Phosphorylation of BLM, Dissociation from Topoisomerase III α , and Colocalization with γ -H2AX after Topoisomerase I-Induced Replication Damage

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Topoisomerase I-associated DNA single-strand breaks selectively trapped by camptothecins are lethal after being converted to double-strand breaks by replication fork collisions. BLM (Bloom's syndrome protein), a RecQ DNA helicase, and topoisomerase III α (Top3 α) appear essential for the resolution of stalled replication forks (Holliday junctions). We investigated the involvement of BLM in the signaling response to Top1-mediated replication DNA damage. In BLM-complemented cells, BLM colocalized with promyelocytic leukemia protein (PML) nuclear bodies and Top3 α . Fibroblasts without BLM showed an increased sensitivity to camptothecin, enhanced formation of Top1-DNA complexes, and delayed histone H2AX phosphorylation (γ -H2AX). Camptothecin also induced nuclear relocalization of BLM, Top3 α , and PML protein and replication-dependent phosphorylation of BLM on threonine 99 (T99p-BLM). T99p-BLM was also observed following replication stress induced by hydroxyurea. Ataxia telangiectasia mutated (ATM) protein and AT- and Rad9-related protein kinases, but not DNA-dependent protein kinase, appeared to play a redundant role in phosphorylating BLM. Following camptothecin treatment, T99p-BLM colocalized with γ -H2AX but not with Top3 α or PML. Thus, BLM appears to dissociate from Top3 α and PML following its phosphorylation and facilitates H2AX phosphorylation in response to replication double-strand breaks induced by Top1. A defect in γ -H2AX signaling in response to unrepaired replication-mediated double-strand breaks might, at least in part, explain the camptothecin-sensitivity of BLM-deficient cells.

DNA topoisomerase I (Top1) is essential for removing DNA supercoiling generated in transcribing and replicating chromatin (11, 67). Top1 relaxes positively and negatively supercoiled DNA by introducing reversible DNA single-strand breaks associated with covalent Top1-DNA complexes. Camptothecin, a natural alkaloid, selectively targets the Top1-DNA complex by stabilizing the covalent Top1-DNA cleavage intermediate (33, 47, 65). Camptothecin and its derivatives, irinotecan and topotecan, are potent anticancer drugs currently being used successfully in the treatment of colon and ovarian cancer (4, 46, 64). The cytotoxic action of camptothecin is manifested when a replication fork encounters the drug-stabilized cleavage complex (31, 34). At these sites, extension of the replicating strand up to the end of the Top1-mediated break in the template strand generates a replication double-strand break ("replication runoff") as demonstrated by ligation-mediated PCR (62) and the induction of γ -H2AX (23) (<http://discover.nci.nih.gov/pommier/pommier.htm>). Camptothecin is, therefore, a well-characterized pharmacological tool for studying the molecular mechanisms involved in cellular responses to replicative stress (23, 48, 59, 62). Top1 cleavage complexes and, therefore, replication double-strand breaks can form in response to common

DNA lesions including abasic sites, mismatches, oxidative base lesions, base adducts, and strand breaks (49, 51).

Histone H2AX phosphorylated on serine 139, termed γ -H2AX, is one of the earliest known markers of camptothecin-induced replication-associated damage (23). More generally, γ -H2AX is a marker of DNA double-strand breaks (45, 54). γ -H2AX has been proposed to anchor the broken chromosome ends together and recruit DNA repair elements (5, 20, 23, 45, 53). We have shown previously that γ -H2AX is critical for the recruitment of the Mre11-Rad50-Nbs1 (MRN) complex in camptothecin-treated cells and that H2AX deficiency renders cells hypersensitive to camptothecin (23, 53). Using aphidicolin, we also showed that blocking replicative polymerases abrogates γ -H2AX formation (23), indicating that γ -H2AX forms in response to replication-associated double-strand breaks induced by camptothecin.

The causative gene of the cancer-predisposing genetic disease Bloom's syndrome, *BLM*, is a member of the RecQ family of DNA helicases (28). BLM is considered a caretaker of the genome (28, 39) and a key component in DNA damage response signaling (22, 52). Evolutionarily conserved and essential for the maintenance of genomic stability, BLM promotes branch migration of Holliday junctions in vitro in an ATP-driven fashion (36, 38, 40, 66, 70). BLM functions in association with topoisomerase III α (Top3 α) (68), a type I class of topoisomerases (11, 37, 67, 69). The BLM-Top3 α complex can resolve recombination intermediates and prevent the collapse of replication forks and consequent DNA double-strand

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FIG. 1. Enhanced sensitivity and Top1-DNA complex formation in BLM-deficient fibroblasts exposed to camptothecin (CPT). (A) Cell survival following camptothecin exposure for 1 h was measured in BLM-deficient (PSNG13) and -complemented (PSNF5) cell lines by colony formation assay. (Inset) BLM and Top3 α protein expression in PSNG13 and PSNF5 cells. (B) Exponentially growing PSNG13 or PSNF5 cells were exposed to 1 μ M camptothecin for 1 h. Cesium chloride fractions from homogenized sarkosyl lysates were immunoblotted for Top1. (C) DNA-containing fractions were combined, serially diluted (2-, 5-, or 10-fold), and immunoblotted for Top1. (D) Quantitation of the serial dilutions of DNA-containing fractions were compared between PSNG13 and PSNF5 following camptothecin treatment ($n = 3$). Bars, standard deviations. (E) Top1 protein expression in PSNG13 and PSNF5 cellular lysates following a 1-h exposure to 1 μ M camptothecin. (F) Proliferative status of control PSNF5 and PSNG13 cells as measured by BrdU staining and fluorescence-activated cell sorting analysis. Scattergrams shown are representative of at least four independent experiments.

breaks (35, 38, 68, 70, 72, 75). In conjunction with BLM, Top3 α is also important for faithful chromosome segregation during anaphase (26) and meiotic recombination (24), possibly unwinding replicating DNA (41) and replication forks restart (27, 55).

Under unperturbed cell growth conditions, BLM is found in promyelocytic leukemia protein (PML) nuclear bodies, where it associates with Top3 α , and in the nucleolus (72). PML is one of the best-characterized molecular partners of BLM (35, 71, 72, 75). The PML gene, originally identified as the translocation site with the retinoic acid receptor- α (RAR α) gene forms

the PML-RAR α fusion protein in promyelocytic leukemia (8, 16, 56, 57). PML is contained in discrete nuclear structures collectively known as PLM nuclear bodies, Kremer bodies, ND-10, or PML oncogenic domains. In addition to BLM, PML nuclear bodies consist of many proteins including Sp100, SUMO-1, p53, TRADD, Top3 α , Rad51, Mre11, NBS1, retinoblastoma, and Daxx (9, 29, 30, 72). The absence of PML disrupts the normal subnuclear localization of BLM and results in an elevation of sister chromatid exchanges (75). While the exact role of PML in DNA damage signal remains to be clarified, its multicomponent association within the nuclear bodies

FIG. 2. Delayed appearance of H2AX phosphorylation (γ -H2AX) following replication-mediated DNA damage in BLM-deficient fibroblasts. (A) BLM-deficient (PSNG13) and -complemented (PSNF5) cells were exposed to 1 μ M camptothecin for the indicated periods. Cells were then permeabilized and fixed using paraformaldehyde and ethanol. Fixed cells were stained with a γ -H2AX antibody and secondary antibody conjugated with a green fluorescent dye (Alexa 488). Reversal of γ -H2AX was examined following a 12-h treatment with 1 μ M camptothecin. Nuclear outlines were traced from a parallel image with propidium iodide staining and superimposed on the γ -H2AX images. (B) Kinetics of appearance of γ -H2AX foci was quantitated in PSNG13 and PSNF5 cells after exposure to 1 μ M camptothecin, 1 mM hydroxyurea (HU), or 1 G ionizing radiation. (C) Levels of γ -H2AX were assayed in BLM-deficient and -complemented cells exposed to 1 μ M camptothecin for indicated times by Western blot analysis. Images are representative of at least three independent experiments. (D) Generation of γ -H2AX foci in primary healthy human fibroblasts (GM00037) and in primary Bloom syndrome patient fibroblasts (GM01492) following 1 μ M camptothecin treatment for indicated times. (E) Quantitation of γ -H2AX foci in GM00037 and GM01492 cells treated with camptothecin. At least 30 nuclei from five separate fields were included for quantitation. CPT, camptothecin.

might be indicative of a storage site function in DNA damage response and the regulation of cell cycle, DNA repair, and cell death (16).

The N-terminal domain of BLM directs its packaging in PML nuclear bodies, while the C-terminal domain appears

essential for nucleolar localization (72). Cells expressing mutants of the N-terminal regions (residues 135 to 235, 241 to 469, or 402 to 600) of BLM fail to show PML colocalization. The N terminus of BLM is also phosphorylated on threonine 99 and 122 in response to replication blockage by hydroxyurea

FIG. 3. Changes in nuclear localization patterns of BLM, PML, and Top3α in BLM-complemented and -deficient fibroblasts. PSNG13 and PSNF5 cells were exposed to 1 μM camptothecin for 1 h or untreated (control). PSNF5 cells were stained with both Top3α (green) and BLM (red) antibodies (A) or with both PML (green) and BLM (red) antibodies (B) or with both PML (green) and Top3α (red) antibodies (C). (D) PSNG13 cells were probed with PML (red) and Top3α (green) antibodies. Note: The majority of PSNG13 cells failed to show punctate Top3α foci. The image presented for PSNG13 cells is representative of a small fraction of nuclei that showed clear Top3α and PML foci. Images displayed from a single nuclear structure are representative of at least three independent experiments. Fig. 7 and Table 1 show the quantitation of foci in response to camptothecin. CPT, camptothecin.

and ionizing radiation by phosphoinositide 3-kinase-related kinases (PIKKs) (2, 7, 15).

The proposed role of BLM in response to replication defects (1, 15, 22, 58, 60) led us to investigate whether human cells deficient in BLM are altered in their sensitivity and responses to camptothecin. Using isogenic human fibroblast cell lines lacking or expressing the BLM (25), we demonstrate enhanced camptothecin sensitivity, enhanced Top1 cleavage complex formation, and delayed appearance of γ-H2AX foci in BLM-deficient cells. We also sought to study the phosphorylation of BLM in response to replication-associated double-strand

breaks and to investigate whether such modifications are PIKK- and replication-dependent and signal a change in localization pattern of BLM. We have established the status and consequence of T99 BLM phosphorylation in response to camptothecin as well as hydroxyurea. We also investigated the molecular interactions of total BLM as well as the T99-phosphorylated form of BLM with Top3α, γ-H2AX, and PML following replicative stress.

MATERIALS AND METHODS

Cell culture. BLM-deficient (PSNG13) and -complemented (PSNF5) fibroblasts (25) were grown in a selection medium consisting of α-minimal essential medium, 10% fetal calf serum, and 350 μg/ml G418. For quantitation of proliferative fraction of cells, exponentially growing cultures were pulse labeled with bromodeoxyuridine (BrdU) and propidium iodide and analyzed by flow cytometry. GM00037 (untransformed normal), GM05849 (ataxia telangiectasia [AT]), GM00637 (simian virus 40-transformed, apparently normal) and GM01492 (untransformed Bloom syndrome) human fibroblasts were obtained from the Coriell Cell Repository (Camden, NJ) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. M059J/Fus1 and M059J/Fus9 cells were donated from Cordula U. Kirchgesner (32) (Stanford University School of Medicine, Stanford, CA) and were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum containing 400 μg/ml G418 (Invitrogen). AT- and Rad3-related kinase dead (ATRkd) cells were donated from William A. Cliby (12) (Mayo Clinic, Rochester, MN) and were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing 400 μg/ml G418.

Drugs, chemicals, and IR exposure. Aphidicolin, hydroxyurea, G418, sodium orthovanadate, and sodium fluoride were purchased from Sigma Co. (St. Louis, MO), and camptothecin was obtained from the Drug Synthesis Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. For exposure

TABLE 1. Quantitative analysis of colocalization in control PSNF5 cells^a

Protein and condition	% Colocalization of protein			
	BLM		PML	
	+	-	+	-
PML				
With	96	2		
Without	2			
Top3				
With	98	NF	99	NF
Without	2		1	

^a Foci were scored for single stain or costaining to indicate single protein or colocalized foci. Analysis includes at least 30 nuclei from three independent experiments. NF, no foci.

TABLE 2. Quantitative analysis of colocalization in PSNF5 cells after camptothecin treatment^a

Protein and condition	% Colocalization of protein							
	T99p-BLM		-H2AX		BLM		PML	
	+	-	+	-	+	-	+	-
γ -H2AX								
With	94	4						
Without	2							
BLM								
With	78	19	74	10				
Without	3		16					
PML								
With	24	22	17	32	81	8		
Without	54		51		11			
Top3 α								
With	28	34	38	28	68	10	72	5
Without	38		41		22		23	

^a Foci were scored for single stain or costaining to indicate single protein or colocalized foci. Analysis includes at least 30 nuclei from three independent experiments.

to ionizing radiation (IR), cells growing on chamber slides were exposed to the indicated dose of IR from a ¹³⁷Cs source in a Mark I irradiator (J. L. Shepherd and Associates, San Fernando, CA). Following drug or IR exposure, cells were incubated at 37°C for indicated times.

Colony formation assay. Cell survival was determined using a colony formation assay after indicated treatments. Monolayers of cells were trypsinized, counted, and plated on six-well, 60-mm sterile polystyrene culture plates. Approximately 100 cells were maintained per well in 3 ml of culture medium and incubated unperturbed for 7 days. Prior to colony counting, culture medium was aspirated, and colonies were treated with 2 ml of fixation solution (50% methanol, 5% acetic acid) for 1 h. After removal of fixation solution, colonies were stained with 3 ml of Wright's Giemsa stain (Sigma Diagnostics, St. Louis, MO) for 1 h. Colonies were counted manually.

Immune-complex of Top1-DNA detection assay. Top1-DNA adducts were detected as described previously (50, 63). Briefly, 10⁶ treated or untreated cells were pelleted and immediately lysed in 1% sarkosyl. Following homogenization with a Dounce homogenizer and pestle B, cell lysates were gently layered on cesium chloride step gradients and centrifuged at 165,000 $\times$ g for 20 h at 20°C. Half-milliliter fractions were collected, diluted with an equal volume of 25 mM sodium phosphate buffer (pH 6.5), and applied to Immobilon-P membranes (Millipore) in a slot-blot vacuum manifold (23). Top1-DNA complexes were detected using the C21 Top1 monoclonal antibody (a kind gift from Yung-Chi Cheng, Yale University, New Haven, CT) using standard Western blotting procedures.

Western blot analysis and antibodies. Cells were washed with phosphate-buffered saline following treatment, and total protein was extracted using RIPA buffer. Total protein was quantitated using the Bradford assay (Bio-Rad, Hercules, CA), and 20 μ g of total protein was used for Western blot analysis.

Aliquots of total protein were boiled with Novex Tris-glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) for 10 min at 95°C and loaded on a Tris-glycine gel for electrophoresis. Fractionated proteins were then transferred onto a nitrocellulose membrane by electroblotting. Nonspecific binding was blocked using 5% nonfat dry milk (in Tris-buffered saline-Tween [TBS-T]). Suitable combinations of antibodies were prepared in 1% nonfat dry milk (in TBS-T). Protein was visualized by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL) and normalized to actin or tubulin levels in each extract.

Antibodies used in Western blot analyses were commercially obtained for γ -H2AX (Upstate Technologies, CA), PML (PG-M3), anti-goat BLM (Santa Cruz Biotech, CA), actin, and tubulin (Ab-4; Neomarkers, Fremont, CA). A polyclonal antibody against phosphorylated T99 BLM was raised in rabbits (Sigma Genosys, Houston, TX). Crude serum from inoculated rabbits was double-affinity purified using a phospho-peptide and non-phospho-peptide-conjugated Sepharose columns and measured for antibody concentration using an

enzyme-linked immunosorbent assay. Antibodies for anti-mouse total BLM (residues 1 to 449) and Top3 α have been described previously (68).

Protein phosphatase treatment. Whole-cell lysates were incubated at 30°C with 2,400 U of λ protein phosphatase for 60 min prior to Western blot analysis (with MnCl₂ in λ -phosphatase buffer [pH 7.5 at 25°C]) and used according to the manufacturer's instructions (New England Biolabs, Beverly, MA). For blockage of phosphatase action, a combination of 10 mM sodium orthovanadate and 50 mM sodium fluoride was added to the protein samples.

Fluorescent confocal microscopy. Cells used for microscopy studies were grown in Nunc chamber slides (Nalgene, Rochester, NY) using 0.5 ml of growth medium. Following treatment, the medium was aspirated out and cells were washed in TBS-T. Cells were then fixed using 2% paraformaldehyde and 70% ethanol at room temperature. To block nonspecific binding, cells were incubated with 8% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Fixed cells were stained overnight with primary antibodies (in 1% bovine serum albumin) as indicated and tagged with fluorescent secondary antibodies for 2 h. Slides were mounted using Vectashield mounting liquid (Vector Labs) and sealed. Slides were shielded from light and stored at 4°C. Slides were visualized using a Nikon Eclipse TE-300 confocal laser scanning microscope system, and images were captured and stored as JPEG files.

RESULTS

Enhanced cell killing and Top1-DNA complex formation by camptothecin in BLM-deficient cells. The isogenic cell lines, distinct in their BLM status (Fig. 1A, inset) (25), were used to measure viability in response to camptothecin using a colony formation assay. The BLM-deficient fibroblast cell line (PSNG13) displayed hypersensitivity to camptothecin in comparison to BLM-complemented cells (PSNF5) (Fig. 1A).

To elucidate the underlying basis for the enhanced sensitivity of BLM-deficient (PSNG13) cells to camptothecin, we measured drug-induced Top1-cleavage complexes following cesium chloride gradient centrifugation and fractionation of sarkosyl-lysed cells (50, 63). Cells lacking BLM (PSNG13) responded to 1 μ M camptothecin for 1 h with a greater level of Top1-DNA complexes than did the cells with complemented BLM (PSNF5) (Fig. 1B). A semiquantitative serial dilution method was used to confirm the difference in Top1-DNA complex formation. The DNA-containing cesium chloride fractions were combined, serially diluted 2-, 5-, or 10-fold, and probed with Top1 antibody (Fig. 1C). Band intensities were quantified using densitometric scanning (Fig. 1D). BLM-deficient (PSNG13) cells formed an approximately fivefold higher level of Top1-DNA complexes than the BLM-complemented (PSNF5) cells. Because differences in levels of Top1-DNA complexes could be due to global changes in Top1 protein expression, cellular Top1 protein levels were measured in both cell lines. The cellular protein expression levels of Top1 enzyme were comparable in both cell lines and did not appear to change following camptothecin treatment in either cell line (Fig. 1E). The PSNG13 cells have a longer doubling time (~18 h) compared to the PSNF5 cells (~12 h). However, using BrdU staining and fluorescence-activated cell sorting analysis, we show that the sensitivity in PSNG13 is not due to a greater proliferative fraction (Fig. 1F, % BrdU-positive cells). The BLM-deficient PSNG13 cells also showed a greater loss in the S-phase fraction of cells in response to camptothecin when assayed by measuring BrdU uptake (not shown). Collectively, these results indicate that BLM-deficient cells produce a higher level of Top1-DNA complexes and are hypersensitive to camptothecin compared to BLM-corrected cells.

FIG. 4. Phosphorylation of BLM on T99 in camptothecin-treated cells. (A) A peptide consisting of the phosphorylated T99 BLM (T99p-BLM) was used to generate antisera in rabbits. Crude serum was double-affinity purified and used in control experiments with extracts from PSNF5 and PSNG13 cells. Preimmunization sera and phosphopeptide column eluate were used as negative controls for PSNF5 lysates from cells treated with 1 mM hydroxyurea for 16 h. Extracts from PSNG13 cells treated with hydroxyurea were used as a negative control for the affinity-purified T99p-BLM antibody. (B) Total protein lysates from PSNF5 cells exposed to 1 μM camptothecin for 1 h or 6 h were probed by Western blotting with the T99p-BLM antibody. The 6-h lysate was also incubated with λ-protein phosphatase (λ-Pase) at 30°C for 60 min with or without protein phosphatase inhibitor (λ-Pase Inh, combination of sodium orthovanadate and sodium fluoride). The lower panel shows lysates probed with BLM antibody. (C) Immunofluorescence images of PSNG13 or PSNF5 cells stained with T99p-BLM antibody after hydroxyurea treatment for 16 h. Nuclear outlines were traced from a parallel image with propidium iodide staining and superimposed on the T99p-BLM images. (D) PSNF5 cells were exposed to 1 μM camptothecin as indicated. (E) Quantitation of T99p-BLM foci per nucleus from experiments using increasing concentrations of either camptothecin or hydroxyurea for indicated times in PSNF5 cells. At least 30 nuclei from three independent experiments were used in the analysis. HU, hydroxyurea; camptothecin, CPT.

Delayed H2AX phosphorylation in BLM-deficient cells treated with camptothecin or hydroxyurea. Phosphorylation of histone H2AX on serine 139 (referred to as γ-H2AX) is an early response to replication-mediated double-strand breaks induced by camptothecin (23). The enhanced sensitivity to camptothecin and Top1-DNA complex formation in BLM-deficient PSNG13 cells led us to hypothesize that BLM might play a role in processing camptothecin-induced Top1-mediated DNA damage. γ-H2AX focus formation in PSNG13 and PSNF5 cells exposed to camptothecin was investigated by confocal microscopy (Fig. 2A). BLM-deficient cells (PSNG13) displayed a consistent (approximately 30 min) delay in γ-H2AX focus formation compared to cells with functional BLM (Fig. 2A and B). We also examined the γ-H2AX foci following camptothecin removal. Following a 12-h exposure to 1 μM camptothecin, γ-H2AX foci reversed similarly in PSNG13 and PSNF5 cells (Fig. 2A). The effects of camptothecin on γ-H2AX were compared to replication damage induced by 1 mM hydroxyurea or to 1 Gy ionizing radiation. A quantification of the appearance of γ-H2AX foci at various time points indicated a delayed phosphorylation of H2AX by hydroxyurea but not ionizing radiation in PSNG13 cells (Fig. 2B). Camptothecin-treated BLM-deficient cells also showed slower formation of γ-H2AX formation than BLM-complemented cells when assayed by Western blot analysis (Fig. 2C). γ-H2AX formation was also examined using normal and Bloom syndrome primary fibroblasts (GM00037 and GM01492, respectively). The BLM-deficient GM01492 cells showed delayed appearance of γ-H2AX foci following camptothecin treatment. Collectively, these results suggest that BLM has a role in the initial DNA damage recognition of Top1-DNA complexes and accurate propagation of the DNA damage signal to PIKKs that modify H2AX following exposure of cells to camptothecin and hydroxyurea.

Nuclear relocalization of BLM, PML, and Top3α in response to camptothecin. To investigate the molecular relationships between BLM and the cellular response to Top1-mediated DNA damage by camptothecin, we analyzed the localization patterns of BLM with PML or Top3α by confocal microscopy (Fig. 3A to C). In untreated BLM-complemented PSNF5 cells, BLM and Top3α were colocalized in nuclear foci together with PML. The number of foci per cell was at an average of eight per nucleus, which is consistent with the pattern previously reported in other cell types with wild-type BLM (Fig. 3A to C and Table 1) (21, 58).

Exposure to camptothecin increased the number of BLM foci, resulting in numerous smaller-size bodies with an average of 33 foci per nucleus (Fig. 3A) (see also Fig. 5B). A similar increase was also observed for Top3α and PML foci after camptothecin. Furthermore, replicative stress changed the localization pattern of BLM with Top3α and PML by slightly reducing the colocalized fraction from the >95% costaining observed in control cells (Fig. 3 and Table 2). Quantitative analysis of representative cell populations at 1 h showed that after replicative stress, approximately 68% of foci counted were positive for both BLM and Top3α, while 10% and 22% of the foci contained either Top3α or BLM only, respectively. Additionally, 81% of the foci counted were positive for both BLM and PML, while 8% and 11% contained either PML or

FIG. 5. Replication-dependent phosphorylation of BLM on T99. (A) Schematic representation of replication double-strand breaks (DSB) induced by camptothecin. The left side of the panel shows reversible Top1 cleavage complex trapped by camptothecin (black rectangle). The right side of the panel shows the Top1 cleavage complex on the replicating leading strand leads to a double-strand break by replication runoff (48, 62). Aphidicolin prevents the formation of replication double-strand breaks. (B) BLM-complemented PSNF5 cells were treated with 1 μ M camptothecin for 1 h with or without pretreatment with 10 μ M aphidicolin for 15 min. Cells were stained with the T99p-BLM antisera (green) and total BLM antibody (red). Nuclear images are representative of at least three independent experiments. (C) Graphical representation of the average number of T99p-BLM or BLM foci per nucleus after camptothecin or aphidicolin and camptothecin treatment in PSNF5 cells. At least 30 nuclei from three independent experiments were used in the analysis. CPT, camptothecin; APH, aphidicolin.

BLM only, respectively. Finally, 72% of the foci counted contained for Top3 α and PML, while 5% and 23% of the foci were positive for Top3 α or PML only, respectively.

An impaired focus formation by Top3 α within PML nuclear bodies in BLM-deficient cells has been reported previously (35, 73). Untreated BLM-deficient PSNG13 cells showed a lower fraction of nuclei with clear Top3 α and PML foci than the BLM-complemented cells (Fig. 3D). Following camptothecin treatment, there was an increase in the number of PML and Top3 α foci with a colocalized fraction of approximately 43% after 1 h. The protein level for PML, but not Top3 α , was also found to increase after 6 h of camptothecin treatment in a BLM-independent manner (Fig. 3E and F).

In summary, Top1-trapping by camptothecin led to an increase in BLM, Top3 α , and PML foci and slightly reduced colocalization of BLM with Top3 α and PML.

Phosphorylation of BLM on T99 in response to replication double-strand breaks induced by camptothecin. Hydroxyurea has been shown previously to influence cellular BLM, most notably by inducing phosphorylation of T99 and T122. Detailed studies on the consequences of such phosphorylation have been limited by the availability of an antibody directed to the T99 phosphorylation site on BLM. Therefore, we first generated T99 phosphorylation-specific antisera in rabbits. Control experiments using crude serum extracted after phos-

pho-peptide inoculation as well as the affinity-purified antibody showed a signal in PSNF5 extracts treated with hydroxyurea (Fig. 4A). In contrast, preimmunization rabbit serum and non-phosphorylated column eluate did not show signal in lysates from hydroxyurea-treated PSNF5 cells. Also, lysates from BLM-deficient PSNG13 cells treated with hydroxyurea did not elicit signal. Cellular lysates obtained from BLM-complemented PSNF5 cells demonstrated T99 phosphorylation in response to camptothecin at 1 and 6 h by Western blotting (Fig. 4B). The signal was lost after lambda protein phosphatase treatment, demonstrating that a phospho-epitope was being recognized.

Immunofluorescence microscopy analysis with the T99p-BLM antibodies showed nuclear focus formation in the PSNF5 cells but not in PSNG13 cells exposed to hydroxyurea (Fig. 4C), confirming the specificity of the antibody. Phosphorylation of BLM on T99 was then examined by microscopy at various times of exposure to camptothecin (Fig. 4D). Figure 4E shows the quantitation for the increased formation of T99p-BLM foci in response to either camptothecin or hydroxyurea. T99p-BLM increased both with time of exposure as well as with concentration of camptothecin or hydroxyurea.

We next investigated whether the formation of phosphorylated BLM foci was related to replication-mediated double-

FIG. 6. Phosphorylation of BLM on T99 in the absence of ATM, ATR, or DNA-PK in response to replication double-strand breaks. Fibroblasts from a healthy (GM00637) and an AT patient (GM05849) (A) or ATRkd and ATR wild-type fibroblasts (B) were compared by confocal microscopic analysis for the kinetics of appearance of T99p-BLM foci by camptothecin. ATRkd cells were pretreated with 1 μ g/ml doxycycline (Doxy +) for 24 h to induce the expression of ATR kinase inactive, resulting in ATR kinase dominant-negative status. (C) The results of a quantitative focus analysis for at least 30 cells from two independent experiments are shown. (D) DNA-PK catalytic subunit (DNA-PKcs)-deficient (MO59J/Fus1) and DNA-PKcs-complemented (MO59J/Fus1) cell lines were also studied for the appearance of T99p-BLM foci.

strand breaks (23, 62). For this purpose, cells were pretreated with aphidicolin, a specific inhibitor of replication polymerases that prevents the formation of camptothecin-induced replication-mediated DNA double-strand breaks (Fig. 5A) (23, 31,

34). As shown in Fig. 5B, T99p-BLM appeared as a subset of total BLM foci in camptothecin-treated PSNF5 fibroblasts. Aphidicolin pretreatment inhibited the formation of T99p-BLM foci in response to camptothecin (Fig. 5B and C). Col-

FIG. 7. Colocalization of BLM phosphorylated on T99 with γ -H2AX and dissociation of T99p-BLM from Top3 α in response to replication double-strand breaks. (A) BLM-complemented PSNF5 cells were costained using T99-phospho-BLM (green) and γ -H2AX (red) antibodies following a 1-h exposure to 1 μ M camptothecin. (B) Costaining with T99-pBLM (green) and PML (red) antibodies. (C) Costaining with T99p-BLM (green) and Top3 α (red). (D) Costaining with total BLM (red) and γ -H2AX (green). (E) Costaining with γ -H2AX (green) and PML (red). (F) Costaining with Top3 α (green) and γ -H2AX (red). Confocal microscopy images are representative of at least three independent experiments. (G) Graphical representation of the percentage fraction of localization of either BLM or T99p-BLM with indicated proteins after camptothecin treatment. At least 30 nuclei were used for the analysis from three independent experiments. CPT, camptothecin.

lectively, these results indicate that camptothecin induces T99 phosphorylation of BLM and focus formation by T99p-BLM in a DNA replication-dependent manner.

Role of ATM, ATR, and DNA-PK in phosphorylating BLM on T99 following replication double-strand breaks induced by camptothecin. We compared the contribution of AT mutated protein (ATM), ATR, and DNA-dependent protein kinase (DNA-PK), for phosphorylation of BLM on T99 by using a panel of genetically modified cell lines deficient in the respective proteins. Using confocal microscopy, we analyzed the generation of T99p-BLM foci following camptothecin exposure in AT (GM05849) and normal fibroblasts (GM00637) (Fig. 6A). The quantitation of foci observed is plotted in Fig. 6C. T99p-BLM foci in AT cells treated for 1 and 2 h with camptothecin

were compared to normal fibroblasts. We also compared the level of focus formation in doxycycline-induced ATRkd and ATR wild-type cells (Fig. 6B). ATRkd cells were treated with 1 μ g/ml doxycycline to induce the expression of ATR kinase inactive, resulting in ATR kinase dominant-negative status (12). Although cells treated with camptothecin for 1 h showed reduced foci in ATRkd cells, at 2- and 3-h time points the phosphorylation of BLM appeared comparable. In contrast, cells deficient for the catalytic subunit of DNA-PK (MO59J/Fus9) and complemented with DNA-PK (MO59J/Fus1) did not show any apparent difference in focus counts after camptothecin (Fig. 6D). Collectively, ATM kinase and, to a lesser extent, ATR are involved in the early phosphorylation of BLM on T99. This result is also indicative of a redundancy between

FIG. 8. Proposed dual role of BLM in DNA repair and signal transduction in response to replication-mediated DNA damage. Molecular Interaction Map (MIM) annotation conventions can be found at <http://discover.nci.nih.gov/mim> (3). (A) In unstrained cells, BLM is bound to Top3α in the PML nuclear bodies (1) (bindings between two molecular species are shown as double-headed arrows). The node on the line corresponds to the bimolecular complex (BLM-Top3α [1]). (B) Camptothecin binds to the Top1 cleavage complexes (Top1 CC) (the green node [2] is the CPT-Top1 CC complex) (47). CPT-trapped Top1 CC induces replication blocks (Repl. Block) (62) (3) (activations are shown as green line with open arrowhead). Replication blocks produce DNA damage (4) (conversions are shown as black arrow) (Repl. Damage is replication-mediated double-strand breaks). BLM-Top3α complexes bind to the blocked replication forks (red node) and prevent replication damage (5) by resolving (repairing) the replication blocks. (C) Replication damage induces the phosphorylation of histone H2AX on Ser 139 (6) and phosphorylation of BLM on Thr 99 (7) (phosphorylations are shown as blue arrows). BLM activates H2AX phosphorylation (8). We propose that phosphorylation of BLM on T99 (red node on the BLM phosphorylation line) inhibits its binding to Top3α (9). CPT, camptothecin.

ATM and ATR for phosphorylating BLM in response to replicative damage.

T99-phosphorylated BLM colocalizes with γ-H2AX and tends to dissociate from Top3α or PML in response to camp-

tothecin. To investigate the role of T99-phosphorylated BLM in the response to replication damage by Top1-DNA complexes induced by camptothecin, we investigated the relative localization of T99p-BLM with γ-H2AX, Top3α, and PML (Fig. 7A to F). γ-H2AX foci mark the sites of replicative double-strand breaks induced by camptothecin (23). T99p-BLM was closely colocalized with γ-H2AX (Fig. 7A, 94% average colocalization; Table 2). However, T99p-BLM appeared at different sites from the PML nuclear bodies (30% average colocalization) (Fig. 7B and Table 2). Also, approximately 58% of T99p-BLM failed to associate with Top3α (Fig. 7C and Table 2). The difference in the colocalization patterns of BLM versus T99p-BLM is represented in Fig. 7G and Table 2. Total BLM and γ-H2AX showed an average of 82% colocalization (Fig. 7D). It is also of note that only 17% of PML (Fig. 7E) and 38% of Top3α (Fig. 7F) were associated with γ-H2AX in response to camptothecin. These results demonstrate coincident induction and colocalization of phosphorylated BLM (T99p-BLM) and H2AX (γ-H2AX). They also indicate dissociation of T99p-BLM from Top3α in response to replication double-strand breaks induced by camptothecin.

DISCUSSION

H2AX phosphorylation following replicative stress is delayed in BLM-deficient cells. We used camptothecin to investigate the changes in BLM associated with replication double-strand breaks (a model is shown in Fig. 5A) (48) and provide a link between the phosphorylation of BLM with the cellular response to replication fork blockage. Our results in the BLM-isogenic human cell line model are consistent with earlier reports showing that BLM-null mouse embryo fibroblasts and *Saccharomyces cerevisiae sgs1* or *Schizosaccharomyces pombe rqh1* mutants are hypersensitive to camptothecin (6, 17). Enhanced camptothecin sensitivity in the PSNG13 human cell line can be explained, at least in part, by the increased steady-state level of Top1 cleavage complexes (Fig. 1). The levels of Top1 protein were, however, comparable in PSNG13 and PSNF5 cell lines. We also observed greater Top2 cleavage complexes in BLM-deficient PSNG13 cells treated with etoposide (data not shown), collectively suggesting enhanced accessibility of chromatin to topoisomerases or reduced rates of removal in BLM-deficient cells.

We observed an unexpected delay in phosphorylation of H2AX in BLM-deficient cells treated with camptothecin (Fig. 2). The delay in H2AX phosphorylation was also apparent with hydroxyurea but not with ionizing radiation, suggesting a replication-dependent defect in signaling for γ-H2AX in BLM-deficient cells. Thus, under replicative stress, BLM might act as a transducer facilitating H2AX phosphorylation in response to replication damage. This proposed role for BLM is consistent with a recent report showing that cells lacking BLM are deficient in activating/phosphorylating the DNA damage sensor kinase ATM at serine 1981 in response to hydroxyurea (14). The absence of BLM has also been shown to impair the focus forming ability of the MRN complex and BRCA1 in response to hydroxyurea (13, 21). It is therefore likely that the signaling for γ-H2AX by BLM is indirect via ATM and PIKKs or the MRN complex. To our knowledge, the only other protein known to similarly promote γ-H2AX formation by the PIKKs

is MDC1 (mediator of DNA damage checkpoint protein 1). MDC1 silencing has been demonstrated to abrogate the phosphorylation of H2AX as well as BRCA1, DNA-PK, and Chk1 and the formation of foci containing Nbs1, 53BP1, and BRCA1 (42, 43, 61). Thus, our results suggest that BLM functions as a transducer facilitating histone H2AX phosphorylation and recruitment of repair factors under replicative stress.

Replication-dependent phosphorylation of BLM (T99p-BLM) and colocalization with γ -H2AX. To further examine the upstream events leading to BLM phosphorylation and the potential interactions between phosphorylated BLM and PML, Top3 α , and γ -H2AX, we generated phosphospecific T99p-BLM polyclonal antibodies. Previously, three kinases, ATM, ATR, and DNA-PK, have been suggested in separate reports to be involved in BLM phosphorylation (7, 15, 44). We first tested cells deficient in the PIKKs (ATM, ATR, and DNA-PK) for their ability to generate T99p-BLM foci and found evidence to suggest an overlapping role for the ATM and ATR kinases in initiating this phosphorylation (Fig. 6A and B). The loss of DNA-PK did not appear to abrogate the phosphorylation of BLM (Fig. 6D). Thus, we conclude that there is redundancy in the PIKK system for phosphorylating BLM on T99. It is likely that the nature of DNA damage and the damage sensors involved could play a role in selecting one or more of these kinases for modifying BLM.

Data obtained with our phospho-specific T99p-BLM antibodies provide evidence for the selective localization of the phosphorylated form of BLM to sites of replication double-strand breaks (γ -H2AX foci) after camptothecin treatment. The T99p-BLM appeared as a fraction of the total BLM nuclear signal. While we observed a strict colocalization between the T99p-BLM and γ -H2AX, T99p-BLM did not colocalize with PML or Top3 α to the extent of BLM (Fig. 7). Most importantly, we conclude that the phosphorylated BLM on T99 is strictly associated with γ -H2AX at the replication damage sites. Using aphidicolin, we demonstrate a replication-dependent phosphorylation of BLM by camptothecin (Fig. 5A). A recent report from Eladad et al. suggests that the intracellular trafficking of BLM to the PML nuclear bodies is regulated by SUMO modification (18). Another component of a BLM complex that could regulate its translocation is the recently identified BLAP75 protein (74). BLAP75 was found to colocalize with BLM, while its deficiency led to the abrogation of BLM phosphorylation and the instability of BLM and Top3 α protein levels after DNA damage. It is therefore possible that the translocation of BLM following replication stress is regulated by phosphorylation-independent events.

Dual function of BLM in replication fork repair and DNA damage signaling. We present a molecular interaction map (3) for the proposed functions of BLM following replication stress in response to Top1-DNA cleavage complexes (Fig. 8). Under healthy conditions, both BLM and Top3 α are found at the potential storage sites of PML nuclear bodies (Fig. 8A) (9, 29, 30, 72). Our finding that total BLM and Top3 α proteins remain associated following DNA damage supports the proposed function of BLM and Top3 α as a repair complex (Fig. 3A to D) (16, 19, 71). The relocalization and diffusion of BLM in association with Top3 α are consistent with a functional role of the BLM-Top3 α complex. The BLM-Top3 α complex has

been proposed to resolve stalled replication forks by resolution of recombination intermediates (double Holliday junctions) (35, 38, 68, 70, 72, 75). It has also been proposed that Top1-induced replication double-strand breaks could be resolved by replication fork regression and formation of double Holliday junctions (48). BLM helicase activity could also restart replication forks following resolution (Fig. 7B) (see also Fig. 6 in reference 45).

If the resolution of the stalled replication fork fails (Fig. 7C), we propose that the replication double-strand breaks are recognized as DNA damage leading to γ -H2AX formation and phosphorylation of BLM on T99 by PIKKs (ATM/ATR) around the break sites. The lack of colocalization between Top3 α and T99p-BLM suggests that phosphorylation of BLM on T99 leads to its dissociation from Top3 α . T99p-BLM might then act independently of Top3 α , possibly as a signaling molecule in replication repair. Collectively, these results suggest that phosphorylation of BLM under replication stress might signal a unique functional switch in response to replication damage. Recent studies utilizing *sgs1 Δ* strains of *S. cerevisiae* have revealed a role for Sgs1p in signaling S-phase DNA damage (10).

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