that histones H2B and H3 can also be monoubiquitinated *in vitro* by BRCA1-(1–639)-BARD1 although to a lesser extent than H2A (data not shown). The notion of BRCA1 being involved in the monoubiquitination of histones in response to DNA damage might be a novel mechanism that could contribute to the DNA repair process.
utination of a protein has been described previously. BRCA1 has been implicated in the up-regulation of the monoubiquitination of Fanconi anemia protein FANC-D2 (18), although the exact function of this modification is not clear.

It is also interesting to note that a subtype of histone H2A, H2AX, which contains a C-terminal extension bearing a phosphatidylinositol 3-OH kinase family phosphorylation motif, is phosphorylated under conditions that generate DNA double strand breaks (32, 33). This phosphorylation event is required for survival under these conditions in Saccharomyces cerevisiae (33). The phosphorylated histone H2AX, γ-H2AX, has been implicated in remodeling higher order chromatin structures (33). In addition, γ-H2AX has also been shown to co-localize with BRCA1 as well as RAD50 and RAD51 after ionizing radiation (22). We did observe that when the recombinant histone H2AX was used in the studies above, it was monoubiquitinated with approximately the same efficiency as histone H2A. Furthermore, when H2AX was phosphorylated with DNA-PK, it could still be monoubiquitinated by BRCA1-(1–639)-BARD1 (data not shown). Ruffner et al. (14) have shown that monoubiquitination of histone H2A can be stimulated by other RING fingers, suggesting a lack of specificity in this reaction. However, the DNA damage-induced association between BRCA1 and H2A/H2AX may significantly elevate the local concentration of BRCA1-BARD1 on chromatin, allowing for specific targeting of H2A/H2AX. Additional studies are required to determine whether BRCA1-BARD1 contributes to the monoubiquitination of H2A/H2AX in a DNA damage-dependent manner.

It also remains possible that the BRCA1-BARD1 heterodimer functions to autoubiquitinate. A significant degree of truncated BRCA1-BARD1 is ubiquitinated in our experiments (Figs. 3 and 6), which occurs rapidly and rather efficiently (Fig. 4). The in vitro autoubiquitination was verified when ubiquitin was co-expressed with BRCA1-(1–300)-BARD1 in 293T cells (Fig. 7), suggesting that this is a modification that BRCA1-BARD1 undergoes inside a cell. The significance of this observation remains to be explored. Future studies will include mapping and mutagenesis studies to define the lysine receptor sites that are being conjugated with ubiquitin.

In light of the observation that BRCA1 and BARD1 stabilize each other (23), we propose that BRCA1-BARD1 autoubiquitination does not serve to target for proteasomal degradation. Instead, it may serve as a signaling event such as in DNA repair or in regulating the BARD1-mediated inhibition of mRNA polyadenylation after DNA damage (34). The observation that BRCA1-(1–639)-BARD1 can covalently attach ubiquitin chains linked through lysine residues other than position 48 supports this notion (Fig. 5A). Polyubiquitin chains have been demonstrated to be involved in non-proteolytic cell-signaling events such as the activation of the IκBα kinase (35) and the inactivation of the yeast transcription factor Met4 (36). In addition, of notable interest, polyubiquitin chains linked through lysine 63, which are assembled by the MMS2/Ubc13 heterodimeric E2 enzyme (37), are believed to play a role in signaling for DNA repair (37, 38). The S. cerevisiae strains containing a mutant ubiquitin in which lysine 63 is substituted with arginine display a UV-sensitive phenotype without affecting protein degradation (38). Furthermore, yeast strains with MMS2 or Ubc13 mutations, either alone or in combination with ubiquitin K63R, exhibit similar phenotypes (37). It would be interesting to test the capacity of MMS2/Ubc13 to synthesize lysine 63-conjugated polyubiquitin chains with BRCA1. Furthermore, if the autoubiquitination chains do serve as part of a signaling platform, it will be important to identify the factors they help recruit.

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