The Bloom syndrome (BS) protein, BLM, is a member of the RecQ DNA helicase family that also includes the Werner syndrome protein, WRN. Inherited mutations in these proteins are associated with cancer predisposition of these patients. We recently discovered that cells from Werner syndrome patients displayed a deficiency in p53-mediated apoptosis and WRN binds to p53. Here, we report that analogous to WRN, BLM also binds to p53 in vivo and in vitro, and the C-terminal domain of p53 is responsible for the interaction. p53-mediated apoptosis is defective in BS fibroblasts and can be rescued by expression of the normal BLM gene. Moreover, lymphoblastoid cell lines (LCLs) derived from BS donors are resistant to both γ-ray and doxorubicin-induced cell killing, and sensitivity can be restored by the stable expression of normal BLM. In contrast, BS cells have a normal Fas-mediated apoptosis, and in response to DNA damage normal accumulation of p53, normal induction of p53 responsive genes, and normal G1-S and G2-M cell cycle arrest. BLM localizes to nuclear foci referred to as PML nuclear bodies (NBs). Cells from Li-Fraumeni syndrome patients carrying p53 germline mutations and LCLs lacking a functional p53 have a decreased accumulation of BLM in NBs, whereas isogenic lines with functional p53 exhibit normal accumulation. Certain BLM mutants (C1055S or Δ133–237) that have a reduced ability to localize to the NBs when expressed in normal cells can impair the localization of wild type BLM to NBs and block p53-mediated apoptosis, suggesting a dominant-negative effect. Taken together, our results indicate both a novel mechanism of p53 function by which p53 mediates nuclear trafficking of BLM to NBs and the cooperation of p53 and BLM to induce apoptosis.

Bloom syndrome (BS) is an autosomal recessive genomic instability syndrome characterized by growth retardation, immune deficiency, and cancer predisposition (1, 2). The gene responsible for the BS has been identified as BLM, which encodes a 1417-residue protein with ATP-dependent 3'-5' DNA helicase activity, and it belongs to the RecQ DNA helicase family (3–5). In addition to BLM, the RecQ family in mammalian cells contains at least four more proteins, i.e. RecQL, WRN, RTS, and RecQ5 (4, 6). Germline mutations in BLM, WRN, and RTS are known to be associated with cancer predisposition syndromes, BS, Werner syndrome (WS), and Rothmund-Thomson syndrome (RTS), respectively (4). Furthermore, both BLM and WRN also contain signature motifs that belong to the DEAH-containing DNA helicase superfamily, which includes XPB and XPD. Germline mutations in p53 are responsible for the cancer predisposing LFS (9). Therefore, both p53 and members of the RecQ family play an essential role in maintaining genomic stability and preventing the risk of cancer.

Apoptosis is an important process in normal development and in stress-mediated signaling pathways. Defective regulation of apoptosis often contributes to the etiology of cancer. Consequently, cells with mutations in tumor suppressor genes such as p53 and the promyelocytic leukemia protein (PML) display a deficiency in stress-induced apoptosis (10–15). BLM protein is a nuclear protein and is present in both foci and a diffuse distribution (16). The nuclear foci have been identified as PML nuclear bodies (NBs) (17–19), structures that also contain PML, hypophosphorylated Rb, SUMO-1, p53, and other proteins (20–22). Cells from PML−/− mice are resistant to apoptosis induced by many stimuli, including Fas, tumor necrosis factor-α, ceramide, and γ-ray radiation (14, 15). These data indicate that NBs may play a role in multiple apoptotic pathways.

We recently reported that cells from XP-B, XP-D, or WS individuals have an attenuated p53-dependent apoptotic pathway and p53 binds to XPB, XPD, and WRN (23–25). In addition, similar to p53-deficient cells, the XP-D LCLs have a deficiency in DNA damage agent-induced apoptosis (23, 25). In this study, we investigated the physical and functional interactions of p53 and BLM in an apoptotic pathway. We hypothesized that similar to WRN, BLM is also a target for p53 and p53-mediated apoptosis. We found that BLM-deficient cells have an attenuated DNA damage-activated and p53-mediated apoptosis. However, induction of p53 and p53-mediated transcriptional activation of downstream targets including Gadd45, Bax, and p21^waf1 was normal. p53 bound to BLM both in vivo and in vitro. Cells that lack p53 had normal numbers of NBs and normal levels of BLM, however, BLM transit to the NBs was reduced and ectopic expression of p53 in these cells restored BLM transit to the NBs to normal.
over, overexpression of BLM mutants that have a deficiency in localization to NBs in normal cells can block wild type BLM localization to NBs and inhibit p53-mediated apoptosis. Our results indicate a novel mechanism of p53-mediated apoptosis involving a p53-BLM-FML signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—GM01310, GM02184, and HG1943 are normal human LCLs and GM3403, GM09960, GM40408, and HG1525 are BS patients-derived LCLs. All the BS LCLs, with the exception of GM040408 (heterozygous with a low sister chromatid exchange (SCE) rate), are homozygous in BLM with a high SCE rate. All of the GM lines were obtained from Coriell Cell repositories (Camden, NJ) while the HG lines were obtained from New York Blood Bank Cell repositories. These cells were maintained in a density greater than 3 × 10^6 cells/ml in RPMI medium supplemented with 15% fetal bovine serum, penicillin, and streptomycin (BIOFLUIDS, Rockville, MD). Primary normal human fibroblasts (GM07532, GM08402, and GM00038) and primary BS fibroblasts (GM04048 and GM01492) were obtained from Coriell Cell Repositories. GM01492 is homozygous for a protein chain-terminating mutation BLMc.2207–2212delATC/ATGAAinsATGCTCC, referred to as blmRAFT (26). Cell culture conditions were described previously (23).

Plasmids, Antibodies, and Western Blot Analysis—pC53SN (gift of B. Vogelstein) encodes a cytomegalovirus-driven human wild type p53 cDNA. pEGFPC1 encodes a green fluorescent protein (GFP) (CLONTECH, Palo Alto, CA). GFP-BLM encodes a wild type BLM cDNA fused with GFP. GFP-C1055S encodes an N-terminal-deleted BLM–133–257 residues). Antibodies used in indirect immunofluorescence and Western blot analyses include polyclonal anti-p53 antibody CM-1 (Signet Laboratory), monoclonal anti-p53 antibody DO-1 (Oncogene Science), affinity-purified polyclonal anti-BLM antibody, and monoclonal anti-actin antibody (ICN). Anti-Pas antibody (C-11) was obtained from MBL International Corp. Western blot analysis was performed similarly as described previously (23).

Indirect Immunofluorescence and Western Blot Analyses—Cells were seeded onto coverslips, fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min, permeabilized with methanol for 20 min, blocked with phosphate-buffered saline plus solution (phosphate-buffered saline, 0.15% glycerin, 0.5% bovine serum albumin), and incubated with primary antibody for 1 h at 37 °C. The slides were then incubated with the corresponding secondary antibodies conjugated with either Texas red or fluorescein isothiocyanate (Vector Laboratories) at a 1:300 dilution for 1 h at room temperature. After being washed with phosphate-buffered saline, coverslips were mounted in VectaShield solution containing 5 μg/ml 4’,6-diamidino-2-phenylindole (Sigma). Cells were examined with a Zeiss Axioskop fluorescence microscope equipped with a high-performance CCD imaging system (IP Lab Spectrum). Alternatively, confocal fluorescence images were collected with a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a ×60 planapochromat lens. Sequential excitation at 488 and 568 nm was provided by a krypton-argon gas laser. Emission filters of 598/40 and 622/39 were used for sequentially collecting red and green fluorescence, respectively, in channels 1 and 2. Z-sections were collected at 0.5-μm intervals for each cell using LaserSharp software. Images were analyzed by Confocal Assistant software. To quantify nuclear BLM foci between NHF and LFS fibroblasts, images that only showed cells with BLM foci were randomly collected. Western blot analysis was performed similarly as described previously (23).

Microinjection, Apoptosis, and Cell Viability Analysis—The microinjection protocol and determination of apoptosis were essentially as described (23). For analyzing the viability of LCLs, exponentially growing cells were plated at a density of 6.25 × 10^5 cells/ml, treated with various doses of either doxorubicin (Sigma) or γ-radiation, and incubated for a designated time. Cell viability was assessed by trypan blue exclusion as described previously (23). The ability of normal LCLs undergoing apoptosis as measured by caspase-mediated cleavage of poly(ADP)-ribose polymerase and annexin V staining as well as by morphologic examination of the nuclei was confirmed previously (23, 25). In the experiments with Fas-mediated apoptosis, a Fas-specific antibody (C3H) at a concentration of 1.4 μg/ml was added to the culture media that contain LCLs. Cell viability was monitored at 4, 8, and 24 h.

RNase Protection, Immunoprecipitation, Cell Cycle Analysis, and Recombinant Protein Binding Assays—RNase protection assay was done essentially as described (25). Expression of p53-responsive genes was determined using the hStress-1 multiprobe template set including bcl-X, bax, bcl-2, caspases, and glucose, and glyceraldehyde-3-phosphate dehydrogenase (PharMingen, San Diego, CA). Quantification of mRNA species was performed by densitometric analysis with MacBas software (FUJIFILM). Immunoprecipitation and recombinant protein binding assays were essentially as described (24).

For flow cytometric analysis, cells were exposed to 5 Gy of γ-radiation and incubated for 4, 8, or 24 h. Prior to harvest, cells were incubated in the presence of bromodeoxyuridine at a final concentration of 10 μM for 30 min. After harvest by trypsinization, cells were fixed in 70% ethanol until the day of analysis. Cells were treated with 0.1% HCl containing 20 μg/ml of RNAse for 30 min at room temperature to extract histones, followed by boiling and rapid cooling to denature the DNA. Cells were then incubated with anti-bromodeoxyuridine-fluorescein isothiocyanate (Pharmingen, San Diego, CA) and counterstained with propidium iodide containing RNase. Samples were run on a Becton-Dickinson FACS Calibur. Data analysis was performed with CellQuest software for the Macintosh (Becton-Dickinson). Cellular debris and fixation artifacts were gated out, and the G1/G0, S, and G2/M fractions were quantified.

RESULTS

p53-mediated Apoptosis Is Attenuated in BS Cells—To investigate the response of BS fibroblasts to p53-dependent apoptosis, we microinjected p53 and BLM cDNA expression constructs separately or together into normal human fibroblasts (NHF) and BS fibroblasts, and then monitored their ability to undergo apoptosis. The BS fibroblasts that were used in these experiments, GM03498 and GM01492, are null for BLM (see “Experimental Procedures”) and exhibit a high SCE phenotype characteristic of BS cells. GM01492 fortuitously is negative for p53 expression (27). p53-mediated apoptosis was attenuated in two BS fibroblasts (GM03498 and GM01492) when compared with three normal NHF examined (GM07532, GM00038, and GM08402) (Table I). Coexpression of a normal BLM expression vector caused a significant increase in p53-mediated apoptosis of GM01492 cells (Fig. 1A), which is evidence of complementation of the attenuated apoptotic phenotype. Expression of a mutant BLM protein that contains a cysteine to serine amino acid substitution at residue
**Interaction of p53 and BLM DNA Helicase in Apoptosis**

1055 (C1055S) and has no detectable helicase and DNA-dependent ATPase activities (28), did not cause an increase in p53-mediated apoptosis. These data indicate that an enzymatically active BLM product is required for the p53-dependent apoptotic effect. In contrast, the mutant BLM (C1055S) acts as a dominant-negative mutant to block p53-mediated apoptosis in NHF (Fig. 1A). Similarly, an N-terminal deletion mutant of BLM (Δ133–237) also blocks p53-mediated apoptosis in NHF (Fig. 1A).

To determine the role of BLM in apoptosis at the physiological level, sensitivity to cell death induced by γ-irradiation or doxorubicin was investigated in four BS LCLs. Three BS LCLs (GM03403, GM09960, and HG1525) feature a high-SCE phenotype and are homozygous for null mutations in BLM. The fourth (GM04408) is a low-SCE cell line in which the BLM gene was spontaneously corrected in vivo by a somatic intragenic recombination event, thereby becoming heterozygous for one of its BLM mutations (3). We have previously shown that cell viability, monitored by trypan blue exclusion, in LCLs treated with γ-irradiation or doxorubicin is tightly correlated with p53-mediated apoptosis (23, 25). Using trypan blue exclusion assay, we found that the three high-SCE BS LCLs examined were less sensitive to both of the genotoxic agents when compared with normal human LCLs (Fig. 1, B and C, and data not shown). In contrast, the low-SCE BS LCL (GM04408) exhibited the same sensitivity to both agents as normal LCLs (Fig. 1, B and C). Expression of a normal BLM cDNA expression vector construct into the high-SCE BS LCL HG1525, which reduces the SCE rate toward normal (16), sensitized the cells to γ-irradiation-induced cell killing (Fig. 1D). BS LCLs exhibit a normal sensitivity to Fas antibody-induced apoptosis (data not shown), indicating that the resistance obtained in BS cells to DNA damage-induced cell killing is not due to a general defect or inability to undergo apoptosis.

**Normal p53-mediated Activation of Its Downstream Genes in BS LCLs**—Because DNA damage-induced cell killing of normal cells is predominantly dependent on the induction of p53 (10–13, 25), we compared p53 levels in normal and BS LCLs following treatment with 5 Gy γ-irradiation or 1 μg/ml doxorubicin. Both normal and BS LCLs had similar expression profiles after the induction of p53 in response to DNA damage (Fig. 2A). Similar results were obtained with another BS LCL GM09960 (data not shown). To determine whether a defect in p53-mediated transcriptional transactivation of downstream genes associated with a loss of BLM, we examined the mRNA levels of p21, Gadd45, Bax, and p53 by RNase protection assay in BS LCLs following treatment with 5 Gy γ-irradiation. Both normal and BS LCLs displayed a similar induction of p21, Gadd45, and Bax (Fig. 2B), indicating that p53-dependent transcription is normal in BS cells. Consistent with this finding, BS LCLs...
FIG. 2. Induction of p53 and p53-responsive genes in BS cells by DNA damaging agents. A, LCLs from normal and BS donors were treated with 5 Gy γ-radiation (lanes 1–4) or 1 μg/ml doxorubicin (lanes 5–8) and incubated for 4, 8, or 24 h. Cells were harvested and equal amounts (40 μg) of total cellular proteins were analyzed by Western blotting with anti-p53 DO-1 antibody. B, RNase protection assay using the 32P-labeled multi-probe template set hStress-1 in normal LCLs (GM01310, HG1525-pREP4-R9, a BLM-rescued revertant) and BS LCLs (GM03403 and HG1525-pREP4, vector only) following 5 Gy γ-radiation and induction of mRNA species relative to levels at 0 h using glyceraldehyde-3-phosphate dehydrogenase as an internal control. C and D, BS LCLs have functional G1-S and G2-M checkpoints. Asynchronous cultures of LCLs from normal and BS donors were irradiated with 5 Gy of γ-radiation and incubated for 4, 8, or 24 h. Cells were pulse-labeled with bromodeoxyuridine for 30 min and harvested for analysis FACS. Representative FACS dot plots of GM01310 and GM03403 are shown in panel C. A fluorescein isothiocyanate-conjugated anti-bromodeoxyuridine antibody was used to identify S phase cells (sample gates shown in first panel). Percentage of cells at G0/G1, S, and G2/M phases was analyzed by fluorescence-activated cell sorter from D.
underwent normal cell cycle arrest at G1 and G2 (Fig. 2, C and D, data not shown) and accumulation of cyclin B1 and hypophosphorylated Rb upon treatment with γ-radiation (data not shown). Collectively, these results indicated that p53-dependent transactivation in response to DNA-damaging agents is normal in BS cells.

**BLM Localization in the PML Nuclear Bodies Is Modulated by p53**—By indirect immunofluorescence microscopy, focal concentrations of BLM can be detected in the NBs in all cells in which BLM protein can be detected (the co-localization is >90%). Because the NBs are known to play an important role in apoptosis and p53-mediated apoptosis was defective in BS cells, we investigated whether BLM localization to the NBs could be modulated by p53 function. To test this possibility, we analyzed three LFS fibroblast cell strains, two heterozygous for missense mutations of p53 at codons 248 and 175, respectively, and one homozygous for a frameshift mutation in p53 (Fig. 3). Because the steady-state levels of BLM are the same regardless of p53 status or radiation treatment (Fig. 3F), the reduction in BLM foci per nucleus was unchanged by γ-irradiation (Fig. 3, B and C), suggesting that post-translational modification or stabilization of a normal p53 does not affect BLM localization to the NBs. Similar results were obtained with γ-irradiated TK6 and WTK1 cells (Fig. 3D). Because the steady-state levels of BLM were the same regardless of p53 status or radiation treatment (Fig. 3F), the reduction in BLM foci per nucleus in the LFS fibroblasts was not explained by decreased BLM protein.

To test whether expression of a normal p53 protein in the mutant fibroblasts could overcome the defect in BLM localization, we examined a 041-derived clone that contains a tetracycline-inducible p53 expression construct (30). Upon induction of p53, the average numbers of BLM foci per nucleus was increased to levels similar to those in normal fibroblasts (Fig. 3F). This result suggests that if p53 functions in the localization of BLM to the NBs.

Expression of a normal GFP-BLM in normal fibroblasts resulted in efficient localization of the protein to the NBs (Fig. 4). Consistent with the results from fibroblasts with a mutant p53, the average numbers of BLM foci per nucleus in WTK1 cells was lower than in TK6 cells (Fig. 3D). The numbers of BLM foci per nucleus in normal fibroblasts was unchanged by γ-irradiation (Fig. 3, B and C), suggesting that post-translational modification or stabilization of a normal p53 does not affect BLM localization to the NBs. Similar results were obtained with γ-irradiated TK6 and WTK1 cells (Fig. 3D). Because the steady-state levels of BLM were the same regardless of p53 status or radiation treatment (Fig. 3F), the reduction in BLM foci in the LFS fibroblasts was not explained by decreased BLM protein.

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of green foci were quantified and data were obtained from a total of 50
incubation, cells were fixed and analyzed under the fluorescence micro-
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due to the disruption of an apoptotic pathway(s) (34). Cell death susceptibility is associated with the recruitment of certain proteins to the NBs (15). NBs are nuclear matrix-associated structures in which PML appears to be the structural entity that recruits other cellular proteins, including Rb, SUMO-1, and UBC9 (20). In addition, both PML and p53 can be modified by SUMO-1, and p53 has been shown to form nuclear foci juxtaposed to NBs (35). Recent studies indicate that NBs contain p53 (21, 22). Furthermore, BLM localizes to NBs (17–19). Because p53-deficient cells have a reduced localization of BLM to the NBs and the reintroduction of wild type p53 increases BLM localization to NBs, p53 may control intranuclear trafficking of BLM to the NBs. A potential role of BLM nuclear trafficking to NBs on p53-mediated apoptosis was further supported by our findings that two BLM mutants (C1055S and Δ133–237) were able to block p53-mediated apoptosis. These dominant negative mutants have a deficiency in the localization in NBs and are able to inhibit wild type BLM trafficking to NBs. Collectively, our results indicate that BLM may be one of the mediators in NBs that senses p53-dependent apoptotic stimuli.

The level of BLM protein is cell cycle dependent, which peaks in S and G2 phases (37–42). However, we showed here that BS cells appear to have intact G1/S and G2/M cell cycle checkpoints in response to γ-radiation. We also showed that there is a decrease in BLM foci formation in p53-deficient cells. In addition, we did not observe any change in BLM levels in normal LCLs and in LFS fibroblasts at 2 h post-γ-radiation. Therefore, it is unlikely that defects of the BLM foci formation in p53-deficient cells are due to cell-cycle-dependent BLM expression.

Interestingly, earlier studies demonstrated that 2 of 11 primary BS fibroblast strains tested showed delayed p53 induction following both UV and γ-radiation exposures (43), and that one strain (GM01492) lacks the p53-dependent G1 cell cycle checkpoint (44). Collister et al. (45) compared the kinetics of DNA damage-induced p53 accumulation in a NHF strain (GM0038) and a fibroblast strain (GM02932) from a BS patient. Exposure of the cells to 2.5 Gy γ-radiation rapidly increased p53 levels as well as p21 and hdm2 to peak values at 2 h in both normal and BS cells, but a slower decline in p53 as well as p21 and hdm2 was shown in the BS cell strain when compared with the fairly rapid declines observed in the NHF. Our results are similar to these published data in that p53 is induced efficiently and peaks at 2 h post-γ-radiation in both normal and BS LCLs. We also observed a relatively high basal level of p53 at 0 or 24 h after DNA damage. However, these differences may not be the same in all the BS cells. Importantly, we showed that the BS cells studied here have both normal p53-dependent induction of p21, Gadd45, and Bax, and normal G1 and G2 cell cycle checkpoint controls in response to γ-radiation.

BLM is a 3’ to 5’ DNA helicase (28, 46–49). It contains a helicase domain that is 40–50% identical to members of the RecQ family, a subfamily of the DExH-containing DNA helicases, which includes WRN, RecQl, RTS, and RecQl5 (3, 6) in mammalian cells, Sgs1 and Rqh1 in yeasts (50), and recQ in bacteria. Similar to its mammalian counterparts, Sgs1 mutant cells show mitotic hyper-recombination (50), which can be partially suppressed by expression of normal BLM and WRN (51). Loss of the helicase activity may be responsible for hyper-recombination in BS cells (28). These results indicate that BLM and WRN may share a similar pathway in the modulation of homologous recombination repair and in controlling genomic stability. Data supporting this hypothesis include a defect in p53-mediated apoptosis in BS and WS cells and a cancer predisposition in both BS and WS patients. Although WRN is predominantly localized in the nucleolus (52), BLM is localized in NBs and in the nucleolus during S phase of the cell cycle (19). In addition, WRN contains an exonuclease motif at its N terminus and shows an exonuclease activity in vitro, a property unique to WRN (36). These data imply that BLM and WRN may utilize different mechanisms and intranuclear locations to regulate genomic stability. Although the relationship between the localization of BLM to the NBs and its potential functional role as a helicase and modulator of homologous recombination remain unclear, our data are consistent with the hypothesis that the cancer-proneness of BS patients may be in part due to the attenuation of an apoptotic pathway involving a p53–BLM-PML signaling cascade.

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