Monopolar Spindle 1 (MPS1) Protein-dependent Phosphorylation of RecQ-mediated Genome Instability Protein 2 (RM12) at Serine 112 Is Essential for BLM-Topo III α-RMI1-RMI2 (BTR) Protein Complex Function upon Spindle Assembly Checkpoint (SAC) Activation during Mitosis

Received for publication, March 19, 2013, and in revised form, October 2, 2013. Published, JBC Papers in Press, October 9, 2013, DOI 10.1074/jbc.M113.470823

Arun Pradhan1, Thiym Raming Singh1‡, Abdullah Mahmood Ali1‡, Kebola Wahengbam1‡ and Amom Ruhikanta Meetei1‡¶

From the 1Experimental Hematology and Cancer Biology and Cancer and Blood Diseases Institute, Cincinnati Children’s Hospital Medical Center (CCHMC), Cincinnati, Ohio 45229, the 2Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229, and the 3Department of Biotechnology, Manipur University, Canchipur 795003, India

Background: The BTR complex is required for genomic stability and is posttranslationally modified in cells arrested at the mitotic spindle assembly checkpoint (SAC).

Results: We identify serine 112 (Ser-112) as an MPS1-dependent phosphorylation site on RM12.

Conclusion: MPS1-dependent phosphorylation of RM12 regulates the chromosomal maintenance upon SAC activation during mitosis.

Significance: Phosphorylation of RM12 regulates SAC checkpoint signaling and maintains genomic stability.

Genomic instability and a predisposition to cancer are hallmarks of Bloom syndrome, an autosomal recessive disease arising from mutations in the BLM gene. BLM is a RecQ helicase component of the BLM-Topo III α-RMI1-RM12 (BTR) complex, which maintains chromosome stability at the spindle assembly checkpoint (SAC). Other members of the BTR complex include Topo IIIα, RM11, and RM12. All members of the BTR complex are essential for maintaining the stable genome. Interestingly, the BTR complex is posttranslationally modified upon SAC activation during mitosis, but its significance remains unknown. In this study, we show that two proteins that interact with BLM, RM11 and RM12, are phosphorylated upon SAC activation, and, like BLM, RM11, and RM12, are phosphorylated in an MPS1-dependent manner. An S112A mutant of RM12 localized normally in cells and was found in SAC-induced co-immunoprecipitations of the BTR complex. However, in RM12-depleted cells, an S112A mutant disrupted the mitotic arrest upon SAC activation. The failure of cells to maintain mitotic arrest, due to lack of phosphorylation at Ser-112, results in high genomic instability characterized by micronuclei, multiple nuclei, and a wide distribution of aberrantly segregating chromosomes. We found that the S112A mutant of RM12 showed defects in redistribution between the nucleoplasm and nuclear matrix. The phosphorylation at Ser-112 of RM12 is independent of BLM and is not required for the stability of the BTR complex, BLM focus formation, and chromatin targeting in response to replication stress.

Overall, this study suggests that the phosphorylation of the BTR complex is essential to maintain a stable genome.

Bloom syndrome is characterized by severe growth retardation, immunodeficiency, reduced fertility, and a predisposition to cancer (1). This autosomal recessive disease that stems from mutations in the BLM,2 a RecQ helicase implicated in DNA replication, repair, recombination, telomere maintenance, and transcription (2).

BLM interacts with several proteins and exists in three well-characterized complexes: 1) the BASC (BRCA1-associated genome surveillance complex), which contains tumor suppressors, DNA damage repair proteins (i.e., MSH2, MSH6, MLH1, ATM, and BLM), as well as the RAD50-MRE11-NBS1 protein complex; 2) the BRAFT complex (BLM, RPA, Fanconi anemia, and Topo IIIα), which consists of the Fanconi anemia core complex, RAM, RPA, Topo IIIα, RM11 (RecQ-mediated genome instability protein 1, BLAP75) and RM12 (RecQ-mediated genome instability protein 2, BLAP18) (3, 4); and 3) the BTR complex, which contains Topo IIIα, RM11, RM12, and the BLM protein (3–6).

The BLM protein resides at the core of the BTR complex that includes Topo IIIα, RM11, and RM12. BLM interacts directly with another BTR component, RM12, an 18-kDa protein containing an OB-fold domain that we and others have recently isolated and characterized (5, 6). A third BTR component, RM11, does not bind BLM directly but also has an OB-fold (oligonucleotide/oligosaccharide binding) domain. The OB-
fold domains of RMI1 and RMI2 heterodimerize, likely bringing RMI2 to the BTR complex (5, 7).

In our previous in vitro studies, the BTR complex was shown to resolve double Holliday junctions (5). The BTR complex was further shown to be unstable in vitro without the RMI2 component, and RMI2-deficient cells under replication stress could not efficiently recruit BLM to chromatin and nuclear foci (5). These data suggest that RMI2 holds key functions in the BTR complex maintenance of chromosome stability.

Consistent with the genomic instability of Bloom syndrome patients, BLM-deficient cells accumulate chromosomal abnormalities: various numbers of chromosomes, a high frequency of chromosomal breaks, and a 10-fold increase in sister chromatid exchanges (SCEs) (2). It is not clear why BLM-deficient cells harbor these abnormalities, but studies of a BLM mutant defective for Ser-144 phosphorylation showed that instabilities were triggered upon activation of the spindle assembly checkpoint (SAC) (8).

The SAC is a mechanism that monitors the proper attachment of chromosomes to the mitotic spindle (the apparatus that pulls chromatids apart toward the opposite poles). Thus, the SAC prevents cells from entering anaphase until the chromosomes have aligned (9). BLM is phosphorylated during mitosis (M) phase and becomes hyperphosphorylated in response to agents that interfere with microtubule assembly (e.g. taxol, nocodazole, and colcemid) (5, 7, 8).

Like BLM deficiency, RMI2 deficiency causes chromosome instability (i.e. chromosomal breaks and an elevated rate of SCEs), suggesting that RMI2 and BLM function in the same pathway to maintain a stable genome (7). Also, like BLM, both RMI1 and RMI2 are phosphorylated in response to agents that interfere in microtubule assembly (5, 7). Given that RMI1 and RMI2 are part of the BLM complex and that cells lacking these proteins show similar phenotypes and similar phosphorylation profiles, it seems that they are likely to be targeted by the same kinase.

Here we sought to determine the identity of this kinase. We demonstrate that mitotic arrest triggers phosphorylation of both RMI1 and RMI2. Moreover, we demonstrate that this phosphorylation is dependent on the mitotic kinase monopolar spindle 1 (MPS1), a mitotic checkpoint kinase that is required for mitotic arrest in the presence of unaligned chromosomes. This observation is consistent with studies showing that MPS1 expression and activity increased during M phase and peaked at SAC activation. Our studies identify an SP motif in RMI2 at Ser-112 and show that Ser-112 phosphorylation is essential for maintaining a stable genome.

EXPERIMENTAL PROCEDURES

Cell Cultures—HeLa, HEK293, and the fibroblasts GM00637 (WT) and GM08505 (BLM−/−) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals) in a humidified atmosphere of 5% CO2 at 37 °C.

Chemicals—Hydroxyurea (Sigma) was suspended in PBS to a stock concentration of 1 mm. Nocodazole and taxol (Sigma) were suspended in DMSO to stock concentrations of 1 mg/ml and 10 mm, respectively. Puromycin (Sigma) was dissolved at a concentration of 10 mg/ml.

Antibodies—Affinity-purified rabbit polyclonal antibodies against RMI2 have been described earlier (5). A polyclonal antibody against BLM was described elsewhere (4), as were Topo III and RMI1 antibodies (7). The goat polyclonal anti-actin antibody and rabbit polyclonal anti-lamin A/C antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-MPS1 was from Bethyl Laboratories, Inc., and the anti-histone H2A antibody was from Millipore.

shRNA or siRNA Knockdown and Generation of Stable Cell Lines—For stable knockdown of endogenous RMI2, we used the pLKO.1 lentiviral system. The pLKO.1:Puro-sh control was described earlier (10). The pLKO.1:Puro-shRMI2 construct was made by cloning the double stranded oligos RMI2-shRNA-Top (5′-CCGCG-CTATGTGGAGACTGTCGTTAAACTC-GAGTTTAACGCAGTTTCCCAATAGGTGTTTGG-3′) and RMI2-shRNA-Bottom (5′-AATTCAAAAC-CTATGTTGG-AACGGCTGTTAAACCTCAGGTGTTAACGACAGTTCCAACATAGG-3′) in the AgeI and EcoRI sites of the pLKO.1 vector. DNA oligos were obtained from Integrated DNA Technologies, Inc. The lentiviral particles were made at the Cincinnati Children’s Research Foundation Core Facility. Stable cell lines were generated by transducing the cells with lentivirus particles as described earlier (5). Puromycin-positive cells were selected at a puromycin concentration of 3 μg/ml.

MPS1 siRNA oligonucleotides were described earlier (8) and were purchased from Dharmacon (Chicago, IL). Cells were transfected with siRNA using Lipofectamine 2000 for 5 h in reduced-serum OptiMEM medium as recommended by the manufacturer (Invitrogen). After 5 h, OptiMEM was removed and replaced by complete DMEM. Cells were harvested 4 days post-transfection and analyzed by immunoblotting.

Mammalian Expression Constructs and Stable Cell Lines—The pMIEG3 retroviral vector and the generation of retroviruses were as described earlier (5). The His-6-FLAG (HF)-tagged RMI2 (HF-RMI2) construct was described earlier (5). HF-RMI2_{S112A} was generated by PCR-mediated site-directed mutagenesis and cloned into the BamHI and Xhol sites of the pMIEG3 vector to generate pMIEG3-HF-RMI2-S112A. Stable cell lines were generated by transducing the cells with lentivirus particles as described earlier (5). GFP-positive cells were sorted and collected using FACs at Cincinnati Children’s FACs facility. GM00637 (WT) and GM08505 cells expressing HF-RMI2 were described earlier (5).

Protein Phosphatase Treatment—HeLa cells expressing HF-RMI2 were either treated with taxol for 16 h or left untreated. Cell lysates were prepared, and HF-RMI2 was immunoprecipitated with anti-M2-agarose. M2-agarose-bound proteins were incubated at 30 °C with 400 units of λ-protein phosphatase (New England Biolabs), either in the presence or absence of phosphatase inhibitors, for 60 min prior to immunoblot analysis. For the inhibition of phosphatase activity, a combination of 10 μM sodium orthovanadate and 50 μM sodium fluoride was added to the protein samples prior to the addition of the λ-protein phosphatase.

Immunoprecipitation and Immunoblotting—Protein complexes associated with HF-RMI2 and HF-RMI2_{S112A} were iso-
labeled by single-step affinity chromatography as described earlier (5). Briefly, cells were washed with PBS and pelleted. Cell pellets were lysed in buffer (40 mM Hepes (pH 7.9), 300 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% IGEPAL, 0.5 mM sodium orthovanadate, 50 mM sodium fluoride, protease inhibitor mixture, and 2 mM PMSF). Lysates were cleared of cellular debris by centrifugation. To purify FLAG-tagged proteins, clarified lysate was incubated with equilibrated anti-FLAG M2-agarose over-night, washed three times with lysis buffer, and eluted with 3× FLAG peptide for 1 h on ice. Protein samples were boiled in SDS buffer, resolved on a 8–16% gradient gel, blotted onto a nitrocellulose membrane, and probed with the respective antibodies as indicated in each figure.

Immunofluorescence—Cells were grown on poly-D-lysine-coated glass coverslips for a minimum of 24 h prior to treatment. Treated or untreated cells were then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, washed with PBS, permeabilized for 5 min with 0.3% Triton X-100 in PBS, and washed again with PBS. To observe tubulin staining and nuclear morphology, cells were left untreated or were exposed to nocodazole (100 ng/ml) for 10 h. Permeabilized cells were incubated (1 h at room temperature) with primary antibody (rabbit anti-α tubulin antibody, 1:200, Abcam). After incubation, cells were washed with PBS (three times, 5 min each) and then incubated with the Rhodamine B-conjugated, donkey anti-rabbit IgG secondary antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA), for 1 h at room temperature.

To stain DNA, cells were washed three times with PBS, mounted over Vectashield-containing DAPI (Vector Laboratories), and sealed with nail polish. Slides were analyzed using a Zeiss Axiovert 200 M microscope (Carl Zeiss, Thornwood, NY). Images were processed into figures using Photoshop (Adobe).

To study BLM focus formation, cells were grown on coverslips and treated with hydroxyurea (2 mM) for 16 h and processed as described earlier (5). To measure the accumulation of the mitotic cell population following nocodazole treatment, phosphorylation on Ser-10 of histone H3 was measured by a method described previously (5).

Metaphase Spread—To analyze gaps/breaks and numbers of chromosomes per cell, exponentially growing cells were treated with colcemid (Invitrogen) (100 ng/ml) for 2 h, harvested, and resuspended in 75 mM KCl. Cells were then incubated for 30 min at 37 °C, fixed with methanol/acetic acid (3:1), and then washed three times for 30 min. The cell suspension was dropped onto ice-cold, wet glass slides and air-dried. The cells were then stained with Giemsa solution for 2 min and examined under light microscopy. SCE analysis was carried out as described previously (8).

Cell Cycle Analysis—Cell cycle analysis was carried out using a method described earlier (10). The DNA content was determined by measuring fluorescence intensities on a FACS at Cincinnati Children’s FACS facility.

Subcellular Fractionation—To track the presence of BLM and RM12 in the nucleoplasm and nuclear matrix, subcellular fractions were made as described earlier (11). For chromatin-targeting studies, cellular fractions were made using a method described earlier (5).

RESULTS

BTR Complex Proteins Are Phosphorylated in Response to SAC Activation—BLM and RM12 are phosphorylated in cells arrested in M phase of the cell cycle in response to drugs that interfere with microtubule assembly (i.e. taxol and nocodazole) (5, 6, 8, 11). Our group reported earlier that, like BLM, both the endogenous and the exogenously expressed RM12 is phosphorylated when cells were treated with these agents (5). To determine which other BTR complex components might be similarly modified, HeLa cells were left untreated or treated with 1 μM taxol or 200 ng/ml nocodazole for 16 h, and the total lysate was immunoblotted to assess the migration of BTR complex members. Immunoblot analysis of total lysates confirmed that taxol- or nocodazole-induced cells do express low-mobility forms of BLM and RM12 (Fig. 1A) because of their phosphorylation, as reported earlier (5). In this analysis, although taxol or nocodazole treatment did not affect the mobility of Topo IIIa, it did cause cells to produce a low-mobility form of RM11. This suggests that, in taxol- or nocodazole-treated cells, another component of the BTR complex is also posttranslationally modified during mitosis, like RM12 and BLM (Fig. 1A).

Taxol or nocodazole treatment results in SAC activation because of disruption of the microtubule assembly. These results show that several members of the BTR complex are phosphorylated upon SAC activation but do not determine whether the modified forms associate. Next we wanted to find out whether the posttranslationally modified forms of BLM, RM11, and RM12 exist in complex. We immunoprecipitated stably expressing His6-FLAG-tagged RM12 (HF-RM12) from HeLa cells using M2-agarose, and the immunoprecipitated sample was immunoblotted for BTR complex members. As shown in Fig. 1B, the posttranslationally modified forms of BLM, RM11, and RM12 exist in the same complex.

Next we wished to determine whether the posttranslationally modified form of RM11 is phosphorylated. We treated the IP samples with α phosphatase both in the absence and presence of phosphatase inhibitor. As shown in Fig. 1B, the slower-migrating form of RM11 disappeared along with BLM and RM12 after the phosphatase treatment. This apparent dephosphorylation was blocked by phosphatase inhibitors. These slower-migrating forms of all three proteins reappeared, which suggests that, like BLM and RM12, RM11 is phosphorylated during mitosis.

Mitotic Phosphorylation of RM11 and RM12 Is Independent of BLM—Because BLM phosphorylation requires RM11 and RM12 (6, 7), we wished to determine whether the phosphorylation of RM11 and RM12 is dependent on BLM. Stably expressed HF-RM12 was immunoprecipitated from BLM-null (GM08505) or wild-type cells (GM00637) using M2-agarose, and immunoprecipitates were immunoblotted for BTR complex members. As shown in Fig. 1C, RM11 and RM12 were phos-
Phosphorylated in both WT and BLM-deficient cells, demonstrating that BLM is not required for RMI1 and RMI2 phosphorylation.

Phosphorylation of RMI1 and RMI2 Requires MPS1—During mitosis, the MPS1 kinase phosphorylates Ser-144 of BLM (8). Because RMI1 and RMI2 are also phosphorylated during mitosis, we asked whether MPS1 might also phosphorylate RMI1 and RMI2. To test this, we used siRNA against MPS1 to knock down MPS1 protein expression in HeLa cells stably expressing HF-RMI2 that were left untreated or treated with taxol (Fig. 2A). Compared with siControl cells, we observed a significant reduction in MPS1 protein upon RNAi in both untreated and taxol-treated cells (Fig. 2A). Next, to observe phosphorylation of BTR complex members, on the basis of mobility shift on SDS-PAGE, HF-RMI2 was immunoprecipitated using M2-agarose, and the IP samples were immunoblotted for the indicated proteins. Lane 1 (Mock) represents FLAG IP done from HeLa cells that were not expressing HF-RMI2. The phosphorylated form of His6-FLAG-tagged RMI2 (pHF-RMI2) is also shown.

To further confirm that the defective phosphorylation of RMI2 is due to MPS1 deficiency and not an off-target effect of MPS1 siRNA, we complemented the MPS1-depleted cells by ectopic expression of MPS1 protein. As shown in Fig. 2C, ectopic expression of MPS1 in cells depleted of endogenous MPS1 (via siRNA knockdown) rescued the defective phosphorylation of RMI2, suggesting that the phosphorylation defect of

![FIGURE 1. Phosphorylation of BTR complex components. A, immunoblot analysis showing the phosphorylation of endogenous BLM (pBLM), Topo IIIa, RMI1 (pRMI1), and RMI2 (pRMI2). Cells were grown in the presence (+) or absence (−) of taxol (1 μM) or nocodazole (200 ng/ml) for 16 h. Cell lysates were prepared using 2× SDS loading buffer and immunoblotted for the indicated proteins. The asterisk represents a cross-reacting band observed in total lysate. B, immunoblot analysis showing the effect of λ-protein phosphatase (λ-PPase) treatment on the slower-migrating forms of BLM, RMI1, and RMI2. Cells were untreated (−) or treated (+) for 16 h, and lysate was immunoprecipitated for RMI2 using M2-agarose. The IP sample was left untreated (−) or treated (+) with λ-protein phosphatase and its inhibitors, eluted using FLAG peptide, and immunoblotted for the indicated proteins. Lane 1 (Mock) represents FLAG IP done from HeLa cells that were not expressing HF-RMI2. The phosphorylated form of His6-FLAG-tagged RMI2 (pHF-RMI2) is also shown. C, phosphorylation of RMI1 and RMI2 is independent on BLM. Fibroblast cell lines GM00637 (WT) or GM08505 (BLM−/−) stably expressing HF-RMI2 were left untreated (−) or treated with taxol for 16 h. RMI2 was immunoprecipitated using M2-agarose, and immunoblotted proteins are indicated. Lane 1 (Mock) represents FLAG IP done from GM00637 cells that were not expressing HF-RMI2.

MPS1-phosphorylates the RMI2 that is in a complex with BLM during M phase (Fig. 2B). Interestingly, the profile of the RMI1 mobility shift was distinct. In MPS1-deficient cells, RMI1 was not completely converted to the low-mobility form and appeared to be an intermediate of that found in the untreated and taxol-treated cell lysates (partially phosphorylated, Fig. 2B). This suggests that, although phosphorylation of RMI2 in the BTR complex requires MPS1, the phosphorylation of RMI1 is only partly dependent on MPS1. Because MPS1-deficient cells still partially phosphorylate RMI1 during SAC arrest, another kinase must also be phosphorylating RMI1 during mitosis.

To further confirm that the defective phosphorylation of RMI2 is due to MPS1 deficiency and not an off-target effect of MPS1 siRNA, we complemented the MPS1-depleted cells by ectopic expression of MPS1 protein. As shown in Fig. 2C, ectopic expression of MPS1 in cells depleted of endogenous MPS1 (via siRNA knockdown) rescued the defective phosphorylation of RMI2, suggesting that the phosphorylation defect of
RMI2 is specifically due to MPS1 deficiency. However, we did not observe any significant increase in RMI2 phosphorylation in cells overexpressing MPS1 kinase (Fig. 2D).

**RMI2 Is Phosphorylated at Serine 112**—BLM is phosphorylated at Ser-144, Ser-1290, and Ser-1296 in an MPS1-dependent manner (8). In vitro kinase assays, Ser-144 was phosphorylated by MPS1 directly. In contrast, Ser-1290 and Ser-1296 were not phosphorylated by MPS1 directly, but knockdown of MPS1 in cultured cells blocked phosphorylation of these residues (8). Interestingly, all three of these serines are part of SP motifs.
Because RMI2 phosphorylation is also dependent on MPS1, we hypothesized that the kinase target might also be an SP motif. An inspection of the RMI2 sequence revealed two SP motifs, represented by serine at positions 20 (Ser-20) and 112 (Ser-112). The motif at Ser-20 is not conserved across all species, whereas that at Ser-112 is conserved in all species examined (Fig. 2E).

To test whether this conserved SP motif is a phosphorylation target of MPS1, it was mutated to alanine (S112A) and stably expressed in HeLa cells as a recombinant, His-FLAG-tagged mutant (HF-RMI2S112A). Cells expressing HF-RMI2WT and HF-RMI2S112A were tested for their response to a 16-hour exposure to taxol. Cell lysates were immunoprecipitated using M2-agarose and immunoblotted for BTR complex members (Fig. 2F). As shown in Fig. 2F, unlike the WT, the mutant form of RMI2 (HF-RMI2S112A) was not phosphorylated. Moreover, in cells expressing the mutant RMI12, phosphorylation of BLM and RMI1 was normal (Fig. 2F, lane 5), suggesting that Ser-112 is the site of M phase-dependent phosphorylation.

The structure of RMI2 is known (12, 13). Computer modeling predicts no obvious structural changes for the Ser-112 mutant, suggesting that the lack of phosphorylation is not due to altered structure (Fig. 2G). Furthermore, the immunoprecipitations show that RMI2S112A forms a complex with other BTR members, so RMI2 phosphorylation is not required for BTR complex assembly.

### Phosphorylation of RMI2 at Ser-112 Is Not Required for BTR Complex Stability

The stability of the BTR complex appears to require RMI2 because cultured cells deficient for RMI2 show a modest decrease in BLM levels and a dramatic decrease in RMI1 and Topo IIIa levels (5, 6). Stability of the BTR complex might similarly require RMI12 phosphorylation at Ser-112. To test this, the expression of endogenous RMI2 was blocked in HeLa cells by expressing a shRNA targeting the 3'-UTR (Fig. 3A). Cells were then transduced with retroviral vectors expressing either recombinant HF-RMI2WT or HF-RMI2S112A. Fig. 3A confirms the previous results, showing that RMI2 deficiency decreases the levels of RMI1 and Topo IIIa, but protein levels were restored by expressing either the WT or the S112A mutant (Fig. 3A). This experiment shows that Ser-112 phosphorylation of RMI2 is not essential for BTR complex stability.

### Phosphorylation of RMI2 at Ser-112 Is Required for Mitotic Arrest upon SAC Activation

SAC activation results in mitotic arrest upon nocodazole treatment, which were further enhanced upon nocodazole treatment. The wild type but not the Ser-112 mutant of RMI2 could rescue the defect observed in these cells, suggesting that phosphorylation at Ser-112 is required for mitotic arrest (Fig. 3, C and D). Failure to phosphorylate RMI2 at Ser-112 results in early exit from metaphase.

### Phosphorylation of RMI2 at Ser-112 Is Required for Chromosome Segregation

Like BLM deficiency, RMI2 deficiency renders cells susceptible to increased SCE, chromosome breaks and gaps, and a wide distribution of chromosomes per cell (5, 6, 8). Therefore, we examined the role of the RMI2 phosphorylation status in regulating SCE and chromosome stability. We did not observe any appreciable difference in SCE frequency between the WT and S112A (data not shown).

For the analysis of chromosome breakage and distribution of chromosome numbers, Giemsa-stained metaphase spreads were made from cells expressing the WT or S112A mutant and scored for chromosome gaps/breaks and chromosome numbers per metaphase. Although neither line showed an appreciable difference in chromosome breakage/gaps, lines expressing the S112A mutant contained a broader distribution of chromosomes per cell (Fig. 3E), suggesting that Ser-112 phosphorylation controls proper chromosome segregation.

### Phosphorylation of RMI2 at Ser-112 Is Not Required for BLM Focus Formation and Chromatin Localization

Both BLM and RMI2 are predominantly nuclear proteins. When the localization of these proteins is visualized under immunofluorescence microscopy, they are both found in promyelocytic leukemia nuclear bodies (5). Upon treatment with hydroxyurea (a replication fork-stalling agent), both BLM and RMI2 redistribute, and BLM forms foci on chromatin that are sites of stalled replication forks (5). RMI2 is required for BLM focus formation (5, 6). We examined whether the phosphorylation of RMI2 at Ser-112 is required for BLM focus formation following treatment with hydroxyurea. The wild type or S112A mutant were expressed in HeLa cells in which endogenous RMI2 was knocked down, and hydroxyurea-induced foci of BLM were detected by immunofluorescence microscopy using anti-BLM antibody. We did not observe any significant difference in BLM focus formation between the wild type and mutant (Fig. 4, A and B).

To examine which cellular compartment contained BTR proteins, extracts from the cells that were untreated or hydroxyurea-treated (Fig. 4C) were fractionated and analyzed. Fractionation confirmed that BLM and RMI2 were normally present in the nucleoplasm, nuclear matrix, and chromatin fractions (11) and that hydroxyurea treatment caused BLM to...
associate with chromatin in all cells tested. Furthermore, cells coexpressing the shRNA and either the recombinant WT or mutant RM12 showed no appreciable difference (Fig. 4C). In all cells, BLM localized to chromatin-associated foci. We therefore conclude that Ser-112 phosphorylation is not required for the recruitment of BLM to stalled replication forks.

Phosphorylation of RM12 at Ser-112 Is Required for Redistribution within the Nucleoplasm and Nuclear Matrix—In exponentially growing cells, BLM and RM12 are observed in both the nucleoplasmic and nuclear matrix fractions (11). Within the nucleus, their pattern of subnuclear localization changes depending on whether cells are growing exponentially or are challenged with agents that interfere with microtubule assembly (11). In mitotic cells, the phosphorylated form of BLM was associated with the nucleoplasm and was not found associated with the nuclear matrix (11). To check the subcellular localization of RM12 in exponentially growing and metaphase-arrested cells, we treated cell lines expressing RM12WT and RM12S112A with either taxol or left them untreated. We found that, in mitotic cells, BLM and RM12 were mostly present in the nucle-
MPS1-dependent Phosphorylation of BTR Complex

SCE (5, 7, 8). Although the increase in SCE and gaps and breaks can be explained by a defective BTR that cannot dissolve Holliday junctions, it does not explain the other defects (i.e., multinucleation, micronuclei, and chromosome number variation). Interestingly, a phosphorylation-defective mutant of BLM at Ser-114 that was proficient in SCE and had no increase levels of chromosome gaps and breaks failed to complement the multinucleation, micronuclei, and chromosome number variation defects upon SAC activation, suggesting that phosphorylation plays a key role in the suppression of these phenotypes (8). Our previous studies showed that RMI2 is also phosphorylated during mitosis upon SAC activation because of interfering with spindle assembly, but its significance remained unknown (5, 7). A better understanding of the composition and posttranslational modifications of BTR complex members provides insight into functions of this complex, thus revealing its role in preventing genomic instability.

Here we further assessed the posttranslational modifications of BTR complex components, revealing that, like BLM, both RM11 and RMI2 are posttranslationally modified upon SAC activation during mitosis. We did not observe any appreciable mobility shift of Topo IIIa, suggesting that it may not be modified upon SAC activation or that the difference in mobility may not be appreciable under the conditions we used to assay for modifications. We demonstrate here that the posttranslational forms of BLM, RM11, and RMI2 (triggered by SAC activation) are due to phosphorylation because treatment of cells with a phosphatase resulted in the disappearance of the slow-migrating bands of BLM, RM11, and RMI2. Moreover, blocking the phosphatase activity resulted in the reappearance of the slow-migrating bands.

We also show that BLM, RM11, and RMI2 phosphorylation does not control BTR complex assembly. Indeed, phosphorylation of RM11 and RMI2 is independent of BLM. Like BLM, however, phosphorylation of RM11 and RMI2 is dependent on MPS1 kinase. We identified the RMI2 site phosphorylated by MPS1 as Ser-112. Although Ser-112 phosphorylation was not required for complex assembly or stability, it was required for metaphase arrest. Cells expressing the S112A mutant exited metaphase prematurely and appeared to enter cytokinesis. This effect was reminiscent of similar experiments in cells stably expressing the S144A mutant of BLM. Cells defective in either phosphorylation event were also defective in cytokinesis and accumulated micronuclei and multinuclei. Also, these cells cannot properly segregate chromosomes to their daughter cells.

The data presented here provide further evidence that the BTR complex is not only essential for preventing SCEs but also for proper chromosome segregation. The phosphorylation of BLM, RM11, and RMI2 during mitosis, which is required for redistribution of the BTR complex between the nuclear matrix and nucleoplasm, which are defective in phosphorylation-defective mutants, is essential for chromosome segregation. Finally, this study supports the hypothesis that the genomic instability of BTR-deficient cells is not solely the effect of impaired Holliday junction dissolution but also triggered by defects in chromosome segregation during mitosis.

DISCUSSION

The BTR core complex consists of BLM, Topo IIIa, RM11, and RMI2. This evolutionarily conserved complex is required for genomic stability (5, 7). Among the BTR members, BLM is the only protein associated with Bloom syndrome, a genetic disease characterized by predisposition to a broad spectrum of cancers (1). Nevertheless, BTR complex deficiency of any member triggers genomic instability (5, 7).

Genomic instability in BTR complex-deficient cells manifests as multinucleation, micronuclei, chromosome number variation, chromosomal gaps or breaks, and an elevated rate of...
Acknowledgments—We thank the Viral Vector Core, DNA Sequencing, and Fluorescent Activated Cell Analyzing and Sorting facility of the Cincinnati Children’s Research Foundation.

REFERENCES

1. German, J., and Ellis, N. A. (2002) in The Genetic Basis of Human Cancer (Vogelstein, B., and Kinzler, K. W., eds.), 2nd Ed., pp. 267–288, McGraw-Hill, New York
2. Chu, W. K., and Hickson, I. D. (2009) RecQ helicases. Multifunctional genome caretakers. Nat. Rev. Cancer 9, 644–654
3. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev. 14, 927–939
4. Meetei, A. R., Sechi, S., Wallisch, M., Yang, D., Young, M. K., Joenje, H., Hoatlin, M. E., and Wang, W. (2003) A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol. Cell Biol. 23, 3417–3426
5. Singh, T. R., Ali, A. M., Basygina, V., Raynard, S., Fan, Q., Du, C. H., Andreassen, P. R., Sung, P., and Meetei, A. R. (2008) BLAP18/RMI2, a novel OB-fold-containing protein, is an essential component of the Bloom helicase-double Holliday junction dissolvasome. Genes Dev. 22, 2856–2868
6. Xu, D., Guo, R., Sobeck, A., Bachrati, C. Z., Yang, J., Enomoto, T., Brown, G. W., Hoatlin, M. E., Hickson, I. D., and Wang, W. (2008) RMI, a new OB-fold complex essential for Bloom syndrome protein to maintain genome stability. Genes Dev. 22, 2843–2855
7. Yin, J., Sobeck, A., Xu, C., Meetei, A. R., Hoatlin, M., Li, L., and Wang, W. (2005) BLAP75, an essential component of Bloom’s syndrome protein complexes that maintain genome integrity. EMBO J. 24, 1465–1476
8. Leng, M., Chan, D. W., Luo, H., Zhu, C., Qin, J., and Wang, Y. (2006) MPS1-dependent mitotic BLM phosphorylation is important for chromosome stability. Proc. Natl. Acad. Sci. U.S.A. 103, 11485–11490
9. Musacchio, A. (2011) Spindle assembly checkpoint. The third decade. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 3595–3604
10. Ali, A. M., Pradhan, A., Singh, T. R., Du, C., Li, J., Wahengbam, K., Grassman, E., Auerbach, A. D., Pang, Q., and Meetei, A. R. (2012) FAAP20. A novel ubiquitin-binding FA nuclear core-complex protein required for functional integrity of the FA-BRCA DNA repair pathway. Blood 119, 3285–3294
11. Dutertre, S., Ababou, M., Onclecrq, R., Delic, J., Chatton, B., Jaulin, C., and Amor-Guéret, M. (2000) Cell cycle regulation of the endogenous wild type Bloom’s syndrome DNA helicase. Oncogene 19, 2731–2738
12. Hoadley, K. A., Xu, D., Xue, Y., Satyshur, K. A., Wang, W., and Keck, J. L. (2010) Structure and cellular roles of the RMI core complex from the Bloom syndrome dissolvasome. Structure 18, 1149–1158
13. Wang, F., Yang, Y., Singh, T. R., Basygina, V., Guo, R., Wan, K., Wang, W., Sung, P., Meetei, A. R., and Lei, M. (2010) Crystal structures of RMI1 and RMI2, two OB-fold regulatory subunits of the BLM complex. Structure 18, 1159–1170
14. Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL workspace. A web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201
15. Kiefer, F., Arnold, K., Künzli, M., Bordoli, L., and Schwede, T. (2009) The SWISS-MODEL Repository and associated resources. Nucleic Acids Res. 37, D387–D392
16. Peitsch, M. C. (1995) Protein modeling by E-mail. Nat. Biotechnol. 13, 658–660