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Phosphorylation of the Bloom's Syndrome Helicase and Its Role in Recovery from S-Phase Arrest

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Bloom's syndrome (BS) is a human genetic disorder associated with cancer predisposition. The BS gene product, BLM, is a member of the RecQ helicase family, which is required for the maintenance of genome stability in all organisms. In budding and fission yeasts, loss of RecQ helicase function confers sensitivity to inhibitors of DNA replication, such as hydroxyurea (HU), by failure to execute normal cell cycle progression following recovery from such an S-phase arrest. We have examined the role of the human BLM protein in recovery from S-phase arrest mediated by HU and have probed whether the stress-activated ATR kinase, which functions in checkpoint signaling during S-phase arrest, plays a role in the regulation of BLM function. We show that, consistent with a role for BLM in protection of human cells against the toxicity associated with arrest of DNA replication, BS cells are hypersensitive to HU. BLM physically associates with ATR (ataxia telangiectasia and *rad3*$^+$ related) protein and is phosphorylated on two residues in the N-terminal domain, Thr-99 and Thr-122, by this kinase. Moreover, BS cells ectopically expressing a BLM protein containing phosphorylation-resistant T99A/T122A substitutions fail to adequately recover from an HU-induced replication blockade, and the cells subsequently arrest at a caffeine-sensitive G_2/M checkpoint. These abnormalities are not associated with a failure of the BLM-T99A/T122A protein to localize to replication foci or to colocalize either with ATR itself or with other proteins that are required for response to DNA damage, such as phosphorylated histone H2AX and RAD51. Our data indicate that RecQ helicases play a conserved role in recovery from perturbations in DNA replication and are consistent with a model in which RecQ helicases act to restore productive DNA replication following S-phase arrest and hence prevent subsequent genomic instability.

The RecQ family of DNA helicases has been highly conserved throughout the history of evolution from bacteria to humans (30, 32, 42). In bacteria and yeasts, there is a single family member in each case (RecQ in *Escherichia coli*, Sgs1 in *Saccharomyces cerevisiae*, and Rqh1 in *Schizosaccharomyces pombe*), while there are at least five for humans. Defects in three of the human RecQ members give rise to defined genetic disorders that are associated with cancer predisposition and/or premature aging. These disorders are Bloom's syndrome (BS), Werner's syndrome, and Rothmund-Thomson syndrome, which are caused by loss of function mutations in *BLM*, *WRN*, and *RECQ4*, respectively (18, 35, 67). At the cellular level, RecQ helicases are rarely essential for viability but seem to be universally required for the maintenance of genome stability.

BS is a rare disorder that is associated with a wide range of abnormalities, including growth retardation, immunodeficiency, reduced fertility, sensitivity to sunlight, and a greatly increased incidence of cancer (25, 30, 60) Indeed, BS is probably unique among cancer predisposition disorders of humans in that it is associated with an increase in the incidence of a diverse range of cancer types, including leukemias, lymphomas, and carcinomas. Cells isolated from BS individuals display elevated frequencies of chromosomal breaks, translocations, and other aberrations, but the hallmark feature, which is used in diagnosis of the disorder, is an approximately 10-fold elevation in the rate of sister chromatid exchanges (SCEs) (12). SCEs are generated by homologous recombination in the late S phase or G_2 phase of the cell cycle (56). Consistent with the notion that BS cells show more general hyperrecombination, BS cells have been reported to show an increase in interchromosomal homologous recombination (25, 26, 30, 60). Moreover, hyperrecombination is a characteristic of yeast *sgs1* and *rqh1* mutants; in the latter case, this outcome is particularly evident in cells that have previously been exposed to hydroxyurea (HU), a ribonucleotide reductase inhibitor that arrests DNA replication (22, 57, 63).

Although alterations in the rates at which genetic recombination events occur are characteristic of RecQ helicase mutants, it is widely thought that this abnormality is a downstream consequence of a primary defect in DNA replication (13, 46). Bacterial, yeast, and human RecQ helicase-deficient cells display abnormalities in DNA replication, which may manifest as an apparently unperturbed S phase or, more frequently, following UV irradiation-induced perturbation of replication, DNA replication inhibitors such as HU, or DNA-damaging drugs. Indeed, many RecQ helicase-deficient mutants are hypersensitive to one or more of these agents and display a defect in responding to replicational stress of this sort. For example, BS cell lines accumulate abnormally sized DNA replication

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intermediates and have a protracted S phase (39). *S. cerevisiae sgs1* mutants are hypersensitive to HU and show a defect specifically in S-phase checkpoint responses to both DNA damage and replication blockade (21). Similarly, *S. pombe rqh1* mutants are HU sensitive and defective in recovery from an S-phase arrest: they are able to complete bulk DNA replication following release from the arrest, but they then undergo an aberrant mitosis (41, 57).

The pattern of expression and subcellular localization of RecQ helicases are also suggestive of a role for these enzymes in DNA replication. Many RecQ helicases, including BLM, accumulate in cells only at or following progression through the G_1/S transition in the cell division cycle (4, 34). Moreover, Sgs1p and BLM (as well as WRN) localize to sites of DNA replication either constitutively (particularly during late S phase) or more strikingly following perturbation of replication (21, 50, 55). These data suggest that there is a regulated translocation of RecQ helicase to sites of damaged or arrested replication forks in order to assist in restoration of DNA synthesis. Numerous models for the role(s) of RecQ helicases in replication fork repair have been proposed (30, 46, 60). These fall broadly into two categories that are not mutually exclusive: those that propose a role in the prevention of replication fork demise (for example, through the removal of aberrant DNA secondary structures from the template to smooth passage of the replisome) and secondly, those that propose a role alongside the homologous recombination machinery in repairing collapsed or damaged forks. The conserved interactions between RecQ helicases and proteins required either for DNA replication, such as replication protein A (6, 7, 16), or for homologous recombination, such as RAD51 (64), are consistent with these proposed roles.

One of the cellular responses to DNA damage and replication blockade is the activation of cell cycle checkpoints that serve to arrest cell cycle progression in order to allow time for the repair or bypass of DNA damage. In general terms, checkpoints comprise three components: the sensors of DNA structural abnormalities, the signal transducers, and the effector molecules (45, 69). Some components, particularly protein kinases, may act both as sensors and as signal transducers by binding (directly or indirectly) to damaged DNA and subsequently phosphorylating other checkpoint proteins (59). One family of protein kinases that has been implicated in such a role is the phosphoinositide 3 (PI-3) kinase family (2, 33, 40, 48, 53). This family comprises proteins that are usually very large (with molecular masses of 200 kDa or greater) and that contain a conserved kinase domain, which is generally located near the C-terminal end of the protein; these proteins also have extended N-terminal regions of various lengths that play roles in binding other proteins or DNA in some cases (2, 33, 40, 48, 53). Among this family are the Mec1 and Tel1 proteins of *S. cerevisiae* and the Rad3 and Tel1 proteins of *S. pombe* (38, 58, 68). In humans, several PI-3 kinases exist, not all of which have been characterized in detail. The closest homologue of $Mec1^{Sc}$ and $Rad3^{Sp}$ is the ATR (ataxia telangiectasia and *rad3*$^+$ related) kinase, while the closest Tel1 homologue is ATM (ataxia telangiectasia mutated) (2, 33, 40, 48, 53). ATR is activated in response to DNA replication blocks and by DNA-damaging agents such as UV light (38, 58, 68). In contrast, ATM appears to be activated primarily by ionizing radiation or radiomimetic agents that generate DNA double-strand breaks (53). Despite this difference in activation, the ATR and ATM kinases phosphorylate many of the same downstream targets, including several that are required for checkpoint functions, such as the Chk1 and Chk2 kinases (33, 38, 58, 68). *ATR* is an essential gene in mammals (as is *MEC1* in budding yeast), although the essential role that it plays remains to be identified (8, 9). *ATM* is not essential for embryonic development in the mouse, but defects in *ATM* in humans give rise to a disorder called ataxia telangiectasia. This rare and debilitating condition is characterized by cerebellar ataxia, oculocutaneous telangiectasias, immune dysfunction, cancer predisposition, and cellular sensitivity to ionizing radiation (40, 53).

It has been reported previously that the BLM protein physically associates with and is a target for the ATM kinase (3). Phosphorylation of BLM on Thr-99 following treatment of cells with ionizing radiation is dependent upon ATM. Moreover, phosphorylation on the same site during an unperturbed mitosis event is partially dependent upon ATM. A second BLM phosphorylation site, Thr-122, may also be targeted by ATM, but to a much lesser extent. Given the proposed role of BLM in S-phase responses to stresses other than DNA double-strand breakage, we and others have analyzed whether BLM is a target for the ATR kinase. Previous work (19) has suggested that BLM is targeted by ATR following exposure of cells to HU, although the consequences of this phosphorylation were not analyzed. We show here that BLM and ATR colocalize to discrete nuclear foci and can be coimmunoprecipitated from cell extracts following arrest of DNA replication. ATR phosphorylates BLM to a similar extent on both Thr-99 and Thr-122. Expression in human BS cells of BLM containing T99A and T122A substitutions leads to a failure to recover normally from S-phase arrest invoked by treatment with HU. This abnormal recovery is associated with a subsequent arrest at a caffeine-sensitive G_2/M checkpoint. Moreover, BS cells are hypersensitive to killing by HU. Our data indicate that BLM is a key downstream target for ATR in the cellular response to inhibition of DNA replication.

MATERIALS AND METHODS

Cell lines. GMO8505 is an SV40-transformed skin fibroblast cell line established from a patient with BS (18). PSNF5 and PSNP2 are stable transfectants expressing a Flag epitope-tagged BLM protein; PSNB2 expresses an untagged BLM protein; and PSNG13, PSNG1, and PSNV4 are vector control transfectants of the same cell line. All of these transfected clones have been described previously (24). Cells were grown in Alpha minimal essential medium (Life Technologies) supplemented with 10% fetal calf serum and 4 mM glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Stable clones were maintained by the addition of 350 μg of G418 (Invitrogen) per ml to the medium. ATR wild-type (WT) and ATR kinase-dead (KD) cells that allow doxycycline-inducible expression of Flag-tagged WT or dominant-negative KD ATR were kindly supplied by P. Nghiem (43) (Howard Hughes Medical Institute, Harvard University, Cambridge, Mass.). Cells expressing WT ATM (GMO4724) or mutated ATM (GMO2782) were obtained from the National Cancer Institute (NCI) registry. Lymphoblastoid cells expressing WT BLM (JB1) were derived from a healthy individual and immortalized by Epstein-Barr virus transformation. GMO3403 is a lymphoblastoid cell line derived from an individual with BS and was obtained from the NCI registry.

In vivo expression of BLM. The *Eco*RI and *Xho*I sites were used to clone BLM N-terminal threonine-to-alanine substitution mutations into the Flag-tagged *BLM* cDNA in pcDNA3, as described in reference 24. GM08505 cells were plated the day before transfection at approximately 2×10^4 per cm^2 and were

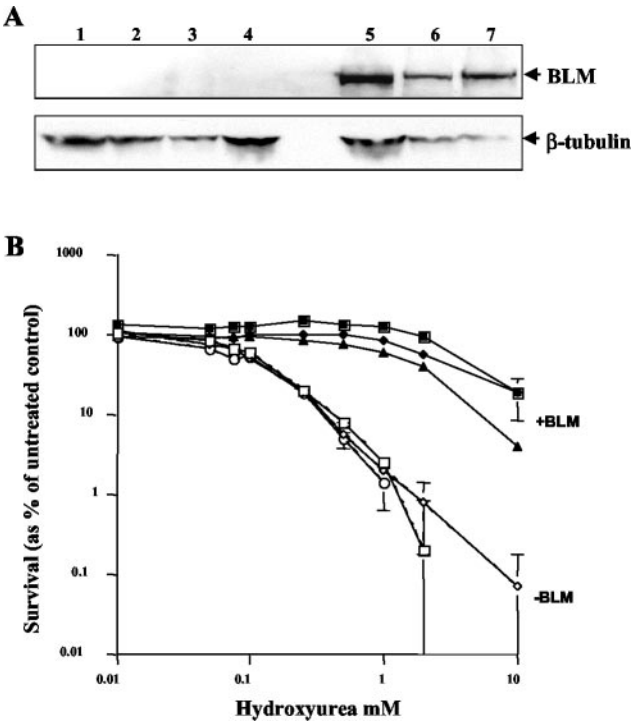

FIG. 1. (A) Western blotting of nuclear extracts to confirm expression of BLM in GMO8505 transfectants. Lane 1, GMO8505 cells; lanes 2 to 4, transfectants of GMO8505 cells containing the pcDNA3 vector only (PSNG1, PSNG13, and PSNV4); lanes 5 to 7, cells expressing the *BLM* cDNA in pcDNA3 (PSNB2, PSNF5, and PSNP2). β-Tubulin was used as a loading control. (B) BS cells are hypersensitive to HU. Clonogenic survival analyses were conducted on three stable transfectants of GMO8505 cells containing either the pcDNA3 vector only (PSNG1, PSNG13, and PSNV4), which are indicated by the open symbols, or cells expressing the *BLM* cDNA in pcDNA3 (PSNB2, PSNF5, and PSNP2), which are indicated by the filled symbols. Cells were exposed to HU for 48 h before washing and being left for 3 weeks to allow colonies to develop. Analyses were performed in triplicate. Error bars represent standard errors of the means.

transfected with BLM cDNA in pcDNA3 vector mutated at the stated phosphorylation sites by using FuGENE (Roche). Stable clones were selected in 750 μg of G418 per ml.

In vitro mutagenesis. Two putative ATM/ATR phosphorylation sites were identified in the N terminus of BLM at residues Thr-99 and Thr-122. Thr-99 was mutated to alanine (T99A) by site-directed mutagenesis of nucleotide 369 (A to **G**), and Thr-122 was mutated to alanine (T122A) by mutation of nucleotide 437 (A to **G**). Sense and antisense strands were generated by PCR using the following primers and subsequently annealed. For T99A, the primers used were 5′-GGA CAGGAA**G**CACAGAGAGGTGGA-3′ and BLMN2 primer with *Xho*I site (5′-GAGACTCGAGTCAGGAGGGTGGAGGCAAATCAG-3′) and 5′-ACCTCT CTGTG**C**TTCCTGTCCTGC-3′ and BLMN5 primer with *Eco*RI site (5′-GAG AGAATTCATGGCTGCTGTTCCTCAAAA-3′); for T122A, the primers used were 5′-GTATGCACT**G**CCCAAAACACACCA-3′ with BLMN2 primer and 5′-TGTGTTTTGGG**C**AGTGCATACAAC -3′ with BLMN5 primer.

Annealed fragments were cloned into pGEX-4T-1 (Amersham Pharmacia Biotech) by using *Eco*RI and *Xho*I sites, as described in reference 65. DNA fragments expressing single mutants were then used as PCR templates to generate double mutants.

Expression and purification of the GST-BLM fragments. Glutathione *S*-transferase (GST) fusion proteins were expressed and purified as described previously by Wu et al. (65), by using BL21 (DE3) cells (New England Biolabs) transformed with pGEX-4T-1 expression plasmids containing WT-BLM, T99A-BLM, T122A-BLM, and T99A/T122A-BLM, all expressing residues 1 to 212 only of BLM.

Antibodies. Anti-BLM mouse monoclonal and rabbit polyclonal antibodies (BFL103 and IHIC33, respectively) as well as rabbit polyclonal antibodies against topoisomerase IIIα (D6) have been described previously (65). Rabbit polyclonal antibodies against RAD51 Ab-1 (Oncogene Research Products), mouse monoclonal antibodies against proliferating cell nuclear antigen (PCNA), rabbit polyclonal antibodies against Chk1 (Santa Cruz Biotechnology), rabbit polyclonal antibodies against phosphorylated Chk1 (Ser345), and phosphorylated H2AX (Upstate Cell Signaling Solutions) were obtained from commercial sources. Mouse monoclonal antibodies against ATM (11G12) were a gift from Malcolm Taylor (University of Birmingham, Birmingham, United Kingdom). Mitotic cells were identified by using rabbit polyclonal antibodies against phosphorylated-Histone H3 (Ser28) (Upstate Cell Signaling Solutions).

TABLE 1. SCE analysis of BS cell transfectants containing the empty pcDNA3 vector (PSNG1 and PSNG13), pcDNA3-WT-BLM (PSNF5 and PSNP2), and pcDNA3-BLM-T99A/T122A (C20, C2.1, and C1.4)

Protein used	Transfectant	No. of chromosomes scored	No. of SCEs	No. of SCEs/ chromosome
−BLM (vector only)	PSNG1	965	1,461	1.51
	PSNG13	437	672	1.54
+BLM (wild-type)	PSNF5	678	188	0.28
	PSNP2	1,066	683	0.64
+BLM (T99A/T122A)	C20	543	245	0.44
	C2.1	544	70	0.13
	C1.4	934	278	0.3

ATR antibody preparation. Either an N-terminal fragment (amino acids 400 to 480) or a C-terminal fragment (amino acids 2341 to 2641) of ATR was expressed as a GST fusion protein in bacteria. The fusion protein was purified and used to inoculate sheep (Diagnostics Scotland, Edinburgh, Scotland) to produce ATR-N and ATR-C antisera. Affinity purification of antibodies was performed using standard procedures. ATR antibodies used for Western blotting (see Fig. 2A) were a kind gift from S. P. Jackson (University of Cambridge), and for use in the procedures depicted in Fig. 2B, antibodies were obtained from Santa Cruz Biotechnology (sc1887). For ATR antibody validation purposes, immunoprecipitations were performed as follows. All steps were performed at 4°C. Sixty micrograms of HeLa nuclear extract was diluted in 250 μl of IPH buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol containing protease inhibitors [Roche]). Extracts were incubated with serum for 1 h prior to addition of protein G Sepharose and incubation with rotation for an additional 1 h. Alternatively, affinity-purified antibodies cross-linked to protein G Sepharose were incubated with extracts for 2 h with rotation. Protein G Sepharose antibody-protein complexes were harvested by centrifugation, and the pellet was washed four times in 250 μl of IPH buffer. Pellets were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and subjected to electrophoresis and Western blotting by standard procedures.

Clonogenic survival assays. Cells were plated at low density for colony formation. The following day, fresh medium containing HU or drug-free medium was added. After 48 h, the medium was removed and cells were washed once in phosphate-buffered saline before colonies were allowed to grow in fresh medium for 14 to 21 days. Colonies were fixed in 3:1 methanol-acetic acid and stained with 1 mg of crystal violet per ml in water. Colonies having more than 50 cells were scored as survivors. The number of colonies at each drug dose was expressed as a percentage of the untreated control.

Kinase assays. ATM kinase assays were performed as described by Canman et al. (10) using kinase immunoprecipitated from cells expressing WT ATM or from ATM-null lymphoblastoid cells. ATR kinase assays were performed using ATR wild-type and ATR KD cell lines. Both kinases were then used to phosphorylate GST-BLM N-terminal fragments according to the procedure described by Cliby et al. (14).

SCE analysis. SCE staining was carried out according to the method of Perry and Wolf (49). Cultures were grown through two cell cycles in the presence of 5 μg of bromodeoxyuridine per ml, and cells were then harvested after the addition of 0.05 μg of Colcemid per ml for the final 2 h.

Metaphase spreads were made after hypotonic treatment using 0.0375 M KCl and fixation with 3:1 (vol/vol) methanol-acetic acid. Slides were stained with Hoechst 33258 at a concentration of 20 μg/ml for 30 min and were then rinsed briefly in distilled water before UV treatment in Sorensen's phosphate buffer (pH 6.8) for 3 h. Slides were rinsed in distilled water and incubated in 2× SSC

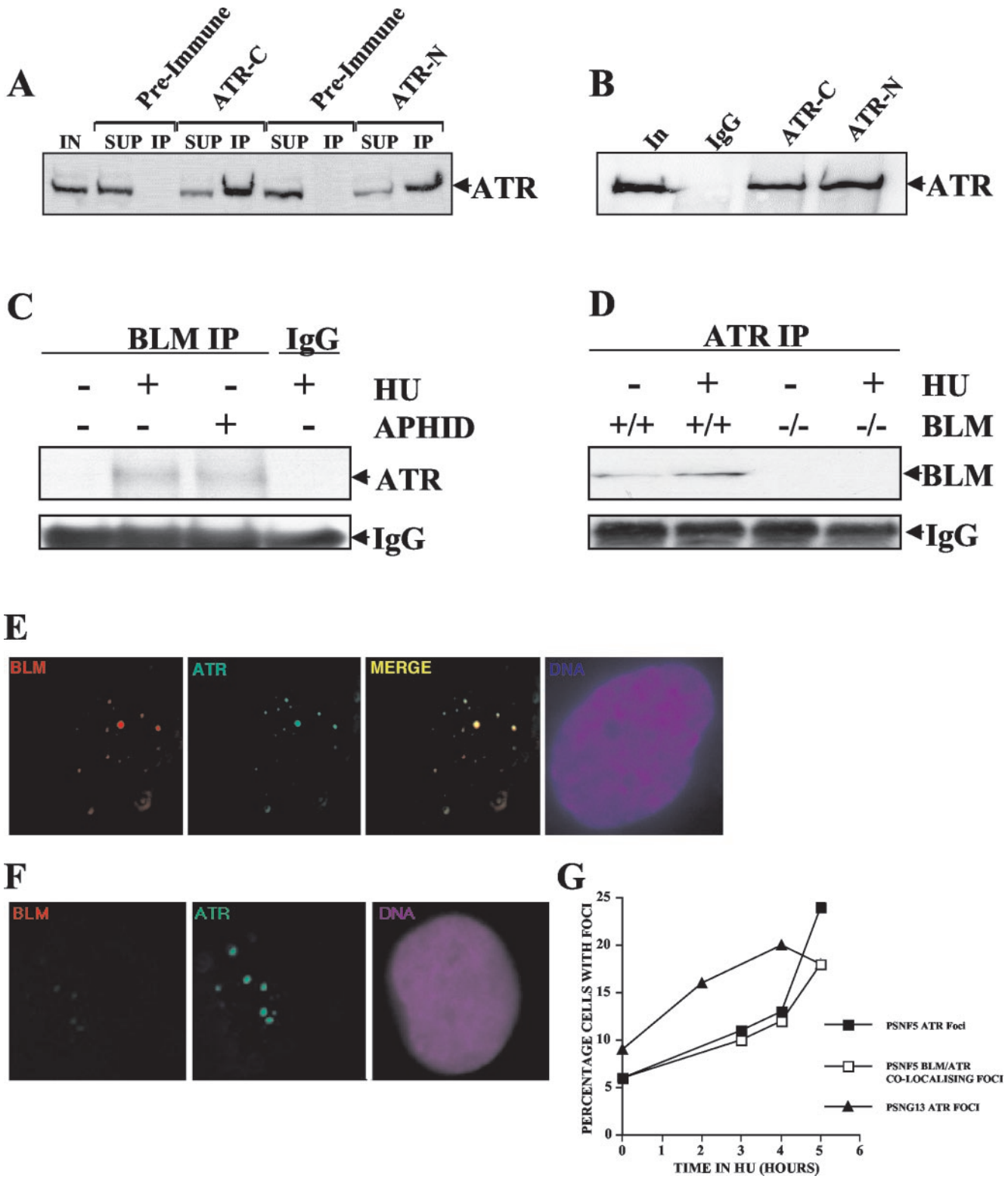

FIG. 2. (A) Validation of anti-ATR antibodies. Immunoprecipitations were carried out by using two independent anti-ATR antibodies raised against the C-terminal region (ATR-C) or the N-terminal region (ATR-N) of ATR. Preimmune antisera were used as negative controls. The immunoprecipitates were Western blotted with an ATR antibody that recognizes the N-terminal domain of ATR (a generous gift of S. P. Jackson, University of Cambridge). ATR was found in the precipitate (IP) when either ATR-C or ATR-N was employed but was found only in the supernatant (SUP) when the preimmune serum was employed. (B) Immunoprecipitations were conducted as described for panel A, except that the Western blotting antibody employed was the commercial anti-ATR FRP1 sc1887 antibody (Santa Cruz Biotechnology). (C) ATR can be coimmunoprecipitated with BLM in cells exposed to replicational stress. Immunoprecipitations were carried out using IHIC33 anti-BLM antibody, or an immunoglobulin G control, on extracts from cells exposed to no treatment (−), HU, or aphidicolin (+), as indicated above the lanes. The

(1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 2 h at 65°C. After being rinsed in distilled water, the chromatids were differentially stained in 3.5% Giemsa in Sorensen's buffer (pH 6.8) for 4 min. More than 30 metaphases were scored for each cell line.

Immunofluorescence studies. Cells to be used for immunofluorescence analysis were fixed and treated as described by Wu et al. (65). To facilitate visualization of ATR foci, soluble proteins were extracted from cells with an ice-cold preextraction buffer (10 mM PIPES, 300 mM sucrose, 3 mM $MgCl_2$, 20 mM NaCl, 0.5% Triton X-100 [pH 6.8]) for 5 min.

Western blotting and immunoprecipitations. Nuclear extracts with a high concentration of salt were prepared from exponentially growing cells and from cells arrested with 2 mM HU or 5 μM aphidicolin for 16 h and were processed as described previously (63). For Chk1 analyses, lysates were prepared as described by Gatei et al. (23).

G_2/M checkpoint analysis. Cells were blocked at the G_1/S boundary by incubating cells for 16 h with 2 mM HU, released into either normal medium or into 4 mM caffeine, and harvested at various time points. Cells were processed for flow cytometric analysis, as described by Xu et al. (66), in order to determine the mitotic index by expression of phosphorylated histone H3. Data were collected by using a Becton Dickinson FACScalibur machine and subsequently analyzed with CellQuest software.

RESULTS

BS cells are hypersensitive to HU. The known defects in DNA replication displayed by BS cells, coupled with the sensitivity of yeast *rqh1* and *sgs1* mutants to HU, led us first to analyze whether BS cell lines may be hypersensitive to HU compared to normal human cells. To study this possibility, we created isogenic cell lines rather than undertaking a comparison of random normal versus BS cell lines. We generated six stably transfected cell lines derived from the SV40-transformed BS fibroblast cell line GMO8505. Three of these lines (designated PSNG1, PSNG13, and PSNV4) were generated by transfection with the empty pcDNA3 vector, and three were produced by transfection with the pcDNA3/BLM construct containing the full-length *BLM* cDNA (designated PSNB2, PSNF5, and PSNP2) (24). For confirmation that transfection with the *BLM* cDNA was able to "correct" the BS phenotype, we demonstrated that the PSNB2, PSNF5, and PSNP2 cell lines expressed BLM protein (Fig. 1A) and had levels of SCEs within a similar range to that seen in WT human cells (Table 1) (24). The six cell lines were then analyzed by using clonogenic survival assays for their relative sensitivity to HU. Figure 1B shows that the three vector-only BS cell transfectants were significantly more sensitive to the cytotoxic effects of HU than were the three corrected cell lines. The degree of sensitivity of the noncorrected BS cells was highly significant, being greater than 10-fold based on D_{37} values.

Association of BLM with the ATR kinase following replication arrest. It is well established that the ATR kinase is activated in human cells following arrest of DNA replication. Given the results presented above, which showed that BS cells are hypersensitive to an agent that causes replication fork arrest, we analyzed whether the BLM protein might associate with ATR. It is known that PI-3 kinase family members can physically associate with at least some of their substrates, and indeed BLM has been shown previously to form a complex with ATM (3). Hence, we analyzed whether BLM and ATR exist in a complex in human cells. This was done by coimmunoprecipitation from either HeLa cells or human lymphoblastoid cell lines. To do this, we generated and validated anti-ATR antibodies for use in both Western blotting and immunoprecipitation analyses (Fig. 2A and B). Despite numerous attempts to demonstrate coimmunoprecipitation of BLM and ATR from nuclear extracts, the results were difficult to replicate consistently (Fig. 2C and data not shown). We reasoned that any association of the two proteins might be dependent upon whether the cells had been exposed to some form of replicational stress prior to performing the immunoprecipitations. We therefore performed coimmunoprecipitation experiments in cells exposed either to HU or to the DNA polymerase inhibitor, aphidicolin. Figure 2C shows that ATR could be readily coimmunoprecipitated with anti-BLM antibodies from HU- or aphidicolin-treated HeLa cell extracts, but not with immunoglobulin G control antibodies. To confirm this result, the reciprocal coimmunoprecipitation experiment was performed (i.e., coimmunoprecipitation with anti-ATR and Western blotting for BLM). BLM could be detected in the ATR immunoprecipitate, and the extent of coimmunoprecipitated BLM was mildly enhanced by prior treatment of the cells with HU (Fig. 2D). As a control of antibody specificity, an identical coimmunoprecipitation experiment was performed using the BS lymphoblastoid cell line GMO3403. As expected, no BLM was detected in the ATR immunoprecipitate in this case. Taken together, these data indicate that ATR and BLM form a complex in human cells and that the extent of complex formation is increased by cellular treatments that cause arrest of DNA replication.

Next, we asked whether BLM and ATR colocalize in the nuclei of human cells. To do this, immunofluorescence studies were performed. In a small percentage (approximately 5%) of PSNF5 corrected BS cells, ATR was found to localize to discrete nuclear foci (Fig. 2E). In cells for which this was observed, these foci colocalized with nuclear foci containing BLM (Fig. 2E). The ability of ATR to localize to these foci was not, however, dependent upon the presence of BLM, since ATR-containing foci were observed in a similar percentage of PSNG13 cells (BS cells transfected with vector only), which lack BLM expression (Fig. 2F). Because of the apparent replication stress-dependent association of BLM and ATR revealed by the coimmunoprecipitation data shown in Fig. 2C

immunoprecipitates were Western blotted for the presence of ATR by using the sheep anti-ATR-N antibodies. (D) BLM can be coimmunoprecipitated with ATR following replicational stress. Immunoprecipitations using the sheep anti-ATR-N antibodies were carried out for normal human lymphoblastoid cells expressing BLM (+/+) or lymphoblastoid BS cells (−/−) and Western blotted for BLM by using the IHIC33 antibody. (E) BLM and ATR colocalize to nuclear foci. Indirect immunofluorescence of BLM (red) and ATR (green) in untreated cells expressing BLM (PSNSF5) are shown. In the merged image, colocalization is shown as yellow. The DNA (blue) panel shows the position of the nucleus, as judged by staining with Hoechst. (F) BLM is not required for the formation of ATR foci. Immunofluorescence analysis was performed as described in panel E, except that the PSNG13 vector-only control BS cells lacking BLM expression were used. (G) The percentage of cells with ATR foci increases in response to HU treatment, and the degree of colocalization of BLM and ATR also increases in response to HU treatment. Cells were incubated with 5 mM HU and fixed for immunofluorescence analyses at the times indicated. The percentage of cells with ATR-, BLM-, and ATR-BLM-colocalizing foci were determined microscopically. A minimum of 100 cells of each type were assessed at each time point.

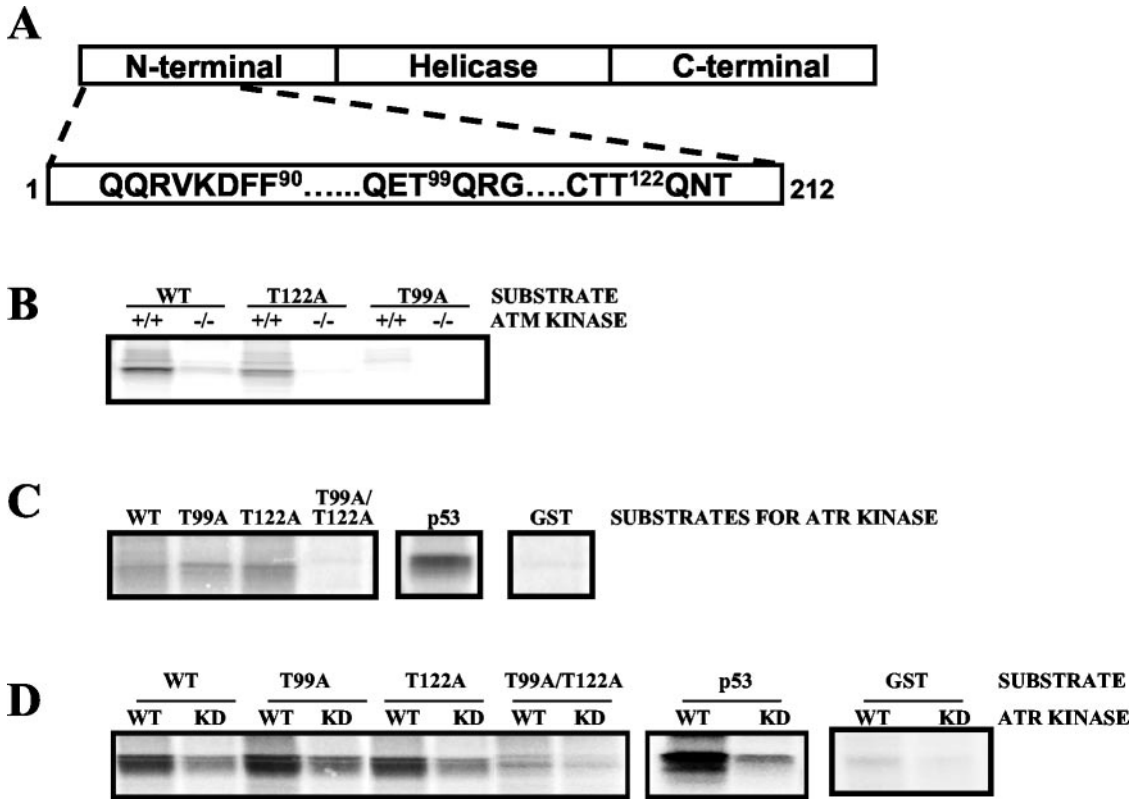

FIG. 3. (A) Schematic representation of the two potential PI-3 kinase recognition sites in the N-terminal region of BLM. The sites at Thr-99 and Thr-122 in each case contain a downstream glutamine residue, as required for recognition by PI-3 kinases. The putative PCNA binding motif QQRVKDFF[90] (conserved residues underlined) is indicated. (B) Phosphorylation of GST-BLM recombinant fragments representing the N-terminal domain between residues 1 and 212. ATM was immunoprecipitated from cells expressing WT ATM (+/+) or from cells expressing mutated ATM (−/−). The immunoprecipitated material was used in phosphorylation reactions containing the GST-BLM-1-212 fragment containing either WT sequence, Thr-99 mutated to alanine (T99A), or Thr-122 mutated to alanine (T122A), as indicated above the lanes. Note the lack of phosphorylation of the T99A protein. (C) ATR kinase assays. ATR was immunoprecipitated from HeLa cell extracts and used to phosphorylate the GST-BLM derivatives depicted in panel B, a doubly substituted derivative (T99A/T122A), and p53 (to act as a positive control) or GST (to act as a negative control). Note the lack of phosphorylation of the T99A/T122A BLM protein. (D) The kinase responsible for phosphorylation of BLM in the immunoprecipitates is ATR. ATR was immunoprecipitated using an anti-Flag antibody from cells expressing inducible WT ATR or a KD version of ATR, and the immunoprecipitate was used to phosphorylate the same proteins as depicted in panel C. Note the considerably reduced phosphorylation of the T99A/T122A BLM derivative.

and D, we asked whether exposure of cells to HU might increase the percentage of cells containing BLM and ATR-colocalizing foci. Indeed, we observed a three- to fourfold increase in the percentage of cells containing ATR foci and a similar fold increase in the percentage of cells showing BLM and ATR-colocalizing foci following exposure of PSNF5 cells to HU for up to 5 h (Fig. 2G). This HU-dependent induction of ATR foci was not dependent on the presence of BLM, as it was also observed in BLM-defective PSNG13 cells (Fig. 2G). These data indicate that ATR and BLM colocalize in human cell nuclei and that the percentage of cells in which colocalization is seen increases significantly following arrest of DNA replication.

BLM is a substrate for ATR. It has been shown previously that BLM is phosphorylated on Thr-99 (and possibly also on Thr-122) by the ATM kinase both in response to gamma irradiation and during apparently unperturbed mitosis (3). To analyze whether these or other sites on BLM were targeted by ATR, we prepared recombinant proteins representing different domains of BLM fused to GST (see Materials and Methods). Because the extreme N-terminal domain of BLM from residues 1 to 212 was the best substrate in vitro for ATM/ATR, we focused on this fragment alone. We created a WT BLM-GST fusion protein as well as derivatives in which residues Thr-99 and Thr-122 were substituted individually or together by alanine (designated T99A and T122A). Figure 3A shows a schematic representation of the region of the BLM protein in which the Thr-99 and Thr-122 residues are located. It should be noted that immediately N terminal to Thr-99 there is a sequence motif that perfectly matches the consensus (61, 62) binding site for the PCNA (see Discussion), which is an essential cofactor in DNA replication. Using these BLM-GST fusion proteins, we first asked if BLM is a substrate for phosphorylation by ATM. For this, ATM was immunoprecipitated from extracts of normal ($ATM^{+/+}$) and ataxia telangiectasia ($ATM^{-/-}$) human cell lines, and the immunoprecipitate was incorporated into kinase assays using either the WT BLM-GST protein as substrate or the T122A or T99A derivatives. In accordance with the results of previous work (3), BLM was a substrate for ATM, and the degree of phosphorylation of the

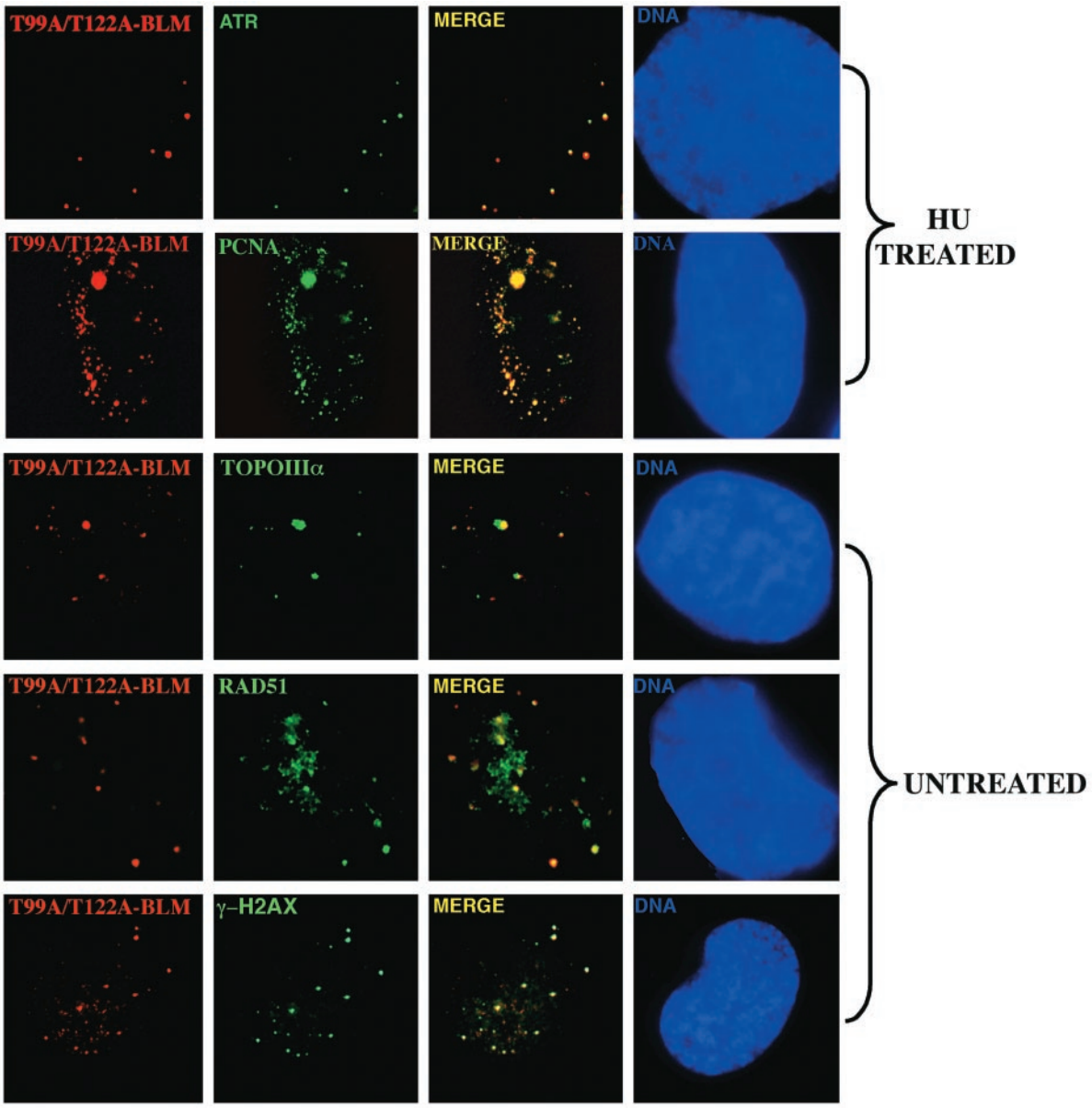

FIG. 4. Formation of BLM foci and colocalization of BLM foci with RAD51, phosphorylated histone H2AX, ATR, PCNA, and topoisomerase IIIα is not dependent upon phosphorylation of BLM on residues Thr-99 or Thr-122. Immunofluorescence analyses were conducted as described in Fig. 2 for cells either untreated (lower 3 rows of panels) or treated with 5 mM HU for 5 h prior to fixation. Colocalization in the merged image is indicated by yellow coloration.

protein was diminished slightly by the T122A substitution but was diminished dramatically by the T99A substitution. Control immunoprecipitations from ATM$^{-/-}$ cells indicated that this phosphorylation was specific for ATM (Fig. 3B). These data confirm that BLM is a substrate for ATM and that the primary target residue for this kinase is Thr-99. Next, we asked whether the N-terminal fragment of BLM is a substrate for the ATR kinase. The data in Fig. 3C show that ATR could phosphorylate the WT BLM fragments as well as each of the single threonine-substituted derivatives. It was only upon substitution of both Thr-99 and Thr-122 (T99A/T122A) that we observed a marked diminution in the extent of phosphorylation of BLM by ATR. Positive and negative controls for the ATR kinase (p53 and GST, respectively) were analyzed in parallel and produced the expected results. These data indicate that BLM is a substrate for the ATR kinase. However, in contrast to the results seen with ATM, both Thr-99 and Thr-122 on BLM appear to be of equal importance quantitatively as targets for ATR. To confirm the specificity of the ATR phosphorylation data, we analyzed phosphorylation of the BLM N-terminal region by ATR immunoprecipitated from cells ectopically expressing either a Flag epitope-tagged WT ATR or a KD version of ATR, as described previously (43, 44). Using p53 as a positive control, we demonstrated that p53 was phosphorylated to a significantly greater extent by the precipitate containing the WT ATR kinase than by a precipitate containing the KD

version of ATR. The background level of phosphorylation seen with the KD version has been observed previously by others using these cell lines (36). The WT ATR kinase was again able to phosphorylate the BLM-1-212 fragment and each of the singly substituted BLM derivatives. However, the level of phosphorylation by ATR of the T99A/T122A double substitution mutant was significantly reduced compared to that seen with either the T99A or the T122A protein. Compared to the kinase reactions utilizing WT ATR immunoprecipitates, the levels of phosphorylation of the BLM-1-212, T99A, and T122A proteins were significantly reduced (and were equivalent to that seen with the p53 control) in immunoprecipitates from cells expressing the KD version of ATR, thereby confirming that the kinase responsible for the bulk of the phosphorylation was ATR. We conclude from this section of the study that BLM is a substrate for the ATR kinase and that both Thr-99 and Thr-122 are apparently targeted to a similar extent.

Substitution of Thr-99 and Thr-122 does not prevent BLM from suppressing SCEs or colocalizing with other replication and repair factors. To analyze the functional effects of the substitution of Thr-99 and Thr-122 in BLM with a nonphosphorylatable residue, we expressed a mutated version of the full-length *BLM* cDNA in GMO8505 cells in which codons for Thr-99 and Thr-122 were altered to become codons for alanine. Four stable transfectants of GMO8505 cells expressing BLM-T99A/T122A were generated and designated C1.4, C1.2, C2.1, and C20. The results of Western blotting for BLM (see Fig. 5A) indicated that these transfectants expressed an equivalent level of BLM to that seen in the PSNF5 cell line transfectant expressing the WT BLM protein from the pcDNA3 expression vector. Next, we analyzed whether expression of BLM-T99A/T122A protein was able to suppress the hyper-SCE phenotype characteristic of BS cells (12). In each case, the transfected clones showed a significantly lower level of SCEs compared to vector-only control BS cell transfectants, indicating that substitution of Thr-99 and Thr-122 by alanine did not prevent BLM from suppressing SCE formation (Table 1 and data not shown).

One possible role of the observed phosphorylation is to influence the ability of BLM to either associate with partner proteins required for DNA repair and replication or to localize to sites of repair and replication. To analyze this possibility, we carried out immunolocalization studies by using fluorescence microscopy. First, we asked whether the substitution of Thr-99 and Thr-122 affected colocalization of BLM with ATR itself in cells exposed to HU. As shown in Fig. 4, the BLM-T99A/T122A protein still colocalized convincingly with ATR. Next, we analyzed colocalization of BLM with PCNA. This was undertaken for two reasons. First, as discussed above, Thr-99 and Thr-122 are located adjacent to a putative PCNA binding motif in the N-terminal domain of BLM, and hence phosphorylation of these residues could influence binding to PCNA. Second, PCNA marks sites of DNA replication and hence permitted us to address the question of whether the nonphosphorylatable BLM was still able to translocate to sites of ongoing DNA replication, as has been shown previously for WT BLM (55). Figure 4 shows that BLM-T99A/T122A was able to colocalize effectively with PCNA in nuclear foci. It has been shown previously that BLM can also colocalize with topoisomerase IIIα and RAD51 in nuclear foci (4, 64, 65). We showed that the BLM-T99A/T122A protein could still colocalize with these two factors, particularly within a subset of the larger nuclear foci (Fig. 4). Another marker of DNA damage responses is the phosphorylation of histone H2AX. It is well established that cells exposed to ionizing radiation or DNA replication inhibitors show focal nuclear staining when an antibody specific for the phosphorylated form of H2AX (termed γH2AX) (51) is used. These foci are widely thought to designate sites of initial DNA damage in the nucleus (52). We found that both WT BLM (not shown) and the BLM-T99A/T122A protein (Fig. 4) colocalized strongly with γH2AX in nuclear foci, indicating that BLM can be found at sites of DNA damage (as suggested previously by the colocalization with RAD51) but that the phosphorylation of Thr-99 or Thr-122 was not essential for this colocalization. Taken together, these immunofluorescence experiments have revealed that BLM protein containing T99A and T122A substitutions is able to accumulate in focal regions of the nucleus and to colocalize with a variety of other proteins that define sites of both DNA damage and ongoing DNA repair as well as of DNA replication.

Substitution of Thr-99 and Thr-122 in BLM affects recovery from replication stress. The most striking phenotype of *S. pombe rqh1* cells, which lack the sole RecQ helicase in that organism, is a failure to undergo productive cell division following release from an HU-induced S-phase arrest (57). To address whether a similar failure to recover from S-phase arrest was seen in the BS cell transfectants expressing BLM-T99A/T122A protein, we treated control cells (PSNF5 transfectants expressing WT BLM) and BLM-T99A/T122A transfectants (clone 1.4) with 2 mM HU for 16 h and then released them from this G_1/S-phase arrest into drug-free medium. Flow cytometry was used to evaluate the degree of S-phase arrest by HU as well as the ability of the cells to recover from this arrest. Recovery was defined by an ability of the cells to progress normally through the remainder of the cell cycle and subsequently to reenter the G_1 phase of the next cell division cycle. As shown in Fig. 5B (left panel), PSNF5 cells arrested normally following treatment with HU for 16 h with the majority of cells at the G_1/S boundary (or very early S phase). Following removal of the HU, these cells progressed synchronously through S phase into G_2/M over the next 16 h and then subsequently reentered the G_1 phase after approximately 18 to 20 h. This is the normal pattern of the cell cycle progression following the release of human cells from an early S-phase arrest. In contrast, the BLM-T99A/T122A cell clones behaved abnormally following HU treatment. There was no obvious defect in the initial response to HU in these cells, since an apparently normal early S-phase arrest was observed. While synchronous progression through the remainder of S phase was evident, with kinetics very similar to those seen in PSNF5 cells, the BLM-T99A/T122A cells entered G_2/M (based on DNA content) but did not progress beyond this point into the subsequent G_1 phase until approximately 24 to 26 h following release from the HU-induced arrest (Fig. 5B, right panel). This pattern of prolonged G_2/M arrest following release was not limited to the clone 1.4 transfectant, since a similar protracted G_2/M phase was seen in three other independent BLM-T99A/T122A-expressing clones following treatment with HU (data not shown).

We next asked whether the cells arrested in G_2/M following

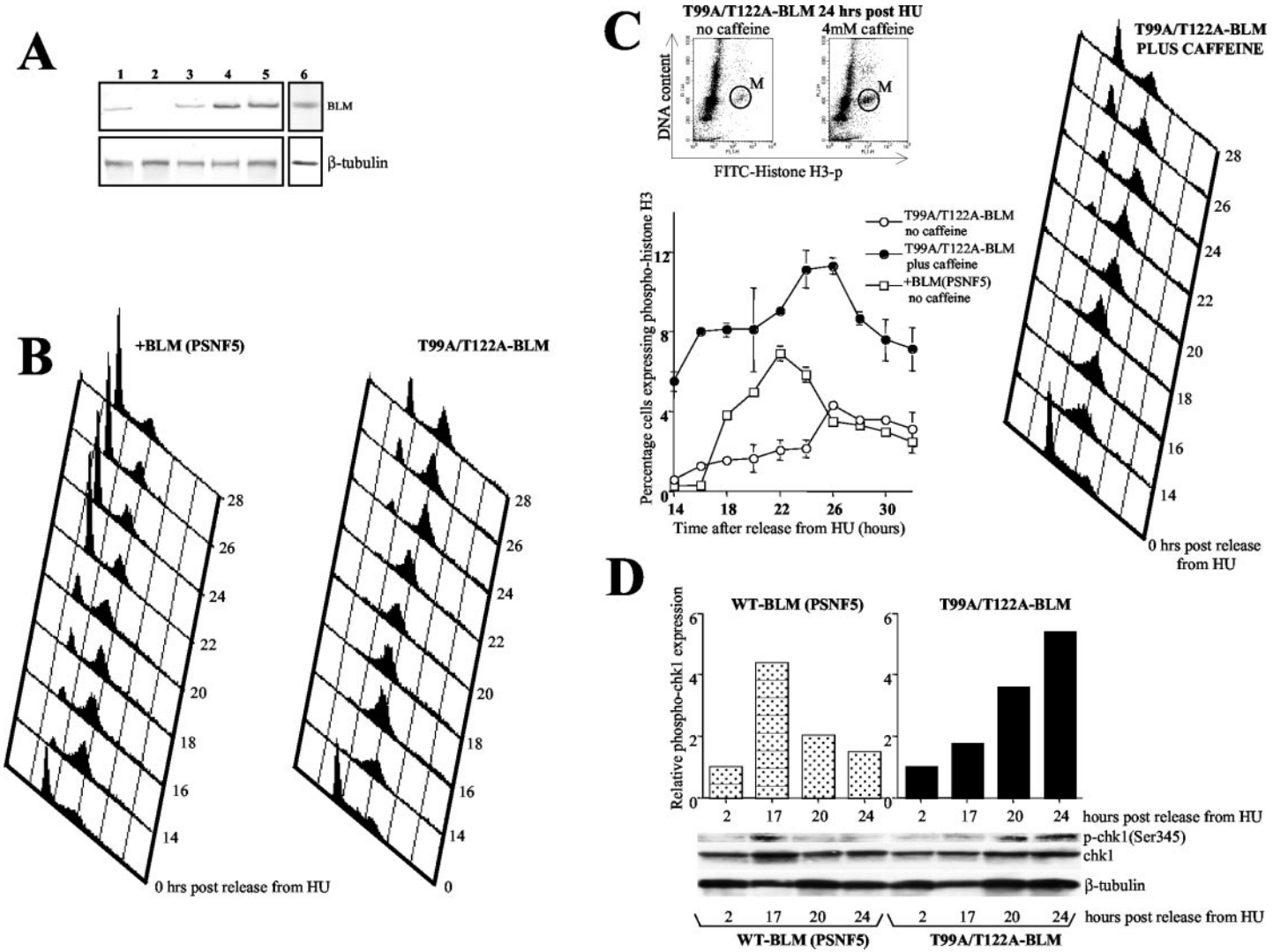

FIG. 5. Cells expressing the ATR phosphorylation site mutants arrest in G_2 following release from replication arrest. (A) Western blotting of crude nuclear extracts to show equivalent BLM expression in control PSNF5 cells (lane 1) and four independent clones of GMO8505 cells expressing BLM-T99A/T122A is shown; clone C2.1 (lane 3), clone C20 (lane 4), clone C1.2 (lane 5) and clone C1.4 (lane 6) are depicted. Lane 2 indicates the untransfected GMO8505 cells for validation of the antibody. β-Tubulin was used as loading control. (B) Exponentially growing cultures of either PSNF5 cells expressing WT BLM or clone 1.4 cells expressing BLM-T99A/T122A were exposed to HU (2 mM) for 16 h and then released into drug-free medium. The DNA content was analyzed by flow cytometry at the times indicated. (C) Analysis of a caffeine-sensitive G_2/M checkpoint in HU-treated cells expressing BLM-T99A/T122A. Cells were treated with HU as before and then released in the presence or absence of 4 mM caffeine. Cells were harvested at the time points indicated and analyzed by flow cytometry for expression of phosphorylated histone H3 to determine the percentage of cells in mitosis (left panel) and for DNA content (right panel). Cells expressing phosphorylated histone H3, and with a 2 N DNA content, are circled and labeled with an M for the 24-h time point. The percentage of cells expressing phosphohistone H3 is shown graphically. Results are expressed as the means of three experiments. Error bars indicate standard errors of the means. (D) Expression of phospho-Chk1. Whole-cell extracts were prepared from cells expressing T99A/T122A-BLM at the times indicated after release from HU block. The extracts were analyzed by Western blotting for total Chk1, phospho-Chk1 (Ser 345), and β-tubulin levels (shown by representative blots). The bar graph indicates quantification of the blotting data (normalized for β-tubulin levels) and represents the means of two determinations.

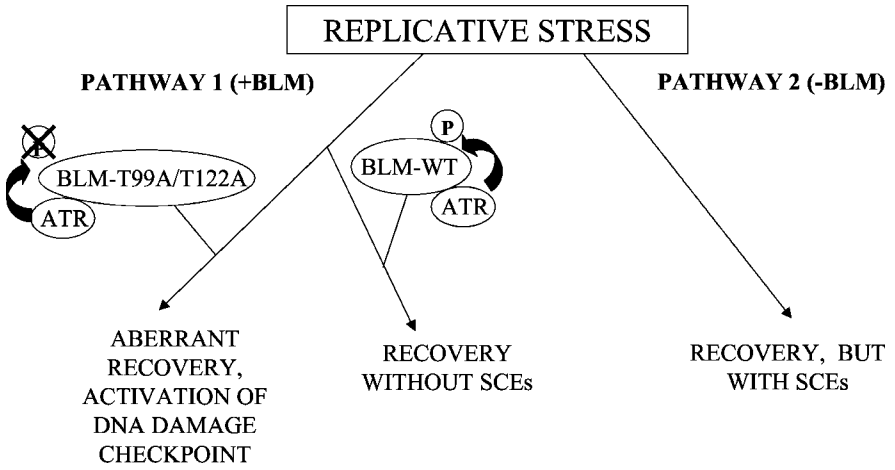

FIG. 6. Model for the role of ATR-mediated phosphorylation of BLM in recovery from replicative stress. Two parallel pathways exist. Pathway 1 (left) is activated in cells expressing BLM protein (+BLM), while pathway 2 (right) is operational in BS cells lacking BLM protein. In pathway 1, cells expressing WT BLM (BLM-WT) display ATR-dependent phosphorylation of Thr-99 and Thr-122 (circled P), and these cells recover from replicative stress via a pathway that avoids SCEs. In cells expressing BLM-T99A/T122A, ATR cannot phosphorylate BLM (circled P with a cross) but are committed to the BLM-dependent pathway, resulting in aberrant recovery associated with G_2 checkpoint arrest.

HU treatment and release were in the G_2 phase or in mitosis. To do this, we stained cells after release from HU arrest for phosphorylated histone H3, a marker of cells in mitosis. Twenty-four hours after release, only 2% of the cells were positive for this marker, suggesting that they were held in G_2 (Fig. 5C, left panel). We reasoned that the abnormal recovery from S-phase arrest in the T99A/T122A cell clones might reflect some form of defective replication following release from the arrest and that this might therefore lead to an accumulation of abnormal DNA structures that subsequently activate a G_2/M checkpoint. To analyze this possibility, we studied whether the checkpoint-overriding drug, caffeine, could drive clone 1.4 cells through the G_2 arrest point into mitosis and hence into the subsequent G_1 phase. Figure 5C shows that, in cells treated with 4 mM caffeine, the G_2 arrest was overridden, as evidenced by an increase in the percentage of histone H3-positive, M-phase cells (up fivefold to over 10%) at the 24-h time point after release from HU arrest and by the accelerated rate of return of cells into G_1 compared to that of clone 1.4 cells not treated with caffeine (compare flow cytometry profiles in Fig. 5B and C). Additional evidence for the existence of a checkpoint-mediated G_2 block following HU-treatment of clone 1.4 was provided by an analysis of the phosphorylation status of Chk1, which was performed by using an antibody specific for phosphoserine 345 (p-Chk1) that detects a modification of Chk1 induced by replication-blocking agents (68). Figure 5D shows that, in PSNF5 cells expressing WT-BLM, p-Chk1 levels peak at 17 h following release from HU treatment and decline at later time points, consistent with their progression through G_2/M and into the next cell cycle. In contrast, in clone 1.4 cells expressing BLM-T99A/T122A, p-Chk1 levels remained elevated even at 24 h after release. Taken together, these data indicate that clone 1.4 BLM-T99A/T122A cells arrest normally in early S phase in the presence of HU, progress synchronously through the remainder of S phase following release into HU-free medium, but then arrest at a caffeine-sensitive G_2/M checkpoint for a protracted period of time (at least 6 to 8 h).

DISCUSSION

We have shown that the BS helicase, BLM, can interact with the ATR kinase and that it is phosphorylated by ATR on residues Thr-99 and Thr-122. BS cells expressing a modified version of BLM, in which the Thr-99 and Thr-122 residues are substituted by alanine, fail to recover adequately from replication arrest.

Previous studies have indicated that BLM is a phosphoprotein in human cells and that phosphorylation induced in response to treatment with ionizing radiation is ATM dependent (3). Similarly, it has been reported that BLM is phosphorylated following treatment of cells with DNA replication inhibitors but that this process is ATM independent (1). Although the kinase responsible for phosphorylation of BLM during times of replicational stress was not identified definitively by previous studies, ATR has, not surprisingly, been proposed as a leading candidate (20), given its known role in responding to S-phase perturbations (14). Our data are fully consistent with this proposal.

It has long been proposed that the genomic instability characteristic of RecQ helicase-deficient mutant cells is a manifestation of a primary defect of some aspect of DNA replication (13, 46). Since RecQ helicase deficiency is rarely associated with a total loss of cell viability, it is extremely unlikely that RecQ helicases are required for the catalysis of an important step in the synthesis of DNA. Nevertheless, it is clear from previous studies of yeast and from the data presented here that loss of RecQ helicase function strongly impairs survival in cells exposed to chemical inhibitors of DNA replication such as HU. Therefore, we and others have proposed that the primary role of RecQ helicases is to promote effective recovery from an S-phase-arrested state (13, 30, 46). Although HU is used routinely to induce robust S-phase arrest, the physiological role of BLM, presumably, is to respond to episodes in the normal cell division cycle in which DNA replication forks encounter "road blocks," i.e., lesions in the DNA that impose a physical block-

ade. Presumably, such events are relatively rare in unperturbed cells, since BLM is not an essential gene for viability in humans. Alternatively, it may be that a parallel pathway for effective replication restoration is operational in cells lacking BLM. There are a number of potential roles for BLM in replication fork maintenance, but most evidence points to participation in the process of homologous recombination-dependent replication restart. Numerous lines of evidence indicate connections between RecQ helicases and recombination, including the physical and genetic connections between *S. cerevisiae* Sgs1p and Rad51p (64) as well as the physical interactions between BLM and both RAD51 (4, 64) and the RAD51 paralog, RAD51D (5), in human cells. Moreover, RecQ helicases efficiently catalyze two processes that may be relevant to a role in homologous recombination: D-loop disruption and Holliday junction branch migration (15, 31, 60).

The PI-3 kinases play a central role during periods when DNA replication is perturbed. In *S. cerevisiae*, Mec1p (which is an essential gene and is the homologue of the ATR kinase) is required for protection against the toxic effects of HU treatment and for the stabilization of arrested forks (47). Similarly, ATR in mammals is essential for viability and is a key player in the protective response of cells to DNA damage arising in S phase (8, 9). Our data point strongly to a functional connection between ATR and BLM in the cellular response to replicational stress. More specifically, we propose that phosphorylation of BLM on Thr-99 and Thr-122 by ATR is an important component of the multifaceted response of cells undergoing replication fork arrest that is subsequently essential for adequate cellular recovery. It should be noted, however, that these sites are poorly conserved in nonhuman BLM homologues. Our finding that DNA replication is apparently completed in cells released from an S-phase-induced arrest (also seen in similarly treated *S. pombe rqh1* mutants [57]) suggests that this defect in recovery has no impact on bulk DNA synthesis. Nevertheless, the finding that cells expressing BLM-T99A/T122A undergo a caffeine-sensitive checkpoint arrest in G_2 following release from an S-phase block suggests that some form of DNA structural abnormality arises during the recovery period that is invisible to the S-phase checkpoint machinery. This DNA structure (see below) is, however, a potent activator of a G_2/M DNA damage checkpoint. Indeed, the protracted G_2/M arrest (of at least 6 h) is suggestive of the accumulation of a DNA structure that is irreparable, at least in G_2, in cells lacking functional BLM. This may indicate a primary or a secondary role for BLM in the G_2 phase in a DNA repair capacity. A similar proposal has been made recently for the Rqh1 helicase (37). This general phenotype is very reminiscent of that seen in HU-treated *rqh1* mutants, although in the case of this yeast mutant, cells recovering from HU-induced arrest complete bulk DNA replication but then exhibit the so-called "cut" phenotype in which the septum bisects the nucleus before normal nuclear division has been effected (57). A key question for the future is the identification of the DNA structure(s) that arises or accumulates during DNA replication that occurs post-HU treatment. Given the known biochemical properties of RecQ helicases, including BLM, and the emerging evidence that phenotypic defects characteristic of RecQ helicase-deficient cells can be suppressed merely by ectopic expression of the bacterial RusA protein (a Holliday junction resolvase enzyme) (17, 54), we propose that some form of unresolved recombination intermediate containing Holliday junctions is a strong candidate for the DNA structure that prevents adequate recovery from S-phase arrest.

ATR acts to maintain genomic integrity through the stabilization of fragile sites in DNA (11). Fragile sites are loci that exhibit breakage or abnormal chromatin condensation in cells that have been exposed to a low dosage of an agent, such as aphidicolin, that induces replicational stress without blocking DNA synthesis per se (28). ATR deficiency results in the appearance of fragile sites under conditions in which they are suppressed in WT cells. Fragile sites are proposed to be regions of the genome that generally replicate very late in S phase and may be fundamentally difficult to replicate due, for example, to their unusual DNA secondary structural features (27). Consequently, these sites may require special attention from the S-phase checkpoint machinery and homologous recombination-dependent replication fork repair. It will be interesting to see if ATR-dependent phosphorylation of BLM is involved in the suppression of fragile sites, since it has been suggested previously that at least some fragile sites may be hot spots for chromosome rearrangements, including SCEs (29), which are present at an elevated frequency in BS cells.

With the use of the T99A/T122A-substituted derivative of BLM, we have been able to reveal a role for the enzyme in facilitating recovery from replication arrest that has escaped attention in previous studies of BS cells. We propose that this difference is a result of the BLM-T99A/T122A protein acting in a dominant-negative mode, a situation encountered frequently with the expression of mutated cDNAs. Indeed, roles for the ATR kinase itself were revealed by utilizing such an approach (43). Our favored hypothesis is that, because multiple pathways seem to exist for the repair of damaged replication forks, decisions regarding pathway choice are determined both by the DNA structural abnormality that requires processing and by the presence or absence of particular repair factors. For the BLM-T99A/T122A protein to act in a dominant-negative fashion suggests that, in cells expressing BLM, commitment to at least one pathway of repair is determined by the BLM protein itself, but in BS cells lacking BLM, an alternative pathway leading to elevated levels of SCEs (Fig. 6) is operational. We propose that the BLM-T99A/T122A protein commits cells to the preferred BLM-dependent pathway, but that once committed, the phosphorylation-resistant BLM is unable to fulfill its role adequately leading to a mutant phenotype that is specific for BLM derivatives defective in phosphorylation of Thr-99 and/or Thr-122. Our present work is aimed at the identification of the key components of the BLM-dependent pathway for replication fork repair.

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