Binding and Melting of D-Loops by the Bloom Syndrome Helicase†

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ABSTRACT: Bloom syndrome is a rare autosomal disorder characterized by predisposition to cancer and genomic instability. *BLM*, the structural gene mutated in individuals with the disorder, encodes a DNA helicase belonging to the RecQ family of helicases. These helicases have been established to serve roles in both promoting and preventing recombination. Mounting evidence has implicated a function for BLM during DNA replication; specifically, BLM might be involved in rescuing stalled or collapsed replication forks by a recombination-based mechanism. We have tested this idea by examining the binding and melting activity of BLM on oligonucleotide substrates containing D-loops, DNA structures that model the presumed initial intermediate formed during homologous recombination. We find that BLM preferentially melts those D-loops that are formed more favorably by the strand exchange protein Rad51, but whose polarity could be less favorable for enabling restoration of an active replication fork. We propose a model in which BLM selectively dissociates recombination intermediates likely to be unfavorable for recombination-promoted replication.

Bloom syndrome is a rare autosomal recessive disorder with clinical manifestations that include short stature, immunodeficiency, and a greatly increased predisposition to developing cancer of many different types (*1*, *2*). Cultured cells from individuals with Bloom syndrome suffer genomic instability that is revealed as hypermutation and elevated somatic recombination (*3*). A high frequency of sister chromatid exchange has long been associated with the disorder (*4*). *BLM*, the gene mutated in individuals with Bloom syndrome, encodes a member of the RecQ family of DNA helicases (*5*), the founding member of which is the product of the *Escherichia coli recQ* gene. Genetic and biochemical analyses have established that RecQ functions in homologous recombination (*6*−*9*) and, in addition, suppresses illegitimate recombination (*10*). Other members of the family include Sgs1 of *Saccharomyces cerevisiae* (*11*, *12*) and Rqh1 of *Schizosaccharomyces pombe* (*13*, *14*), which also serve to suppress inappropriate recombination. Among at least four additional RecQ family members in humans is WRN, the helicase defective in Werner syndrome, another genome instability disorder (*15*, *16*). While a defining function of helicases is the unwinding of DNA duplexes, recent work with several RecQ family members including BLM (*17*) indicates activity on alternative DNA structures. In particular, BLM can unwind DNA tetraplexes which can form in stretches of G rich DNA (*18*). This finding has raised the notion that one function of BLM is to dissociate aberrant DNA structures arising as a consequence of damage, torsional stress, or repetitive or simple-sequence based conformational shifting, to prevent effects that could jeopardize or endanger the integrity of the genome (*19*).

Several lines of experimentation point to a role for BLM in DNA replication. Bloom syndrome cells show retarded S-phase progression and accumulation of abnormal replication intermediates (*20*−*22*). Growing evidence also indicates that replication fork collapse and regression might be common occurrences resulting from encounters by the replication machinery with lesions, strand breaks, aberrant structures, and even collisions with transcription and repair complexes (*23*−*25*). A mechanism to salvage this potentially disastrous situation could proceed by utilizing the recombinational repair machinery to reattach chromatid arms broken from the collapsed or regressed replication fork. Since BLM and the RecQ helicases are thought to prevent inappropriate recombination, the idea has been raised that BLM might act at stalled replication forks to prevent inadvertent or promiscuous recombination. One model holds that by recognizing a Holliday junction formed by a regressed replication fork and reversing it through catalyzed branch migration (Figure 1, steps c→a), BLM could restore the fork and enable replication to proceed (*26*, *27*). Preempting cleavage of the Holliday junction would minimize recombination and prevent activation of a cell cycle checkpoint mechanism that would ensue with the formation of double-stranded DNA ends.

It was recently demonstrated that BLM can bind selectively to Holliday junctions and promote branch migration (*26*). These findings were taken as support for the idea that

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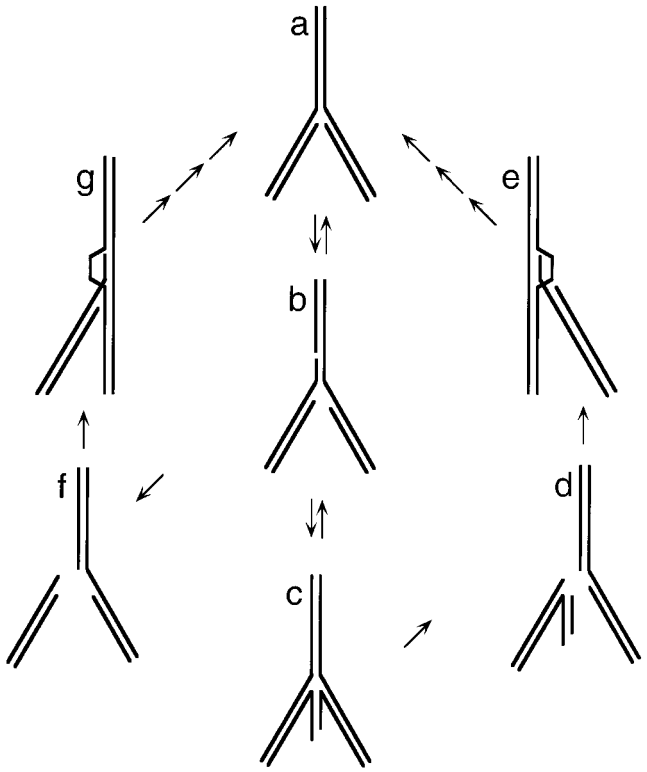

FIGURE 1: Rescue of collapsed or regressed replication forks by recombination mechanisms. An active replication fork (a) encounters a disruption in the phosphodiester backbone of one strand or suffers an event that exposes a single-stranded region (b). The fork regresses by pairing of the complementary newly synthesized strands and branch migration to a four-stranded X-form or Holliday junction (c) that can be reversed by BLM or cleaved by a structure-specific endonuclease to release a broken arm (d). Collapse of the fork by unwinding to the phosphodiester interruption (f) or by endonucleolytic cleavage at the single-stranded region would release a broken arm with a double-strand break at the terminus. Recombination mediated by the homologous pairing activity of Rad51 forms D-loop intermediates e and g. By further processing of the D-loop the replication fork can be restored. It is assumed that DNA ligases and exonucleases could serve in various unspecified steps in the model to seal nicks and to process DNA ends so as to render them suitable for further processing.

BLM reverses regressed replication forks. Although the model is attractive, the broad substrate specificity of BLM in unwinding alternative DNA structures suggests that the protein might have additional functions in preventing inappropriate recombination. Recombinational repair of duplex ends would be necessary to reestablish replication after fork collapse following an encounter with a strand interruption or after double-strand break formation from cleavage of a regressed fork. Such repair would presumably be initiated by D-loop formation promoted by the homologous pairing activity of Rad51 (Figure 1, e and g).

In this study, we investigated the action of BLM on D-loop DNA. We report that BLM recognizes and binds D-loops tightly and has a distinct preference in melting those D-loops that are formed with the polarity favored by the Rad51 strand exchange protein. We offer a model for BLM functioning in an additional role in suppressing unproductive recombination events during replication fork reassembly.

EXPERIMENTAL PROCEDURES

BLM Expression Plasmid. The BLM gene was modified for expression in *S. cerevisiae* in a similar manner as described by Karow et al. (*17*) such that a yeast Kozak sequence and the first five codons from the *S. cerevisiae TOP2* gene were added replacing the *BLM* initiation codon, and a tag encoding a C-terminal hexahistidine sequence was added. DNA fragments with these modifications were prepared by polymerase chain reaction and then used to replace the corresponding DNA sequences in pB3, a pBluescript plasmid derivative containing the full-length *BLM* cDNA (*5*). The C-terminal modification was created in a polymerase chain reaction with pB3 using primer Y210 (5′-TCTCGAG**TTA***GTGATGGTGGTGATGATG*TGAGAATG-CATATGAAGG-3′), which incorporates a *Xho*I site (underlined), a stop codon (bold-faced type), and six contiguous histidine codons (italic type) and primer HG3 corresponding to nucleotides 3979−3999 of the *BLM* cDNA open reading frame which spans an *Eco*RV site. The 378 bp[1] PCR product was prepared for ligation by digesting with *Eco*RV and *Xho*I and then used to replace the corresponding *Eco*RV/*Xho*I fragment in pB3. This modified *BLM* gene was then excised as a 4.4 kbp *Eag*I−*Xho*I fragment and introduced into a yeast expression vector pYES (Stratagene, La Jolla, CA) opened by cutting with *Not*I and *Xho*I to yield pYES-BLM-6H. The N-terminal modification was created in a PCR reaction with pB3 using primer Y248 (5′-AGAGAGGGTACCC**TAAC-C***ATGTCAACTGAACCG*GCTGCTGTTCCTCAAAATAAT-3′), which incorporates a *KpnI* site (underlined), a yeast Kozak sequence (bold-faced type), and five yeast *TOP2* codons (italic type), and primer BC18 corresponding to the complementary sequence of nucleotides 1500−1479 of the *BLM* cDNA open reading frame which spans an *Eco*RI site. The 1458 bp PCR product was digested with *Kpn*I and *Eco*RI and inserted into pYES BLM-6H opened with the corresponding restriction enzymes to yield pYBLM. DNA sequencing confirmed that the recombinant gene was correctly constructed and that no mutations had been introduced by the PCR procedure.

BLM Protein. Recombinant BLM protein was purified after overexpression of the modified *BLM* gene in *S. cerevisiae* by a procedure similar to that described by Karow et al. (*17*). *S. cerevisiae* strain JEL-1 (α *leu2 trp1 ura3-52 prb1-1122 pep4-3* Δ*his::pGAL10-GAL4*, obtained from Dr. J. C. Wang, Harvard University) transformed with pYBLM was grown in 6 L of synthetic medium (*28*) lacking uracil for maintenance of the plasmid and containing 2% raffinose at 30 °C to a density of $\sim 1 \times 10^7$ cells/mL. The cultures were shifted to 25 °C and induced by addition of galactose to 2%. After an additional 20 h cells were harvested by centrifugation, yielding 50 g of packed cells which were washed in 50 mM potassium phosphate, pH 7.0, and resuspended in 50 mL of 50 mM potassium phosphate buffer, pH 7.0, with 0.5 M KCl, 10% glycerol and 50 μL/g of packed cells protease inhibitor cocktail (P8215, Sigma Chem. Co., St. Louis, MO). Cells were crushed by passage through a French pressure cell at 20 000 psi and cleared by centrifugation at 20 000 rpm for 30 min in the Sorvall SS34 rotor. The supernatant was decanted, split into two portions, and

[1] Abbreviations: ATPγS, adenosine 5′-*O*-(thiotriphosphate); bp, base pair; BSA, bovine serum albumin; dsDNA, double-stranded DNA; DTT, dithiothreitol; FPLC, fast-protein liquid chromatography; nt, nucleotide; NTA, nitrilotriacetate; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA.

Table 1: Oligonucleotides

oligonucleotide	sense	length (nt)	sequence (5′ → 3′)
DL-1	bottom[a]	61	GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCT-GCAGGTTCACCC
DL-2	top	61	GGGTGAACCTGCAGGTGGGCGGCTGCTCATCGTAGGTTAGTTGGTAGA-ATTCGGCAGCGTC
DL-3	invading(no tail)	21	AAAGATGTCCTAGCAAGGCAC
DL-4	invading(20 nt 5′ tail)	41	TAAGAGCAAGATGTTCTATAAAAGATGTCCTAGCAAGGCAC
DL-5	invading(20 nt 3′ tail)	41	AAAGATGTCCTAGCAAGGCACGATCGACCGGATATCTATGA
HJ-1	top	48	ACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCC
Sub HJ-4	bottom	18	GCCAGAATTCGGCAGCGT
Sub HJ-2b	bottom	30	GGGTCAACGTGGGCAAAGATGTCCTAGCAA
Sub HJ-2c	bottom	34	GGGTCAACGTGGGCAAAGATGTCCTAGCAATGTA
HJ-2	bottom	48	GGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTATGACGT
HJ-3	top	48	ACGTCATAGACGATTACATTGCTAGGACATGCTGTCTAGAGACTATCG
HJ-4	bottom	48	CGATAGTCTCTAGACAGCATGTCCTAGCAAGCCAGAATTCGGCAGCGT
M13 oligo	top	90	ACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGT-CGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAAT
98-mer	top	98	GAATACAAGCTTGGGCTGCAGGTCGACTCTAGAGGATCCCCGGGCGAG-CTCGAATTCGGGTCTCCCTATAGTGAGTCGTATTAATTTCGATAAGCCAG
38-mer	bottom	38	GAATACACGGAATTCGAGCTCGCCCGGGGATCCTCTAG
5′ end	bottom	30	AGAGTCGACCTGCAGCCCAAGCTTGTATTC
3′ end	bottom	30	CTGGCTTATCGAAATTAATACGACTCACTA
5′ end tailed	bottom	38	TCGAAGAGAGAGTCGACCTGCAGCCCAAGCTTGTATTC

[a] Bottom or top refers to the orientation of one in a pair of complementary sequences.

each loaded in separate batches onto an FPLC column (5 mL bed volume) containing nitrilotriacetate-agarose (Qiagen, Inc., Chatsworth, CA) charged by washing with 10 column volumes of 10 mM $NiCl_2$ then equilibrated with P buffer (50 mM potassium phosphate buffer, pH 7.0, with 0.25 M KCl) containing 50 mM imidazole. After washing with 75 mL of the equilibration buffer the column was developed with a 40 mL linear gradient (50 to 1500 mM imidazole). Fractions (1 mL) were assayed for ATPase activity as described below, and those containing the peak of activity eluting at ~300 mM imidazole were pooled and loaded directly onto a heparin Sepharose column (1 mL, HiTrap Heparin, Amersham Pharmacia Biotech, Piscataway, NJ) prepared by washing with H buffer (60 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) containing 0.25 M KCl. The column was washed with 5 mL of the same buffer then developed with a 5 mL linear gradient (0.25 to 1.2 M KCl). Fractions of 0.25 mL were collected and assayed for ATPase activity. The peak fractions were pooled, dialyzed against buffer H containing 100 mM KCl, 10% glycerol, and stored at −80 °C. BLM preparations used in this study hydrolyzed 2.0×10^4 nmol of ATP/min/mg of protein at 37 °C. Protein concentrations were determined by using the Coomassie dye-binding method (*29*) or by visual comparison to fixed amounts of known standards after SDS−gel electrophoresis. As reported by Karow et al. (*17*) the nitrilotriacetate-agarose column fraction contained BLM protein with a mass of 170 kDa that was essentially homogeneous (>95%) as determined by Coomassie blue staining of samples analyzed by SDS−gel electrophoresis. The heparin Sepharose column removed trace contaminants.

ATPase. ATPase activity was determined by measuring the release of ^{32}P inorganic phosphate from [γ-^{32}P]ATP (*30*). Reaction mixtures (50 μL) contained 50 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP (~4×10^3 cpm/nmol), 1 mM DTT, 50 mM NaCl, 50 μg/mL BSA, and 3.8 nmol (as nucleotide) of sheared salmon sperm DNA and BLM. After incubation at 37 °C for 1 h, reactions were quenched by addition of 100 μL of acetone, 10 μL of 10 mM KH_2PO_4, 100 μL of 1% ammonium molybdate in 1 M H_2SO_4, and 700 μL of isobutanol:benzene (1:1). The mixture was vortexed vigorously for 5 min, centrifuged at 14 000 rpm for 5 min in a microcentrifuge, and one-half of the organic phase was collected and the radioactivity determined in a scintillation counter.

DNA Substrates. Oligonucleotides were labeled at the 5′-terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase and unincorporated label was removed by passage through a 1 mL Sephadex G25 column. In preparing heteroduplex combinations, only one oligonucleotide utilized was radiolabeled. Heteroduplex combinations were prepared by annealing equimolar amounts of each oligonucleotide in reactions containing 10 mM Tris-HCl, pH 8.0, and 0.2 M NaCl. Reactions (100 μL) were heated at 85 °C for 10 min, held at T_m−20 °C for 1 h, then cooled on ice. Radiolabeled heteroduplex DNA was purified by gel electrophoresis. After addition of tracking dye, mixtures were laid on top of a 12% polyacrylamide gel (14 × 14 × 0.1 cm) containing 45 mM Tris, 45 mM boric acid, 1 mM EDTA, and electrophoresed at 15 mA for 1 h until a xylene cyanol tracking dye had migrated 5−6 cm. The radiolabeled DNA was located by autoradiographic exposure to a piece of X-ray film and a gel strip containing the DNA was excised with a razor blade. The gel strip was ground with a micropestle, and the DNA was leached out by diffusion for 2 h into 0.4 mL of 0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS. After precipitation with ethanol, the DNA was redissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Concentrations of DNA were determined by measuring the specific activity of a known concentration of each labeled oligonucleotide prior to the construction of the substrate and the final activity of the purified substrate, and are expressed in moles of substrate unless indicated otherwise. Table 1 indicates the oligonucleotides that were utilized in this study. Substrate design for D-loops and Holliday junctions was based on the sequences used by McGlynn et al. (*31*); oligonucleotide partial duplexes with 5′ or 3′ blunt ends or internal heteroduplex regions were based on sequences used by Kelman et al. (*32*); single-

stranded M13 DNA heteroduplexed with a 91-mer was based on Karow et al. (*17*).

Unwinding Assays. Reactions (30 μL) containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 50 mM NaCl, 100 μg/mL BSA, 50 fmol of ^{32}P-labeled DNA substrate, were prewarmed at 37° for 5 min, then started by addition of BLM in the range of 1−25 nM. After appropriate incubation, reactions were stopped by addition of 0.2% SDS, 15 mM EDTA, and 3% glycerol and loaded onto 12% polyacrylamide gels (14 × 14 × 0.1 cm) containing 45 mM Tris, 45 mM boric acid, and 1 mM EDTA, and electrophoresed. Gels were dried, and the radiolabeled DNA was imaged using a Molecular Dynamics PhosphorImager and quantitated using ImageQuant software.

Binding Assays. Reactions (50 μL) containing 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM ATPγS, 1 mM DTT, 100 μg/mL BSA, 25 fmol of ^{32}P-labeled DNA substrate, and BLM in the range of 1−15 nM. After 15 min, reaction mixtures were spotted directly onto a Gelman GN-6 nitrocellulose filter (25 mm) retained in a vacuum manifold and allowed to pass through the filter. Filters were washed with one 2 mL rinse of 30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, dried under a heat lamp, and counted in Ecolume (ICN) scintillation fluid. Filters were pretreated by heating in a boiling solution of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA as described previously (*33*). Background retention of single-stranded and double-stranded oligonucleotides with no BLM protein added was <0.5% of the input total. The efficiency of retention of protein−DNA complexes was determined to be 85% when histone H1 was used as a positive control for DNA binding without regard to protein conformation. Filter binding data were analyzed after Hill plot transformation as described (*34*).

RESULTS

BLM Exhibits Broad Specificity in DNA Binding. Karow et al. (*17*) demonstrated that BLM can bind tightly to synthetic four-armed Holliday junctions and also dissociate α-structures formed by RecA-mediated strand exchange between gapped circular and linear duplex molecules containing a region of shared homology. They proposed that BLM might act to promote branch migration of Holliday junctions formed by regression of replication forks thereby preventing Holliday junction cleavage and further processing of the resulting DNA double-strand breaks (see Figure 1). We considered the notion that BLM might function directly in suppressing recombination by disrupting joint molecules formed as early intermediates in the homologous pairing step of recombinational repair. Studies on the *E. coli* RecA strand exchange protein and its human homologue Rad51 indicate that an early intermediate in the homologous pairing phase preceding strand exchange is a D-loop in which one strand of a duplex DNA molecule is displaced concomitant with formation of Watson−Crick base pairing between the complementary strand and an incoming single strand (*35*−*37*). We decided to examine the interaction between BLM and D-loops to investigate any potential anti-recombinational activity.

We studied the binding specificity of BLM to various DNA structures to learn if there was a preference for D-loops.

Table 2: DNA-Binding and Unwinding Substrates

substrate	oligonucleotides	K_d[a]	*n*
single-stranded DNA	DL-1[b]	17 nM	1.9
bubble	DL-1,[b] DL-2	4 nM	1.2
D-loop without tail	DL-1, DL-2, DL-3[b]	7 nM	1.1
D-loop with 5′ tail	DL-1, DL-2, DL-4[b]	10 nM	0.9
D-loop with 3′ tail	DL-1, DL-2, DL-5[b]	12 nM	0.9
nicked	HJ-1, SubHJ-4, Sub HJ-2b[b]	3.7 μM	2.0
Holliday junction	HJ-1,[b] HJ-2, HJ-3, HJ-4	12 nM	1.1

[a] Binding curves were performed 2−5 times for each DNA substrate. [b] Indicates which oligonucleotide was 5′ end-labeled with ^{32}P.

Binding was measured directly using a membrane filter assay (*38*). In this procedure, DNA bound with protein can be selectively retained on nitrocellulose filters while protein-free DNA passes through. With the use of ^{32}P-labeled oligonucleotide substrates prepared by annealing various combinations of complementary or partially complementary single-stranded oligonucleotides (see Table 2), we found that BLM formed complexes with D-loops in a reaction corresponding to a classical saturation process (Figure 2). The binding curves showed hyperbolic isotherms for DNA with little sign of sigmoidicity. The D-loop substrates in this case were prepared by annealing three oligonucleotides designed to form a single molecule 61 nucleotide residues in length with a central noncomplementary bubble of 21 residues flanked by 20 nucleotide residues of duplex to either side and in which the third strand invades and base pairs with one of the looped out strands in the 21 nucleotide bubble region. Binding reactions were performed with ATPγS as a nonhydrolyzable cofactor to prevent any dissociation of the substrates through helicase action of BLM. As seen in Figure 2A, there was a pronounced preference for BLM binding to the D-loop structure compared with duplex DNA of 48 nucleotide base pairs which in this case contained an internal nick in one strand. Analysis of the data after Hill plot transformation indicated an apparent K_d for D-loops of 7 nM compared with 3.7 μM for duplex DNA, a 500-fold difference (Table 2). We note that the Hill coefficient (*n*) was unity in the case of BLM binding to D-loop DNA but was ∼2 with linear duplex DNA, indicating there was positive cooperativity in the binding to the latter substrate.

We examined other DNA structures to test what feature of the D-loop was recognized by BLM. In their studies on BLM interactions with Holliday junctions using a gel mobility shift assay, Karow et al. (*26*) estimated that BLM bound four-armed X-junctions 15 times tighter than single-stranded or double-stranded DNA oligonucleotides and concluded that BLM interacts specifically with the crossover junction. However, using the filter retention assay, we determined the binding constants between BLM and a synthetic Holliday junction and BLM and a 61 residue single-stranded oligomer to be similar to what we observed with the D-loop substrate (Figure 2; Table 2). We also examined binding of BLM to duplex DNA with an internal bubble of 21 noncomplementary base pairs as well as additional D-loop structures as above but with 20 nucleotide 5′ or 3′ tails. BLM has high affinity for all the structures we tested that have single-stranded-duplex junctions, with the highest affinity being for the bubble structure (Table 2). This bubble structure could be considered similar to a partially opened X-junction with two flayed open duplex ends. On the basis of our

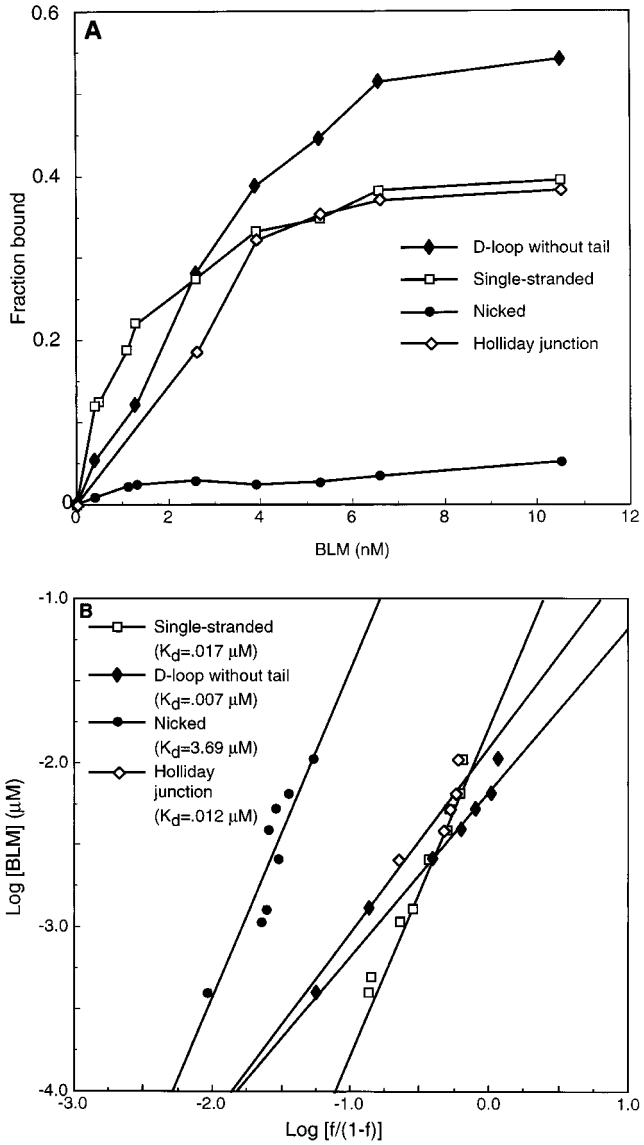

FIGURE 2: BLM binds tightly to D-loop DNA. (A) Binding of BLM to D-loop DNA (filled diamonds), single-stranded DNA (open squares), nicked DNA (filled circles), and Holliday junction DNA (open diamonds) was determined by a filter-binding assay. The fraction of DNA bound to the filter with increasing amounts of BLM is plotted and represents a typical experimental determination. (B) The filter-binding data was analyzed after Hill plot transformation. *f* indicates the fraction of oligonucleotide bound to the total amount present in the reaction. The equilibrium dissociation constant (K_d) was determined from the *y*-intercept. The Hill coefficient (*n*) was determined from the slope. The fractions bound were calculated based on total input DNA without correction to reflect the efficiency of retention (0.85) of DNA−protein complexes.

findings, it would appear that BLM can recognize a single-strand conformation in addition to a junction structure. Alternatively, it is possible that BLM recognizes junction structures as well as certain conformations due to secondary structure, possibly resembling junctions, in the single-stranded DNA oligonucleotide we tested.

In all of the additional substrates utilized above, the Hill coefficient was found to be unity (Table 2) with the exception of single-stranded DNA where it was ~2 (see Figure 2 and Table 2). Thus, it would appear that there is cooperativity in the binding of BLM to some forms of DNA, but not to others. The mere presence of single-stranded DNA does not appear to be sufficient to effect the change in binding mechanism reflected by the Hill coefficient. The molecular basis for this difference is not clear, but it may reflect some step in the assembly of BLM onto DNA in different conformational states perhaps due to different quaternary forms assumed by BLM in solution (*39*, *40*). It is also unclear why the relative affinities for different DNAs as determined by us and by Karow et al. (*26*) are not in complete agreement. In particular there is a widespread disparity in the binding affinity with single-stranded DNA. The differences may be due to the use of two very different types of assay systems, both of which have the drawback of being nonequilibrium methods. Further investigation with more sophisticated binding assays should resolve the discrepancies.

Specificity of BLM for Melting D-Loops. To test the idea that BLM suppresses recombination by disrupting joint molecules formed as early intermediates in homologous pairing, we wished to examine the ability of BLM to melt D-loops. We were interested in using oligonucleotides to model recombination intermediates, but were concerned about extraneous unwinding effects due to the action of BLM at DNA ends. Previous studies in other laboratories demonstrated that in the absence of a single-stranded binding protein to sequester melted single strands and prevent their reassociation, BLM could efficiently melt heteroduplexes formed by annealing oligonucleotides of up to 91 residues in length (*17*, *18*, *41*). However, in the absence of a protruding 3′-single-stranded stretch, melting activity was found to be very low even with heteroduplexes of only a few dozen base pairs.

We tested the melting activity of BLM on a variety of DNA heteroduplexes and found, in accordance with others, that a heteroduplex formed with a 91-mer oligonucleotide annealed to single-stranded circular M13 DNA could be melted in the absence of a single-stranded DNA binding protein (Figure 3A). A heteroduplex formed with a 30-mer annealed flush to the 5′ end of a 98-mer was melted easily by BLM. When a flayed end was present, both the rate and extent of melting were higher (Figure 3B). A 30-mer oligonucleotide annealed to an internal region of a 98-mer and containing an unpaired 5′ protruding tail of four nucleotides was melted extremely efficiently. In contrast, heteroduplex formed with a 30-mer annealed flush to the 3′ end was refractory to melting (Figure 3A). A 48-mer with blunt ends but with an internal nick 18 residues from one end was also refractory to melting, even at a 4-fold higher concentration of BLM than was able to unwind the other substrates (Figure 3A). In addition we found that a blunt-ended duplex 83-mer was completely refractory to melting at a BLM to DNA molar ratio of 1:4 (data not shown). These findings indicated that as long as DNA ends were kept flush, there would be minimal complications in interpreting melting activity. Therefore, we adopted a strategy for substrate design using oligonucleotides to model the structures formed by RecA and Rad51 in homologous pairing reactions.

Heteroduplex 61-mer oligonucleotides were prepared as substrates as above with an invading third strand complementary to 21 internal nucleotides of one 61-mer and a corresponding single-stranded loop in the other 61-mer (see Figure 4 and Table 2). In addition to a basic D-loop in which the third strand was completely complementary over its length, we also examined D-loops with protruding 5′ or 3′

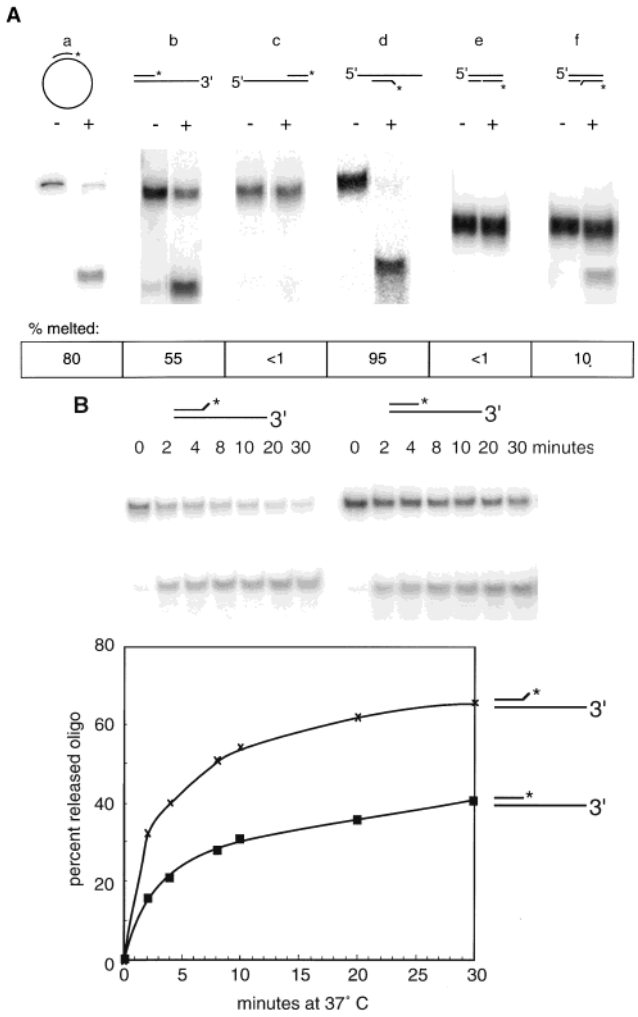

FIGURE 3: BLM unwinding activity as a function of DNA conformation. (A) Unwinding assays showing BLM helicase activity on the following heteroduplexes: (a) 91-mer annealed to single-stranded circular M13 (see Table 1; the M13 oligo was annealed to M13 and extended one nucleotide by incorporation of [α-^{32}P]-dCTP by DNA polymerase I Klenow fragment); (b) 30-mer annealed flush to the 5′ end of a 98-mer (see Table 1; the 5′ end oligo was annealed to the 98-mer oligo); (c) 30-mer annealed flush to the 3′ end of a 98-mer (see Table 1; the 3′ end oligo was annealed to the 98-mer oligo); (d) flayed 38-mer annealed to the internal region of a 98-mer (see Table 1; the 38-mer oligo was annealed to the 98-mer oligo); (e) nicked double-stranded 48 bp substrate (see Table 1; HJ-1 was annealed to Sub HJ-4 and Sub HJ-2b); (f) substrate e with 4 nt 3′ tail at the nick (see Table 1; HJ-1 was annealed to Sub HJ-4 and Sub HJ-2c). The asterisk represents the oligonucleotide that is labeled at the 5′ end. Reactions were performed as described in Experimental Procedures. (−) lanes contained no BLM; (+) lanes contained the following: (a) 20 ng substrate and 5 nM BLM; (b) and (c) 50 fmol of substrate and 6.3 nM BLM; (d) 50 fmol of substrate and 5nM BLM; (e) 50 fmol of substrate and 22 nM BLM; (f) 50 fmol of substrate and 4.4 nM BLM. The percent melted after reaction for 20 min was determined by quantitating the radioactivity of the released oligo as compared to the radioactivity of the annealed substrate plus the released oligo, normalized to the (−) lane. For substrates a, d, and f, the percent melted at the concentrations of BLM shown (5, 5, and 4.4 nM, respectively) did not increase with increasing concentrations of BLM. (B) Time course and kinetics of unwinding by BLM of a flayed substrate (see Table 1; 5′ end tailed oligo was annealed to the 98-mer oligo) as compared to a flush substrate (substrate b in Figure 3A). The oligonucleotide that is labeled at the 5′ end is indicated by the asterisk. Each lane contains 1.7 nM substrate and 6.3 nM BLM. The kinetics of unwinding were determined by phosphorimaging.

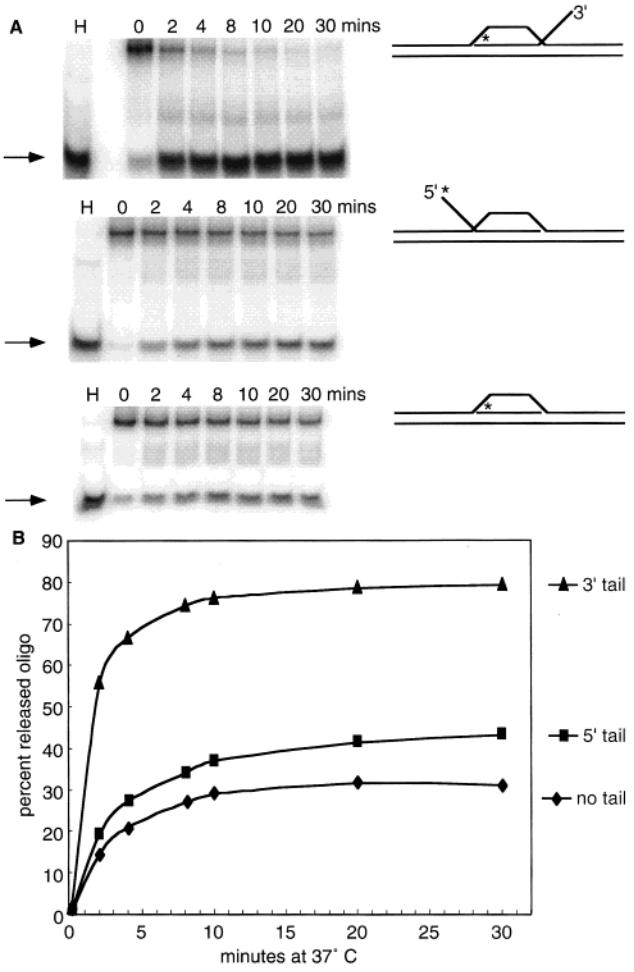

FIGURE 4: BLM preferentially unwinds D-loops with a 3′ single-stranded tail. (A) Time course of unwinding by BLM of synthetic D-loops with a 3′ single-stranded tail, 5′ single-stranded tail, or no tail. In the schematics, the oligonucleotide that is labeled at the 5′ end is indicated by the asterisk. The lane indicated by H contains the substrate heated at 100 °C for 10 min. The arrow indicates the migration of the released oligonucleotide. Each lane contains 1.7 nM substrate and 1.8 nM purified BLM protein and reactions were performed as described in Experimental Procedures. (B) The kinetics of unwinding of synthetic D-loops by BLM were determined by quantifying the gels in panel A using a Storm PhosphorImager (Molecular Dynamics). The percent released oligo was determined by quantitating the hybridization of the released oligo as compared to the hybridization of the annealed substrate plus the released oligo. All time points were normalized to the zero time point.

single-stranded tails of 20 residues extending from the invading third strand. In each case, the invading third strand was ^{32}P-labeled so that dissociation could be monitored. At a molar ratio of BLM to DNA of 1:1, all three of the D-loops were melted (Figure 4), but there was strong preference for the structure with the 3′ protruding tail, in both the initial rate and the extent of unwinding.

The possibility was considered that the observed melting was not a direct consequence of BLM acting on the D-loop, but resulted from a secondary action following melting outward to the duplex ends from the internal looped-out strand. To investigate this, we tested BLM for melting activity on DNA with an internal bubble. Melting activity was observed but the initial rate was approximately one-fourth of the rate seen with D-loop DNA with no tail (Figure 5). Thus, it seems likely that the preferential melting of

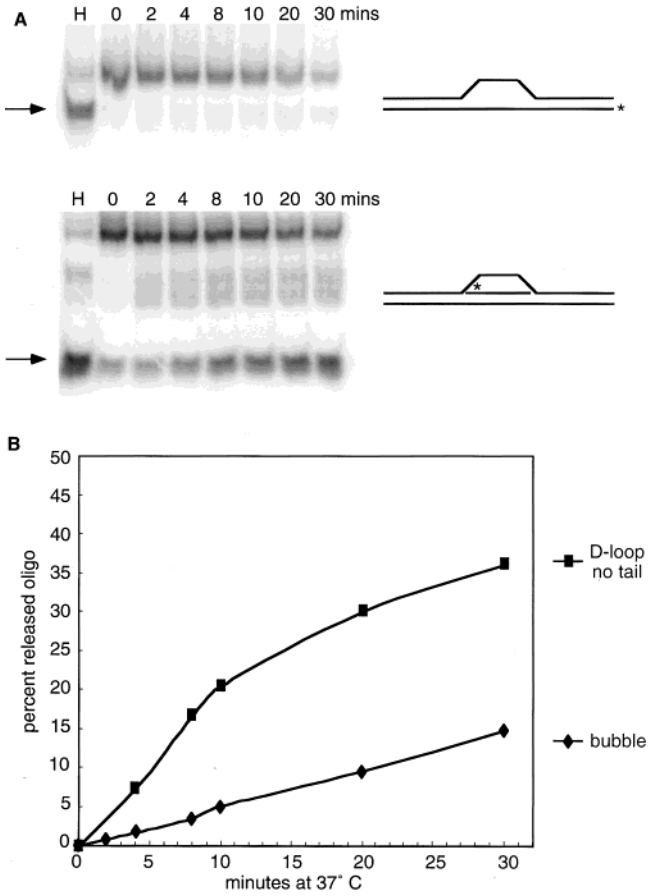

FIGURE 5: BLM unwinds a bubble structure less efficiently than a D-loop. (A) Time course of unwinding by BLM of a bubble substrate consisting of the two longer oligonucleotides used in the D-loops in Figure 4 compared to a D-loop without a tail. In the schematics, the oligonucleotide that is labeled at the 5′ end is indicated by the asterisk. The lane indicated by H contains the substrate heated at 100 °C for 10 min. The arrow indicates the migration of the released oligonucleotide. Each lane contains 1.7 nM substrate and 1.8 nM purified BLM protein and reactions were performed as described in Experimental Procedures. (B) The kinetics of unwinding of the bubble structure and the D-loop without a tail were determined by quantifying the gels in panel A using a Storm PhosphorImager (Molecular Dynamics). The percent released oligo was determined by quantitating the hybridization of the released oligo as compared to the hybridization of the annealed substrate plus the released oligo. All time points were normalized to the zero time point.

D-loops arises from an inherent preference of BLM for a structural feature of the three-stranded complex.

The duplex formed by the invading third strand in the D-loop structures is likely to be cohelical with the duplex stretches formed by the flanking sequences. However, there is a discontinuity in the phosphodiester backbone at the site of annealing with the invading third strand, and we considered whether such a discontinuity might account for the activity of BLM in melting the third strand. We tested this idea by assessing melting activity on duplex DNA with an internal nick. No activity was observed (Figure 3A), but when a short single-stranded tail of only four nucleotides was added there was significant unwinding activity. Since the unpaired single-stranded tail is probably bent away from the axis of the helix, it seems likely that BLM recognizes the structure at the junction where the invading single strand wraps plectonemically around its complementary sequence.

DISCUSSION

Studies on the *E. coli* RecQ protein, the prototype and founding member of the family of helicases that includes BLM, reveal a multifunctional role in recombination. Genetic and biochemical evidence supports the idea that RecQ can act as an initiator in the early steps of recombination and as a disrupter of later steps (*6*, *42*, *43*). Inactivation of RecQ has no strong effect on recombination or repair unless the host cell is already compromised by additional defects in recombinational processes. This finding is not surprising given that RecQ is only one of several different helicases in the cell with similar biochemical activities and possibly partially overlapping functions (*7*, *8*). Thus, a role of one helicase can be unmasked but only in the absence of compensatory functions. Biochemical evidence has mounted, indicating that RecQ has a relaxed substrate specificity and is free to unwind many DNA substrates presented. In addition, RecQ also appears to act as a suppressor of illegitimate recombination (*10*). Thus, its role in homologous recombination may be through regulating a pool of substrates whose level and composition are governed dynamically at the intersection of several genetic pathways.

Given the genetic complexities of its prototype, it would not be unexpected to find that BLM operates in multifarious systems to maintain genomic stability. The studies reported in this investigation provide additional support for the idea that BLM can perform multiple functions in regulating recombination. We provide evidence that BLM recognizes and melts D-loop structures which are thought to be formed as early intermediates during homologous pairing and DNA strand exchange. Thus, in addition to promoting the branch migration of Holliday junctions and reversal of four-stranded-X structures speculated by Karow et al. (*26*) to arise from regressed replication forks, BLM could also act in regulating recombination by dissociating initiation events.

During DNA synthesis, recombination may play an essential role in ensuring that replication is completed even in the face of deleterious events such as replication fork encounters with DNA damage, stalling of the fork, or dissociation of the replication machinery resulting in disruption of the process and endonucleolytic breakdown. One mechanism for restoring replication is a recombination repair process dedicated to repairing collapsed replication forks (*23*−*25*, *44*). This process could occur through several branches in the model outlined in Figure 1. In this model a replication fork (a) is pictured as encountering a nick or structural blockade revealing a single-stranded region on one arm (b). By unwinding to the site of the nick or cleavage of the exposed single-stranded DNA, an arm could be broken off (f). Regression of the fork by pairing of newly replicated complementary strands could lead to a Holliday structure or X-form (c), which if cleaved by a structure specific endonuclease would also result in a broken arm (d). Reversing the regressed replication fork by branch migration as pictured in steps c→a or restarting replication through recombination by the pathways pictured with g and e as intermediates could salvage the process and rescue the cell from death resulting from incomplete synthesis of the genome.

While recombination would likely be initiated once DNA double strand ends were exposed, not all recombination

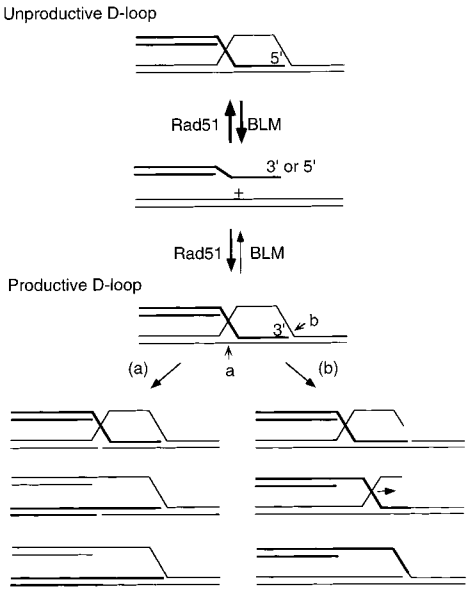

FIGURE 6: Model for BLM and Rad51 in antagonistic roles. After collapse of a replication fork, the broken, partially completed sister chromatid with a protruding single-stranded tail is paired by the action of Rad51 at the homologous locus in the other sister chromatid. Rad51 prefentially pairs 5′-tailed molecules to form joint molecules with a D-loop. The invading strand within these D-loops forms heteroduplex with 5′ → 3′ polarity proximal to the invasive end and is unpaired distally. BLM preferentially melts D-loop with an unpaired protruding 3′-invading strand. Thus, D-loops with invading 3′-tailed molecules have a higher probability of escaping dissociation by the melting activity of BLM. Preferential reactions are indicated schematically by the boldness of the arrows. Nicking at site **a** generates a structure resembling a replication fork in which the invading 3′-tail now is in the correct configuration to resume leading strand synthesis. Similarly, nicking at site **b**, followed by branch migration to the end of the heteroduplex (shown by the arrow) also generates a structure resembling a replication fork. By either pathway an invading 3′-single-stranded tailed molecule will generate the structure imagined to represent an active replication fork. Unspecified nicks are assumed to be repaired by the action of DNA ligase.

intermediates would necessarily be productive in terms of salvaging the replication fork. It has been established in studies on Rad51 that the 5′ end of a single-stranded DNA is more invasive than the 3′ end (*45*). Thus, as illustrated in Figure 6, Rad51 strand exchange protein would favor pairing of the tailed molecule with a protruding 5′ single-stranded region; the joint molecule formed, however, would not have the correct polarity for reinitiation of DNA replication. As we have demonstrated in this work, BLM preferentially dissociates joint molecules of the polarity favored by Rad51, e.g., D-loops with the 5′ end of the invading strand paired and the 3′ end free. This finding suggests that BLM might act during DNA replication to provide a mechanism for dissociating recombinational intermediates that are not productive for replicational restart. As shown in the model, if the invading single-stranded tail was the 3′ end, then by simple nicking of the D-loop at a or b the joint molecule could be resolved to form a structure resembling a replication fork with the polarity correct for resuming replication. In the absence of BLM function, it would be expected that somatic recombination would be elevated due to the ungoverned formation of joint molecules mediated by Rad51 and that replication would be slowed and beset with abnormalities.

BLM appears to be regulated with the cell cycle, rising during S-phase and G2 and diminishing after the onset of mitosis (*46*, *47*). This periodic mode of expression is consistent with a model accounting for the genomic instability associated with Bloom syndrome as a manifestation of a defective replication salvage system. The recent findings documenting association and interaction of BLM with toposiomerase III (*48*, *49*), plus a group of proteins that associate with BRCA1 (*50*) as well as the synaptonemal complex during meiosis (*51*, *52*), suggest additional roles for BLM in maintaining the integrity of the genome that remain to be detailed at the molecular level.

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