Bloom’s syndrome (BS) is a rare autosomal recessive genetic disorder associated with genomic instability and an elevated risk of cancer. Cellular features of BS include an accumulation of abnormal replication intermediates and increased sister chromatid exchange. Although it has been suggested that the underlying defect responsible for hyper-recombination in BS cells is a temporal delay in the maturation of DNA replication intermediates, the precise role of the BS gene product, BLM, in DNA metabolism remains elusive. We report here a novel interaction of the BLM protein with the human 5′-flap endonuclease/5′-3′ exonuclease (FEN-1), a genome stability factor involved in Okazaki fragment processing and DNA repair. BLM protein stimulates both the endonucleytic and exonucleolytic cleavage activity of FEN-1 and this functional interaction is independent of BLM catalytic activity. BLM and FEN-1 are associated with each other in human nuclei as shown by their reciprocal co-immunoprecipitation from HeLa nuclear extracts. The BLM-FEN1 physical interaction is mediated through a region of the BLM C-terminal domain that shares homology with the FEN1 interaction domain of the Werner syndrome protein, a RecQ helicase family member homologous to BLM. This study provides the first evidence for a direct interaction of BLM with a human nuclease enzyme. We suggest that functional interactions between RecQ helicases and Rad2 family nucleases serve to process DNA substrates that are intermediates in DNA replication and repair.

Stimulation of Flap Endonuclease-1 by the Bloom’s Syndrome Protein*  

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2 The abbreviations used are: BS, Bloom’s syndrome; MBP, maltose-binding protein; BSA, bovine serum albumin; EtBr, ethidium bromide; ELISA, enzyme-linked immunoassay; ss, single-stranded; nt, nucleotide.

3 The region of BLM that contains these seven motifs exhibits ~40% homology to other proteins in the RecQ family of DNA helicases (6). The region of BLM that contains these seven motifs exhibits ~40% homology to other proteins in the RecQ family of DNA helicases. A 150 amino acid region of extended homology C-terminal to the helicase domain is found in most, but not all, RecQ helicases. Other members of the RecQ helicase family include Escherichia coli RecQ (7), Saccharomyces cerevisiae Sgs1 (8, 9), Schizosaccharomyces pombe Rqh1 (9, 10), and five human homologs (BLM, WRN, RecQ4, RecQ5, and RecQL) (5). Of the human RecQ homologs, three, including BLM, are associated with genetic disorders characterized by chromosomal instability. The WRN gene is mutated in Werner syndrome, a premature aging disorder with elevated cancer risk (11). Mutations in the RecQ4 gene have been found in patients with Rothmund-Thomson syndrome, another rare premature aging and cancer-predisposition disorder (12). The linkage of mutations in human RecQ helicases with genomic instability, cancer, and premature aging suggests that this class of enzymes have important caretaker roles in specialized pathways of DNA metabolism (5).

The BLM protein is a DNA-dependent ATPase and unwind B-form DNA in a 3′ to 5′ direction with respect to the strand that the helicase is presumed to translocate on (13). BLM is able to unwind an array of DNA structures including recombination intermediates (Holliday Junctions and D-loops) (14, 15) and alternate DNA structures (triplexes and tetraplexes) (16, 17). Electron microscopic studies have provided evidence that BLM forms oligomeric ring-like structures in solution (18); however, the assembly state of BLM (and other RecQ helicases) that is important for DNA unwinding activity is open to debate, since recent evidence indicated that a catalytically active, truncated form of BLM was monomeric (19). BLM has been shown to interact physically and/or functionally with a number of proteins (p53, Refs. 20 and 21; WRN, Ref. 22; MLH-1, Refs. 23 and 24; RAD51, Ref. 25; RPA, Ref. 26; and TOP3a, Refs. 27–29) involved in various aspects of DNA metabolism (30). In addition, BLM resides in complexes containing caretaker proteins that include BRCA1, ATM, MRE11-RAD50-NBS1, and Fanconi anemia (FA) proteins (31, 32). Based on the diverse set of replication intermediates compared with normal cells (3, 4). BS cells exhibit chromosomal instability characterized by elevated rates of sister chromatid exchanges (SCEs), insertions, deletions, loss of heterozygosity, telomere associations, and quadriradials (for review, see Ref. 5). Despite the interest in the molecular pathology of BS, the fundamental defects underlying the genomic instability and cellular dysfunction characteristic of the disease are poorly understood.
of DNA substrates upon which BLM can act and the multiple protein interactions involving BLM, it seems likely that BLM shares homology with the domain of WRN responsible for the physical/functional interaction with FEN-1/EXO-1. The functional interaction is mediated by a C-terminal region of BLM that shares sequence homology with the BLM protein and repair (39–42). WRN stimulates FEN-1 cleavage activity by a physical interaction with a C-terminal domain of the WRN protein that shares sequence homology with the BLM protein (34). The sequence homology between BLM and WRN in the region that mediates the physical and functional interaction between WRN and FEN-1/EXO-1 may point to a similar biochemical function of BLM to stimulate the Rad2 nuclease cleavage reaction. One hypothesis is that WRN or BLM may share responsibility of functioning with the Rad2 nucleases during replication to ensure efficient and accurate processing of specific DNA structures that arise during DNA synthesis. Alternatively, WRN and BLM may have separate roles in their interactions with cellular DNA nucleases, such as FEN-1 or EXO-1, that contribute to their unique forms of genomic instability as well as their distinctive cellular and clinical phenotypes. These two hypotheses are not mutually exclusive.

FEN-1 haploinsufficiency in mice can lead to tumor progression (43), suggesting that FEN-1, like BLM, is a tumor suppressor (44). The cancer predisposition and replication defects of BS may be at least partially explained by the lack of a BLM-FEN-1 interaction, since FEN-1 has an essential role in DNA replication and repair. Evidence is presented in this study that BLM and FEN-1 are associated in a complex in human cells as demonstrated by their reciprocal co-immunoprecipitation. Purified recombinant BLM protein interacts physically with human FEN-1, and stimulates FEN-1 cleavage activities on 5′-flap and nicked duplex DNA substrates that are proposed intermediates in DNA replication and repair. The functional interaction is mediated by a C-terminal region of BLM that shares homology with the domain of WRN responsible for the physical/functional interaction with FEN-1/EXO-1. The physical and functional interaction of human RecQ helicases, including BLM and WRN, with human Rad2 nucleases is likely to be important for the roles of these proteins in the maintenance of genome stability.

MATERIALS AND METHODS

Proteins—Hexahistidine-tagged recombinant human BLM protein was overexpressed in S. cerevisiae and purified as described elsewhere (18). MBP fusion peptides of BLM were expressed in BL21 (DE3) cells and purified as described previously (28). Recombinant histidine-tagged WRN protein was overexpressed using a baculovirus/Sf9 insect system and purified as described elsewhere (34).

FEN-1, encoded by a plasmid kindly provided by Dr. M. Lieber (University of Southern California), was overexpressed in E. coli by isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) induction of mid-log phase cells for 4 h at 37 °C. The bacterial cell pellets were resuspended in 5–10 mM Tris containing 8–16% glycerol and 0.5 mM PMSF. After overexpression FEN-1 protein was resuspended in 50 ml of FEN-1 Purification Buffer (25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 1% glycerol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml chymostatin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin). Lysozyme was added to a concentration of 100 μg/ml, and the lysozyme was incubated in ice for 20 min. The suspension was then sonicated briefly, quickly frozen on dry ice, and allowed to thaw on ice, before clarification by centrifugation at 40,000 × g for 15 min at 4 °C. Imidazole (5 mM final concentration) was added to the supernatant, and the material was loaded onto a 5 ml HisTrap column (Amersham Biosciences) using an AKTA PPLC system (Amersham Biosciences). The column was washed successively with FEN-1 Purification Buffer containing 40 and 100 mM imidazole, respectively. FEN-1 was eluted with FEN-1 Purification Buffer containing 250 mM imidazole. Fractions collected were analyzed by SDS-polyacrylamide gel electrophoresis, and those fractions containing FEN-1 were pooled and dialyzed against 25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 1% glycerol, and 1 mM dithiothreitol. Aliquots of recombinant FEN-1 were frozen in liquid nitrogen and stored at −80 °C. The purified FEN-1 recombinant protein was judged to be 98% pure from analysis on Coomassie-stained SDS-polyacrylamide gels.

BLM-FEN-1 Co-immunoprecipitation Experiments—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO2. GM08505, an SV40-transformed fibroblast cell line from a Bloom’s syndrome patient obtained from Coriell Institute for Medical Research, was grown in MEM-Eagle supplemented with 15% fetal bovine serum at 37 °C in 5% CO2. Nuclear extracts were prepared from exponentially growing HeLa and GM08505 cells as described previously (45). For co-immunoprecipitation experiments, nuclear extracts (500 μg) was incubated with either rabbit polyclonal anti-FEN-1 antibody (1:400, Ref. 34) or anti-BLM antibodies IHC1133 (1:50) raised in rabbits against a chimeric protein consisting of MBP fused to residues 1–449 of BLM (IHC1133, as described in Ref. 28) in buffer D (50 mM HEPES, pH 7.5, 100 mM KCl, 1% glycerol) for 4 h at 4 °C. The mixture was then sonicated with 20 μl of protein G-agarose (Roche Applied Science) at 4 °C overnight. The beads were then washed three times with buffer D supplemented with 0.1% Tween 20. Proteins were eluted by boiling in SDS sample buffer, and half of the eluate was resolved on 10% polyacrylamide Tris-glycine SDS gels, and transferred to polyvinylidine difluoride membranes (Amersham Biosciences). The membranes were blocked with nonfat dry milk in TBS containing 0.1% Tween 20, and proteins were eluted by boiling with SDS Sample buffer and resolved on 5–7.5% gradient polyacrylamide Tris-glycine SDS gels. After blotting, membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline and incubated with rabbit polyclonal anti-FEN-1 antibodies (1:1000 dilution) or rabbit polyclonal anti-BLM antibodies IHIC1133 (1:500) overnight. The blots were incubated with either goat-anti-rabbit IgG secondary antibody conjugated to horseshad peroxidase (HRP) (Santa Cruz Biotechnology), BLM and FEN-1 on immunoblots were detected using ECL Plus (Amersham Biosciences).

MBP-BLM-Amylese Pull-down Experiments—Maltose-binding protein (MBP) fusion peptides were expressed in BL21(DE3) cells (New England Biolabs) transformed with the pMAL-C2 expression plasmids containing various portions of the BLM cDNA (MBP-BLM (1–447), MBP-BLM (968–1417)) (28). BL21(DE3) cells transformed with plasmid MBP or MBP-BLM were kindly gift from Dr. Bollinger (NIH) and were used for control experiments. Overnight transformed bacterial cultures were used to inoculate 50 ml of LB supplemented with 2% glucose and 100 μg/ml ampicillin at a 1:100 dilution, and the cultures were grown at 37 °C to an OD600 of ~0.5. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.4 mM, and the cultures were grown at 37 °C to an OD600 of ~0.5. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.4 mM, and the cultures were grown at 37 °C to an OD600 of ~0.5. Isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) induced MBP-BLM (968–1417) (28). Bacterial cell lysates were extensively washed by sonication and the lysate was clarified by centrifugation at 42,000 rpm in a Beckman JA10 rotor for 30 min at 4 °C. For the pull-down assay, 200 μl of the clarified lysate was incubated for 2 h at 4 °C with 50 μl of binding buffer (50 mM Tris, pH 8.0, 10% glycerol, 100 mM NaCl, 0.01% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 5 μg/ml leupeptin) containing 100 ng of purified recombinant FEN-1. Resin was washed three times with binding buffer supplemented with 0.1% Tween 20, and proteins were eluted by boiling with SDS Sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. After transferring the proteins to a polyvinylidene difluoride membrane, the membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20, and probed with either anti-FEN-1 or anti-BLM antibodies as described above.

ELISA for Detection of BLM-FEN-1 Protein-Protein Interaction—Recombinant BLM protein was purified as described above. The purified recombinant BLM protein was dialyzed to a concentration of 1 ng/ml in carbonate buffer (0.016 M Na2CO3, 0.034 M NaHCO3, pH 9.6), and were added to appropriate wells of a 96-well microtiter plate (50 μl/well), which was incubated at 4 °C. Bovine serum albumin (BSA) was used in the coating step for control reactions. The samples were aspirated, and the wells were blocked for 2 h at 30 °C with 2% BSA (phosphatase and proteinase inhibitor-free; 0.02% BSA). The procedure was repeated. FEN-1 was diluted to 1.0 ng/ml in blocking buffer, and was added to the appropriate wells of the ELISA plate (50 μl/well), which was incubated for 1 h at 30 °C. For ethidium bromide (EtBr) treatment, 50 μg/ml EtBr was included in the incubation.
tion with FEN-1 during the binding step in the corresponding wells. The samples were aspirated, and the wells were washed five times before addition of anti-FEN-1 antibody diluted 1:10,000 in Blocking buffer and incubated at 30 °C for 1 h. Following three washings, horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:10,000) was added to the wells, and the samples were incubated for 30 min at 30 °C. After washing five times, any WRN or BLM bound to the FEN-1 was detected using OPD substrate (Sigma). The reaction was terminated after 3 min with 3 N H2SO4, and absorbance readings were taken at 490 nm.

**ELISA Data Analysis**—The fraction of the immobilized BLM or WRN bound to the microtiter well that was specifically bound by FEN-1 protein was determined from the ELISA. A Hill plot was used to obtain the Hill coefficient and the corresponding binding constant. The standard deviation was calculated for each point in the ELISA graph. The mean and standard deviation of the absorbance values were determined for the ELISA data from at least three independent experiments with standard deviations shown by error bars.

**Results**

**Physical Interaction between BLM and FEN-1**—Previously, we reported a physical interaction between the human WRN protein and FEN-1 that is mediated by a C-terminal region of the WRN protein. This region of WRN shares sequence homology with a similar region of the human BLM protein. To explore the possibility that endogenous BLM and FEN-1 reside in a complex together in vivo, co-immunoprecipitation experiments from human nuclear extracts were performed using polyclonal antibodies directed against either purified human recombinant FEN-1 (34) or BLM (28) protein. Anti-BLM antibody precipitated both BLM and FEN-1 proteins from normal human nuclear extract (Fig. 1A, upper and lower panels, lane 2).

Approximately 35% of the FEN-1 from the HeLa nuclear extract input (Fig. 1A, lower panel, lane 1) was co-immunoprecipitated with BLM using the anti-BLM antibody. Neither BLM nor FEN-1 was precipitated when rabbit preimmune serum was used (Fig. 1A, lane 3, upper and lower panels, respectively). The specificity of the BLM antibody was demonstrated in control experiments using nuclear extracts from the BLM-/- cell line GM08505. FEN-1 failed to be precipitated by the BLM antibody (Fig. 1A, lower panel, lane 5) despite its presence in BLM-/- nuclear extracts (Fig. 1A, lower panel, lane 4).

In immunoprecipitation experiments using anti-FEN-1 antibody, both FEN-1 and BLM proteins were co-precipitated from HeLa nuclear extract (Fig. 1B, upper and lower panels, lane 3). Approximately 25% of the BLM from the HeLa nuclear extract input (Fig. 1B, upper panel, lane 1) was co-immunoprecipitated with FEN-1 using the anti-FEN-1 antibody. BLM protein was not precipitated by the anti-FEN-1 antibody from the BLM-/- nuclear extracts (Fig. 1B, upper panel, lane 4) in which BLM was absent (Fig. 1B, upper panel, lane 2). These results demonstrate that BLM and FEN-1 can be reciprocally co-immunoprecipitated from human nuclear extracts using FEN-1 antibody or BLM antibody, indicating that BLM and FEN-1 are associated with each other in vivo.

Our earlier studies demonstrated that the interaction between WRN protein and FEN-1 was mediated by a region of WRN, residues 949–1042, sharing homology to residues 1076–1217 of BLM. To determine if a C-terminal region of BLM harboring this region mediates the interaction with FEN-1, we performed affinity pull-down experiments using MBP fusion BLM fragments coated on to amyllose resin, together with purified recombinant FEN-1 protein. FEN-1 was effectively precipitated by a C-terminal fragment of BLM (MBP-BLM-(966–1417)) (Fig. 1C, lane 4) whereas the N-terminal BLM fragment (MBP-BLM-(1–447)) failed to precipitate FEN-1 (Fig. 1C, lane 3). In control experiments, FEN-1 was not precipitated by affinity beads with MBP bound (Fig. 1C, lane 2) or MBP fused to HuR, an RNA-binding protein not known to interact with BLM (Fig. 1C, lane 5). MBP fusion proteins (MBP, MBP-HuR, MBP-BLM-(1–447), MBP-BLM-(966–1417)) bound to amyllose resin used as input were detected by Western blot analysis using anti-MBP antibody (New England Biolabs) (data not shown). These results demonstrate that FEN-1 can be specifically precipitated by the MBP-BLM-(966–1417) fragment.

**Physical Analysis of the BLM-FEN-1 Protein Interaction**—Results from the immunoprecipitation experiments and BLM fragment affinity pull-down studies suggested that BLM and FEN-1 physically interact with each other. ELISA studies were performed to explore the specificity and nature of the suggested BLM-FEN-1 interaction. FEN-1 protein (0–24 μt) was incubated in the presence of 3% BSA with BLM (6 μt) that had been immobilized on microtiter wells. Bound FEN-1 was detected using anti-FEN-1 antibodies. The specificity of this interaction was demonstrated by very low absorbance values (0.043 OD490) for wells that had been precoated with BSA compared with the intense signal obtained with BLM (Fig. 2). In control experiments, a very weak absorbance signal from anti-FEN-1 antibody was detected in wells containing immobilized BLM protein but lacking FEN-1 input (data not shown).
The colorimetric signal from the BLM-FEN-1 interaction was resistant to the presence of ethidium bromide (data not shown), indicating that a contaminating DNA bridge was not responsible for the positive signal.

As shown in Fig. 2, the colorimetric signal from the BLM-FEN-1 interaction was both dose-dependent and saturable. Since FEN-1 also interacts with WRN we performed ELISA experiments to compare the binding efficiency of FEN-1 to BLM versus WRN (Fig. 2). Specific binding of FEN-1 to BLM-coated or WRN-coated wells was analyzed according to Scat-
BLM and FEN-1 form a complex by direct physical interaction. BSA (filled circle), purified recombinant BLM (open circle), or WRN (filled triangle) was coated onto ELISA plates (6 nM application). Following blocking with 3% BSA, the wells were incubated with increasing concentrations of purified recombinant FEN-1 (0–24 nM) for 1 h at 30 °C. Wells were aspirated and washed three times, and bound FEN-1 was detected by ELISA using a rabbit polyclonal antibody against FEN-1. The values represent the mean of three independent experiments performed in duplicate with S.D. indicated by error bars.

BLM Stimulates FEN-1 Cleavage of a 1-Nucleotide 5′-Flap Substrate—The physical interaction between BLM and FEN-1 suggested that BLM might modulate the cleavage activities of human FEN-1. To characterize the effect of BLM on FEN-1 cleavage, we initially utilized a 19-bp DNA substrate with a single unannealed 5′-nucleotide adjacent to an upstream 25-bp duplex (1-ni 5′- flap). The 1-ni 5′- flap substrate was susceptible to FEN-1 cleavage that generated primarily the 2-ni product, and to a lesser extent the 1-ni product (Fig. 3A, lanes 2–6). In the presence of 10 fmol of purified FEN-1, ~2% of the substrate was incised (Fig. 3A, lane 3 and Fig. 3B) and at 20 fmol of FEN-1, ~6% incision was detected (Fig. 3A, lanes 4 and B). In the presence of BLM (100 fmol), FEN-1 incised 13 and 28% of the 1-ni flap substrate molecules at 10 and 20 fmol, respectively (Fig. 3A, lanes 9 and 10, and B). BLM stimulation of FEN-1 cleavage activity was also observed in reactions containing 40 fmol of FEN-1 where 47% of the DNA substrate was incised by FEN-1 in the presence of BLM (100 fmol) as compared with 19% incision by FEN-1 alone (Fig. 3A, lanes 5 and 11, and B). Based on initial slopes (% incision/FEN-1 (fmol)) (Fig. 3B), it was calculated that BLM stimulated FEN-1 4.7-fold to incise the 1-ni flap substrate.

We next performed a BLM protein titration at a limiting amount of FEN-1 (5 fmol) that incised ~2% of the 1-ni 5′- flap substrate (Fig. 3, C, lane 2, and D). FEN-1 cleavage was stimulated 2-fold in the presence of 25 fmol BLM (Fig. 3, C, lane 5, and D) and 3-fold in the presence of 50 fmol of BLM (Fig. 3, C, lane 6, and D). FEN-1 cleavage was stimulated ~6-fold when 100 fmol of BLM was present (Fig. 3, C, lane 7, and D). At 200 fmol of BLM, FEN-1 incision activity reached a plateau (~14% incision) (Fig. 3, C, lane 8, and D).

BLM Stimulates FEN-1 Endonucleolytic Cleavage of a 26-Nucleotide 5′-Flap Substrate—5′-ssDNA flap substrates with longer 5′-ssDNA tails are relevant in a biological context because they are proposed intermediates in Okazaki fragment processing during lagging strand DNA replication, and also in DNA repair pathways such as base excision repair. The 5′- flap arises during strand displacement synthesis by a DNA polymerase; moreover, a helicase may facilitate the creation and unwinding of the 5′- flap by its unwinding function. Consistent with this notion, we reported that WRN unwinds 5′-flaps with greater efficiency as the length of the 5′-ssDNA tract increases up to at least 26 nt (46). BLM, like WRN is able to unwind a 5′- flap substrate with a 26-nt 5′-ssDNA tail in a reaction dependent on ATP hydrolysis (data not shown). The ability of BLM to recognize and unwind the 5′- flap substrate with a 26-nt 5′- tail suggested that the protein may stimulate FEN-1 cleavage of the longer 5′- flap DNA structure. Recently, we reported that FEN-1 cleavage efficiency decreases substantially with increasing length of the 5′-ssDNA flap (47). The average size of an Okazaki fragment (100–150 nt) (48) may require that additional proteins facilitate cleavage of longer flap structures by FEN-1 or other nucleases. We examined, therefore, the ability of BLM to stimulate FEN-1 cleavage of a 26 ni 5′- flap substrate. FEN-1 (5 fmol) incised 13% of the 26-nt flap substrate (Fig. 4A) to yield the 27-nt product and to a lesser extent, the 26-nt product (data not shown). In the presence of 100 fmol of BLM, a level determined to achieve maximal stimulation of FEN-1 incision of the 1-ni 5′- flap substrate, FEN-1 (5 fmol) incised 63% of the 5′- flap substrate molecules (Fig. 4A), a 4.8-fold stimulation. At 10 fmol of FEN-1, 74% of the DNA substrate was cleaved by FEN-1 in the presence of BLM compared with 24% by FEN-1 alone (Fig. 4A). Stimulation of the FEN-1 cleavage reaction by BLM was also observed when 20 fmol of FEN-1 was used, although the effect was not as great since a plateau was reached. These results demonstrate that BLM can readily stimulate FEN-1 cleavage of the 26-nt 5′- flap substrate and that the patterns of cleavage products are not altered by the presence of BLM.

Kinetic analysis of FEN-1 cleavage reactions on the 26-nt 5′- flap DNA substrate showed a significant effect of BLM on the rate of FEN-1 cleavage (Fig. 4, B and C). Under the reaction conditions used, 10 fmol of FEN-1 incised ~15% of 26-nt 5′- flap substrate in a 15-min reaction in the absence of BLM (Fig. 4B, lane 7). In the presence of 100 fmol of BLM, FEN-1 incision was stimulated significantly at the earliest time point (1 min) (Fig. 4B, lane 8) and throughout the time course (Fig. 4C). At 6 min, FEN-1 incision in the absence of BLM was 7.5%; however, in the presence of BLM, FEN-1 incised 45.5% of the substrate (Fig. 4B, lanes 4 and 10). FEN-1 cleavage in the presence and absence of BLM is linear with respect to time from 1–6 min ($R^2 = 0.99$ and 0.99, respectively). Linear regression analyses yielded reaction rates of 7.18 and 0.83 fmol product/min for the BLM + FEN-1 and FEN-1 only reactions, respectively. This represents an 8.7-fold rate increase when BLM is present. At 15 min, the FEN-1 cleavage reaction conducted in the presence of BLM approached a plateau of 65% substrate incised. In contrast, FEN-1 alone cleaved 15% of the substrate by the end of 15 min (Fig. 4C).

BLM Stimulates FEN-1 Cleavage of Duplex DNA Containing a Nick—FEN-1 has been shown to catalyze a 5′- to 3′- exonuclease reaction on nicked duplex DNA, an intermediate structure of DNA repair that may arise at the site of a damaged base after incision by a nuclease (49). We tested the effect of BLM protein on FEN-1 cleavage of a nicked duplex substrate with the 5′-32P label on the 19-nt downstream primer of the nicked duplex DNA molecule. FEN-1 (40 fmol) alone incised 12% of the nicked DNA duplex substrate molecules (Fig. 5, A, lane 2 and B). In the presence of BLM (25 fmol), FEN-1 incised ~20% of the nicked duplex substrate molecules (Fig. 5, A, lane 5 and B).
In the presence of 200 fmol of BLM, FEN-1 incision was 38% (Fig. 5, A, lane 8 and B), a 3-fold stimulation of cleavage activity. In reactions containing BLM (200 fmol) alone, no incision product was detected (data not shown). Using a limiting amount of FEN-1 (10 fmol), −1.5% of the nicked duplex substrate was incised in the absence of BLM (Fig. 5B, inset). FEN-1 incision activity was stimulated by BLM over a range of BLM levels tested. In the presence of 50 fmol of BLM, FEN-1 cleavage was increased over 4-fold (Fig. 5B, inset). Further increase in FEN-1 incision of nicked duplex was observed at
BLM stimuliates FEN-1 cleavage activity on a 26-nt 5’-flap substrate. Panel A, reactions (20 μl) containing 10 fmol of a 26-nt 5’-flap DNA substrate (indicated above the lanes), 100 fmol of BLM, and indicated amounts of FEN-1 were incubated at 37 °C for 15 min under standard conditions. Substrate and cleavage products were resolved on denaturing polyacrylamide gels as described under “Materials and Methods.” Percent incision (mean value of three experiments) with S.D. indicated by error bars is shown. Filled circles, FEN-1; open circles, FEN-1 + BLM. Panel B, kinetics of FEN-1 cleavage of the 26-nt 5’-flap substrate in the presence or absence of BLM. Reactions (160 μl) containing 80 fmol of 26-nt 5’-flap DNA substrate and 65 fmol of FEN-1 were incubated at 37 °C under standard conditions, and aliquots were removed at 1, 3, 6, 9, 12, and 15 min. The reactions conducted in the presence of BLM contained 600 fmol of BLM protein. Phosphorimaging of a typical gel from a kinetic experiment is shown. Increasing times of incubation (0–15 min) for the FEN-1 cleavage reactions conducted in the absence of BLM (lanes 2–6) or the presence of BLM (lanes 7–13) are indicated. Fifteen minute incubations conducted in the absence of BLM + FEN-1 or FEN-1 are shown in lanes 1 and 14, respectively. Panel C, percent incision from the data shown in panel B (mean value of three experiments) with S.D. indicated by error bars is shown. Filled circles, FEN-1 alone; open circles, FEN-1 + BLM.

DISCUSSION

RecQ DNA helicases play key roles in the maintenance of genome integrity and have been proposed to act in several aspects of DNA replication, repair and recombination. How-
ever, the nature of their contribution to genomic stability is not clearly understood. Various RecQ-deficient eukaryotic cells display elevated levels of recombination (10, 50–57), suggesting an anti-recombinase role for RecQ helicases. The peculiar recombination phenotype of BS is an increased rate of reciprocal exchanges between both sister chromatids and homologous chromosomes (2). Based on the replication defects associated with BS, the elevated recombination may result from the accumulation of aberrant DNA structures at replication forks due to impaired fork progression and/or a failure to rescue stalled or collapsed replication forks (5, 58). Concerted action of BLM, and WRN, and perhaps other RecQ helicases with structure-specific nucleases on DNA structures associated with the replication fork may be important to prevent fork demise and the resulting genomic instability. We find that WRN and FEN-1 form a complex \textit{in vivo} that co-localizes in foci associated with arrested replication forks and that the two proteins function together to process a regressed replication fork intermediate and double flap structures associated with replication (59).

![Fig. 5. BLM stimulates FEN-1 incision of a nicked duplex DNA substrate.](image)

A C-terminal fragment of BLM retains the ability to stimulate the FEN-1 cleavage of duplex DNA substrates containing a 5'-flap or a nick. Reactions (20 μl) containing 10 fmol of 26-nt 5'-flap substrate or a nicked duplex substrate and FEN-1 (5 fmol) were incubated with the specified amounts of MBP or MBP-BLM-(966–1417) at 37 °C for 15 min under standard conditions. Percent incision (mean value of three independent experiments with S.D. indicated by error bars) for reactions containing 26-nt flap substrate (panel A) or nicked duplex substrate (panel B) with FEN-1 and either MBP (filled circles) or MBP-BLM-(966–1417) (open circles) is shown.

![Fig. 6. A C-terminal fragment of BLM retains the ability to stimulate the FEN-1 cleavage of duplex DNA substrates containing a 5'-flap or a nick.](image)
Although mounting evidence suggests that WRN and Rad2 family nucleases function together, an interaction between BLM and a human nuclease has not been identified previously.

In this study, we have demonstrated that BLM and FEN-1 reside together in a complex in vitro as evidenced by their reciprocal co-immunoprecipitation from human nuclear extracts; moreover, results from ELISA indicate a direct interaction between the purified recombinant BLM and FEN-1 proteins. The BLM-FEN-1 interaction is a high affinity one, as evidenced by a $K_d$ of 1.7 nM, similar to the value obtained for the WRN-FEN-1 interaction ($K_d$ = 1.4 nM). The BLM-FEN-1 $K_d$ value is also similar to those reported for the BLM-RPA ($K_d$ = 1.3 nM) (26) and BLM-TRF2 ($K_d$ = 2.5 nM) (60) interactions, suggesting that BLM binds equally well to FEN-1, RPA, and TRF2. Importantly, the BLM-FEN-1 physical interaction mediates a functional interaction. BLM stimulates FEN-1 endonucleolytic and exonucleolytic cleavage activities on a variety of DNA substrates that are proposed intermediates in DNA replication and repair. The ability of BLM to stimulate FEN-1 cleavage of short 5'-flap and nicked duplex DNA substrates may be important in DNA repair related processes. In addition, BLM stimulates FEN-1 cleavage of longer 5'-flap substrates that potentially arise during Okazaki fragment processing in DNA replication. Importantly, for the first time, BLM is directly linked to nucleolytic processing of DNA metabolic intermediates by its interaction with an essential genome stability factor, FEN-1.

A key finding of this study is that the helicase activity of BLM is not essential for the stimulation of FEN-1 nuclease activity in vitro. Our data indicate that this stimulation results from the binding of BLM protein to FEN-1 and not from the creation of a FEN-1 substrate by the helicase activity of BLM. Nevertheless, although a short non-catalytic region of the BLM protein is sufficient for binding to FEN-1 and stimulation of its cleavage activity, evidence indicates that BLM catalytic activity may be of importance in vivo. Genetic rescue experiments using BS cell lines that had been stably transfected with BLM alleles encoding wild-type or mutated BLM protein that lacked helicase activity demonstrated that the DNA helicase activity of BLM is necessary for the correction of the genomic instability of BS cells (61). Similarly, the hydroxyurea-sensitive phenotypes of yeast sgs1 top3 (61) or dna2-1 (62) mutants can be complemented by the expression of normal BLM protein but not by BLM missense mutants that are devoid of helicase activity. Despite a genetic requirement for the helicase activity of BLM, evidence also suggests that the N-terminal and C-terminal regions of BLM may be important for subcellular localization and genetic function (63). The C-terminal domain of BLM is also essential for the maintenance of chromosomal stability and for nucleolar localization in human cells.

The elevated sister chromatid exchange in BS may be a consequence of a replication defect, suggesting a direct role for BLM in DNA replication. Stimulation of FEN-1 cleavage of 5'-flap substrates may be an important role for BLM during Okazaki fragment processing. Pol alpha/PCNA displacement of the RNA/DNA chimeric fragment synthesized by pol alpha/primase may serve to eliminate the replication errors introduced by pol alpha, since it lacks a proofreading subunit. It is tempting to speculate that the ability of BLM (or WRN) helicase to unwind 5'-flap structures may facilitate the process of strand displacement to ensure that the nucleotides synthesized by pol alpha/primase are removed by structure-specific nucleases. Maturation of long 5'-flaps to which RPA can bind requires the sequential action of Dna2 and FEN-1 (48, 64). Thus the RecQ helicase may work in conjunction with a polymerase during strand displacement and with the nuclease during cleavage of the 5'-flap to keep the 5'-ssDNA tract relatively short to prevent the formation of secondary structures that are resistant to FEN-1 cleavage.

The critical roles of FEN-1 in the maintenance of genomic stability reflect the functions of the enzyme in DNA repair (base excision repair and the non-homologous end-joining pathway of double strand break repair) and in Okazaki fragment processing that occurs during DNA replication. FEN-1 has recently been described as a tumor suppressor protein (44) with the demonstration that FEN-1 haploinsufficiency in mice can lead to elevated tumor incidence (43). The elevated cancer and genomic instability of BS has prompted researchers to also consider BLM helicase as a so-called caretaker tumor-suppressor protein (5, 58). Consistent with this notion, BLM has been shown to be associated with other caretaker proteins in several multiprotein nuclear complexes including the BRCA1-associated genome surveillance complex (31) and the BRAFT complex that contains five of the Fanconi anemia complementation group proteins (32). Although FEN-1 was not reported to be a member of either of these complexes, a number of other DNA-associated proteins (e.g. topoisomerase III alpha, RPA, MLH1, RAD50-MRE11-NBS1, and p53) were found to co-reside in specific BLM complexes, and direct physical interactions between some of these proteins and BLM have been demonstrated. Of the BLM protein interactions, RPA dramatically stimulates BLM helicase activity (26) and BLM stimulates the DNA relaxation activity of human topoisomerase III alpha (29). Moreover, BLM and topoisomerase III alpha together catalyze the dissolution of DNA molecules containing a double Holliday junction, a structure that can arise during homologous recombination processes (65). The biological importance of these and other BLM protein interactions remains under investigation. No direct interaction between BLM and a human endonuclease has been identified previously. It was proposed that at least one function of BLM in a multiprotein complex may be to act in a concerted fashion with FA proteins or associated proteins to modify DNA structures during DNA repair (32). Interestingly, a DNA endonuclease involved in DNA damage repair was identified in the BLM-FA complex (32). Our studies would suggest that FEN-1 is a strong candidate for direct interaction with BLM within a complex that participates in DNA repair and/or at the replication fork.

Very recently, it was reported that ectopically expressed human BLM suppressed the temperature-sensitive growth defect and DNA damage sensitivity of the S. cerevisiae DNA replication mutant dna2-1 (62). The dna2-1 mutant is defective in a helicase-nuclease that is believed to function in concert with scFEN-1 nuclease to process 5'-flap structures that arise from strand displacement during lagging strand DNA synthesis (66). According to a proposed model for eukaryotic Okazaki fragment processing, Dna2 helicase/nuclease cleaves the RNA containing 5'-flap leaving a short 5'-flap DNA intermediate that is subsequently acted upon by FEN-1 (48). Results from in vitro reconstitution of the processing reactions that take place during Okazaki fragment maturation have suggested that Dna2 is likely to be essential only in cases where FEN-1 activity is somehow impaired (48, 64). This idea is supported by the observation that overproduction of scFEN-1 suppresses the mutant cellular phenotypes of the dna2-1 strain (66). Our finding that human BLM directly stimulates FEN-1 cleavage activity on 5'-flap and nicked duplex substrates suggests that the enhancement of endogenous scFEN-1 cleavage activity by human BLM may be the basis for the genetic complementation of the dna2-1 mutant. The demonstration that yeast-expressed human BLM was co-immunoprecipitated with scFEN-1-myc (62) is consistent with the hypothesis that BLM and FEN-1 directly interact with each other and that this interaction is
responsible for the genetic rescue of the dna2-1 mutant phenotypes. Taken together, the evidence suggests that BLM plays a direct role in DNA replication by promoting replication intermediate processing through its interaction with FEN-1 and/or a related structure-specific nuclease (EXO-1).

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Stimulation of Flap Endonuclease-1 by the Bloom's Syndrome Protein
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