Redistribution of BRCA1 among Four Different Protein Complexes following Replication Blockage*

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Natsuko Chiba and Jeffrey D. Parvin‡
From the Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

The BRCA1 protein is known to participate in multiple cellular processes. In these experiments, we resolved four distinct BRCA1-containing complexes. We found BRCA1 associated with the RNA polymerase II holoenzyme (holo-pol), a large mass complex called the fraction 5 complex, the Rad50-Mre11-Nbs1 complex, and a complex that has not been described previously. We observed this new complex after treating cells with hydroxyurea, suggesting that the hydroxyurea-induced complex (HUIC) is involved with the response to DNA replication blockage. After hydroxyurea treatment of cells, BRCA1 content decreased in the holo-pol and the fraction 5 complex, and BRCA1 was redistributed to the HUIC. The HUIC was shown not to contain a number of holo-pol components or the Rad50-Mre11-Nbs1 complex but was associated with the BRCA1-associated RING domain protein BARD1. These data suggest that BRCA1 participates in multiple cellular processes by multiple protein complexes and that the BRCA1 content of these complexes is dynamically altered after DNA replication blockage.

Mutations in the BRCA1 tumor suppressor gene are associated with about 4% of all breast cancers and about 50% of all familial cases (1, 2). Emerging data indicate that BRCA1 is likely to serve as an important central component in multiple biological pathways that regulate transcription, repair of DNA damage, the cell cycle, polyadenylation of mRNAs, and chromatin remodeling (3–7). It is not clear whether all of these processes are due to one biochemical mechanism or to multiple mechanisms with BRCA1 functioning in multiple protein complexes.

BRCA1 has been found to be associated with multiple polypeptides, including BARD1, which binds to the amino terminus of BRCA1 (8). Both BRCA1 and BARD1 proteins contain a RING finger motif and BRCT repeat. The BRCA1-BARD1 complex interacts with a polyadenylation factor, CstF50 (cleavage stimulation factor), suggesting a link between the regulation of polyadenylation of mRNA and DNA repair (4). BRCA1 has ubiquitin ligase activity, and, in association with BARD1, ubiquitin ligase activity is high (9–11). Direct specific interactions between BRCA1 and other polypeptides include the transcriptional regulators Pol II,1 RNA helicase A, p53, STAT1, myc, and CtIP and repair mediators including Rad50 and BACH1 (12–21).

Several BRCA1-containing complexes have been purified using different methods. BRCA1 involvement in transcription is indicated by its association with the RNA polymerase II holoenzyme (holo-pol) and by activation of transcription by BRCA1 in cell free reactions (12, 22–24). BRCA1 is associated with the chromatin-remodeling SWI-SNF (mating type switch/sucrose non-fermenters) complex, either in association with holo-pol (23) or independent of Pol II (25). BRCA1 association with Rad50-Mre11-Nbs1 may contribute to repair of DNA damage. The BRCA1-associated genome surveillance complex contains various proteins for DNA repair, including the Rad50 complex, cell cycle check point, and DNA repair factors (26). Because BRCA1-associated genome surveillance complex is derived from a single-step immunoprecipitation (IP) from unpurified nuclear extracts, it is unclear whether it represents multiple complexes or a single complex.

BRCA1 protein dynamically changes its subcellular localization, depending on the cell cycle or whether the genome has been damaged. In S phase, BRCA1 localizes to discrete nuclear foci (27), but treatment with hydroxyurea (HU), UV irradiation, or γ-irradiation leads to dispersal of these BRCA1 foci (28). After HU and UV treatment, BRCA1 colocalizes with BARD1 and RAD51 in proliferating cell nuclear antigen-containing replication structures (29). After HU treatment or irradiation, BRCA1 forms a complex with Rad50, Mre11, and Nbs1 in discrete nuclear foci (irradiation-induced foci) (19, 20). It is unknown whether these changes in subcellular position reflect changes in BRCA1 protein complexes.

In this study, we observed BRCA1 associated with three protein complexes in asynchronously cycling cells, and BRCA1 shifted to a fourth protein complex after cells were treated with HU. These data support a concept that the multiple processes with which BRCA1 is involved reflect multiple protein complexes with which it associates.

MATERIALS AND METHODS

Cell Culture and Biochemical Purification—HeLa-S3 cells and 293S cells were passaged in suspension culture using standard procedures. About 5 × 10⁶ cells were infected with recombinant adenovirus at a multiplicity of infection of about 1–2 plaque-forming units/cell, and cells were harvested 44 h after infection. The purification from whole-cell extracts by chromatography on a Biorex70 ion exchange matrix and sucrose gradient sedimentation have been described previously (22, 23).

Adenovirus Construction—HA epitope-tagged full-length and deleted mutant BRCA1 were inserted into AdEasy (Quantum Biotechnology, Massachusetts 02115). The abbreviations used are: Pol II, RNA polymerase II; holo-pol, RNA polymerase II holoenzyme; HU, hydroxyurea; HUIC, hydroxyurea-induced complex; IP, immunoprecipitation; HA, hemagglutinin.
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Inc.) shuttle vectors such that the BRCA1 gene would be under the control of the cytomegalovirus promoter. Full-length HA epitope-tagged BRCA1 (HA epitope tagged to the amino terminus, HA-BRCA1; HA epitope tagged to the carboxyl terminus, BRCA1-HA) was subcloned from constructs in the pC1DNA vector (27). The 775–1292 deletion was constructed by digestion of KpnI and NheI from HA-BRCA1 followed by ligated insertion of the following forward and reverse linkers: forward, 5′-TCGGTGGACGACAAAGAAGCAGGTAAGACGGTT-3′; and reverse, 5′-CTACGACCCTGCTCTGCTCTTCTTGGTCCCAGCCACG-AC-3′. This fragment was inserted into the AdEasy shuttle vector. With each shuttle vector construct, recombination occurred in bacteria to recover adenoviral genomic DNA with the BRCA1 gene, and virus was recovered after transfection into 293A cells.

**Immunoprecipitation—**400–500 µl of protein from sucrose gradient fraction or 150–180 µl of eluted protein from the Biorex70 column was immunoprecipitated with the specific antibody for the HA epitope (12CA5), Med17, or Mre11. 450–750 µl of binding reactions was incubated with rotation for 4 h at 4 °C in buffer H (20 mM Tris-OAc, pH 7.9, 1 mM EDTA, and 5% glycerol), 0.12 × KOAc, 0.1% Nonidet P-40, 0.1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, and 0.5 mM phenylmethylsulfonyl fluoride in the presence of protein extract, 3–5 µl of antibody, and 20 µl of protein A beads. When affinity-purified anti-Med17 antibody (30, 31) was used, these steps were performed with or without antigenic peptide (0.1 mg/ml). With all IPs, supernatant was removed, and protein beads were then washed three times using 450–800 µl of wash buffer (120 or 300 mM KOAc, 20 mM Tris-OAc, pH 7.9, 0.1% Nonidet P-40, 0.1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). For Western blot analysis, samples were subjected to electrophoresis in 5% or 6% SDS-polyacrylamide gels and immunoblotted using the indicated antibodies.

**RESULTS**

**Identification of the Hydroxyurea-induced Complex (HUIC).**—We have previously identified two separate protein complexes containing BRCA1 in HeLa cell extracts (22, 23). One complex was identified as the holo-pol, and the second complex was a more massive complex, which may have contained a nucleic acid component (23). A third BRCA1-containing complex has been described that contains Rad50-Mre11-Nbs1 along with BRCA1 (20) or contains other repair factors (26). Data have suggested that BRCA1 shifts its subnuclear localization and perhaps associates with new protein complexes after HU-mediated replication block or exposure to DNA-damaging agents in the S phase (26, 28). To induce a replication block in a given cell in the cell cycle, this incubation time was sufficient to cyclin E, suggesting that the HU-treated extract was indeed consistent with previously published observations (22, 23), the polo. Whereas the observations with the untreated extracts are consistent with those fractions containing the holo-pol (Fig. 1C). It is thus inferred that this third peak seen only after the addition of HU is not associated with the holo-pol. Whereas the observations with the untreated extracts are consistent with previously published observations (22, 29), the HUIC has not yet been described.

To further characterize the HUIC as well as other BRCA1-containing protein complexes, we established a system for overexpressing epitope-tagged BRCA1 in preparative extracts.

**Overexpressed BRCA1Copurifies with holo-pol.—**Recombinant adenoviruses, which express full-length BRCA1 with an amino-terminal HA tag, were used to infect ~5 × 10⁹ CV-1 cells in suspension culture. Whole-cell extracts were prepared by standard procedures (23) and chromatographed on a Biorex70 column. For Western blot analysis, samples were subjected to electrophoresis in 5% or 6% SDS-polyacrylamide gels and immunoblotted using the indicated antibodies.

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![FIG. 1. Identification of the HUIC. A, hydroxyurea blocks the cell cycle in S phase. Whole-cell extracts from asynchronous HeLa cell culture (lane 1) or from HeLa cell culture blocked in S phase by hydroxyurea (lane 2) were analyzed for the presence of the S-phase-specific cyclin A (top panel) or the G1-S-phase-specific cyclin E (bottom panel). The migration of the 66-kDa marker is indicated at the left. B, BRCA1 from hydroxyurea-treated HeLa cells copurifies with Pol II on Biorex70 matrix. Whole-cell extract from hydroxyurea-treated cells was chromatographed on Biorex70 and immunoblotted for BRCA1 and Pol II. Samples represent load (L), flow-through at 0.15 M KOAc (FT), protein peak from the 0.3 × KOAc wash (0.3), protein peak from the 0.6 × KOAc wash (0.6), and protein peak from the 1.5 × KOAc wash (1.5). C, identification of HUIC by sucrose gradient sedimentation. The protein peak eluted from Biorex70 at 0.6 × KOAc was subjected to sucrose gradient sedimentation. Immunoblots of these fractions were stained for BRCA1, Pol II large subunit, and cyclC. In fractions 17–21 of the HU-treated sample, a new peak was observed. Fractions on the right (high numbers) represent low molecular mass complexes. Approximate sedimentation coefficients were determined by analyzing eukaryotic and bacterial ribosomes on similar sucrose gradients.](image-url)
Characterization of the HUIC and Association with BRCA1—We applied the overexpression of tagged BRCA1 to the analysis of the HUIC. After infecting 293S cells with HA-tagged BRCA1, whole-cell extracts were chromatographed on Biorex70, and eluted proteins were analyzed by immunoblotting for the HA epitope and Pol II. C, the 0.6 m KOAc fractions were subjected to sucrose gradient sedimentation, and fractions were immunoblotted for the HA epitope and the Pol II large subunit.

To confirm that overexpressed HA-BRCA1 interacts with Pol II, sucrose gradient-purified holo-pol (fraction 14) was epitope-purified using the HA epitope-specific antibody (lane 4). The same fraction of an uninfected sample was used as a control (lane 3). In lanes 1 and 2, 10% of the respective input samples were analyzed.

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Overexpressed BRCA1 copurifies with the holo-pol. A, scheme for purification of HA-BRCA1 from adenovirus-infected cell extracts. B, after infecting 293S cells with HA-tagged BRCA1, whole-cell extracts were chromatographed on Biorex70, and eluted proteins were analyzed by immunoblotting for the HA epitope and Pol II. C, the 0.6 m KOAc fractions were subjected to sucrose gradient sedimentation, and fractions were immunoblotted for the HA epitope and the Pol II large subunit. D, HA-BRCA1 is associated with holo-pol. Sucrose gradient-purified holo-pol (fraction 14) was epitope-purified using the HA epitope-specific antibody (lane 4). The same fraction of an uninfected sample was used as a control (lane 3). In lanes 1 and 2, 10% of the respective input samples were analyzed.
consistent with the HUIC (Fig. 3B). This pattern of redistribution of BRCA1 content after HU treatment from complexes of higher mass to the HUIC, which was originally observed in HeLa cells, was repeated in 293S cells. We analyzed these fractions for BARD1 and for Rad50 because these proteins are known to interact with BRCA1. We found that the sedimentation of BARD1 did not change after HU treatment. BARD1 was present throughout the gradient, but with peaks consistent with the fraction 5 and the HUIC (Fig. 3B). Rad50 sedimented in fractions at the top of the gradient regardless of HU treatment (Fig. 3B), suggesting that either BARD1 or Rad50 may be associated with BRCA1 in the HUIC. By comparison, Pol II did not cosediment with the HUIC (Fig. 1; data not shown).

We tested whether the HUIC was a derivative of the holo-pol by immunoprecipitation using the holoenzyme-specific affinity-purified antibody directed against Med17 (30) (Fig. 4A). Fractions 5, 17, and 25 containing HA-BRCA1Δ775–1292 (from Fig. 3B) were immunoprecipitated with the Med17-specific antibody, and the blot was stained for the HA epitope (Fig. 4A). These fractions were chosen because they represent the peaks of the fraction 5, holo-pol, and HUIC complexes. When analyzing fractions 5, 17, and 25 containing HA-BRCA1Δ775–1292 with the holo-pol, significant purification of HA-BRCA1 by the Med17-specific antibody was observed (lanes 4–6), indicating association of the HA-BRCA1Δ775–1292 with the holo-pol. By contrast, the HA-BRCA1Δ775–1292 in fraction 25, the HUIC-containing fraction, was not at all associated with Med17 (lanes 7–9). The very faint band in lane 9 probably resulted from contamination of the HUIC by the holo-pol, which sedimented in adjacent fractions. This result suggests that the HUIC is a distinct complex from the holo-pol. The results of the IP from fraction 5 were weakly positive (lanes 1–3), suggesting that the protein complex in this sample may be derived from the holo-pol.

BRCA1 has been reported to interact with the Rad50-Mre11-Nbs1 repair complex and also with BARD1 (8, 20), and these proteins all copurify with the HUIC (Fig. 3B). We tested whether the HUIC is the same as the BRCA1-Rad50-Mre11-Nbs1 complex by immunopurification using Mre11-specific antiserum. Whereas the Mre11 antibody could purify Mre11 and Rad50, HA-BRCA1Δ775–1292 and BARD1 were not detected (Fig. 4B). Thus, the HUIC is not the same as the Rad50-containing complex. By contrast, we have identified a BRCA1-associated protein in the HUIC. Anti-HA epitope immunoprecipitation from the HUIC-containing samples revealed that BARD1 was present in this complex (Fig. 4C).

BRCA1–Rad50–Mre11–Nbs1 Complex—It has been reported that the Rad50-Mre11-Nbs1 complex associates with BRCA1 (20), but we did not observe this complex in HUIC fractions even though the proteins cosediment. A small pool of BRCA1 was observed in the 0.3 M KOAc elution of the Biores70 column (Fig. 2B), and most of the total Rad50 in the extract was in the 0.3 M KOAc elution (data not shown). The 0.3 M KOAc Biorex70 eluate was analyzed by sucrose gradient sedimentation (Fig. 5A). Samples expressing full-length HA-BRCA1 were subjected to Western blot analysis and stained for the HA epitope, Rad50, Mre11, and Nbs1. These patterns are similar to Rad50, Mre11, and Nbs1 sedimentation in fractions 15–23. HA-BRCA1 was present in these samples but also shifted to a higher molecular mass and fractionated in fractions 13–23. This shift in BRCA1 content was likely due to contamination by holo-pol (data not shown). Next, we tested whether BRCA1 is associated with Rad50 in this 0.3 M KOAc chromatographic fraction using antibody specific for Mre11 antibody. In this fraction, full-length HA-BRCA1 was associated with Mre11, as was Rad50 (Fig. 5B). Finally, we tested whether HU, which causes damaged DNA, would stimulate association of endogenous BRCA1 with the Rad50-Mre11-

FIG. 4. Characterization of the HUIC from HA-BRCA1Δ775–1292-expressing cells. A, the HUIC is not associated with Med17. Three sucrose gradient fractions, 5, 17, and 25, of HA-BRCA1Δ775–1292-expressing cells were immunoprecipitated by pol-holo-specific antibody directed against Med17. Samples are arranged in threes with 10% input (lanes 1, 4, and 7), Med17 IP with peptide block (–; lanes 2, 5, and 8), and Med17 IP without peptide block (–; lanes 3, 6, and 9). B, the HUIC does not contain the Rad50 complex. Immunoprecipitation from the HUIC-containing samples in HU-treated cells infected with HA-BRCA1Δ775–1292 using an antibody specific for Mre11 reveals purification of Rad50 and Mre11, but not of HA-BRCA1Δ775–1292 or BARD1. C, BARD1 is a component of the HUIC. The HUIC-containing samples in HU-treated cells infected with HA-BRCA1Δ775–1292 (lanes 3 and 4) or matched samples from uninfected HU-treated cells (lanes 1 and 2) were immunoprecipitated with anti-HA antibody (lanes 3 and 4) and analyzed by Western blot using antibody specific to BARD1 and HA epitope.

FIG. 5. Rad50 complex is associated with BRCA1 in the 0.3 M KOAc protein peak eluted from the Biorex70 matrix. A, BRCA1 in the 0.3 M KOAc Biorex70 protein peak copurifies with Rad50 complex on sucrose gradient sedimentation. The 0.3 M KOAc Biorex70 fraction from cells overexpressing HA-tagged full-length BRCA1 was subjected to sucrose gradient sedimentation, and fractions were immunoblotted for HA-epitope, Rad50, Mre11, and Nbs1. B, immunoprecipitation, using anti-Mre11 antibody, of the Biorex70 0.3 M eluate containing full-length HA-BRCA1. Immunoblot was stained using antibody specific for the HA epitope (top panel) and Rad50 (bottom panel). C, immunoprecipitation from the Biorex70 0.3 M eluate chromatography of uninfected samples using antibody specific for Mre11. The samples were derived from cells not treated with HU (lanes 1–3) or from HU-treated 293S cells (lanes 4–6). Immunoblots were stained using antibody specific for BRCA1, Rad50, Mre11, and Nbs1.
is also dynamically shifting from this complex to the HUIC. Current data have not yet determined whether the BRCA1-Rad50-Mre11-Nbs1 complex we have identified is the same as the BRCA1-associated genome surveillance complex.

BRCA1-BARD1 interactions are demonstrated in the most abundant BRCA1-containing complexes. As mentioned above, the redistribution of BRCA1 among protein complexes is consistent with changes noted in subnuclear localization (28, 29). We observed clearly that BRCA1 content shifted from the holo-pol complex and fraction 5 complex to the HUIC after HU treatment of cells. This shift was less apparent for the BRCA1-Rad50 complex. Is the HUIC a product derived from the holo-pol complex or from the fraction 5 complex? We found that the HUIC does not contain the holo-pol components Med17, Pol II, and BRG1 (data not shown), and in sucrose gradient sedimentation of the 0.6 M KOAc elution, BARD1 fractionated broadly throughout the gradient, including two peaks consistent with the fraction 5 complex and the HUIC. It is possible that HU treatment leads to degradation of holo-pol and the fraction 5 complexes, leaving a residual BRCA1-BARD1-containing complex in the HUIC. This hypothesis is consistent with a model in which transcription functions in surveillance for DNA damage by transcription-coupled repair and recombination (6). Upon encountering DNA damage, the BRCA1-BARD1 ubiquitin ligase activity destroys the holo-pol, leaving the HUIC at the site of damage, and the HUIC then recruits repair factors.

BRCA1 is a dynamic protein because it is present in untreated cycling cells in three complexes, but after replication blockage by HU treatment, the BRCA1 content shifts to a new protein complex, the HUIC. It is likely that BRCA1 protein participates in multiple cellular pathways by different functions in different complexes.

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