The BRCA1/BARD1 Heterodimer Assembles Polyubiquitin Chains through an Unconventional Linkage Involving Lysine Residue K6 of Ubiquitin*

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The BRCA1 tumor suppressor forms a heterodimer with the BARD1 protein, and the resulting complex functions as an E3 ubiquitin ligase that catalyzes the synthesis of polyubiquitin chains. In theory, polyubiquitination can occur by isopeptide bond formation at any of the seven lysine residues of ubiquitin. The isopeptide linkage of a polyubiquitin chain is a particularly important determinant of its cellular function, such that K48-linked chains commonly target proteins for proteasomal degradation, while K63 chains serve non-proteolytic roles in various signaling pathways. To determine the isopeptide linkage formed by BRCA1/BARD1-dependent polyubiquitination, we purified a full-length BRCA1/BARD1 heterodimer in protein ubiquitination (8–15). Here we have used mutation analysis to establish the linkage specificity with that of E6-AP, an E3 ligase known to induce proteolysis of its cellular substrates. Using a comprehensive mutation analysis, we found that E6-AP catalyzes the synthesis of K48-linked polyubiquitin chains. In contrast, however, the BRCA1/BARD1 heterodimer directs polymerization of ubiquitin primarily through an unconventional linkage involving lysine residue K6. Although heterologous substrates of BRCA1/BARD1 are not known, BRCA1 autoubiquitination occurs principally by conjugation with K6-linked polymers. The ability of BRCA1/BARD1 to form K6-linked polyubiquitin chains suggests that it may impart unique cellular properties to its natural enzymatic substrates.

The BRCA1 tumor suppressor gene has been implicated in various cellular processes that include DNA repair, transcriptional regulation, and cell cycle checkpoint control (1). Its protein product contains an NH2-terminal RING domain and two COOH-terminal BRCT repeats. In vivo, BRCA1 exists as a heterodimer with BARD1, a related protein that displays an analogous configuration of RING and BRCT motifs (2). The BRCA1/BARD1 heterodimer is stabilized by a 4-helix bundle formed by α-helices that immediately flank the RING domains of both polypeptides (3). Since most cellular BRCA1 polypeptides are found in association with BARD1 (4, 5), the BRCA1/BARD1 heterodimer is likely to be an essential mediator of BRCA1 function (6). This conclusion is strongly supported by analysis of Bard1-null mice, which display a characteristic phenotype that is essentially indistinguishable from that of Brca1-null animals (7).

Recent studies have uncovered an enzymatic role for the BRCA1/BARD1 heterodimer in protein ubiquitination (8–15). Ubiquitination occurs through a sequential process involving ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (16, 17). In theory, polyubiquitin chains are linked at one or more of the seven lysine residues of ubiquitin. The isopeptide bond with lysine residue 48 on the previously attached monomer (18). These proteins are usually targeted for degradation, presumably because 26 S proteasomes specifically recognize ubiquitin conjugated with K48 (19). In addition, chains in which each consecutive ubiquitin monomer forms an isopeptide bond with lysine residue 48 on the previously attached monomer (18). These proteins are usually targeted for degradation, presumably because 26 S proteasomes specifically recognize the K48 isopeptide linkage (19). In addition, chains assembled through either K29 or K63 have also been observed in vivo, and chains linked at these and other sites can be generated in vitro (20–22). While K48- and K29-linked chains target proteins for proteasomal destruction, K63-linked chains confer non-proteolytic signals that control various pathways, including post-replicative DNA repair and NF-κB activation (23). Here we have used mutation analysis to establish the linkage specificity of BRCA1/BARD1-dependent polyubiquitination. Surprisingly, BRCA1/BARD1 catalyzes ubiquitin polymerization primarily through lysine residue 6 to form an unconventional type of polyubiquitin chain.

The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; GST, glutathione S-transferase; Ni-NTA, nickel-nitrilotriacetic acid; Ub, ubiquitin.

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EXPERIMENTAL PROCEDURES

Plasmids—Baculoviral transfer vectors for expression of wild type (pBac-Brca1–6H-wt/pVL1392) and mutant (pBac-Brca1–6H-mt/pVL1392) Brca1 were constructed by inserting cDNA sequences encoding full-length human Brca1 with an N2-terminal FLAG tag (NH2-MADYKDDDDK) and a COOH-terminal hexahistidine (His6) tag (HHHHHHHAS-COOH) into the pVL1392 plasmid (Pharmingen). The baculoviral vector for expression of full-length GST-Brca1 (pGST-Brca1/pVL1392) was prepared by isolating cDNA sequences encoding glutathione-S-transferase and full-length human Brca1 into pVL1392. A vector (6H-E6-AP/pET14b) for expression of E6-AP with an N2-terminal His tag (NH2-M6AHHHHH) was generated by inserting cDNA sequences encoding full-length human UbcH5c (Image clone ID number 3447673) into 6H-pET14b. To generate a plasmid (U6/pET14+) encoding native wild type ubiquitin, the cDNA sequence for a single ubiquitin monomer was PCR amplified from MT123, a plasmid kindly encoded native wild type ubiquitin, the cDNA sequence for a single number 3447673) into 6H-pET14b. To generate a plasmid (Ub/pET14*) containing the His6-tagged UbcH5c (6H-UbcH5c/pET14b) was generated by inserting cDNA sequences encoding full-length human UbcH5c (Image clone ID number 3447673) into 6H-pET14b. To generate a plasmid (Ub/pET14*) encoding native wild type ubiquitin, the cDNA sequence for a single ubiquitin monomer was PCR amplified from MT123, a plasmid kindly encoded native wild type ubiquitin, the cDNA sequence for a single number 3447673) into 6H-pET14b.

Isolation of Full-length BRCA1/BARD1 Complexes—To generate recombinant baculoviruses encoding the FLAG-Brca1–6H polyepitide, S9 cells were transfected with the pF-Brca1–6H-wt/pVL1392 vector using the Baculogold transfection kit (Pharmingen). Recombinant viruses encoding GST-Bard1 were obtained by transfecting S9 cells with the GST-Bard1/pVL1392 vector. For expression of the Brca1/Bard1 heterodimer, 500 ml of S9 spinner cells were co-infected with Brca1 and Bard1 recombinant baculoviruses at a multiplicity of infection of ~2 plaque-forming units/cell for each. Cells were harvested 72 h after infection, and the cell pellet was resuspended in lysis buffer (50 mM Hepes, pH 7.9, 250 mM NaCl, 0.1% Nonidet P-40, 10 mM 2-mercaptoethanol, 10% glycerol) with Complete EDTA-free protease inhibitor mixture (Roche Diagnostics). After 15 min on ice, the resuspended cells were sonicated, the cell debris removed by centrifugation at 30 K for 45 min at 4 °C, and the supernatant filtered through a 0.45-μm polyethersulfone filter (Nalgene). To purify the Brca1/Bard1 complex, the filtered lysate was incubated with Ni-Nta agarose resin (Qiagen) for 2 h at 4 °C. The resin was then washed twice with lysis buffer (with protease inhibitors) and twice with “Ni2+-wash buffer” (25 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol, protease inhibitors) containing 10 mM imidazole, packed into a column, and eluted sequentially with Ni2+ wash containing 30 mM and then 25 mM imidazole. The 25 mM imidazole eluate was pooled, adjusted to 250 mM NaCl and 0.1% Nonidet P-40, and incubated with glutathione-agarose beads (Sigma). After mixing for 3 h at 4 °C, the beads were washed twice with “GST wash buffer” (25 mM Tris-HCl, pH 7.9, 250 mM NaCl, 10 mM 2-mercaptoethanol, 10 mM NAD, 0.1% Nonidet P-40, and 1 mM diethiothreitol, 20% glycerol, protease inhibitors) and twice with “storage buffer” (25 mM Tris-HCl pH 7.9, 50 mM NaCl, 0.02% Nonidet P-40, 1 mM diethiothreitol, 20% glycerol, protease inhibitors). The beads were then packed into a column, eluted with storage buffer containing 10 mM glutathione, and the peak protein fractions were pooled and stored in aliquots at –80 °C. The yield of Brca1/Bard1 heterodimer was determined by comparing the intensity of Coomassie staining with that of known quantities of bovine serum albumin and ubiquitin (Affinity Research).

In Vitro Ubiquitination—Ubiquitination reactions were conducted at 37 °C for 40 min in a 30-μl volume containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 2 mM NaF, 10 μM okadaic acid, 2 mM ATP, 0.6 mM E1, and 20 ng rabbit E1 (Affinity Research). Most reactions were performed with 1 μg of ubiquitin monomer, 0.25 μg of 6H-UbcH5c, and a defined quantity of the purified Brca1/Bard1 heterodimer (i.e. an amount containing 20 μg of the Brca1 subunit) (Figs. 1B and 2A). However, experiments with the Ub-K ubiquitin series (Figs. 1C and 3A) and the Brca1 autoubiquitination assays (Fig. 4) were conducted using 0.125 μg 6H-UbcH5c and 0.25 μg ubiquitin. The E6-AP-dependent reactions were conducted with 240 ng of 6H-E6-AP, 0.6 μg of 6H-UbcH5c, and 1 μg of ubiquitin (Fig. 2B) or 300 ng of 6H-E6-AP, 1.25 μg of 6H-UbcH5c, and 1 μg of ubiquitin (Fig. 3B). After terminating the reactions with 6 μl of 5× SDS loading buffer (0.3 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 25% 2-mercaptoethanol, 0.05% bromophenol blue), the reaction products were separated by SDS-PAGE and detected by immunoblotting. The ubiquitin-specific (FL-76 polyclonal and P4D1 monoclonal) and Brca1-specific (C20 polyclonal) antibodies were purchased from Santa Cruz Biotechnology.

RESULTS

To express full-length Brca1/Bard1 heterodimers, S9 infected cells were co-infected with baculoviruses encoding FLAG-Brca1–6H, a full-length Brca1 polypeptide with an amino-terminal FLAG epitope and a COOH-terminal hexahistidine tag, and GST-Bard1, a fusion polypeptide containing GST linked to full-length Bard1. The Brca1/Bard1 heterodimer was purified from S9 lysates by sequential affinity chromatography on a Ni-Nta resin and glutathione-agarose beads (Fig. 1A, lane 2). In addition, a heterodimer containing the tumor-associated C61G mutation of Brca1 was also expressed and purified (Fig. 1A, lane 3). Both were then tested for their ability to catalyze ubiquitin polymerization in the presence of UbcH5c, one of three closely related E2 enzymes (UbcH15a, UbcH5b/ Ubc4, and UbcH5c) known to support Brca1-dependent ubiquitination (8–14). The ubiquitination reactions were fractionated by SDS-PAGE, and the formation of polyubiquitin was assessed by immunoblotting with ubiquitin-specific antibodies. As expected, high molecular mass ubiquitinated products were generated in an ATP-dependent manner by the wild type (Fig. 1B, lane 3), but not the mutant (lane 4). Brca1/Bard1 heterodimer Ubiquitination reactions were also conducted using Ub-K0, a lysineless derivative of ubiquitin in which all seven lysines have been substituted with arginine. As such, Ub-K0 retains the potential for monoubiquitination but lacks the conjugation sites necessary for polyubiquitination. As shown in Fig. 1C, reactions with Ub-K0 only generate ubiquitinated products that migrate close to the molecular mass of FLAG-Brca1–6H (lane 7) and presumably represent monoubiquitination of FLAG-Brca1–6H at one or a few sites. This result is consistent with previous reports of Brca1 autoubiquitination (11–13) and indicates that the more slowly migrating products generated with wild type ubiquitin (Fig. 1C, lane 6) contain true polyubiquitin chains.

To evaluate the chain specificity of Brca1/Bard1-dependent polyubiquitination, we used a panel of mutant ubiquitin molecules in which arginine was substituted for one of the seven lysine residues: Ub-R6, Ub-R11, Ub-R27, Ub-R29, Ub-R33, Ub-R48, and Ub-R63. For example, in Ub-R6, only lysine 6 has been changed to arginine. As shown in (Fig. 2A) polyubiquitination...
biquitination was abolished in reactions conducted with Ub-R6, suggesting that BRCA1/BARD1 catalyzes the formation primarily of K6-linked ubiquitin polymers. Consistent with a previous study (12), the levels of polyubiquitination were reduced in Ub-R48 and Ub-R63 reactions, indicating that BRCA1/BARD1 may also induce, to a lesser extent, formation of K48- and K63-linked chains. As a control, we examined the linkage specificity of E6-AP, a ubiquitin ligase that can induce ubiquitination and degradation of the p53 tumor suppressor (25) and, like BRCA1, can function in collaboration with the UbcH5 family of E2 enzymes (26). As shown in Fig. 2B, E6-AP polyubiquitination was completely ablated in the Ub-K48 reaction, implying that E6-AP directs the synthesis of K48-linked chains.

To confirm the linkage specificities of BRCA1/BARD1 and E6-AP, in vitro reactions were also conducted using a panel of ubiquitin mutants in which all but one of the seven lysine residues are substituted with arginine: Ub-K6, Ub-K11, Ub-K27, Ub-K29, Ub-K33, Ub-K48, and Ub-K63. For example, in Ub-K6, all the lysines are changed to arginine with the exception of residue K6. As shown in Fig. 3A, robust polyubiquitination by BRCA1/BARD1 was only observed in reactions containing Ub-K6. On long exposures of the immunoblots, significantly lower levels of polyubiquitination were also detected in reactions conducted with Ub-K27, Ub-K48, and Ub-K63 (data not shown). These results confirm that while BRCA1/BARD1 preferentially catalyzes the formation of K6-linked polymers, it also has some capacity to induce synthesis of chains with other linkages. As shown in Fig. 3B, only Ub-K48 supported polymer formation by E6-AP, confirming that K48-linked chains are the major products of E6-AP-dependent ubiquitination.

Although heterologous substrates of BRCA1/BARD1-mediated polyubiquitination have not yet been identified, several groups have shown that the BRCA1 subunit of the heterodimer undergoes autoubiquitination in vitro (11–13). To determine whether K6-linked chains are conjugated to BRCA1 during autoubiquitination, BRCA1/BARD1-dependent reactions were conducted and the resulting ubiquitin-conjugates of BRCA1 were visualized by immunoblotting with BRCA1-specific antibodies (Fig. 4). As expected, only the unmodified FLAG- BRCA1–6H polypeptide was detected in reactions conducted in the absence of ubiquitin (lanes 1 and 4). In the presence of wildtype ubiquitin, high molecular mass species of polyubiquitinated BRCA1 were readily generated (lane 2), consistent with previous reports of BRCA1 autoubiquitination (11–13).
contrast, however, polyubiquitinated BRCA1 was not observed in reactions conducted with Ub-R6 (lane 3), indicating that autoubiquitination primarily produces BRCA1 conjugates with K6-linked chains. The ability of BRCA1/BARD1 to induce K6-linked polyubiquitinated BRCA1 was confirmed by the observation that BRCA1 polyubiquitination occurs in reactions conducted with Ub-K6 (lane 6), the ubiquitin mutant that harbors a single lysine at residue 6, but not with the lysineless ubiquitin Ub-K0 (lane 5).

DISCUSSION

The identification of K6, an unconventional site for ubiquitin polymerization, as the principal linkage generated by BRCA1/BARD1 was unexpected. Nevertheless, several precautions were taken to ensure the proper fidelity of linkage formation during in vitro polyubiquitination. First, the reactions were conducted with a full-length heterodimer that contains all the critical domains, and all ubiquitin monomers were expressed in a bacterial strain supplemented with exogenous tRNAs that recognizes the minimal codon (CGC) that is not susceptible to misincorporation, and all ubiquitin monomers were expressed in a bacterial strain supplemented with exogenous tRNAs that recognize the minimal codon (CGC) that is not susceptible to misincorporation, and all ubiquitin monomers were expressed in a bacterial strain supplemented with exogenous tRNAs that recognize the minimal codon (CGC) that is not susceptible to misincorporation. Third, to avoid misincorporation of lysine in the ubiquitin mutants, a known hazard of ubiquitin synthesis in bacteria (27), all arginine substitutions were generated by site-directed mutagenesis to a codon (CGC) that is not susceptible to misincorporation, and all ubiquitin monomers were expressed in a bacterial strain supplemented with exogenous tRNAs that recognize the minimal codon (CGC) that is not susceptible to misincorporation. Third, to avoid misincorporation of lysine in the ubiquitin mutants, a known hazard of ubiquitin synthesis in bacteria (27), all arginine substitutions were generated by site-directed mutagenesis to a codon (CGC) that is not susceptible to misincorporation, and all ubiquitin monomers were expressed in a bacterial strain supplemented with exogenous tRNAs that recognize the minimal codon (CGC) that is not susceptible to misincorporation. Third, to avoid misincorporation of lysine in the ubiquitin mutants, a known hazard of ubiquitin synthesis in bacteria (27), all arginine substitutions were generated by site-directed mutagenesis to a codon (CGC) that is not susceptible to misincorporation.

While K29- and K48-linked chains can target their conjugated substrates for proteasomal degradation, K63 chains are believed to serve diverse non-proteolytic functions (23). In contrast, however, the cellular functions of K6 polyubiquitin chains are not known. To date, the production of K6 chains has only been observed in E3-independent reactions catalyzed by Rad6, the yeast ortholog of UbcH2 (22). Interestingly, although Rad6 forms exclusively K6 linkages in E3-independent reactions, it generates K48 chains in the presence of an appropriate E3 (22). This suggests that the chain linkage of ubiquitination can be influenced by the combined specificities of the collaborating E2 and E3 enzymes. A similar phenomenon is observed here, in that UbcH5 generates primarily K48 chains in the presence of E6-AP and K6 chains in the presence of BRCA1/BARD1.

A recent study using a selected panel of ubiquitin mutants (Ub-A48, Ub-A63, and Ub-A48/A63) indicated that BRCA1/BARD1 can potentially generate K48- and K63-linked polymers (12). While our data are consistent with these observations, the use of a comprehensive panel of ubiquitin mutants reveals that K6 is the preferred linkage specificity of BRCA1/BARD1. Nonetheless, if BRCA1/BARD1 does catalyze, even to a modest extent, the formation of K48 or K63 linkages, then branched polymers could potentially be generated during BRCA1/BARD1-dependent ubiquitination. At present, the physiological functions of K6-linked chains, whether branched or not, have not been defined.

Important insights into the molecular functions of BRCA1 should emerge once the natural substrates of BRCA1/BARD1-dependent ubiquitination are identified. The only potential polyubiquitination substrate reported to date is BRCA1 itself, which undergoes autoubiquitination in vitro (11–13). Here we have shown that autoubiquitination results in conjugation of mostly K6 link chains to BRCA1. In light of this, it may be significant that the BRCA1 and BARD1 polypeptides appear to stabilize each other upon co-expression in vivo (7, 10, 28). Since BRCA1 autoubiquitination is catalyzed much more efficiently by the BRCA1/BARD1 heterodimer than by BRCA1 alone (11–13), it seems unlikely that autoubiquitination, if indeed it occurs in vivo, serves to target BRCA1 for proteasomal degradation. This in turn suggests that K6-linked chains may not promote proteasome-dependent proteolysis. Clearly, further studies will be necessary to determine the physiological functions of K6-linked polyubiquitin and its role in BRCA1-mediated tumor suppression.

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REFERENCES

1. Scully, R., and Livingston, D. M. (2000) Nature 408, 429–432
2. Wu, L. C., Wang, Z. W., Tsan, J. T., Spillman, M. A., Phung, A., Xu, X. L., Yang, M.-C. W., Hwang, L.-Y., Bowcock, A. M., and Baer, R. (1996) Nat. Genet. 14, 40–49
3. Brzozowicz, P. S., Rajagopal, P., Hoyer, D. W., King, M.-C., and Klevit, R. E. (2001) Nat. Struct. Biol. 8, 833–837
4. Yu, X., and Baer, R. (2000) J. Biol. Chem. 275, 18541–18549
5. Chiba, N., and Parvin, J. D. (2002) Cancer Res. 62, 4222–4228
6. Baer, R., and Ludwig, T. (2002) Curr. Opin. Genet. Dev. 12, 86–91
7. McCarthy, E. E., Calebi, J. T., Baer, R., and Ludwig, T. (2003) Mol. Cell. Biol. 23, 5566–5583
8. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11364–11369
9. Raffner, H., Joazeiro, C. A., Hemmati, D., Hunter, T., and Verma, I. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5134–5139
10. Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohita, T. (2001) J. Biol. Chem. 276, 14577–14580
11. Chen, A., Kleinman, F. E., Manley, J. L., Ouchi, T., and Pan, Z. Q. (2002) J. Biol. Chem. 277, 22085–22092
12. Xia, Y., Pan, G. M., Chen, H. W., Verma, I. M., and Hunter, T. (2003) J. Biol. Chem. 278, 5255–5263
13. Mallery, D. L., Vandenberg, C. J., and Hiom, K. (2002) EMBO J. 21, 6755–6762
14. Brzozowicz, P. S., Reife, J. R., Nishikawa, H., Miyamoto, K., Fox, D. I., Fukuda, M., Ohita, T., and Klevit, R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5646–5651
15. Kennett, A., Gordon, R. E., and Borden, K. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15404–15409
16. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–533
17. Weissman, A. M. (2001) Nat. Rev. Mol. Cell. Biol. 2, 169–178
18. Chau, V., Tobias, J. W., Bachmair, A., Marrietti, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1583
19. Gregori, L., Piosch, M. S., Cousins, G., and Chau, V. (1990) J. Biol. Chem. 265, 8354–8357
20. Ararison, T., and Ellisien, M. J. (1994) Mol. Cell. Biol. 14, 7676–7683
21. Spence, J., Sadas, S., Haas, A. L., and Finley, D. (1995) Mol. Cell. Biol. 15, 1263–1273
22. Babesina, D. V., and Haas, A. L. (1996) J. Biol. Chem. 271, 2823–2831
23. Pickart, C. M. (2000) Trends Biochem. Sci. 25, 544–548
24. Treier, M., Staewelski, L. M., and Bohnmann, D. (1994) Cell 78, 779–798
25. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) Cell 75, 495–505
26. Scheffner, M., Huibregtse, J. M., and Howley, P. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8797–8801
27. You, J., Cohen, R. E., and Pickart, C. M. (1999) BioTechniques 27, 956–954
28. Joukov, V., Chen, J., Fox, E. A., Green, J. B., and Livingston, D. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12078–12083