Intra-nuclear trafficking of the BLM helicase to DNA damage-induced foci is regulated by SUMO modification

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The Bloom syndrome gene, BLM, encodes a RecQ DNA helicase that when absent from the cell results in genomic instability and cancer predisposition. We show here that BLM is a substrate for small ubiquitin-like modifier (SUMO) modification, with lysines at K317, K331, K334 and K347 being preferred sites of modification. Unlike normal BLM, a double mutant BLM protein with lysine to arginine substitutions at residues 317 and 331 was not modified by SUMO, and it failed to localize efficiently to the PML nuclear bodies. Rather, double mutant BLM protein induced the formation of DNA damage-induced foci (DDI) that contained BRCA1 protein and phosphorylated histone H2AX. Double mutant BLM only partially complemented the genomic instability phenotypes of Bloom syndrome cells as assessed by sister-chromatid exchange and micronuclei formation assays. These results constitute evidence that BLM is a DNA damage sensor that signals the formation of DDI, and they establish SUMO modification as a negative regulator of BLM’s signaling function.

INTRODUCTION

The RecQ family of DNA helicases functions in DNA recombination and maintenance of genomic integrity (1). RecQ family members include the single-copy genes in Escherichia coli recQ, Saccharomyces cerevisiae SGS1 and Schizosaccharomyces pombe rgh1+ and in higher eukaryotes a five-gene family that consists of RECQL, BLM, WRN, RTS and RECQL5 (2). As DNA helicases, RecQ proteins possess DNA-dependent ATPase and ATP-dependent DNA unwinding activities with a 3’ to 5’ directionality (3,4), and they exhibit a marked preference for DNA substrates that resemble recombinational intermediates, including G4 tetraplex DNAs (5,6), Holliday junctions (7–9) and D-loop structures (10).

The BLM gene is mutated in the rare autosomal recessive disorder Bloom syndrome (BS). BS is characterized by proportional growth deficiency, a sun-sensitive facial erythema and predisposition to the development of cancers of many kinds (11). BS cells feature a striking genomic instability, which includes excessive chromosomal breakage, hyper-recombination and an elevated rate of locus-specific mutations. BS cells are also hypersensitive to genotoxic agents, especially when these agents are administered to cells synchronized in S phase (12). The hyper-recombination of BS cells is characterized by an increased frequency of exchanges between homologous chromosomes and high sister-chromatid exchanges (SCEs). The increased rate of somatic mutations in concert with the increased rate of recombination between homologs in BS cells probably explains the high incidence of cancers in persons with the syndrome.

BLM is a nuclear protein the levels of which are regulated during the cell cycle, being lowest in early G1 and highest in late S phase (13,14). In unsynchronized, untreated cells, BLM is distributed throughout the nucleoplasm in fine granules and in focal concentrations known as the promyeloctytic leukemia (PML) nuclear bodies (PML-NBs) (15,16). The PML-NBs number from 10 to 30 per nucleus, and they range in size from 0.2 to 1 μm (17–19). Besides BLM, the PML-NBs

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contain other proteins important in the response to DNA damage, including topoisomerase IIIα, p53 and the Rad50 complex (RAD50/MRE11/NBS1) (20–22).

In cells treated with DNA damaging agents, including γ-irradiation, DNA crosslinking agents and ultraviolet irradiation, BLM migrates out of the PML-NBs and into foci that contain proteins that assist in DNA repair (12,23). Foci can also be induced by treatment of cells with inhibitors of DNA replication such as hydroxyurea, which stalls DNA polymerase on the template by limiting the polymerase for nucleotides. These foci contain proteins such as phosphorylated histone H2AX (γH2AX), the RAD50 complex, RAD51, FANCD2, BRCA1 and BLM (12,23–27). Because the foci that form by these different treatments have many factors in common, we will hereafter refer to these foci as DNA damage-induced (DDI) foci.

The mechanisms that cause the DDI foci to form are not well understood (28); however, several lines of evidence indicate that BLM may have important roles in regulating their assembly. Mono-ubiquitylation of the Fanconi anemia (FA) protein FANCD2 (Ub-FANCD2) and migration of Ub-FANCD2 to the DDI foci is one of the earliest events to occur in response to treatment with genotoxic agents (26,29). Factors that regulate FANCD2 ubiquitylation include a complex of FA proteins that also contains BLM (30,31). In addition, BLM preferentially associates with Ub-FANCD2 (32). Cells deficient in one or another FA protein exhibit many of the cytogenetic defects of BS cells when treated with crosslinking agents, including increased DNA breaks and an increased frequency of homologous exchanges (33), further implying a functional relationship between BLM and FA proteins. Phosphorylation of histone H2AX is another early event; its phosphorylation occurs in the chromatin adjacent to the site of DNA damage (34). BLM rapidly associates with γH2AX in DDI foci in response to DNA damage (12,35). Intriguingly, mouse H2AX knock-outs have similar defects in recombinational repair of double-strand breaks and elevated SCEs as seen in BS cells (36). In addition, BLM is phosphorylated by the ATR and ATM kinases, which are activated in response to DNA damage (37,38). Interaction between BLM and ATR is associated with movement to DDI foci and recruitment of the p53 binding protein 53BP1 to the DDI foci (22,39). Thus, BLM is one of the earliest components to associate with DDI foci, and it is intimately associated with the major factors known to be involved in DDI focus formation.

BLM also associates directly with other factors that accumulate in DDI foci, including a complex of proteins containing BRCA1, the RAD50 complex and p53. Transit of BRCA1 and the RAD50 complex to the DDI foci is partially dependent on BLM, because the recruitment of these proteins occurs with slower kinetics in BLM-deficient BS cells (12,35). BLM also recruits p53 to the DDI foci (23). If p53 is degraded in BS cells with HPV E6 protein, the BRCA1 and RAD50 complex localization defect is alleviated (12). These data show that BLM also plays an important role in the maturation of DDI foci.

Given its potentially important role in DDI foci formation and maturation, we have investigated the mechanisms that regulate the localization of BLM to PML-NBs in undamaged cells and the mechanisms that regulate its trafficking between these bodies and DDI foci in response to DNA damage. Previous studies have demonstrated that the small ubiquitin-like modifier (SUMO) regulates the localization of PML to PML-NBs (40,41). There are three human SUMO genes that encode SUMO-1, SUMO-2 and SUMO-3. These proteins have ~20% identity to ubiquitin (42). Although the levels of SUMO-modified PML are relatively low (10%), experiments with mouse PML knock-out cells have shown that SUMO modification of PML is essential for the formation of the PML-NBs (40,43). In addition, several viral immediate-early proteins (e.g. ICP0 and Vmw110) induce the loss of SUMO-modified forms of PML, which disrupts the PML-NBs (44–46). Conversely, treating cells with arsenate induces hyper-modification of PML, which enlarges the PML-NBs (47). Other protein components of the PML-NBs also are substrates for SUMO modification (e.g. Sp100, Daxx and p53), suggesting that the SUMO pathway plays an integral role in the dynamics and functions of these nuclear structures, including pathways important in the control of genome integrity (48).

Sumoylation occurs by a process that is similar to ubiquitylation. A SUMO protease cleaves the C-terminal end to expose diglycine residues, and E1 protein loads SUMO onto the E2 conjugating enzyme UBC9, which in turn transfers SUMO to its substrates (42). The modification occurs by an isopeptide bond formed between the C-terminal carboxyl group of SUMO and the ε-amino group of lysines in SUMO substrates. For some substrates, sumoylation by UBC9 is stimulated by interaction with SUMO ligases (E3s, PIAS proteins) (49). Desumoylation is catalyzed by SUMO proteases (e.g. SENP1, SuPr-1, etc.). The dynamic nature of SUMO modification and demodification may explain why the ratio of modified to unmodified proteins is usually very low.

In the present report, we show that BLM is a substrate for SUMO and that SUMO modification of BLM regulates its intra-nuclear trafficking. BLM proteins with mutations of its SUMO modification sites not only failed to localize to the PML-NBs but also induced the formation of DDI foci in the absence of treatment with DNA damaging agents. The mutations also impaired BLM’s function in the maintenance of genomic integrity. These results constitute evidence that BLM is a DNA damage sensor that signals the formation of DDI foci, and they establish SUMO modification as a negative regulator of BLM’s signaling function.

RESULTS

Amino acids 133–458 mediate BLM’s localization to the PML-NBs

To identify the region of BLM that mediates its localization to the PML-NBs, we transiently expressed a series of expression constructs for full-length and deleted BLM proteins with green fluorescent protein (GFP) fused to its N-terminus. After 24 h, the cells were fixed, stained with anti-PML and examined by fluorescence microscopy. As previously shown (21), full-length GFP–BLM was present in the nucleoplasm, and in 60% of transfected cells, it was also detected in nuclear foci, >90% of which co-localized with PML (Fig. 1). The fragment 1–458/NLS with a C-terminal deletion of amino acids 459–1417 of BLM, excluding the C-terminal NLS that is essential for nuclear localization, localized to the PML-NBs, whereas a
fragment with an N-terminal deletion of amino acids 1–487 (Δ1–487) failed to localize there. Deletion of amino acids 336–458 (1–335/NLS) from the 1–458/NLS construct severely diminished BLM’s ability to localize to the PML-NBs, whereas deletion of amino acids 1–132 (133–458/NLS) had no effect. Thus, the region 133–458 is necessary and sufficient for BLM’s localization to the PML-NBs. Because a deletion of amino acids 212–237 within this region disrupted BLM’s localization to the PML-NBs (Fig. 1), the results suggested that binding of a factor to this region was required for targeting BLM to the PML-NBs.

**BLM is a SUMO substrate**

To understand the role of the N-terminus of BLM in PML-NB localization, we performed a yeast two-hybrid screen with the N-terminus to identify protein partners that interact with this region. We identified the three BLM-interacting proteins UBC9, SUMO-1 and SUMO-2 that are part of the sumoylation pathway (Table 1). Interaction between BLM and UBC9 was previously observed in a yeast two-hybrid assay (50). These data suggested that BLM is a substrate for SUMO modification.

To test the interaction between BLM and UBC9, we expressed by transient transfection GFP–UBC9 or GFP in SV40-transformed normal fibroblasts (GM00637) or SV40-transformed BS fibroblasts (GM08505) and performed immunoprecipitations with anti-BLM. GFP–UBC9 could be immunoprecipitated with anti-BLM from normal cells, which contain BLM, but neither from control BLM-deficient BS cells nor from control GFP-transfected normal cells (Fig. 2A). We concluded that BLM interacts with UBC9 in vivo.

**Figure 1.** BLM amino acids 133–458 mediate localization to the PML-NBs. Immunofluorescence analysis of BLM localization was performed with HeLa cells that transiently expressed GFP–BLM and deletion derivatives of GFP–BLM. (A) Schematic representation of GFP–BLM, the deletion derivatives tested, and a summary of the observed results. The clone names on left indicate the amino acid residues that are present in the construct or, when preceded by the Greek letter Δ, the residues deleted from the full-length GFP–BLM. With normal GFP–BLM, all the cells that expressed GFP–BLM exhibited diffuse nucleoplasmic protein and 60% of the cells exhibited green fluorescent foci. There was an average of ~10 PML-NBs/cell as determined by indirect immunofluorescence with anti-PML antibodies. The localization of GFP–BLM foci in the PML-NBs was >90%. For the constructs shown, the GFP and PML foci were counted in a total 415 cells, constituting an average of 69 cells examined for each construct (range 23–144). A plus sign indicates that the distribution of GFP in the nucleus was similar to that found for normal GFP–BLM. A minus sign indicates that no GFP foci were detected. An intermediate pattern of localization (+/-) was detected with the GFP–BLM construct 1–335/NLS, as determined by the examination of 144 cells in which a total of 21 GFP foci were detected in 12 cells. These few GFP foci co-localized with PML. GFP, green fluorescent protein (green box); acidic stretches, short runs of acidic amino acids (yellow boxes)—the region 212–237 is a strongly acidic region; helicase domain (red box); C-ter, the C-terminal extended homology region (purple box); HRDC, helicase RNAseD C-terminal domain (blue box); NLS, nuclear localization signal (black box). (B) Representative cells from the transient transfection experiments.
Table 1. BLM interacting proteins identified in a yeast two-hybrid screen

<table>
<thead>
<tr>
<th>Candidates (GenBank accession no.)</th>
<th>Incidence</th>
<th>β-galactosidase units</th>
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<tr>
<td>UBC9 (U29092)</td>
<td>22</td>
<td>0.20+/− 0.048</td>
</tr>
<tr>
<td>Synapsin Ia homologb</td>
<td>11</td>
<td>Not done</td>
</tr>
<tr>
<td>SUMO-1 (U83117)</td>
<td>7</td>
<td>0.25+/− 0.086</td>
</tr>
<tr>
<td>SUMO-2 (X99585)</td>
<td>4</td>
<td>5.93+/− 0.74</td>
</tr>
<tr>
<td>MSPE58 (AF015308)</td>
<td>3</td>
<td>0.92+/− 0.36</td>
</tr>
<tr>
<td>p58 (D67052)</td>
<td>2</td>
<td>5.21+/− 0.28</td>
</tr>
<tr>
<td>SR-like protein (U49056)</td>
<td>2</td>
<td>Not done</td>
</tr>
</tbody>
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The N-terminal 520 amino acids of BLM were fused to the GAL4 DNA binding domain, and this construct was used as bait to isolate clones from a HeLa GAL4 transactivation-domain fusion library. Approximately \(8\times10^6\) cDNAs were screened and 101 positive clones were isolated. Shown are those proteins that were identified two or more times in the screen, with the exception of ribosomal proteins and repeat sequence DNA positive clones, which were excluded.

To obtain \textit{in vivo} evidence that BLM is a SUMO substrate, we immunoprecipitated BLM from HeLa cells and performed western blot analysis of whole cell lysates and immune complexes with antibodies raised against BLM, SUMO-1 and SUMO-2 (Fig. 2B). With anti-BLM, a major band was detected with an apparent molecular weight of \(\sim 180\text{ kDa}\) that constituted unmodified BLM, and a smear of minor bands was detected \(20–40\text{ kDa}\) larger than the major BLM band. With anti-SUMO-1 and anti-SUMO-2 antibodies, a similar smear of bands was detected with mobilities indistinguishable from those detected with anti-BLM. In addition, higher molecular weight SUMO-BLM species were detected with SUMO-2 antibodies. The levels of SUMO-2-modified BLM were higher than the levels of SUMO-1-modified BLM. These data indicated that BLM is a SUMO substrate; however, the steady-state levels of sumoylated BLM were low (<5%), which indicated that either sumoylation is a transient event or only a specific subpopulation of BLM is modified by SUMO.

To further characterize the SUMO modification of BLM, we transcribed and translated the full-length protein in rabbit reticulocyte lysates in the presence of \(^{35}\text{S}\)methionine and assayed for its ability to be modified by SUMO-1 or SUMO-2 using an \textit{in vitro} modification system (Fig. 2C). All modification reactions contained purified recombinant SUMO E1 activating and E2 conjugating enzymes and either no SUMO or purified recombinant SUMO-1 or SUMO-2. \textit{In vitro} transcription and translation of full-length BLM produced protein fragments of the expected molecular mass of \(180\text{ kDa}\) and lower due to premature termination of translation. In reactions containing no SUMO, only unmodified BLM was detected. When incubated in the presence of SUMO-1 or SUMO-2, unmodified BLM disappeared completely, and a smear of high molecular weight protein was detected at the top of the gel. Consistent with the \textit{in vivo} data, SUMO-2 more readily modified BLM than SUMO-1. MSH2 (an unrelated mismatch repair protein) was not modified by either SUMO-1 or SUMO-2 using identical conditions, demonstrating the specificity of these reactions. These results demonstrated that BLM can be modified by both SUMO-1 and SUMO-2 and they suggested that modification may occur at multiple different lysines.

The interaction between BLM and SUMO in the yeast two-hybrid assay was detected with the N-terminal 520 amino acids of BLM. Consequently, we tested a purified fragment of recombinant BLM protein consisting of amino acids 1–431 (51) as substrate in the SUMO modification assay. By sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), the unmodified form of BLM 1–431 migrated with an apparent molecular weight of \(\sim 80\text{ kDa}\) (Fig. 2D). In the sumoylation reaction, additional bands were detected at early time points that migrated with apparent molecular weights from 100 to 120 kDa, representing SUMO-modified forms of the BLM 1–431 fragment. At later time points, and in reactions containing excess amounts of SUMO-1 or SUMO-2, modification of the BLM 1–431 fragment was so extensive that the protein barely migrated into the gel (Fig. 2E). These data suggested that there are at least two preferred sites of modification on BLM and that additional, less efficiently modified sites also exist. In addition, because purified BLM could be sumoylated with purified recombinant E1, UBC9 and SUMO proteins, UBC9 binding alone was sufficient for modification \textit{in vitro}.

Deletion of BLM amino acids 212–237 disrupts sumoylation

To identify regions of BLM required for sumoylation, an N-terminal fragment of BLM (1–447) was cloned into the pCITE vector and deletion mutants derived from it were tested in the \textit{in vitro} assay, using protein produced by \textit{in vitro} transcription and translation. In the \textit{in vitro} reaction, BLM 1–447 was more readily modified by SUMO-2 than by SUMO-1 (Fig. 3A, lanes labeled 1 and 2, respectively), similar to what was observed \textit{in vitro} and \textit{in vivo} with the full-length protein. The reaction products with SUMO-2 also more readily formed high molecular weight smears, which was most likely due, in part, to the ability of SUMO-2 to form polymeric chains (52). The formation of several discrete higher molecular weight species in reactions containing SUMO-1 again suggested the presence of two or more preferred modification sites in BLM. With deletions of amino acids 1–132, 155–211, 238–339, 316–355 and 340–446, SUMO modification of BLM was detected at or close to normal levels, whereas with deletions of amino acids 194–236 and 212–237, SUMO modification of BLM was >95% reduced (Fig. 3A). These data indicated that either the major SUMO-modified lysines in BLM reside between amino acids 212–237 or this region is important for interactions with UBC9. It is interesting to note that the same deletion disrupts BLM’s ability to localize to the PML-NBs, which suggests a functional relationship between sumoylation of BLM and its targeting to PML-NBs.
To identify the sites of BLM that were modified by SUMO, we substituted arginines for individual or multiple lysines throughout the BLM 1–447 fragment, produced the mutant proteins by in vitro transcription and translation and tested them in the in vitro assay. Unlike other previously characterized SUMO substrates, we were unable to detect modification site(s) in BLM by arginine substitutions for single lysines or pairs of lysines. No change in sumoylation pattern was observed with any mutant except the mutant K331R (data not shown). These data suggested that BLM is likely to be...
which allowed comparison of the K-back mutants with different from the migration of the internally modified BLM, migration of the presumptive N-terminus-modified BLM was of the normal and K-less 131–447 BLM fragment, the terminus of the protein. In comparisons of the modification on a substrate lacking lysines, the observation indicated that, was still modified (Fig. 3B). Because modification occurred corresponding to mono-sumoylated K-back mutants. 

fication reactions. The asterisks indicate the positions of these bands temporarily migrating high molecular weight bands not detected in the K-less modification. Individual or multiple arginines were subsequently changed back to lysines in these proteins was determined by the presence of differentially modifying high molecular weight bands not detected in the K-less modification sites in BLM is complex and may be less con-

modified at multiple lysine residues and that the selection of modification sites in BLM is complex and may be less con-

Because analysis of the single mutants did not identify defini-

itively the BLM SUMO sites, we took an alternative approach. We prepared a fragment of BLM 131–447 in which all 26 lysines were mutated to arginines (131–447 K-less mutant) and then back-mutated the arginines individually or in pairs to lysine (K-back mutants). Testing the 131–447 K-less mutant in the in vitro system, we found BLM was still modified (Fig. 3B). Because modification occurred on a substrate lacking lysines, the observation indicated that, in the in vitro system, SUMO could be attached to the N-terminus of the protein. In comparisons of the modification of the normal and K-less 131–447 BLM fragment, the migration of the presumptive N-terminus-modified BLM was different from the migration of the internally modified BLM, which allowed comparison of the K-back mutants with normal BLM modification. Analysis of each K-back mutant showed that modification could occur at residues K317, K344, K347 and most prominently at K331 (Fig. 3B), but not at other sites (e.g. K167). The sequence context of the lysines at these four sites (SKCL, EKMS, SKPE and RKED, respectively) bore little or no resemblance to the consensus sequence (εKxε) for a site of modification (53,54). Furthermore, because no SUMO sites were identified in the region 212–237, we concluded that the 212–237 region is important for UBC9 binding.

To confirm sites of SUMO modification directly, we produced several micrograms of the mono-sumoylated form of BLM 1–431 in vitro, isolated the protein by SDS–PAGE, subjected it to trypsin digestion and analysis by mass spectrometry. In this analysis, we identified a peptide with a mass consistent with modification at lysine 331 (data not shown). Other sites of modification, however, could not be identified by this approach. Altogether, these data indicated that BLM is modified at several preferred sites and that K331 is a major site of SUMO modification.

Figure 3. Determination of domain requirements for sumoylation and of specific BLM SUMO sites. (A) Deletion analysis. BLM 1–447 protein (lanes marked ‘normal’) and its deleted derivatives were produced by in vitro transcription and translation. SUMO modification was performed with recombinant E1, UBC9 and SUMO-1 (1) or SUMO-2 (2). The minus sign indicates that the reaction did not include exogenously added SUMO. The deletion constructs 194–236 and 212–237 were not sumoylated by either SUMO-1 or SUMO-2, indicating that either the major SUMO modified lysines in BLM reside between amino acids 212–237 or this region is important for interactions with UBC9. The migrations of proteins of known molecular weights are indicated at left. (B) Mapping of BLM SUMO sites in the K-back mutants. SUMO modification was performed with all recombinant proteins as in (A). A fragment of BLM 131–447 was produced in which every lysine was changed to an arginine (K-less). Sumoylation of the K-less protein (confirmed by comparing the migration of modified BLM reaction products generated using Hist- and GST-tagged SUMO-1; data not shown) indicated that modification could occur at the N-terminus of the BLM fragment. Individual or multiple arginines were subsequently changed back to lysines (K317, K331, etc.) in the K-less protein. Modification of the internal lysine residues in these proteins was determined by the presence of differentially migrating high molecular weight bands not detected in the K-less modification reactions. The asterisks indicate the positions of these bands corresponding to mono-sumoylated K-back mutants.

SUMO modification regulates BLM’s intra-nuclear localization and the formation of DDI foci

Because amino acids 133–458 were sufficient for BLM’s localization to the PML-NBs and the presumptive UBC9 binding site and preferred SUMO sites were within this region, we investigated whether sumoylation could influence BLM’s intra-nuclear localization. To address this question, we generated full-length GFP–BLM expression constructs that contained the single lysine to arginine mutations K317R and K331R and the double mutant K317R/K331R (GFP–BLM DM). HeLa cells were transiently transfected with each of the mutant and normal BLM constructs, and 24 h later the cells were fixed, stained with anti-PML antibody and analyzed by fluorescence microscopy. As observed previously, in cells that expressed normal GFP–BLM, ~60% of the cells that expressed GFP–BLM contained nuclear BLM foci, of which >90% co-localized with PML in the PML-NBs (Fig. 4A). In cells that expressed the K317R mutant, 48% of the nuclei contained BLM foci but only 38% of the BLM foci co-localized with PML, and in cells that expressed the K331R mutant, 30% of the nuclei contained BLM foci but only 47% of the BLM foci co-localized with PML (Fig. 4A). Even more striking, however, was expression of the double mutant GFP–BLM DM. The protein induced the appearance of numerous BLM foci in 68% of the transfected cells, of which <6% co-localized with PML foci (Fig. 4A and B). Thus, expression of BLM molecules that contained SUMO-site mutations disrupted BLM’s localization to the PML-NBs, and concomitantly, BLM appeared in novel focal concentrations.

In addition to the formation of novel foci, we noted that, in HeLa cells that transiently expressed single or double mutant GFP–BLM, the numbers of PML-NBs were reduced by half or more when compared with the numbers detected in cells that expressed normal GFP–BLM (Fig. 4A). For example, in cells that expressed GFP–BLM DM, there were on average 0.7 PML-NBs per nucleus, whereas in cells that expressed GFP–BLM, there were on average 10.6 PML-NBs per nucleus. These results suggested that expression of
BLM SUMO-site mutants destabilized the formation of the PML-NBs, possibly by sequestration of a factor that stabilizes the PML-NBs.

To determine whether SUMO modification could restore the normal localization of GFP–BLM DM, we transfected normal GFP–BLM or GFP–BLM DM with SUMO-1 fused to the N-terminus of the GFP domain. Fusion of SUMO-1 to GFP–BLM DM resulted in substantial re-localization of the mutant BLM to the PML-NBs, whereas fusion of SUMO-1 to normal GFP–BLM did not affect its localization to the PML-NBs (Fig. 4A and C).

Because in response to DNA damage, normal BLM leaves the PML-NBs and localizes to the DDI foci (12,23,35,39), we hypothesized that the novel foci in which GFP–BLM DM localized might be equivalent to the DDI foci. To test this hypothesis, we transfected HeLa cells with either the GFP–BLM or GFP–BLM DM constructs, and after 24 h the cells were stained with anti-BRCA1 or anti-γH2AX antibodies. In cells that expressed GFP–BLM, BRCA1 protein was present predominantly in a diffuse form; in contrast, in cells that expressed GFP–BLM DM, BRCA1 protein often co-localized with GFP–BLM DM, being focally concentrated in approximately half of the novel BLM foci (Fig. 5A). Similarly, in cells that expressed GFP–BLM DM, γH2AX co-localized with GFP–BLM DM, being focally concentrated in >95% of the novel BLM foci (Fig. 5B). These data showed that the novel foci induced by GFP–BLM DM were similar in protein content and appearance to the DDI foci that normally form after cells have been treated with genotoxic agents. However, GFP–BLM DM induces DDI foci in the absence of exogenously induced DNA lesions. Altogether, these data indicated that SUMO modification negatively regulates BLM localization to the DDI foci and directs BLM to the PML-NBs.

Increased genomic instability in cells that express double mutant BLM

To define a functional link between BLM and its ability to be modified by SUMO, we stably expressed normal GFP–BLM, GFP–BLM DM or GFP alone in the BLM-deficient BS cell line GM08505 and assessed SUMO modification and genomic stability in several clones. GFP–BLM and GFP–BLM DM proteins were expressed at similar levels in each of the clones examined as determined by western blot analysis (data not shown). These levels of GFP–BLM and GFP–BLM DM proteins were expressed at similar levels in each of the clones examined as determined by western blot analysis (data not shown). These levels of GFP–BLM and GFP–BLM DM proteins were expressed at similar levels in each of the clones examined as determined by western blot analysis (data not shown). These levels of GFP–BLM and GFP–BLM DM proteins were expressed at similar levels in each of the clones examined as determined by western blot analysis (data not shown). These levels of GFP–BLM and GFP–BLM DM proteins were expressed at similar levels in each of the clones examined as determined by western blot analysis (data not shown). These levels of GFP–BLM and GFP–BLM DM proteins were expressed at similar levels in each of the clones examined as determined by western blot analysis (data not shown). These levels of GFP–BLM and GFP–BLM DM proteins were expressed at similar levels in each of the clones examined as determined by western blot analysis (data not shown).
with anti-BLM, in cells that expressed normal GFP–BLM, we detected a major band with an apparent molecular weight of 200 kDa, which represented GFP–BLM, and a smear of minor bands with mobilities 20–40 kDa larger than the major band, whereas in cells that expressed GFP–BLM DM, the smear of minor bands was not detected (Fig. 6A). With anti-SUMO-2 antibodies, molecules with molecular weights indistinguishable from the minor bands were detected. In addition, higher molecular weight species were detected in cells that expressed GFP–BLM that were not detected in cells that expressed GFP–BLM DM. These data indicated that GFP–BLM DM is not detectably modified by SUMO in vivo.

As we had observed in the transient transfection experiments (1) clones that expressed GFP–BLM DM contained twice the numbers of GFP foci when compared with cells that expressed normal GFP–BLM, (2) the percentage of GFP–BLM DM foci that co-localized with PML was very low and (3) cells that expressed GFP–BLM DM had less than half the number of PML-NBs per nucleus compared with cells that expressed GFP–BLM (Fig. 6B and C). Similarly, the percentage of co-localization of GFP–BLM DM with BRCA1 or γH2AX was ~50 and 95%, respectively (Fig. 6D and E). These data showed that in cells that stably expressed GFP–BLM DM, DDI foci were present constitutively.

Two clones of cells that expressed GFP–BLM DM were selected for SCE tests and compared with a clone that expressed GFP–BLM or GFP alone. Cells were cultured in the presence of BrdU for two rounds of DNA synthesis, metaphase chromosomes were prepared and the sister chromatids were differentially stained. Similar to results we reported previously (21,55), in cells that expressed GFP the average SCE frequency was 34.7 SCEs/46 chromosomes ± 10.6, whereas in cells that expressed normal GFP–BLM the average SCE frequency was 5.6 SCEs/46 chromosomes ± 4.0 (Table 2). In the two clones tested that expressed GFP–BLM DM, the average SCE frequency was 8.8 SCEs/46 chromosomes ± 8.6. The distributions of SCEs in cells that expressed GFP–BLM DM and GFP–BLM were statistically different (P < 0.001; Student’s t-test). In addition, ~5.3% of cells that expressed GFP–BLM DM exhibited levels of SCEs two standard deviations above normal (>25 SCEs/46 chromosomes). The difference in mean values between cells that expressed GFP–BLM and GFP–BLM DM was still significant even when these high-SCE cells were excluded from the analysis. These findings indicated that the double mutant BLM protein only partially complemented the high SCEs of BS cells; moreover, in some cells of the population, GFP–BLM DM failed to suppress SCEs entirely.

To determine whether spontaneous chromosome breakage was increased, we counted the numbers of micronuclei and nuclear blebs produced by cells that expressed the GFP–BLM, GFP–BLM DM or GFP constructs (Fig. 7). The average frequency of micronuclei per cell that formed in BS cells that expressed GFP was 24.0 ± 6.2%, whereas in cells that expressed GFP–BLM the average frequency was 6.4 ± 0.66%. In the two clones that expressed GFP–BLM DM, the average frequencies of micronuclei was 20.4 ± 4.4%. A less striking difference between GFP–BLM and GFP–BLM DM was detected in the frequency of nuclear blebs. Altogether, these data indicated that mutation of BLM SUMO-sites impaired BLM’s normal function in the maintenance of genomic integrity.

**DISCUSSION**

**Sumoylation and BLM intra-nuclear trafficking**

We have shown here that a double mutation of BLM that prevents its sumoylation in vivo induces the formation of DDI foci in the absence of treatment with genotoxic agents and further that the double mutation impairs BLM’s function in maintenance of genomic integrity. These findings constitute two equally important and novel discoveries. First, the effects of expressing the double mutant demonstrate that BLM has a direct role in regulating the formation of DDI foci, and they therefore implicate BLM as a DNA damage sensor. Secondly, the data show that SUMO modification functions to recruit BLM to PML-NBs, but most importantly, they show that this recruitment is central to regulating the formation of DDI foci. The role of SUMO modification in regulating the BLM function and the formation of DDI foci has broad implications on how SUMO modification may regulate other cellular processes. PML-NBs contain many proteins and a significant number of these are SUMO substrates (48). It is possible that SUMO modification of these other proteins similarly regulates their ability to traffic between the PML-NBs and multi-protein complexes outside of the PML-NBs.
Sumoylation of PML is essential for its localization to the PML-NBs (40,43). In addition, a significant number of DNA repair factors are SUMO modified and they may also be regulated in a fashion similar to BLM. SUMO modification of WRN has been proposed to regulate its localization to the nucleolus (56).

Several characteristics of SUMO-modification of BLM were unusual. First, we found that the sites of modification did not conform to the consensus sequence, which defines the modification sites in most known SUMO substrates (53,54). Secondly, we found that BLM was more efficiently modified by SUMO-2 than SUMO-1 (Figs 2 and 3). The functional significance of this observation remains to be determined. Thirdly, in vitro modification with SUMO-1 or SUMO-2 did not occur at just one site, but occurred at several preferred sites in the BLM N-terminus (Fig. 2). Under certain in vitro conditions, particularly in the presence of SUMO-2, hyper-modified forms of BLM were detected. That modification under these conditions could occur simultaneously at multiple sites was confirmed using a mutant form of SUMO with no lysines and therefore unable to form polymeric chains (data not shown). Although the in vitro modification properties of BLM were unusual and had characteristics suggestive of a SUMO E3 ligase (57–59), we were able to identify two specific modification sites that proved to be functionally important in vivo.

Two models can be proposed to explain the abnormal formation and persistence of DDI foci in cells that expressed BLM DM. In one model, the DDI foci form due to the presence of endogenous DNA damage. We note that the levels of SCEs (Table 2), micronuclei formation (Fig. 7) and other cytogenetic abnormalities, including DNA breaks, gaps, rings

Figure 6. Analysis of GFP–BLM DM clones. (A) BLM-DM is not modified by SUMO-2. Clones of GM08505 cells were isolated that stably expressed GFP–BLM (BLM) or GFP–BLM DM (DM3). Whole cell lysates (lanes labeled ‘L’) were prepared and immune complexes were isolated with anti-BLM (lanes labeled with a plus sign) or rabbit IgG (lanes labeled with a minus sign). The immune complexes were analyzed by probing western blots with anti-BLM (BLM panel) or anti-SUMO-2 (SUMO-2 panel). A light smear of bands above the major BLM band was detected in lysates and immune complexes isolated from a clone that expressed BLM but not from a clone that expressed BLM DM. The same smear was detected with anti-SUMO-2 antibodies. These bands and additional higher molecular weight species are labeled by asterisks. A ‘c’ indicates a constant band found also in BS cells (c.f. Fig. 2). The levels of GFP–BLM and GFP–BLM DM expressed in GM08505 cells was ~10-fold greater than a comparable SV40-transformed cell line that contained normal BLM (data not shown). (B) Graph representing the numbers of observed BLM foci, PML foci, and co-incident BLM-PML foci. Green foci and PML foci were quantified in GM08505 cells that stably expressed GFP–BLM (BLM) or GFP–BLM DM (DM3 and DM18). The total number of nuclei scored is indicated in parentheses. Foci were scored in ~100 cells in each of three independent experiments, as described in the legend to Figure 4. (C) Absence of co-localization of BLM DM and PML in the PML-NBs. The coincidence of BLM (green fluorescence) and PML (red fluorescence) foci was detected in cells that expressed normal GFP–BLM (BLM) but not in cells that expressed double mutant GFP–BLM (BLM DM). Nuclei were identified with DAPI. (D) In contrast, BRCA1 and BLM co-localized in 50% of the novel foci formed by GFP–BLM DM. (E) Similarly, γH2AX and BLM co-localized in >90% of the novel foci formed by GFP–BLM DM.
and acentric fragments (data not shown), were less abundant in cells that expressed BLM DM when compared with untreated BS cells, and BS cells do not have abnormally high numbers of DDI foci (12). Consequently, it is unlikely that the DDI foci present in cells that express DM form as a result of increased DNA damage in the cell. It is possible, however, that the DM-induced DDI form due to normal levels of endogenous DNA damage, but they persist because they fail to disassemble at the normal rate. In this case, SUMO modification of BLM functions to disassemble the DDI foci and to facilitate BLM’s return to PML-NBs.

A more compelling model is that BLM functions as a DNA damage sensor, which can directly signal the induction of DDI foci (Fig. 8). Normally, BLM leaves the PML-NBs to survey the nucleoplasm for its preferred DNA substrates (e.g. stalled replication forks, D-loops or Holliday junctions). If BLM does not encounter one of these substrates, it is modified by SUMO and returned to the PML-NBs, where it is rapidly desumoylated. If BLM encounters one of its substrates, it becomes resistant to SUMO modification. Unmodified, nucleoplasmic BLM then binds a key factor(s) that stimulates the assembly of DDI foci. Perhaps, BLM recruits and activates a kinase that phosphorylates histone H2AX. By this model, nucleoplasmic BLM then binds a key factor(s) that stimulates the assembly of DDI foci (12). Consequently, it is unlikely that the DDI foci present in cells that express DM form as a result of increased DNA damage in the cell. It is possible, however, that the DM-induced DDI form due to normal levels of endogenous DNA damage, but they persist because they fail to disassemble at the normal rate. In this case, SUMO modification of BLM functions to disassemble the DDI foci and to facilitate BLM’s return to PML-NBs.

Table 2. The distributions of the numbers of SCEs per 46 chromosomes in GM08505 cells that stably expressed the GFP–BLM, GFP–BLM DM or GFP constructs

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Each entry in the table records the number of metaphases with the indicated number of SCEs per 46 chromosomes. Blanks indicate no metaphases were scored with the indicated number of SCEs per 46 chromosomes. BLM18, clone that expressed GFP–BLM; DM31 and DM18, clones that expressed GFP–BLM DM; GFP5, clone that expressed GFP. Mean is the average frequency of SCEs per 46 chromosomes per cell. Cells indicate the total number of cells examined. Chrom. indicates average number of chromosomes per metaphase.

In summary, SUMO modification negatively regulates BLM’s ability to induce DDI foci. Moreover, the abnormal formation of DDI foci in cells that express BLM
DM disrupts DNA repair, possibly due to the sequestration of repair factors. These findings have general implications for how SUMO modification regulates the functions of its substrates, many of which are also known to associate dynamically with large protein complexes. BLM SUMO-site mutants provide a useful model system for the study of the regulation of DNA damage surveillance, for the identification and characterization of factors in the DDI foci and of the mechanism by which they form and for the investigation of the role of sumoylation in DNA repair.

MATERIALS AND METHODS

Expression constructs

For expression of BLM proteins in cultured human cells, we cloned the full-length BLM cDNA into the EGFP-C1 vector (CLONtech), which produced a GFP–BLM fusion protein with GFP appended to the N-terminus of BLM, as described (21). Deletion and amino acid substitution mutants were derived from GFP–BLM by standard polymerase chain reaction (PCR)-based procedures. The SUMO-GFP–BLM and SUMO-GFP–BLM DM constructs were prepared by combining SUMO and GFP–BLM PCR fragments engineered with a short stretch of overlap, followed by PCR to obtain the SUMO-GFP–BLM fragment. This fragment was then used to replace the same region in GFP–BLM and GFP–BLM DM. Human UBC9 obtained from the yeast two-hybrid screen was also cloned in the EGFP-C1 vector.

For the yeast two-hybrid screen, we fused the GAL4 DNA binding (DB) domain to the N-terminal 520 amino acids of BLM (pGBT9–BLM 1–520) using the vector pGBT9 (CLONtech) using PCR. A cDNA library prepared with HeLa cell mRNA, cloned into the GAL4 transactivation (TA) domain using the vector pGAD-GH, was purchased from CLONtech.

For in vitro transcription and translation, BLM amino acids 1–447 were cloned into the vector pCITE1 (Invitrogen), and deletion and substitution mutants were derived therefrom by PCR. Recombinant E1, UBC9 and SUMO-1 proteins were expressed and purified from E. coli as described earlier (61).

Figure 7. Frequencies of micronuclei and nuclear blebs in GM08505 cells that stably expressed the GFP–BLM (BLM18 and BLM22), GFP–BLM DM (DM3 and DM18) or GFP (GFP5) constructs. Each bar represents the average of three independent experiments. The total numbers of cells counted for each cell line are shown in parentheses.

Expression and purification of BLM 1–431 were performed as described previously (51). All vector constructs were sequence verified.

Cell lines

The SV40-transformed fibroblast cell line GM08505 derived from a person with BS, who was homozygous for the mutation BLMc.2207-2212delATCTGAinsTAGATTC, and a control normal SV40-transformed fibroblast cell line GM00637 were obtained from the Coriell Institute for Medical Research via J. German of Cornell Medical College. HeLa cells were obtained from American Type Culture Collection. The cells were grown at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) with high glucose content and 10% fetal bovine serum (Life Technologies) and 200 U/mL penicillin/streptomycin. Transformation of HeLa and GM08505 cells with GFP–BLM plasmid.
constructs was performed as described previously (21). Clones that stably expressed different GFP–BLM constructs were cultured in the same conditions, except that 0.2 mg/ml gentamycin (G418, Invitrogen) was added to the medium to maintain selection.

**Antibodies**

Rabbit polyclonal anti-BLM serum was raised and antibodies purified as previously described (21). Mouse monoclonal anti-SUMO-1 was prepared as previously described (62). Mouse monoclonal antibodies to SUMO-2 were prepared using recombinant SUMO-2 as antigen. Commercial antibodies used in these studies included mouse monoclonal anti-PML (PG-M3, Santa Cruz Biotechnology Inc.), mouse monoclonal anti-GFP (CLONtech Laboratories, Inc.), mouse monoclonal anti-BRCA1 (Ab-1, Oncogene Research Products) and rabbit polyclonal anti-γH2AX (H-124, Santa Cruz Biotechnology Inc.). The secondary antibodies used in this study were goat anti-mouse IgG (Alexa fluor 568) or goat anti-rabbit IgG (Alexa Fluor 594) from Molecular Probes.

**Yeast two-hybrid screen**

The GAL4-DB domain BLM 1–520 fusion construct and GAL4-TA HeLa cDNA library were co-transformed into yeast H77C cells using the lithium chloride method. LEU+, TRP+ and HIS+ transformants were selected in the presence of 5 mM aminotrizole (Sigma). An estimate of the number of TRP containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl and 1% instructions. After 24 h, cells were washed in phosphate buf-

**Analysis of BLM SUMO modification in vivo**

For analysis of BLM’s interaction with UBC9, 2 × 10⁶ GM08505 or GM00637 cells were seeded overnight, then the cells were transfected with the GFP–UBC9 construct or the GFP vector (2–10 μg) using the Lipofectamine Plus reagent kit (Life Technologies) following the manufacturer’s instructions. After 24 h, cells were washed in phosphate buffered saline (PBS) and lysed in ice-cold NP-40 lysis buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl and 1% NP-40 supplemented with complete protease inhibitor cocktail (Boehringer Manheim). The lysate was cleared by centrifugation in a microfuge at 15 000 r.p.m. for 10 min at 4°C. Immunoprecipitation was performed as described with anti-

**Analysis of SUMO modification of BLM in vitro**

Labeled proteins were produced by in vitro T7 RNA polymerase transcription with pCITE- or Bluescript-cloned cDNAs or cDNA fragments coupled to translation with the rabbit reticulocyte lysate system in the presence of [³⁵S]methionine according to the manufacturer’s instructions (Promega Corp. Madison, WI, USA). Modification reactions were performed with recombinant E1, UBC9, SUMO-1 or SUMO-2. Unless otherwise noted, 2 μl of product from the reticulocyte lysate reaction was added to a 20 μl modification reaction containing 1 mM ATP, 5 mM phosphocreatine, 20 U/ml creatine phosphokinase, 500 nM recombinant E1 enzyme, 600 nM recombinant UBC9 and 10 μM recombinant SUMO-1 or SUMO-2. Reactions were incubated for 1 h at 37°C and stopped by the addition of SDS–PAGE sample buffer. Samples were analyzed by SDS–PAGE followed by autoradiography. In addition, purified recombinant BLM 1–431 was SUMO modified using the same reaction conditions and analyzed by SDS–PAGE followed by Coomassie blue staining. Hypermodified BLM was produced using similar assay conditions in which the ratio of recombinant SUMO to BLM was increased from 1 μg SUMO:4 μg BLM fragment to 4 μg SUMO:1 μg BLM fragment.

A SUMO site was identified by in vitro modification of purified recombinant BLM followed by mass spectrometric
Analysis of cells that transiently expressed GFP-tagged BLM proteins

Approximately $2 \times 10^4$ HeLa cells were seeded overnight in four-well chamber slides (Lab-Tek, Nalge Nunc International) and transiently transfected with $1 \mu g$ of different GFP–BLM constructs using transfection reagent (Qiagen) according to the manufacturer’s instructions. After 24 h, cells were fixed in 4% paraformaldehyde (PFA, Fisher Scientific) and 0.1% Triton X-100 for 10 min at 4°C, washed in PBS and blocked in 10% normal goat serum (Vector Laboratories) and 0.1 M glycine (Sigma) for 1 h at room temperature. Slides were then incubated with the primary antibody diluted in blocking buffer for 1 h at room temperature or overnight at 4°C, washed extensively in PBS and stained with the appropriate secondary antibody diluted in blocking buffer for 1–1.5 h at room temperature. The slides were washed in PBS and mounted in Vectashield (Vector Laboratories). To visualize the DNA, the last wash contained DAPI (Sigma) at 0.4 μg/ml.

Cells that expressed high or intermediate levels of nuclear GFP, as determined by the intensity of the green fluorescence in DAPI labeled cells, were scored as cells that expressed GFP–BLM constructs, and BLM foci (GFP; green foci) were counted in up to 100 cells. The PML-NBs (PML; red foci) were visualized by staining with anti-PML antibodies and counterstaining with a secondary antibody labeled with Alexa fluor. For studies using antibodies to BRCA1 and γH2AX, cells were fixed with 3% PFA, 2% sucrose and 0.5% Triton X-100 for 10 min. Blocking was performed in 3% BSA (Vector Laboratories), 2% normal goat serum (Vector Laboratories) and 0.1 M glycine for 1–1.5 h. Staining with secondary antibodies alone was used to evaluate background staining. Cells were viewed by epifluorescence at 100× magnification and images captured with a CCD camera and IPLabs Version 3.5 (Scanalytics) software in the Sloan-Kettering Institute Molecular Cytology core facility. All images were processed with Adobe Photoshop software. A focus was defined as a spherical body between 0.2 and 1 μ in size containing greater concentrations of object protein compared to that protein’s diffuse or background nuclear staining as evidenced by its increased focal fluorescence intensity. Co-localization was determined by the observation of focal concentrations of two coincident proteins as exhibited by orange/yellow coloration under a double- or triple-band pass filter. The percentage of cells that contained BLM in the PML-NBs was evaluated with the following formula: Percentage = (number of nuclei that expressed BLM and contained coincident BLM and PML foci)/(number of nuclei that expressed BLM foci) × 100. All numbers reported were the average of at least three independent experiments. Transfection with the GFP vector alone was used as a negative control.

Analysis of BS cells that stably expressed GFP–BLM and GFP–BLM DM

Approximately $1 \times 10^6$ GM08505 BS cells were seeded in a six-well plate prior to being transfected with 1.5 μg of GFP–BLM or GFP–BLM DM using Lipofectamin (Invitrogen) according to the manufacturer’s instructions. After 48 h, the efficiency of the transfection was verified by examination of cells under an inverted fluorescence microscope, then the cells were transferred to a 10 mm dish in DMEM containing 0.2 mg/ml genetin. About 3 weeks later, clones were picked and expanded under selection. Three to five clones were isolated for each construct. The clones were analyzed for GFP expression by flow cytometry using a FACScan cytometer with Cellquest software (BD Biosciences, San Jose, CA, USA). For western blot analysis of BLM levels, cells were lysed in NP-40 lysis buffer, and for analysis of SUMO-modification of BLM, cells were lysed as described earlier. Proteins were separated by SDS–PAGE electrophoresis through a 4–15% gradient and were transferred to a nylon membrane and processed for western blot analysis using anti-BLM and anti-SUMO antibodies as described previously. Indirect immunofluorescence was performed as described above.

Cytogenetic analyses

For SCE analysis, GM08505 cells that stably expressed the GFP–BLM, GFP–BLM DM or GFP construct were cultured with 10 μM BrdU (Sigma) at 37°C in 5% CO2. After 48 h, the cells were incubated with 0.1 μg/μl Colcemid (Invitrogen) for up to 3 h at 37°C and 5% CO2, harvested and processed as described earlier (55). The slides were examined under the microscope at 100×, acceptable metaphases were captured with a CCD camera and SCEs were counted.

For micronuclei studies, DAPI stained cells were analyzed at 100× magnification and scored for micronuclei formation or nuclear blebs. A micronucleus was defined as a DAPI-fluorescent body under one third the size of the nucleus. A nuclear bleb was defined as a small DAPI-fluorescent body emerging from the nucleus and still attached to it.

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Conflict of Interest statement. None declared
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