The breast and ovarian cancer-specific tumor suppressor RING finger protein BRCA1 has been identified as an E3 ubiquitin (Ub) ligase through in vitro studies, which demonstrated that its RING finger domain can auto-ubiquitylate and monoubiquitylate histone H2A when supplied with Ub, E1, and UBC4 (E2). Here we report that the E3 ligase activity of the N-terminal 110 amino acid residues of BRCA1, which encodes a stable domain containing the RING finger, as well as that of the full-length BRCA1, was significantly enhanced by the BARD1 protein (residues 8–142), whose RING finger domain itself lacked Ub ligase activity in vitro. The results of mutagenesis studies indicate that the enhancement of BRCA1 E3 ligase activity by BARD1 depends on direct interaction between the two proteins. Using K48A and K63A Ub mutants, we found that BARD1 stimulated the formation of both Lys48- and Lys63-linked poly-Ub chains. However, the enhancement of BRCA1 auto-ubiquitylation by BARD1 mostly resulted in poly-Ub chains linked through Lys63, which could potentially activate biological pathways other than BRCA1 degradation. We also found that co-expression of BRCA1 and BARD1 in living cells increased the abundance and stability of both proteins and that this depended on their ability to heterodimerize.

BRCA1 is a tumor suppressor gene that is mutated in 50–90% of hereditary breast and ovarian cancers (1, 2). The human BRCA1 gene encodes a large protein of 1863 amino acids, which contains an N-terminal RING-finger domain and two C-terminal BRCT domains (3, 4). To date, BRCA1 has been implicated in interactions with more than 20 proteins and involved in a remarkable range of cellular processes from transcriptional regulation to DNA damage repair (5–10). It has been suggested that BRCA1 may regulate various biological pathways via a common mechanism such as chromatin remodeling (11–14).

20% of the clinically relevant mutations of BRCA1 occur within the N-terminal 100 residues, which contain the RING motif (residues 23–76) (15). Recently, RING domains have been documented to have E3 ubiquitin ligase activity (16, 17). The RING E3s function as adaptors to recruit substrates and a Ub-conjugating enzyme (E2), and to mediate the transfer of Ub from E2 to substrate proteins (16–18). Ub conjugation (ubiquitylation) is well known as a signal for protein degradation and is involved in multiple biological pathways (19). The RING domain of BRCA1 exhibits E3 ligase activity in vitro (20–22), and all of the cancer predisposing mutations in the RING domain have been tested inactivate BRCA1 E3 Ub ligase activity (21, 22).

Human BARD1 (BRCA1-associated RING domain 1) encodes a protein of 777 amino acids and contains an N-terminal RING domain (residues 49–100) and two C-terminal BRCT motifs (15, 23). In living cells, BRCA1 exists mostly as a heterodimeric complex with BARD1 (23, 24). The recently reported NMR structure of a BRCA1-BARD1 heterodimeric complex reveals that the α-helices flanking the central RING motif of BRCA1 and BARD1 form a stable four-helix bundle that acts as the major heterodimerization interface between the two proteins (15). Several lines of evidence suggest that BARD1 is involved in BRCA1-mediated tumor suppression. BARD1 mutations have been detected in breast, ovarian, and uterine tumors (25), and inhibition of BARD1 expression in cultured cells results in a premalignant phenotype (26). The BRCA1-BARD1 complex has been shown to interact with the polyadenylation factor CstF-50 (27), presumably to inhibit mRNA processing at sites of DNA damage (28). BRCA1-BARD1 co-localize with DNA replication and repair factors in response to DNA damage (29–32). Importantly, it has been reported that BRCA1-BARD1 heterodimers exhibit significant E3 Ub ligase activity and that the BARD1 RING finger domain greatly potentiates the ligase activity of the BRCA1 RING finger (22, 33). However, BARD1 may also have BRCA1-independent functions, since it can act as an apoptosis inducer in a BRCA1-independent manner (34).

Here we report that BARD1 can significantly enhance BRCA1 E3 Ub ligase activity by directly binding BRCA1, although BARD1 itself does not exhibit E3 ligase activity in vitro. BARD1 and BRCA1 form heterodimers in living cells and mutually control each other’s abundance and stability. The enhancement of BRCA1 autoubiquitylation by BARD1 mostly results in poly-Ub chains linked through Lys63, which could be involved in pathways related to DNA damage response and repair rather than signaling BRCA1 degradation.

**Experimental Procedures**

Plasmid Constructs—A DNA fragment containing the N-terminal 110 amino acids of BRCA1 was PCR-amplified from pBluescript II

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Yan Xia§§, Gerald M. Pao¶, Hong-Wu Chen¶, Inder M. Verma**, and Tony Hunter‡ ‡‡

From the §Molecular and Cell Biology Laboratory and ¶Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, California 92037 and the **Department of Biological Chemistry, UCD Cancer Center/Basic Science, University of California at Davis, Sacramento, California 95817

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SK (+) 73.1 (35) using the primers (5′-GCCCATGCTTTTCCGGAGTATC-3′) and (5′-GCCGATCTGTTTCACTTATGAACT-3′). The fragment was cloned using BanHI and EcoRI into pGEX-KG and pIX8 to create pYN122 and pYN131, respectively. A DNA fragment containing the N-terminal 308 amino acids of BRCA1 was PCR-amplified from pHIV CSC vector from pBluescript 73.1, giving pHIV CSC. The plasmid pHIV CSC was constructed by introducing pYN151 (I69A), pYN152 (C83G), pYN153 (C50G/C83G), pYN154 (C50G/C83G), pYN155 (H68A), pYN156 (L44R), and pYN158 (I105D) were created using the QuickChange site-directed mutagenesis kit (Stratagene). A DNA fragment containing the N-terminal 8 residues of BRCA1 (43) was part of a larger proteolysis-resistant structural domain containing the first 110 residues of BRCA1 (45). A plasmid expressing GST-BRCA1-(1–110) was constructed, and bacterially expressed protein was purified using glutathione beads. Purified GST-BRCA1-(1–110) (data not shown) was assayed for its ability to mediate the transfer of Ub and stimulate the synthesis of stable Ub conjugates in an in vitro ubiquitylation assay, using blotting with an anti-Ub monoclonal antibody to detect ubiquitylated products. As shown in Fig. 1B, the BRCA1 RING domain exhibited E3 Ub ligase activity in an E1- and E2-dependent manner, consistent with previously published results (21). The polyubiquitylated conjugates include ubiquitylated BRCA1 and His-E1/Ub (data not shown). Purified His-BRCA1-(1–110) had E3 ligase activity similar to that obtained with GST-BRCA1-(1–110) (data not shown). Histone H2A could also be monoubiquitylated by GST-BRCA1-(1–110) (data not shown) and by full-length BRCA1 (Fig. 1C, lane 7). The band was identified as monoubiquitylated H2A, based on its reactivity with both anti-H2A and anti-Ub antibodies. When the same membrane was probed with polyclonal anti-H2A, anti-H2B, anti-H3, and anti-H4 antibodies (Novus Biologicals, Inc.) antibodies. RESULTS

The E3 Ubiquitin Ligase Activity of BRCA1 Is Enhanced in the Presence of BARD1—The BRCA1 central RING motif, which encompasses residues 23–76 (15), is part of a larger proteolysis-resistant structural domain containing the first 110 residues of BRCA1 (45). A plasmid expressing GST-BRCA1-(1–110) was constructed, and bacterially expressed protein was purified using glutathione beads. Purified GST-BRCA1-(1–110) was assayed for its ability to mediate the transfer of Ub and stimulate the synthesis of stable Ub conjugates in an in vitro ubiquitylation assay, using blotting with an anti-Ub monoclonal antibody to detect ubiquitylated products. As shown in Fig. 1B, the BRCA1 RING domain exhibited E3 Ub ligase activity in an E1- and E2-dependent manner, consistent with previously published results (21). The polyubiquitylated conjugates include ubiquitylated BRCA1 and His-E1/Ub (data not shown). Purified His-BRCA1-(1–110) had E3 ligase activity similar to that obtained with GST-BRCA1-(1–110) (data not shown). Histone H2A could also be monoubiquitylated by GST-BRCA1-(1–110) (data not shown) and by full-length BRCA1 (Fig. 1C, lane 7). The band was identified as monoubiquitylated H2A, based on its reactivity with both anti-H2A and anti-Ub antibodies. When the same membrane was probed with polyclonal anti-H2A, anti-H2B, anti-H3, and anti-H4 antibodies, no extra slower migrating bands were observed (data not shown), indicating that H2A is specifically monoubiquitylated by BRCA1 in vitro.

To examine whether BARD1, which is itself a RING protein, might affect BRCA1 E3 Ub ligase activity, increasing amounts of purified GST-BRCA1-(8–142) containing the RING domain (residues 49–100) were incubated together with GST-BRCA1-(1–110), His-E1, His-E2 (Ub4c), and GST-Ub. BRCA1 significantly enhanced GST-BRCA1 E3 Ub ligase activity (Fig. 1B), as had previously been reported in analogous studies by Hashizume et al. (22) and Chen et al. (33). His-BRCA1-(1–110) E3 ligase activity was also greatly stimulated by BARD1 (data not shown). The GST-BRCA1-(8–142) protein lacked E3 ligase ac-
BARD1 cooperate in the monoubiquitylation of histone H2A. Polyubiquitylated conjugates and GST-Ub are indicated on the membrane, which was probed with anti-Ub antibody. Polyubiquitylated conjugates and GST-Ub are indicated on the right, markers, in kDa, are shown on the left.

The enhancement of BRCA1 E3 Ligase Activity by BARD1

Depends on Direct Interaction between the Two Proteins—To determine whether the enhancement of BRCA1 E3 Ub ligase activity by BARD1 depends on the integrity of the BARD1 RING domain and the interaction between the two proteins, the RING consensus residues Cys50, Cys83, and His68, in the BARD1 RING domain, were mutated to Gly or Ala; the nonconserved Arg58 and Ile69 were also mutated to Ala (Fig. 2A). The conserved Cys and His residues are necessary for the integrity of the BARD1 RING domain, which is in turn required for the proper orientation of the N- and C-terminal helices that form the four-helix bundle, and therefore these mutations might be expected to affect the interaction between BRCA1 and BARD1. The mutant GST-BARD1-(8–142) proteins were then purified and examined for their ability to bind BRCA1. GST protein and WT GST-BARD1-(8–142) were used as negative and positive controls, respectively. As shown in Fig. 2B, WT GST-BARD1-(8–142) bound strongly to His-BRCA1-(1–110), whereas GST itself did not. The R58A and I69A mutant proteins bound equally well to BRCA1 when compared with WT BARD1. The C50G, H68A, and C83G single mutant proteins still bound to BRCA1 weakly, whereas the C50G/H68A, C50G/C83G, and H68A/C83G double mutant proteins only exhibited extremely low binding activity in the GST pull-down assay (Fig. 2B). To test the importance of the hydrophobic interactions in the four-helix bundle that stabilize the BRCA1-BARD1 heterodimer, we also constructed two BARD1 mutants with mutations in critical hydrophobic residues in the N- or C-terminal α-helix, L44R (N-terminal α-helix) and I105D (C-terminal α-helix, L44R (N-terminal α-helix) and I105D (C-terminal α-helix, L44R (N-terminal α-helix) and I105D (C-terminal α-helix, L44R (N-terminal α-helix) and I105D (C-terminal α-helix, L44R (N-terminal α-helix) and I105D (C-terminal α-helix), L44R (N-terminal α-helix) and I105D (C-terminal α-helix). The R58A and I69A mutant proteins bound equally well to BRCA1 when compared with WT BARD1. The C50G, H68A, and C83G single mutant proteins still bound to BRCA1 weakly, whereas the C50G/H68A, C50G/C83G, and H68A/C83G double mutant proteins only exhibited extremely low binding activity in the GST pull-down assay (Fig. 2B). To test the importance of the hydrophobic interactions in the four-helix bundle that stabilize the BRCA1-BARD1 heterodimer, we also constructed two BARD1 mutants with mutations in critical hydrophobic residues in the N- or C-terminal α-helix, L44R (N-terminal α-helix) and I105D (C-terminal α-helix).
alpha-helix), which contain an intact RING domain but fail to bind BRCA1 in the yeast two-hybrid assay (15, 44). Consistently, the L44R mutant protein showed no detectable binding, and the I105D mutant protein showed only extremely weak binding to BRCA1 in the GST pull-down assay (Fig. 2C).

Next, equal amounts of purified WT or mutant BARD1 proteins were tested for their effects on BRCA1 E3 Ub ligase activity using GST-Ub, and ubiquitylated products were detected by blotting with anti-GST monoclonal antibody. As shown in Fig. 3, WT GST-BARD1 (8–142) and the R58A and I69A mutant proteins significantly enhanced BRCA1 E3 Ub ligase activity (lanes 4, 6, and 8). The C50G, H68A, and C83G mutant proteins also enhanced BRCA1 E3 ligase activity, although relatively weakly (lanes 5, 7, and 9). The I105D, C50G/H68A, and C50G/C83G mutant proteins had only very small stimulatory effects (lanes 12–14), and the L44R mutant protein had almost no detectable effect on BRCA1 E3 Ub ligase activity (Fig. 3, lane 11). In addition, the same amount of purified GST-BARD1 (8–142) had only an extremely weak stimulatory effect on the E3 ligase activity of GST-BRCA1 (1–78) (Fig. 3, lanes 15 and 16), which lacks the C-terminal alpha-helix (residues 81–96) flanking the central RING motif (15), consistent with the previous report that BARD1 does not interact stably with the N-terminal 71 residues of BRCA1 (23) and the fact that this helix is critical for the four-helix bundle (15). These results indicate that a direct stable interaction between BARD1 and BRCA1 is required for BARD1 to enhance BRCA1 E3 Ub ligase activity and that the integrity of the BARD1 RING domain is not so important.

**BRCA1 and BARD1 Mutually Stabilize Each Other in Vivo**—To further investigate the molecular basis of BRCA1 and BARD1 cooperation, expression plasmids for FLAG-tagged human BRCA1 (full-length or N-terminal 308 amino acids) and FLAG-tagged WT or mutant human BARD1 (full-length or aa 8–142) were co-transfected into human 293T cells. As shown in Fig. 4A, the levels of FLAG-BRCA1 (1–308) protein were dramatically increased in the presence of increasing amounts of FLAG-tagged WT BARD1 (full-length or aa 8–142). To test the effect of BARD1 on full-length BRCA1, pHIV CSC BRCA1, which expresses full-length human BRCA1 in the third generation lentiviral vector, was co-transfected with FLAG-tagged BARD1 (full-length) expression plasmid or pBluescript II (KS+)(Stratagene) control plasmid. pCMX LacZ (45) and pEGFP C-2 (Clontech) were also co-transfected as controls for transfection efficiency. The level of transfected full-length BRCA1 protein was significantly elevated when full-length FLAG-BARD1 was co-expressed (Fig. 4B). The endogenous BRCA1 protein level was also increased by transfected BARD1 (data not shown).

Next, the effects of mutant BARD1 proteins on BRCA1 abundance were assessed. As shown in Fig. 4C, the levels of FLAG-BRCA1 (1–308) protein were dramatically increased when full-length WT BARD1 or the R58A mutant was co-expressed (lanes 2, 4, and 13). In contrast, the C50G, H68A, and C83G single mutant proteins caused a relatively small increase in BRCA1 levels (lanes 3, 5, and 6), whereas the C50G/H68A, C50G/C83G, and H68A/C83G double mutant and L44R and I105D mutant proteins only had an extremely small stimulatory effect on BRCA1 abundance (Fig. 4C, lanes 7–9, 11, and 12). Mutant BARD1 (8–142) proteins behaved similarly to mutant BARD1 (full-length) (data not shown). The effect of BRCA1 on BARD1 abundance was also assayed. As shown in Fig. 4D, the levels of WT FLAG-BARD1 (full-length or aa 8–142) proteins were sig-
BRCA1 E3 ubiquitin ligase activity.

0.5 μg of purified GST-BRCA1-(1–110) or GST-BRCA1-(1–78) were incubated with purified His-E1, His-E2 (Ubc4), and GST-Ub, in the absence or presence of 1 μg of purified WT or mutant GST-BARD1-(8–142) (lanes 3–10). lane 1 shows a reaction containing only E1 and E2, whereas lane 2 shows a reaction containing only BRCA1-(1–110) and BARD1, in addition to GST-Ub. The reactions were separated by SDS-PAGE and analyzed by immunoblotting with anti-GST antibody. GST-Ub and polyubiquitylated conjugates are indicated on the right, and the molecular mass markers, in kDa, are shown on the left. The reactions were done in duplicate (lanes 1–9, 15, and 16) or separately with controls (lanes 10–14). (data not shown). When BARD1 or BRCA1 were co-transfected with vector control pFLAG, the levels of both proteins were very low, and they were rapidly degraded (Fig. 5, lanes 1–5 and 16–20). The results suggest that BRCA1 and BARD1 stabilize each other through their direct interaction.

**BARD1 Enhances BRCA1 Autoubiquitylation Involving Polyubiquitin Chains Linked through LysK63**—It would be paradoxical if the enhancement of BRCA1 autoubiquitylation by BARD1 led to the degradation of BRCA1, since our data indicated that these proteins actually stabilize each other. Poly-Ub chains can be linked through Lys residues 11, 29, 48, and 63 in vivo (46). LysK48-linked chains target proteins to the proteasome, whereas LysK63-linked chains function to signal biological processes other than proteasome-mediated degradation (46–48). To investigate whether BRCA1 E3 ligase activity promotes formation of LysK48- or LysK63-linked poly-Ub chains and whether this is influenced by BARD1, K48A, K63A, and K48A/K63A GST-Ub mutants were generated. BRCA1 E3 Ub ligase activity was assayed in the absence or presence of GST-BARD1-(8–142) using equal amounts of purified GST-tagged wild type Ub, Ub(K48A), Ub(K63A), or Ub(K48A/K63A) in each reaction. The levels of poly-Ub chains were assayed by blotting with anti-BRCA1 (A) antibodies and then reprobing with anti-Ub antibody after stripping. Double mutant GST-Ub(K48A/K63A) was almost inactive in the in vitro ubiquitylation assay (Fig. 6, lanes 9 and 10), indicating that most of the poly-Ub chains were linked through LysK48 or LysK63. With GST-Ub and GST-Ub(K63A), the signals for poly-Ub conjugates were much stronger in the presence of GST-BARD1-(8–142), when the membrane was probed with anti-Ub antibody (Fig. 6, left panel, lanes 3, 4, 7, and 8). For GST-Ub(K48A), the signals for poly-Ub proteins were also stronger with GST-BARD1-(8–142) although much weaker compared with those for GST-Ub and GST-Ub(K63A) (Fig. 6, left panel, lanes 5 and 6). This indicated that BARD1 dramatically enhanced BRCA1 E3 Ub ligase activity and that formation of both LysK48 and LysK63 poly-Ub chains was stimulated by BARD1, although most of the poly-Ub chains detected in the reaction were linked through LysK63 (Fig. 6, left panel). A different picture emerged when the membrane was probed with anti-BRCA1 (A) antibodies. In the absence of BARD1, signals for BRCA1-(GST-Ub), conjugates were much stronger with GST-Ub (WT) and GST-Ub (K63A) than with GST-Ub (K48A), indicating that most of the poly-Ub chains attached to BRCA1 were linked through LysK48 in the absence of BARD1 (Fig. 6, right panel, lanes 3, 5, and 7). However, the signals obtained with GST-Ub(K48A) and GST-Ub(K63A) in the presence of GST-BARD1-(8–142) were nearly equivalent, and the signal observed with GST-Ub(K63A) was only weakly stimulated by GST-BARD1-(8–142), whereas that observed with GST-Ub(K48A) was strongly stimulated by GST-BARD1-(8–142) (Fig. 6, right panel, lanes 5–8). This suggests that BARD1 stimulation of BRCA1 autoubiquitylation results in LysK48-linked poly-Ub chains, although a significant fraction of total BRCA1 autoubiquitylation occurs through LysK63-linked poly-Ub chains, although a significant fraction of total BRCA1 autoubiquitylation occurs through LysK48 linkages. BRCA1-stimulated ubiquitylation of other proteins in the reaction results in formation of both LysK48- and LysK63-linked poly-Ub chains, but this occurs primarily on LysK48. Probing the membrane with anti-His monoclonal antibody showed that the poly-Ub conjugates included ubiquitylated His-E1/E2 in addition to ubiquitylated BRCA1 (data not shown), which in part explains the different patterns obtained when the membrane was probed with anti-Ub and anti-BRCA1 (A) antibodies (Fig. 6). It is also to be expected that anti-Ub antibody will detect polyubiquitylated protein/BRCA1 more efficiently than anti-BRCA1 (A), because each polyubiquitylated BRCA1 molecule has multiple sites for binding Ub antibody but only one site for binding BRCA1 antibodies (the BRCA1 (A) antibodies...
used in the assay was directed against BRCA1 amino acids 2–20). To rule out the possibility that GST itself is the target for poly-Ub chain attachment, the GST-tagged wild type and mutant Ub were incubated with thrombin to cleave off the GST tag. WT and mutant Ub proteins without GST tags were then used in the in vitro ubiquitylation assay. The results were similar to those obtained with GST-tagged Ub (data not shown). This result, plus the fact that GST-BARD1 cannot be polyubiquitylated in vitro by BRCA1 (data not shown), indicated that GST is not a substrate for BRCA1 E3 Ub ligase.

DISCUSSION

The RING finger domain is a zinc-binding motif, characterized by a set of spatially conserved Cys and His residues that
follow the linear order C3HC/HC3 within the primary amino acid sequence (49). RING domains can specifically interact with E2 Ub-conjugating enzymes, thereby promoting ubiquitylation (16, 17). Previously, the BRCA1 RING finger was demonstrated to have in vitro E3 ligase activity that depended on an intact RING finger structure (20–22), and the E3 ligase activity of the BRCA1 RING finger was shown to be greatly enhanced by the BARD1 RING finger domain by Ohta et al. (15). The enhancement of BRCA1 E3 ligase activity was shown to be dependent on the stable complex formed between these two domains (15, 50). In the present study, we showed that the BARD1 RING domain protein enhances BRCA1 RING domain E3 Ub ligase activity through direct interaction between the two proteins. Moreover, we found that the BARD1 enhancement of BRCA1 autoubiquitylation mostly resulted in the formation of Lys63-linked Ub chains, whose significance is discussed below. Consistent with previous results, GST-BRCA1(1–110), which encodes a stable domain containing the RING motif, exhibited E3 Ub ligase activity in vitro, which mediated autoubiquitylation or mono-ubiquitylation of histone H2A (Fig. 1, B and C). However, the RING domain of BARD1 protein lacked E3 ligase activity in vitro, even when used at much higher concentrations than BRCA1 (Fig. 1D). The NMR structure of BARD1 in association with BRCA1, recently reported by Klevit’s group (15), reveals that BARD1 lacks the central α-helix in its RING domain that is important for the c-Cbl RING (18) and Rbx1 RING (51) to interact with E2, which may explain its lack of E3 ligase activity. Our in vitro studies show that the BARD1 RING domain and surrounding helices have a different function, namely to enhance the BRCA1 E3 activity. In support of this, we found that both autoubiquitylation of BRCA1(1–110) and monoubiquitylation of histone H2A by full-length BRCA1 were significantly enhanced by BARD1 (Fig. 1, B and C), consistent with the recent report from Pan’s group (33). Our results could explain the Ohta group’s finding that BARD1 immunocomplexes have E3 ligase activity, since the transfected Myc-BARD1 may have formed a complex with endogenous BRCA1 and stimulated its activity (22).

Analysis of BARD1 RING finger mutants indicated that the conserved Cys and His residues in the RING domain are important for BARD1 binding to BRCA1 (Fig. 2B), probably because an intact RING domain is needed to orient the N- and C-terminal helices involved in heterodimerization, and that the enhancement of BRCA1 E3 Ub ligase activity by BARD1 is completely dependent on direct interaction between the two proteins (Fig. 3). This conclusion is supported by the deleterious effects of mutating L44R and I105D, which are part of the hydrophobic core forming the BARD1:BRCA1 interface and one of the intramolecular helical packing contacts, respectively, and whose mutation abolishes BARD1 binding to BRCA1 (15, 44). Moreover, the direct interaction between BARD1 and BRCA1 appears to be more important than the integrity of the BARD1 RING domain for enhancement of BRCA1 E3 ligase activity (Fig. 3). The enhancement of BRCA1 E3 ligase activity by BARD1 could result from a conformational change in the BARD1 RING finger induced upon BARD1 binding that promotes a functional interaction with the E2 Ub-conjugating enzyme.

In transient expression studies, we found that BRCA1 levels were dramatically elevated by co-expressed BARD1 (Fig. 4, A and B) and that the stimulatory effect was dependent on the interaction between the two proteins (Fig. 4C). Likewise, BARD1 levels were elevated by co-expressed BRCA1 (Fig. 4D), which is consistent with the report from Ohta’s group (22). The
increased abundance of both proteins was due to increased stability, and this effect required direct interaction between BRCA1 and BARD1 (Fig. 5). This may explain why BRCA1 exists in vivo in the form of a BRCA1/BARD1 heterodimer (23, 24). Our results are in agreement with the report from Livingston’s group, in which they showed that Xenopus laevis BRCA1 and BARD1 mutually stabilize each other through their interaction (52). The stabilization of both proteins seems likely to be due to the highly stable conformation of the heterodimer. Stabilization of one RING domain protein by another has been observed previously; the MDM2 E3 Ub ligase and the structurally related protein MDMX interact through their RING finger domains, and this interaction stabilizes MDM2 and prevents it from degradation (53, 54). These examples suggest an interesting possibility that RING-RING interactions might be a general regulatory feature of RING finger E3 Ub ligases.

The fact that BARD1 binds BRCA1 and stabilizes it in vivo and yet enhances its E3 Ub ligase activity would seem contradictory if the enhanced BRCA1 autoubiquitylation led to its degradation. It is now clear that poly-Ub chains can be assembled through lysines other than Lys48 and some of the resulting chains function in distinct biological processes (47). In the most striking examples, poly-Ub chains linked through Lys63 have been reported to be involved in DNA repair (55–58, 67, 69). Both the yeast Mms2/Ubc13 and UEV1a/Ubc13 are involved in catalyzing the assembly of Lys63 chains (58, 59, 67, 69). Both the yeast Mms2/Ubc13 and human UEV1a/Ubc13 interact with their RING partner proteins Rad5 (56) and Traff (70), which may be either a cognate E3 or a substrate of the respective E2/UEV heterodimers. It remains to be determined what the Ub-conjugating enzyme(s) for BRCA1 E3 ligase activity are in vivo (Ub4c has been reported to catalyze formation of Lys63 chains in yeast), how BRCA1 functions in the recognition and assembly of Lys63 chains together with E2, and what the possible function(s) of these BRCA1 attached Lys63 chains are. Our future work will aim to elucidate the functional mechanism of BRCA1 as an E3 Ub ligase and its role in tumor suppression, which might provide new approaches for the treatment and prevention of breast and ovarian cancers.

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