Research Article
BRCA1 Forms a Functional Complex with γ-H2AX as a Late Response to Genotoxic Stress

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1. Introduction
One of the first observable responses to DNA damage is activation of DNA-PK family kinases and resulting phosphorylation of the histone variant H2AX at serine 139 by the ATM/ATR family of kinases. The tumor suppressor BRCA1, also phosphorylated by ATM/ATR kinases, is one of several proteins that colocalize with phospho-H2AX (γ-H2AX) at sites of active DNA repair. Both the precise mechanism and the purpose of BRCA1 recruitment to sites of DNA damage are unknown. Here we show that BRCA1 and γ-H2AX form an acid-stable biochemical complex on chromatin after DNA damage. Maximal association of BRCA1 with γ-H2AX correlates with reduced global γ-H2AX levels on chromatin late in the repair process. Since BRCA1 is known to have E3 ubiquitin ligase activity in vitro, we examined H2AX for evidence of ubiquitination. We found that H2AX is ubiquitinated at lysines 119 and 119 in vivo and that blockage of 26S proteasome function stabilizes γ-H2AX within cells. When BRCA1 levels were reduced, ubiquitination of H2AX was also reduced, and the cells retained higher levels of phosphorylated H2AX. These results indicate that BRCA1 is recruited into stable complexes with γ-H2AX and that the complex is involved in attenuation of the γ-H2AX repair signal after DNA damage.
and other types of genotoxic stress [18, 19]. Although the role of BRCA1 in DNA repair is not known, the N-terminal ring finger of BRCA1 interacts with the ring finger of BARD1 [20], and the complex has been shown to possess E3 ubiquitin ligase activity in vitro [21]. The E2 ubiquitin-conjugating enzyme (UbcH5c) has been shown to associate with this complex [21, 22], and several in vitro substrates have been identified, including monoubiquitinated histones H2AX, H2A, H2B, H3, and H4 (but not H1) [22]. So far, in vivo targets of the complex have been less clearly defined.

BRCA1 association with chromatin is properly considered an intermediate or late event in chromatin repair [1, 23]. Here we demonstrate by differential fractionation of chromatin bound BRCA1 complexes that BRCA1 and γ-H2AX form a biochemical complex in the chromatin fraction of cells as a late event following DNA damage. The complex is resistant to nonionic detergent extraction and is dependent on wortmannin-sensitive kinases, features that are distinct from BRCA1 prior to genomic stress. We show that a phosphomimetic of H2AX (H2AX-E139) is ubiquitinated in vivo, and the major site of ubiquitination is on K118 and/or K119. When BRCA1 levels were reduced using an antisense morpholino knockdown strategy, we observed substantially reduction in H2AX ubiquitination and increased amounts of H2AX S139 phosphorylation. These results are consistent with the hypothesis that BRCA1 is present in non-chromatin-associated complexes (including processive RNA polymerase II) prior to genotoxic stress; it becomes phosphorylated, moves into a stable chromatin-associated complex containing γ-H2AX, and then targets γ-H2AX for turnover as a late phase of DNA repair.

2. Materials and Methods

2.1. Plasmids. H2AX, H2AX-A139, and H2AX-E139, were generated by RT-PCR from human RNA using primers, described in the supplemental data (see Table 1 in Supplementary Material available online at doi: 10.4061/2011/801594), and cloned into pcDNA3.1D-V5-H6 (Invitrogen) to generate epitope-tagged variants. PCR-mediated mutagenesis was performed [24] using primers described in the supplemental data to generate additional mutations. pMT-HA-Ub (hemagglutinin tagged ubiquitin) was the generous gift of Dr. Dirk Bohmann (University of Rochester).

2.2. Cell Culture. 293T, MCF-7, and HBL100 were maintained as described in [25].

2.3. DNA Damage and Wortmannin. Damage—randomly cycling HBL100 cells were treated with 4 μM adriamycin or were exposed to 10 Gy ionizing radiation using a 137Cs source (Mark 1 irradiator, Shepherd and Associates). Following treatments, cells were returned to a 5% CO2 incubator for the indicated amount of time. Cells treated with wortmannin were first pretreated with 100 μM wortmannin for 15 minutes [3].

2.4. Immunoprecipitations and Immunoblotting. Nuclei were prepared by extracting cells in EBC buffer (50 mM Tris pH 8.0, 120 mM NaCl, and 0.5% NP-40) as described in [25]. Nuclei were then treated with 0.1 M HCl for 30 minutes and neutralized with 0.1 M NaOH. This acid soluble “chromatin fraction” was centrifuged for 5 minutes at 14,000 RPM to remove insoluble material. Lysates were precleared with protein A or G beads and then incubated overnight with primary antibodies to γ-H2AX (Upstate Biotechnology) or BRCA1 (Ab-4, EMD Biosciences). Extracts were run on a 5% or 14% SDS-PAGE gel. Proteins were then transferred to nitrocellulose membranes, blocked in 5% nonfat dry milk, and incubated with a primary antibody generated against BRCA1 (Ab-4, EMD Biosciences), γ-H2AX (Upstate Biotechnology), H2A (H-124, Santa Cruz Biotechnology), RNA polymerase II (N-20, Santa Cruz Biotechnology or 8WG16 (provided by Dr. Michael Carey (UCLA)), FCP1 (provided by Dr. Michael E. Dahmus (UC Davis)), or β-actin (Sigma), followed by a goat antirabbit (or anti-mouse) horseradish peroxidase-conjugated secondary antibody (Pierce). Blots were developed with a chemiluminescence detection substrate (SuperSignal, Pierce).

2.5. Immunocytochemistry. Cells were fixed in 4% paraformaldehyde (PFA) for 30 minutes on ice. γ-H2AX was detected with an antibody from Upstate Biotechnology. Alexa-488-conjugated secondary antibodies (Molecular Probes) were used to visualize immune complexes, and photomicrographs were prepared as described in [25]. Quantitation of fluorescent γ-H2AX foci was accomplished using a Laser Scanning Cytometer (LSC, CompuCyte). Potential autofluorescence was carefully gated by tuning the lasers to optimize signal.

2.6. Proteasome and Phosphatase Inhibitors. Human MCF-7 or 293T cells were treated with 5 μM adriamycin for 1 hour. Following adriamycin treatment, the cells were washed then treated with or without lactacystin (Sigma) or a serine/threonine phosphatase inhibitor cocktail [(−)β-Bromotetramisole Oxalate, Cantharidin and Microcystin-LR] (EMD Biosciences) for 4 hours, after which the cells were fixed in 4% PFA. γ-H2AX was immunostained as described above. Quantitation of fluorescent γ-H2AX foci was accomplished using a Laser Scanning Cytometer (LSC, CompuCyte).

2.7. Antisense Morpholino Oligos. Two antisense morpholino oligos (Gene Tools, Inc.) were designed against BRCA1 (AS1 5′-GGCGAAGAGCAGATGATCATTCTTCT-3′ and AS2 5′-TGTGTGACTTACAGATGGGACAC-3′). 20 μL of each of the two 500 μM morpholino antisense oligos were delivered into the cells using the EPEI delivery solution according to the manufacturer’s protocol. The remaining cells were treated with a serum-free media control, EPEI delivery solution (Gene Tools, Inc.), or 40 μL of a 500 μM scrambled control oligo (5′-CCTTCTACACGTGTTCAATTATA-3′). After a two-hour treatment, the media were replaced with serum-containing media. The cells were allowed to
3. Results

3.1. BRCA1 Interacts with γ-H2AX. BRCA1 and γ-H2AX form a physical complex on chromatin following DNA damage. Before DNA damage, small amounts of BRCA1 are present in the chromatin fraction, consistent with the percentage of S-phase nuclei in this population of cells [4, 7, 26]. After treatment with the DNA-damaging agent adriamycin, there is a substantial increase in acid-stable nuclear BRCA1:γ-H2AX complexes (Figure 1(a)). Stable interaction is minimal at early times following DNA damage then increases to the maximum at about 60 minutes. The recover for 24 hours at which time they were lysed with EBC buffer. Data related to dose and effectiveness are provided (see supplementary data).
association is blocked by the ATM/ATR inhibitor wortmannin (Figure 1(b)). This data supports the hypothesis that interaction of BRCA1 and H2AX occurs well after DNA damage, requires phosphorylation by ATM or ATR [3, 27], and provides evidence that the interaction requires the formation of a stable complex.

3.2. Time Course of the BRCA1 Complex Formation. In order to analyze the kinetics of BRCA1 complexes present in undamaged cells and in cells responding to DNA damage. In undamaged cells, we had previously demonstrated that BRCA1 interacts with the phosphorylated, transcriptionally active form of RNA polymerase II (RNA pol II) [25]. Following γ-irradiation, we found that the BRCA1:γ-H2AX complex was enhanced (Figure 1(a)) whereas the BRCA1:RNA pol II interaction is disrupted (Figure 1(c)). This shift accompanies the formation of a stable complex.

Further analysis of the timing of the BRCA1:γ-H2AX complex shows that this complex coincides with a decreased level of cellular γ-H2AX. When the BRCA1:γ-H2AX complex is at its highest level (about 60 minutes after DNA damage), the level of γ-H2AX has begun to decrease, as demonstrated by immunoblot of the total chromatin extract (Figure 1(e)).

3.3. H2AX Is Ubiquitinated In Vivo. Because BRCA1 is an E3 ubiquitin ligase, we hypothesized that the reduction of γ-H2AX seen following association with BRCA1 could be the result of ubiquitin-mediated proteasomal degradation. If this were true, then proteasome inhibitors should result in stabilized levels of γ-H2AX. BRCA1 has been shown to ubiquitinate H2AX in vitro [22]; therefore, we wanted to determine if γ-H2AX turnover was proteasome mediated. To test whether γ-H2AX was degraded by the 26S proteasome, MCF-7 mammary epithelial cells were treated with the DNA-damaging agent adriamycin then treated with or without the proteasome inhibitor lactacystin for an additional hour before fixation. Cells were fixed and immunostained to identify γ-H2AX high and γ-H2AX low cells. As shown in Figure 2(a), lactacystin increased the percentage of γ-H2AX high cells in the population of cells recovering from adriamycin treatment.

By this γ-H2AX immunofluorescence assay, 20% of untreated cells were found to be positive for high levels of γ-H2AX (Figure 2(b)). When cells were treated with adriamycin for 1 hour followed by 1 hour recovery, γ-H2AX immunofluorescence showed a 2-fold increase over control cells, consistent with the view that H2AX was dynamically phosphorylated in response to DNA damage. The proteasome inhibitor lactacystin had a similar effect as adriamycin, when added alone, consistent with the hypothesis that γ-H2AX formation in normal cycling cells could be stabilized by proteasome inhibitors. When adriamycin-induced damage was followed by lactacystin treatment, there was a fourfold increase in the amount of γ-H2AX over control, resulting in over 80% of cells containing high levels of γ-H2AX by two hours. Addition of a phosphatase inhibitor cocktail also stabilized γ-H2AX level, suggesting that there may be additional ways to attenuate H2AX phosphorylation in cells. However, the phosphatase inhibitors alone did not cause stabilization or activation of γ-H2AX, indicating that proteasome-mediated turnover was a general mechanism to be addressed in further detail. These results provide evidence that γ-H2AX is degraded by the proteasome and potentially by other mechanisms requiring dephosphorylation of one or more components after DNA damage.

3.4. H2AX Is Ubiquitinated at Lysine 118 and/or 119. Previous reports have shown that histone H2A is polyubiquitinated on K119, and H2B had shown evidence of ubiquitination on K120 [28]. To examine the role of ubiquitin modifications in γ-H2AX turnover we created a series of modifications intended to model phosphorylation at S139 and the roles of various lysine (K) residues in the C terminus (Figure 3(a) and supplementary Figure 1). H2AX-V5-H6 was first mutagenized to replace S139 with alanine (A) or glutamic acid (E), to mimic the negative charge of phosphorylation at S139. As predicted, H2AX-S139-V5-H6 localized to chromatin but was moderately less stable than the wild-type H2AX-V5-H6 or A139 mutation products (not shown). Cotransfection of H2AX-S139-V5-H6 with a cytosolic form of BRCA1 lacking a nuclear localization sequence (BRCA1-ΔN11-GFP) resulted in nuclear localization of both proteins and rapid turnover (not shown). For these reasons, we hypothesized that H2AX-S139 was mimicking S139 phosphorylation and recruiting binding of BRCA1.

We then replaced each lysine (K) residue found in the conserved C-terminus of H2AX-E139 with arginine (R) and tested for ubiquitination in vivo (Figure 3). K118 and K119 (which align with K119 and K120 of H2A) were mutated individually (Figure 3(c), lanes 6 and 7) and in combination (lane 8). K133 and K134 were also mutated individually (Figure 3(c), lanes 9 and 10) and in combination (lane 11) to arginine. Mutation of K132 to R132 was also prepared as part of this series but had no effect (data not shown). 293T cells were transfected with wild-type or mutant H2AX-pcDNA3-H2AX-V5-H6 in addition to HA-tagged ubiquitin. H6-tagged complexes were purified using nickel-chelated beads, and bound proteins were analyzed by immunoblotting for the presence of HA-tagged H2AX (Ub-H2AX). As shown, V5 immunoblotting identified modified and ubiquitinated H2AX only when extracts from cotransfected cells were purified and blotted (Figure 3(b)). Reprobing these blots with anti-HA antisera revealed that the upper band contained ubiquitin (Figure 3(b)). While the
major band migrated in a fashion consistent with that of monoubiquitinated H2AX, there were additional bands of immunoreactivity at higher molecular weights that suggested polyubiquitination as well (Figure 3(b) arrowheads). H2AX mutated at both K$_{118}$ and K$_{119}$ showed no ubiquitination (lane 8), indicating that H2AX is ubiquitinated at either K$_{118}$ or K$_{119}$, whereas other point mutations showed levels of ubiquitination similar to wild type. These results show that H2AX-E$_{139}$ is ubiquitinated at either K$_{118}$ or K$_{119}$.

3.5. BRCA1 Knockdown Increases Steady State Levels of γ-H2AX Buy Reduction of Ubiquitination in Cells Undergoing Replicative Stress. To functionally examine the interaction between BRCA1 and γ-H2AX, we used antisense morpholino oligonucleotides to knockdown BRCA1 expression. Treatment of cells with BRCA1 antisense oligonucleotides reduced the amount of BRCA1 to under 3% of normal levels (Figure 4(a), and supplemental data). Reduction of BRCA1 protein resulted in increased levels of γ-H2AX expression by both immunofluorescence (Figure 4(a)) and immunoblotting (Figure 4(b)).

Cells treated with antisense oligos directed at BRCA1 mRNA were transfected with H2AX-V5-H6 and HA-tagged ubiquitin. The epitope-tagged H2AX product was then isolated using nickel-chelated beads and analyzed by immunoblotting for the presence of HA-tagged ubiquitin. Cells treated with BRCA1 antisense oligos had a reduction in the global amount of H2AX ubiquitination detected (Figure 4(c)). Efforts to examine the role of double-strand break repair on this process are underway but are complicated by the extreme sensitivity of BRCA1 deficient cells to genotoxic agents.

It has been shown previously that BRCA1 deficiency is associated with G2/M checkpoint and other defects [29–32]. To examine the relationship between BRCA1 and genotoxic stress more carefully, we examined cells treated with BRCA1
fluorescence, had normal γ-H2AX staining patterns. (c) H2AX variants were transfected with HA-tagged ubiquitin. Arrows indicate unmodified and ubiquitinated H2AX.

**Figure 3**: H2AX-E139 is ubiquitinated at K118 or K119. (a) Critical amino acids within the C-terminus are shown. (b) HA-tagged ubiquitin (HA-Ub) was transfected into cells with or without H2AX-V5-H6. His-tagged (H6) proteins were purified from chromatin fractions then probed sequentially for the presence of HA-ubiquitin and V5 epitopes (WB). 10% of the unfractionated extract was blotted directly and probed for β-actin. Histidine-tagged proteins were purified from chromatin fractions then probed sequentially for the presence of HA-ubiquitin and V5 epitopes. 10% of the unfractionated extract was blotted directly and probed for γ-H2AX. These results suggest that BRCA1 deficiency is associated with defective clearance of γ-H2AX from cells following replication and other types of genotoxic stress (Figure 5). We propose that BRCA1 interacts with processive RNA pol II in undamaged cells as part of a role in genomic surveillance (Figure 5(a)) [25, 33]. Following genotoxic stress, phosphorylation of BRCA1 by ATM/ATR and potentially by chk1 results in its dissociation from stalled RNA pol II complexes. We propose that an early repair complex forms on DNA as a consequence of ATM/ATR phosphorylation. Early targets for ATM/ATR include phosphorylation of H2AX to form γ-H2AX [34, 35]. γ-H2AX then serves as a template to aid in the recruitment of early and late components of the repair machinery, including 53BP1 (Figure 5(b)) [36]. We propose that BRCA1 is recruited at later times, potentially after break repair has been affected. One target for BRCA1 recruitment is γ-H2AX, which is directly or indirectly ubiquitinated on K118 or K119 and degraded through the actions of the 26S proteasome (Figure 5(c)).

We have shown that BRCA1 is in a biochemical complex with γ-H2AX after DNA damage. This interaction is dependent on the DNA-PK family of kinases (ATM and/or ATR), as the interaction is disrupted by the inhibitor wortmannin. This data agrees with previous data that BRCA1 colocalizes with repair proteins and suggests a function for BRCA1 in the chromatin fraction of nuclei. BRCA1 becomes part of the BRCA1-associated surveillance complex (BASC), which includes many proteins involved in DNA repair, including MSH2, MSH6, MLH1, ATM, BLM, and the RAD50-MRE11-NBS1 protein complex [37]. BRCA1 and the BASC complex are localized at the site of DNA damage (nuclear foci). The repair factors are thus at the site of damage where they can perform their particular enzymatic activities and repair the DNA.

Using a mutant from of H2AX (H2AX-E139) designed to mimic phosphorylation at S139 in γ-H2AX, we performed mutagenesis of several conserved lysine residues in the C-terminal end of H2AX. Results from these experiments suggest that ubiquitination is suppressed following mutation of both K118 and K119, but not by mutation of other lysines either alone or in combination. Previously reported also showed that BRCA1 was capable of supporting ubiquitination of H2AX, but those studies were carried out in cell-free reactions in vitro [22]. Ubiquitination of K118 and K119 agrees with tryptic peptide data [38] that showed ubiquitination between residues 118 and 127.

We propose that a major function of BRCA1 is to decrease the levels of γ-H2AX in cells as a mechanism for signaling an end to early events in DNA repair. BRCA1 activity is then critical for timely attenuation of active repair phase. Defective production of BRCA1 in tumor cells would be expected to result in less ordered dimunition of the repair signal and lead to problems in progression though G2/M phases of the cell cycle.

**4. Discussion**

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Figure 4: BRCA1 knockdown stabilizes γ-H2AX and reduces ubiquitination of γ-H2AX. (a) Cells treated with different amounts of antisense morpholino oligos (AS1 and AS2) or a scrambled antisense morpholino (scrAS) were stained with DAPI or immunostained for γ-H2AX. A second set of morpholino-treated cells was immunostained for BRCA1 to show dose-dependent knockdown of BRCA1 protein. (b) Cells treated with scrambled antisense morpholino (lane 1, scrAS) or increasing amounts of antisense morpholino oligos (AS1/2, lanes 2 and 3) were lysed and separated by SDS-PAGE and probed (WB) for BRCA1, γ-H2AX, β-actin, or β-tubulin. (c) 293T cells were treated with control (scrambled antisense (scrAS) or BRCA1 antisense morpholino oligos (AS1/2) and then transfected with control vector, HA-ubiquitin alone, and/or H2AX-E139-V5-H6. Histidine- (H6-) tagged H2AX was purified from chromatin fractions then probed for HA-ubiquitin. The blot was stripped and reprobed for V5-tag on H2AX. (d) Cells treated with a scrambled antisense morpholino (scrAS) or anti-BRCA1 morpholino oligos (AS1/2) were stained with DAPI (i–iv) or immunostained for γ-H2AX (i’–iv’). Micrographs were captured with either 10x (i–iii) or 60x (iv) objectives. Final magnification was 100x or 600x.

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Figure 5: Model of BRCA1 interactions with RNA polymerase II and γ-H2AX in distinct biochemical complexes. See text for details.

References


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