

Replication Protein A Physically Interacts with the Bloom's Syndrome Protein and Stimulates Its Helicase Activity*

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Bloom's syndrome is a rare autosomal recessive disorder characterized by genomic instability and predisposition to cancer. *BLM*, the gene defective in Bloom's syndrome, encodes a 159-kDa protein possessing DNA-stimulated ATPase and ATP-dependent DNA helicase activities. We have examined mechanistic aspects of the catalytic functions of purified recombinant BLM protein. Through analyzing the effects of different lengths of DNA cofactor on ATPase activity, we provide evidence to suggest that BLM translocates along single-stranded DNA in a processive manner. The helicase reaction catalyzed by BLM protein was examined as a function of duplex DNA length. We show that BLM catalyzes unwinding of short DNA duplexes (≤ 71 base pairs (bp)) but is severely compromised on longer DNA duplexes (≥ 259 -bp). The presence of the human single-stranded DNA-binding protein (human replication protein A (hRPA)) stimulates the BLM unwinding reaction on the 259-bp partial duplex DNA substrate. Heterologous single-stranded DNA-binding proteins fail to stimulate similarly the helicase activity of BLM protein. This is the first demonstration of a functional interaction between BLM and another protein. Consistent with a functional interaction between hRPA and the BLM helicase, we demonstrate a direct physical interaction between the two proteins mediated by the 70-kDa subunit of RPA. The interactions between BLM and hRPA suggest that the two proteins function together *in vivo* to unwind DNA duplexes during replication, recombination, or repair.

Bloom's syndrome (BS)¹ is a rare autosomal recessive disorder characterized by pre- and postnatal growth retardation, immunodeficiency, sun-induced facial erythema, and a greatly increased predisposition to a wide range of malignant cancers

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¹ The abbreviations used are: BS, Bloom's syndrome; bp, base pair; RPA, replication protein A; hRPA, human RPA; SSB, single-stranded binding protein; ESSB, *E. coli* SSB; scrPA *S. cerevisiae* replication protein A; ssDNA, single-stranded DNA; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; nt, nucleotide.

(1). The most characteristic feature of cells from BS patients is genomic instability (for review, see Ref. 2). This is manifested predominantly as an elevated frequency of chromosome breaks and exchanges (1, 3, 4) as well as a characteristic increase in the level of reciprocal exchanges between sister chromatids (5). BS cells exhibit hyper-recombination (1, 6, 7) and abnormalities in DNA replication that include an extended S phase and accumulation of abnormal replication intermediates compared with normal cells (8–10).

The gene defective in BS, designated *BLM*, encodes a protein of 1417 amino acids with the seven conserved motifs found in RNA and DNA helicases (7). By sequence alignment, the BLM gene product belongs to the RecQ subfamily of DNA helicases that includes a single *Escherichia coli* DNA helicase named RecQ, a protein required for the RecF pathway of genetic recombination (11) and for suppression of illegitimate recombination (12). Two yeast proteins, *Saccharomyces cerevisiae* Sgs1p (13, 14) and *Schizosaccharomyces pombe* Rqh1p (15), belong to the RecQ subfamily and have proposed roles in recombination and possibly replication. At present, five human members of the RecQ subfamily have been identified, including BLM (7), WRN (16), RecQL (17), RecQL4 (18), and RecQL5 (18). Mutations in the WRN gene are responsible for the premature aging disorder Werner's syndrome (16). Most recently, it was demonstrated that mutations in the *RecQL4* gene result in some cases of Rothmund-Thomson's syndrome (19). Both Werner's syndrome (20, 21) and Rothmund-Thomson's syndrome (22), like BS, are characterized by chromosomal instability suggesting that DNA helicases are important caretakers of the human genome with specialized roles in pathways of DNA metabolism.

Biochemical studies have shown that the BLM protein is a DNA-stimulated ATPase and ATP-dependent helicase, catalyzing strand displacement of short and medium length oligonucleotides (≤ 91 bp) from partial duplex substrates with a 3' to 5' polarity (23). BLM helicase preferentially unwinds a G4 DNA substrate consisting of four guanine-rich strands stabilized by Hoogsteen bonding (24). Electron microscopy analysis has shown that BLM protein forms oligomeric rings in solution (25). Size exclusion chromatography data indicate that the majority of enzymatically active BLM has an apparent molecular mass of >700 kDa, which is consistent with an oligomeric structure for BLM (25).

Aside from these structural and biochemical data, molecular details of the interactions of BLM protein with other proteins and biological DNA substrates remain to be defined. The molecular deficiencies involved in the clinical phenotype of BS presumably reflect an impaired function of the BLM protein in a pathway of nucleic acid metabolism. Transfection of the wild-

type *BLM* gene into BS cells reduces the frequency of sister chromatid exchanges (26). Mutant alleles of *BLM* found in individuals with clinical BS encode BLM protein that is devoid of DNA helicase activity and fails to reduce the high sister chromatid exchanges in transfected BS cells (26, 27). These studies provide evidence that the enzymatic activity of BLM is important for its cellular function.

In an effort to better understand the mechanistic aspects of the BLM catalytic activities, we have further characterized the catalytic activities of the BLM protein. Our results show that BLM unwinds short DNA duplexes (≤ 71 bp) but is severely compromised on DNA duplexes ≥ 259 bp. The poor unwinding of BLM helicase on relatively long DNA duplex substrates suggested to us that additional protein factor(s) might convert the helicase into a more processive enzyme. A good candidate to serve as an accessory factor to BLM helicase is the heterotrimeric single-stranded DNA-binding protein RPA that has been implicated in replication, recombination, and DNA repair (28). Evidence indicates that RPA modulates these processes by specific protein-protein and protein-DNA interactions. We have recently demonstrated that a specific functional and physical interaction exists between human RPA and WRN helicase (29). WRN helicase was found to be capable of unwinding long DNA duplexes up to 849 bp in a reaction dependent on hRPA. The notion that RPA may coordinately function with BLM helicase *in vivo* is supported by the recent finding that BLM colocalizes with RPA in meiotic prophase nuclei of mammalian spermatocytes (30). Colocalization of BLM and RPA at the synaptonemal complex of homologously synapsed autosomal bivalents suggests that these foci mark sites of ssDNA synaptic-related meiotic activity. The interactive roles of BLM and hRPA on synapsed meiotic bivalents are undefined but likely to involve the catalytic activity of BLM protein.

In this study we have shown that RPA is required to support BLM helicase activity on a relatively long DNA duplex of 259-bp. Two heterologous SSBs, ESSB and gp32, failed to substitute for RPA. This functional interaction was further substantiated by the demonstration of a physical interaction between hRPA and BLM. This interaction, and colocalization of the two proteins in meiotic cells, suggests that BLM and RPA function together in a pathway of DNA metabolism such as recombination or replication.

MATERIALS AND METHODS

Proteins—Recombinant hexahistidine-tagged BLM protein was overexpressed in *Saccharomyces cerevisiae* and purified as described previously (25). hRPA containing all three subunits (RPA70, RPA32, and RPA14) was purified as described previously (31). *S. cerevisiae* replication protein A (scrPA) was a generous gift of Drs. Dan Bean and Steven Matson, University of North Carolina, Chapel Hill. ESSB was purchased from Promega. T4 gp32 was from U. S. Biochemical Corp. Restriction endonuclease *Hae*III was obtained from New England Biolabs. Klenow enzyme was obtained from Roche Molecular Biochemicals. DNase I was from Roche Molecular Biochemicals. BSA type V was from ICN Biochemicals.

Nucleotides and DNA—M13mp18 ssDNA was from New England Biolabs. The 28-mer oligonucleotide 5'-TCCAGTCACGACGTTGTA-AAACGACGG-3' was from Life Technologies, Inc. M13mp18 RFI was prepared as described previously (32). (dT)₋₂₆₃ and ATP were from Amersham Pharmacia Biotech. (dT)₋₉₀₀ was from Midland Certified Reagent Co. [³H]ATP was from Amersham Pharmacia Biotech, and [α -³²P]dCTP was from NEN Life Science Products.

DNA Helicase Substrates—The 71-, 259-, and 851-bp M13mp18 partial duplex substrates were constructed as described previously (29, 33) with the following modifications. Duplex DNA fragments (69-, 257-, and 849-bp) from the *Hae*III digest of M13mp18 replicative form were purified by polyacrylamide gel electrophoresis and electroelution. The desired restriction fragment (100 ng) and M13mp18 ssDNA circle (2 μ g) were incubated together in an annealing reaction. The resulting partial duplex was labeled at its 3' terminus in a fill-in reaction with [³²P]dCTP and Klenow enzyme. The 30-bp M13mp18 partial duplex substrate was

constructed with a 28-mer complementary to positions 6296–6323 in M13mp18. The 28-mer was annealed to M13mp18 ssDNA circle and labeled at its 3' end as described above. Partial duplex DNA substrates were purified by gel filtration column chromatography using Bio-Gel A-5M resin (Bio-Rad).

DNA Helicase Assay—Helicase assay reaction mixtures (20 μ l) contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 5 mM ATP, 100 μ g/ml bovine serum albumin, 50 mM NaCl, and the indicated amounts of BLM helicase and/or single-stranded DNA-binding protein. The concentration of the 30-, 71-, 259-, and 851-bp partial duplex helicase substrates in the reaction mixture was approximately 2 μ M (nucleotide). Reactions were initiated by the addition of BLM protein and incubated at 37 °C for the indicated times. Reactions were terminated by the addition of 10 μ l of 50 mM EDTA, 40% glycerol, 0.9% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol. The products of helicase reactions with the 30-, 71-, 259-, and 851-bp partial duplex substrates were resolved on 12, 8, 6, and 6% nondenaturing polyacrylamide gels, respectively, as described previously (29). Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager or film autoradiography and quantitated using the ImageQuant software (Molecular Dynamics). The percent helicase substrate unwound was calculated by the following formula: % displacement = $100 \times P/(S + P)$. P is the product volume and S is the substrate volume. The values for P and S have been corrected after subtracting background values in the no enzyme and heat-denatured controls, respectively. All helicase data represent the average of at least three independent determinations.

ATPase Assay—Standard ATPase assay reaction mixtures (30 μ l) contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, the indicated ssDNA effector (30 μ M nucleotide), 0.8 mM [³H]ATP (42 cpm/pmol), 13 nM BLM protein, and the indicated amounts of hRPA. Reactions were initiated by the addition of BLM protein and incubated at 37 °C. Samples (5 μ l) were removed at 2-min intervals and evaluated by thin layer chromatography as described previously (34). Less than 20% of the substrate ATP was consumed in the reaction over the entire time course of the experiment. The kinetic rate constant (k_{cat}) values were expressed as the mean of at least three independent determinations.

ELISA Method for Detection of BLM-hRPA Protein-Protein Interaction—hRPA was diluted to a concentration of 1.65 ng/ μ l in Carbonate Buffer (0.016 M Na₂CO₃, 0.034 M NaHCO₃ (pH 9.6)). hRPA was then added to the appropriate wells of a 96-well ELISA plate (100 μ l/well) and allowed to incubate for 2 h at 24 °C. For control experiments, BSA was substituted for hRPA in the coating step. Wells were aspirated and washed three times with Wash Buffer (PBS, 0.5% Tween 20). Blocking Buffer (PBS, 0.5% Tween 20, 3% BSA) was added to appropriate wells and allowed to incubate 2 h at 24 °C. Wells were aspirated and washed one time with Blocking Buffer. BLM protein was diluted to 1.0 ng/ μ l in Binding Buffer (50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 5 mM ATP, 100 μ g/ml BSA, and 50 mM NaCl, or the indicated NaCl concentration). The diluted BLM protein was then added to appropriate wells of the ELISA plate (100 μ l/well) and allowed to incubate for 30 min at 24 °C. Wells were aspirated and washed three times with Binding Buffer. Primary antibody (rabbit polyclonal IgG against BLM protein) was diluted 1:1000 in Blocking Buffer, added to appropriate wells, and allowed to incubate 1 h at 24 °C. Wells were aspirated and washed four times with Blocking Buffer. Secondary antibody (goat anti-rabbit IgG-horseradish peroxidase) (Jackson ImmunoResearch) was diluted 1:10,000 in Conjugate Buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20, 1% BSA), added to appropriate wells, and allowed to incubate 30 min at 24 °C. Wells were aspirated and washed five times with Conjugate Buffer. Complexes were detected using K-Blue substrate (Biogen Corp.). The reaction was terminated after 1 min with 1 N sulfuric acid. Absorbance readings were taken at 450 nm. The A₄₅₀ values, corrected for background signal in the presence of BSA, are expressed as the mean of three independent determinations.

For DNase I treatment, both BLM protein and hRPA were pretreated with 20 units of DNase I in Binding Buffer at 37 °C for 15 min. The proteins were subsequently used in the ELISA as described above. Under the conditions used for DNase I treatment, 250 ng of control DNA standard (DNA Molecular Weight Marker II, Roche Molecular Biochemicals) was completely degraded (<2.5 ng, detectable limit) as evidenced by SYBR Green Stain (FMC Bioproducts) detection of DNA electrophoresed on a 1% agarose gel.

Data Analysis—The fraction of the immobilized hRPA bound to the microtiter well that was specifically bound by BLM protein was determined from the ELISAs. A Hill plot was used to analyze the data (Equations 1 and 2).

TABLE I
Hydrolysis of ATP (k_{cat}) catalyzed by BLM in the presence of various DNA effectors

The BLM concentration was 13 nM (monomer).

DNA effector ^a	k_{cat} <i>min</i> ⁻¹
None	ND ^b
(dT) ₁₆	141 ± 27
(dT) _{~263}	822 ± 216
(dT) _{~900}	956 ± 57
M13mp18 ssDNA circle	1163 ± 358
(dT) _{~900} + dT ₁₆ ^c	921 ± 82
M13mp18 ssDNA + (dT) ₁₆ ^c	1022 ± 183

^a DNA effector concentration was 30 μM nucleotide phosphate unless specified otherwise.

^b ND, not detectable.

^c Concentration of (dT)₁₆ was 90 μM nucleotide phosphate.

$$K_d = \frac{(1-f)[\text{Pt}]}{f} \quad (\text{Eq. 1})$$

$$\log [\text{Pt}] = \log (f/(1-f)) + \log K_d \quad (\text{Eq. 2})$$

K_d is the dissociation constant of the BLM·hRPA complex, [Pt] is the total concentration of BLM protein present in the reaction, and f is the ratio of the amount of the bound hRPA over the total amount of hRPA in the reaction. The logarithm of [Pt] was plotted against the logarithm of $(f/(1-f))$, and the y intercept represented the logarithm of K_d .

Far Western Blotting—Far Western blotting analysis was conducted essentially as described by Wu *et al.* (35). Previously, a physical interaction between BLM and hTOPIII α was demonstrated (35). In these studies, the BLM-hTOPIII α interaction served as a positive control in experiments to detect a BLM-hRPA interaction. Briefly, 0.2–1.0 μg of each polypeptide was subjected to SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL filters (Amersham Pharmacia Biotech). All subsequent steps were performed at 4 °C. Filters were immersed twice in denaturation buffer (6 M guanidine HCl in PBSA) for 10 min followed by 6 times for 10 min in serial dilutions (1:1) of denaturation buffer supplemented with 1 mM dithiothreitol. Filters were blocked in TBS containing 10% powdered milk, 0.3% Tween 20 for 30 min before being incubated in BLM (0.5 μg/ml) in TBS supplemented with 0.25% powdered milk, 0.3% Tween 20, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride for 60 min. Filters were washed for 4 times for 10 min in TBS containing 0.3% Tween 20, 0.25% powdered milk. The second wash contained 0.0001% glutaraldehyde. Conventional Western analysis was then performed to detect the presence of BLM using BFL-103 (35) as primary antibody. Anti-mouse IgG/horse-radish peroxidase conjugate (Sigma) was used as secondary antibody at a 1:10,000 dilution and detected using ECL (Amersham Pharmacia Biotech) following the manufacturer's instructions.

RESULTS

Characterization of ATP Hydrolysis Catalyzed by Bloom Protein—BLM helicase, like all helicases characterized to date, hydrolyzes nucleoside triphosphate as an energy source for the unwinding reaction (36). We studied the DNA-stimulated ATPase activity of BLM protein in the presence of DNA cofactors of varying lengths. Results of these assays are summarized in Table I. In the absence of a DNA effector, little or no ATP hydrolysis by BLM protein could be detected using a thin layer chromatography method to measure conversion of [³H]ATP to [³H]ADP. These results are consistent with the strong stimulation of ATP hydrolysis by DNA effectors reported by Karow *et al.* (23). With the very short (dT)₁₆ oligonucleotide, the turnover rate constant k_{cat} for ATP hydrolysis was 141 min⁻¹. As the length of the dT tract was increased to an average length of 263 nt, the k_{cat} increased nearly 6-fold. Increasing the dT tract from 263 to 900 nt resulted in only a modest 1.2-fold increase in the k_{cat} for ATP hydrolysis. By using M13 ssDNA circles as an infinitely long DNA effector, the increase in k_{cat} to 1163 min⁻¹ was similarly modest (1.2-fold). However, we cannot rule out that very long ssDNA molecules would result in a significantly

greater ATPase activity of BLM since the M13 ssDNA circle contains secondary structure. These data indicate that the stimulatory effect of DNA molecules on BLM ATP hydrolysis begins to plateau at a poly(dT) tract length of approximately 263 nt.

The stimulation of BLM protein ATPase activity by a given DNA concentration (30 μM nucleotide phosphate) was much greater for long dT tracts (263 and 900). To investigate if the BLM ATPase reaction was saturated with respect to each DNA effector, we examined BLM protein ATP hydrolysis in the presence of a 3-fold greater concentration of nucleotide phosphate (90 μM) for each DNA effector. The initial rates of BLM ATP hydrolysis were not increased with the elevated nucleotide phosphate concentration for each of the DNA effectors (data not shown). These data provide evidence that the ATPase reaction is saturated with respect to ssDNA for each DNA effector under the reaction conditions used.

The data in Table I might be interpreted to suggest that the free ends of the DNA effector molecules are inhibitory to the ATP hydrolysis reaction of BLM protein. To address this possibility, we mixed (dT)₁₆ with either (dT)_{~900} or M13 ssDNA at a 3:1 molar ratio of nucleotide phosphate and tested the mixture in BLM ATPase reactions. The k_{cat} values for BLM-catalyzed ATP hydrolysis in the presence of the (dT)_{~900} + (dT)₁₆ mixture or M13 ssDNA + (dT)₁₆ mixture were 921 and 1022 min⁻¹, respectively (Table I). These data indicate that the BLM ATPase reaction stimulated by (dT)_{~900} or M13 ssDNA was not inhibited by the presence of the short (dT)₁₆ molecules, suggesting that the DNA ends do not inhibit ATP hydrolysis.

Effect of Duplex Length on the BLM Helicase Reaction—Helicases can be classified by the macroscopic reaction mechanism for unwinding (36). Duplex unwinding as a function of duplex length is an important property of each helicase and may yield insights into the biochemical role of the enzyme in the cell. By using a variety of DNA substrates, biochemical studies of purified helicases *in vitro* have demonstrated that each DNA unwinding enzyme exhibits its own characteristic dependence of unwinding on DNA duplex length.

To characterize the effect of duplex length on the unwinding activity of BLM helicase, we tested partial duplex substrates of varying length in a strand displacement assay. Unwinding of M13 partial duplex DNA substrate molecules of 30, 71, 259, and 851 bp was measured as a function of BLM protein concentration (Fig. 1). The figures for percent unwinding of the 30- (Fig. 1A) and 71-bp (Fig. 1B) substrates rose to 70 and 37% with 4 nM BLM and 92 and 65% with 16 nM BLM (Fig. 1C). Further increase in amount of BLM helicase in the reaction did not result in an increase in the percent of DNA unwound for either substrate. The molecular explanation for the inability to achieve a greater percentage of the 71-bp partial duplex DNA substrates unwound is not clear. This phenomenon has been previously observed for UvrD helicase (33) and may reflect strand reannealing during the unwinding reaction (discussed below).

These results demonstrate that BLM helicase is not a very processive helicase even on short DNA duplexes, since the fraction of partial duplex molecules unwound exhibits a strict dependence on length of duplex. Rather, the amount of BLM helicase that is required in the unwinding reaction for the 30- and 71-bp partial duplex substrates is proportional to the length of DNA duplex to be unwound.

It is useful to express the strand displacement data as a rate of base pairs unwound per min per BLM helicase monomer (bp/min/BLM monomer). At a BLM concentration of 2 nM, the rates of duplex unwinding were 0.033 bp/min/BLM monomer for the 30-bp duplex substrate and 0.039 bp/min/BLM mono-

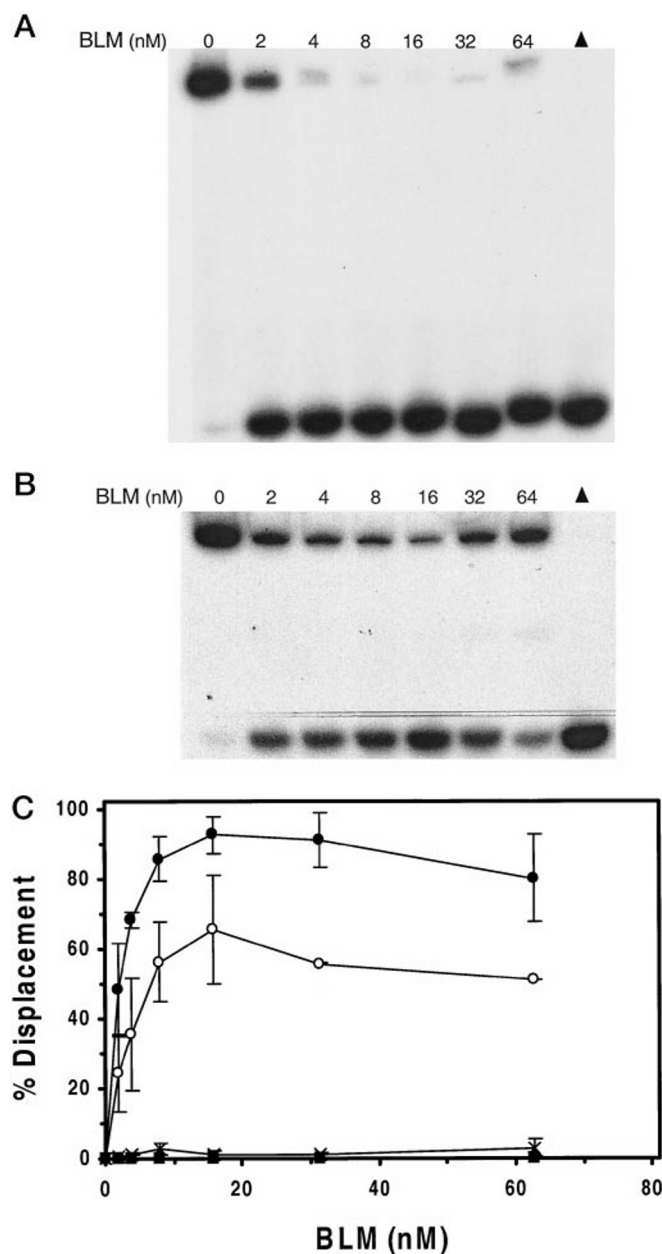


FIG. 1. BLM helicase catalyzes a limited unwinding reaction. Helicase assays were as described under "Materials and Methods" using the indicated concentration of BLM protein and M13mp18 partial duplex substrates of 30 bp (A), 71 bp (B), 259 bp (not shown), and 851 bp (not shown). Incubation was at 37 °C for 1 h. Reaction products were analyzed by nondenaturing gel electrophoresis. ▲, heat-denatured control. C, quantitation of results from helicase assays. ●, 30 bp; ○, 71 bp; ×, 259 bp; ■, 851 bp. Percent displacement is expressed as a function of BLM protein concentration.

mer for the 71-bp duplex substrate. At a BLM concentration of 4 nM, the rate of unwinding was 0.024 bp/min/BLM monomer for the 30-bp duplex substrate and 0.030 bp/min/BLM monomer for the 71-bp duplex substrate. Thus, the rates of unwinding (bp unwound/min/BLM monomer) were very similar for both the 30- and 71-bp partial duplex. Thus the number of base pairs unwound by BLM helicase for short (≤ 71 bp) partial duplex DNA substrates depends on the amount of BLM protein in the reaction as opposed to the length of the duplex unwound.

In contrast to the data described above, the 259- and 851-bp partial duplex substrates were very poorly unwound by BLM helicase at all protein concentrations tested. BLM protein displaced less than 2% of the 259-mer DNA fragments on the

helicase substrate (Fig. 1C) and produced no detectable unwinding of the 851-bp partial duplex substrate (Fig. 1C). Increasing the BLM concentration did not result in a proportional increase of percent substrate unwound (Fig. 1C). The maximum rate of unwinding (bp unwound/min/BLM monomer) was 13-fold less for the 259-bp partial duplex compared with the 71-bp partial duplex. These results indicate that BLM helicase unwinds short DNA duplexes (≤ 71 bp) efficiently but is severely compromised in its ability to unwind longer DNA duplexes. We conclude that BLM helicase catalyzes a limited unwinding reaction *in vitro*.

Specific Stimulation of BLM Helicase Activity by RPA—The limited unwinding reaction catalyzed by BLM helicase suggests that the enzyme encounters some type of kinetic barrier that prevents the unwinding of long duplex DNA tracts. Although there are a number of possible explanations for this result, we sought to test the effect of SSBs on the unwinding reaction. Previously, we demonstrated that a specific functional and physical interaction exists between WRN helicase and human replication protein A, which allows the enzyme to catalyze efficient unwinding of long DNA duplexes (29). Hence, an additional protein factor such as hRPA may be required to convert BLM helicase into a more processive enzyme. Alternatively, the poor ability of BLM helicase to unwind long duplex regions may be due to reannealing of the two strands of the DNA behind the advancing helicase. In the latter case, an SSB of any source may be suitable for stimulation of BLM helicase activity on long DNA duplexes.

To test the effect of the single-stranded DNA-binding protein, hRPA, on BLM unwinding activity, BLM protein was incubated with the 259-bp partial duplex in the presence of different concentrations of hRPA (Fig. 2). BLM protein (32 nM) alone catalyzed very little detectable unwinding ($\sim 3\%$) of the 259-bp partial duplex DNA substrate. In control reactions, hRPA (384 nM heterotrimer) alone also did not denature the 259-bp partial duplex DNA substrate (Fig. 2A). However, activation of BLM helicase activity on the 259-bp partial duplex could be detected at hRPA concentrations as low as 96 nM (heterotrimer) ($\sim 32\%$ substrate unwound) (Fig. 2, A and B). A 1.5-fold increase in hRPA concentration (144 nM, heterotrimer) resulted in stimulating BLM helicase activity to 75% substrate unwound. Maximal unwinding of the 259-bp partial duplex (82%) was achieved at an hRPA concentration of 192 nM heterotrimer.

To determine if the stimulatory effect of hRPA on BLM helicase activity reflected a specific functional interaction between the two molecules, the effects of ESSB and T4 gp32 on BLM-catalyzed unwinding of the 259-bp partial duplex were tested. In control reactions, ESSB (1456 nM homotetramer) or T4 gp32 (6042 nM monomer) alone did not denature the 259-bp partial duplex DNA substrate (data not shown). At all concentrations tested, neither ESSB (0–1456 nM homotetramer) nor T4 gp32 (0–6042 nM monomer) stimulated BLM helicase to unwind the 259-bp duplex over background (data not shown).

To gain insight into the mechanism of stimulation of BLM helicase activity by hRPA, strand displacement was expressed as a function of the ratio (R) of SSB-binding units per DNA-binding site. This analysis takes into account the fact that one ESSB homotetramer binds 35 nt (37); one gp32 monomer binds 7 nt (37), and one hRPA heterotrimer binds 30 nt (28). The stimulation of BLM helicase activity on the 259-bp partial duplex substrate was first detectable at a 1.5-fold excess of hRPA heterotrimer binding units compared with ssDNA-binding sites for hRPA ($r = 1.5$) (Fig. 2B, inset). At an R value of 2, the hRPA-stimulated BLM unwinding reaction attained a value of 75% substrate unwound, approaching the maximum.

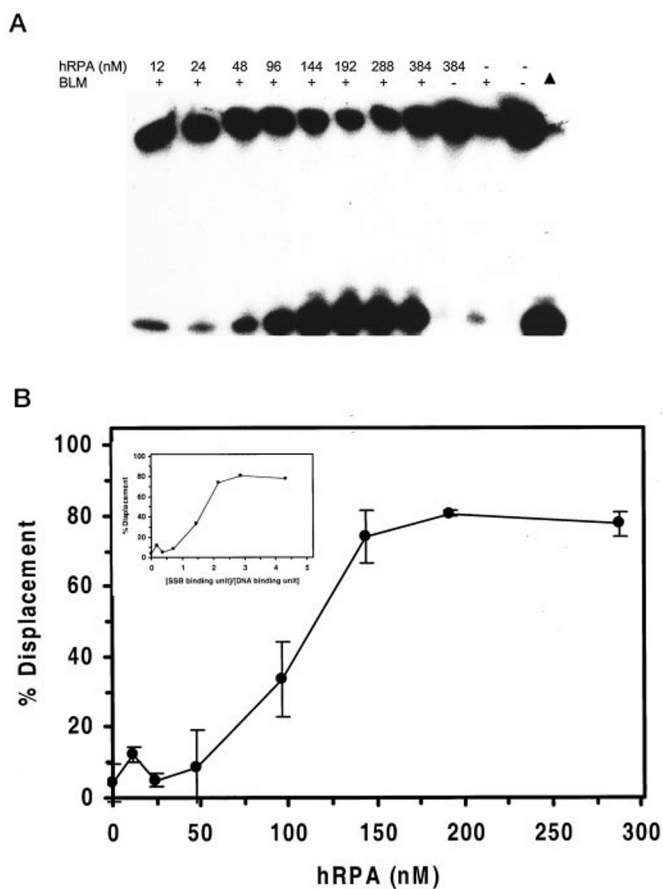


FIG. 2. Stimulation of BLM helicase activity on a 259-bp partial duplex DNA substrate by hRPA. A, BLM protein (32 nM) was incubated with the 259-bp partial duplex in the presence of the indicated concentrations of hRPA under standard helicase reaction conditions. Incubation was at 37 °C for 1 h. B, quantitation of results obtained in A. Percent displacement is expressed as a function of hRPA concentration. *Inset*, quantitation of results obtained in A. Percent displacement is expressed as a function of the ratio, (SSB binding unit)/(DNA binding unit).

In contrast, ESSB failed to stimulate BLM helicase activity at a 25-fold excess of ESSB binding equivalents over ESSB-binding sites (data not shown). Likewise, gp32 failed to stimulate BLM helicase activity at a 21-fold excess of binding units compared with binding sites (data not shown). The fact that both ESSB and T4 gp32 failed to stimulate unwinding of the long DNA duplex suggests that a specific interaction between BLM helicase and hRPA might be responsible for the observed unwinding of the 259-bp DNA duplex. The unique requirement for RPA to stimulate BLM-catalyzed unwinding of a long DNA duplex suggests that this functional interaction may also be important *in vivo*.

Effect of *S. cerevisiae* RPA on BLM Helicase Activity—The evolutionary conservation of eukaryotic RPA homologues suggests that RPA may have similar functions in DNA metabolism in eukaryotic organisms (28). However, a number of *in vitro* studies have demonstrated that RPA homologues are not functionally equivalent (28, 38). It is presumed that species-specific interactions of RPA with DNA and other proteins are responsible for the differences. To examine this issue in the context of BLM function, we tested RPA from *S. cerevisiae* for its effect on the BLM helicase reaction. BLM protein (32 nM, monomer) was incubated with the 259-bp partial duplex in the presence of the indicated concentrations of scRPA (Fig. 3). In control reactions, scRPA (300 nM, heterotrimer) alone did not denature the 259-bp partial duplex substrate. At an scRPA concentration of

12.5 nM heterotrimer, approximately 40% of the 259-bp partial duplex substrate was unwound. The percent substrate unwound achieved a maximum of approximately 70% at an scRPA concentration of 300 nM heterotrimer. These results indicate that scRPA can effectively substitute for hRPA in the stimulation of BLM helicase activity on a long 259-bp DNA duplex. Indeed, on a mole for mole basis, scRPA was more effective than hRPA in stimulating BLM helicase activity at significantly lower concentrations than hRPA. There are a number of possible explanations for the observed differences; for example, hRPA was overexpressed in *E. coli*, whereas scRPA was overexpressed in its native organism, potentially influencing the specific activity of the two proteins.

Effect of Duplex Length on BLM Helicase Reaction in the Presence of hRPA—As described above, at concentrations of BLM protein ≤ 4 nM, the rates of unwinding the 30- and the 71-bp partial duplex substrates were very similar in the absence of hRPA (Fig. 1). However, for longer duplexes (259 and 851 bp), helicase activity was hardly detectable. One of the possible explanations for the ability of hRPA to stimulate BLM helicase activity on the 259-bp partial duplex is that hRPA converts BLM helicase into a more processive enzyme. To examine more closely the stimulatory effect of hRPA on the BLM unwinding reaction, we tested the effect of hRPA on BLM helicase activity using the different length partial duplex DNA substrates (Fig. 4). The same levels of BLM protein were used in these experiments as were used to measure unwinding in the absence of hRPA (Fig. 1). The concentration of hRPA in these studies was 144 nM heterotrimers, a 2-fold excess of hRPA binding equivalents over binding sites on the helicase substrate. This concentration of hRPA was chosen because BLM helicase activity on the 259-bp partial duplex was stimulated to a near maximum (75% substrate unwound) at this level (Fig. 2).

The results of BLM protein titrations on the 30-, 71-, 259-, and 851-bp partial duplex substrates in the presence of hRPA are shown in Fig. 4. By using the 30-bp partial duplex, significant unwinding ($\sim 77\%$) was achieved at a relatively low concentration of BLM protein (2 nM, monomer). At the same concentration of BLM protein, 27% of the 71-bp partial duplex was unwound. These results show that BLM helicase still exhibits a protein concentration-dependent reaction mechanism even on short duplexes since the percent partial duplex substrate unwound at a given BLM protein concentration is clearly less for the 71-bp duplex than for the 30-bp duplex. This finding was further supported by an analysis of the helicase data using the 259-bp partial duplex. Very little unwinding of the 259-bp partial duplex ($< 1\%$) could be detected at a BLM concentration of 2 nM. Thus, even in the presence of hRPA, the percent fragment displaced by BLM helicase was significantly reduced for the 259-bp duplex compared with the shorter 30- and 71-bp duplexes. These results suggest that even in the presence of hRPA, the BLM unwinding reaction is compromised on long DNA duplexes. However, at higher concentrations of BLM protein, the presence of hRPA stimulates BLM helicase to more robustly unwind the 259-bp duplex, a finding consistent with the results presented in Fig. 2. The percent of 259-bp partial duplex substrate unwound increased proportionately with BLM protein concentrations. At the highest concentration of BLM tested, 64 nM monomer, 56% of the 259-bp partial duplex substrate molecules in the reaction was unwound.

In these experiments, the rate of helicase activity on the 259-bp partial duplex substrate achieves a maximum of 0.014 bp/min/BLM monomer at a BLM protein concentration of 32 nM. This rate is closely matched by similar values of 0.012 and 0.011 bp/min/BLM monomer at BLM concentrations of 16 and

FIG. 3. *S. cerevisiae* RPA stimulates BLM helicase activity. BLM protein (32 nM) was incubated with the 259-bp partial duplex in the presence of the indicated concentrations of scRPA under standard helicase reaction conditions. Incubation was at 37 °C for 1 h. Percent displacement is expressed as a function of scRPA concentration. *Inset*, quantitation of results. Percent displacement is expressed as a function of the ratio, (SSB binding unit)/(DNA binding unit).

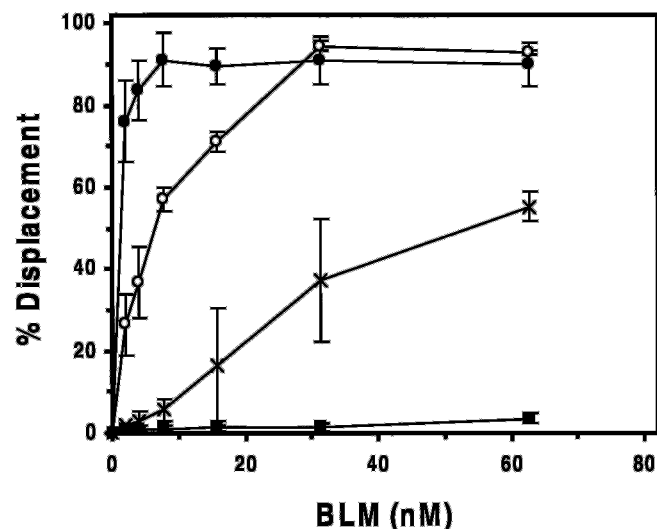
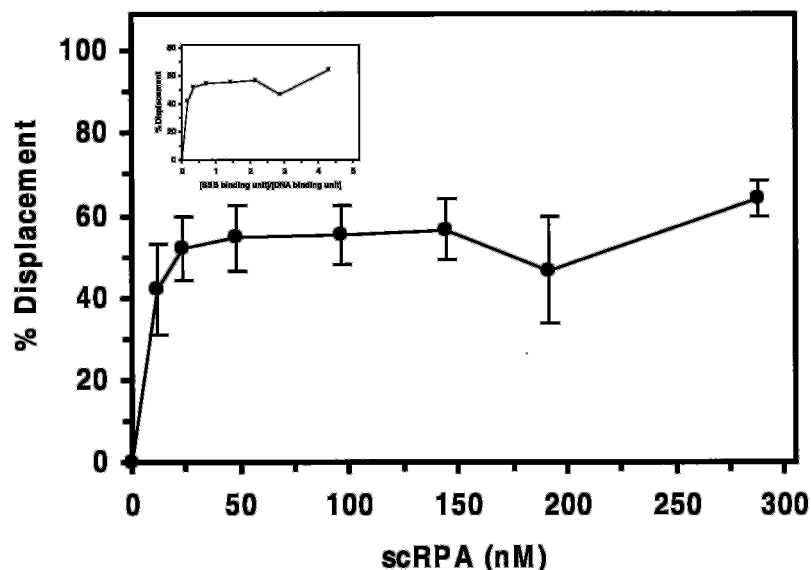


FIG. 4. Stimulatory effect of hRPA on BLM helicase activity is dependent on duplex length. BLM protein (indicated concentrations) was incubated with the 30- (●), 71- (○), 259- (×), and 851-bp (■) partial duplex DNA substrates in the presence of hRPA (145 nM heterotrimer) under standard helicase reaction conditions. Incubation was at 37 °C for 1 h. Reaction products were analyzed by nondenaturing gel electrophoresis. Percent displacement is expressed as a function of BLM protein concentration.

64 nM, respectively. The maximal rates of BLM helicase activity in the presence of hRPA on the 30- and 71-bp partial duplex substrates were 0.053 and 0.044 bp/min/BLM monomer. This rate analysis demonstrates a 3.8- and 3.1-fold enhancement of unwinding the short 30- and 71-bp duplex substrates, respectively, compared with the longer 259-bp substrate. These data suggest that the efficiency of BLM-catalyzed unwinding is reduced even in the presence of hRPA on longer DNA duplex substrates. However, the presence of hRPA is required to support BLM helicase activity on DNA duplexes of at least 259 bp.

In contrast to the results with the 259-bp duplex, hRPA had only a very minor effect on the unwinding activity of BLM on the 851-bp duplex even at the highest concentration of BLM tested, 64 nM (Fig. 4). These results suggest that BLM helicase, even in the presence of hRPA, fails to appreciably unwind very long DNA duplexes.

BLM Helicase Poorly Unwinds the 851-bp Duplex Over Prolonged Incubation—Previously, we demonstrated that hRPA

TABLE II
The effect of hRPA on the K_{cat} for ATP hydrolysis catalyzed by BLM
The concentration of BLM in each reaction was 13 nM.

hRPA nM	ssDNA effector	
	M13mp18	Poly(dT) ₋₂₆₃
	k_{cat} (min^{-1})	
0	1163 ± 358	822 ± 216
32	1300 ± 278	1204 ± 282
64	1407 ± 72	1182 ± 163
128	830 ± 266	862 ± 76
256	288 ± 46	812 ± 115

enables WRN helicase to unwind a long 849-bp partial duplex DNA substrate in a time-dependent manner (29). To explore the possibility that BLM helicase could effectively unwind longer DNA duplexes than 259 bp given sufficiently long periods of incubation, the BLM helicase was tested for unwinding of the 851-bp partial duplex over a 3-h period in the presence of hRPA. However, this kinetic analysis of the BLM helicase reaction demonstrated only slight displacement of the 851-mer (~4%) in reactions containing up to 40 nM BLM monomer (data not shown).

Effect of hRPA on ATPase Activity—To address the mechanism by which hRPA stimulates BLM helicase activity, we measured the effect of hRPA on ssDNA-stimulated ATPase activity of BLM (Table II). The turnover rate constant k_{cat} for ATP hydrolysis by BLM helicase was determined at various concentrations of hRPA protein and compared with the k_{cat} value obtained in the absence of hRPA. As shown in Table II, there was minimal effect of hRPA on k_{cat} values for BLM-catalyzed ATPase activity using the DNA effector (dT)₋₂₆₃. A modest increase of approximately 1.5-fold was detected at hRPA concentrations of 32 and 64 nM heterotrimer. Likewise, a minor increase in BLM-specific ATPase activity was also observed at these concentrations of hRPA using M13mp18 ssDNA circles as the effector. These results suggest that hRPA does not significantly increase the specific ATPase activity of BLM protein. At the highest concentration of hRPA tested, there is a significant decrease in the ATPase reaction rate using M13mp18 ssDNA as the effector. This inhibition may reflect competition between hRPA and BLM helicase for ssDNA-binding sites on the M13 ssDNA, or inhibition of translocation of BLM helicase along ssDNA (see "Discussion"). Alternatively, a physical interaction between BLM and hRPA may inhibit BLM ATPase activity in the presence of M13 ssDNA, although this

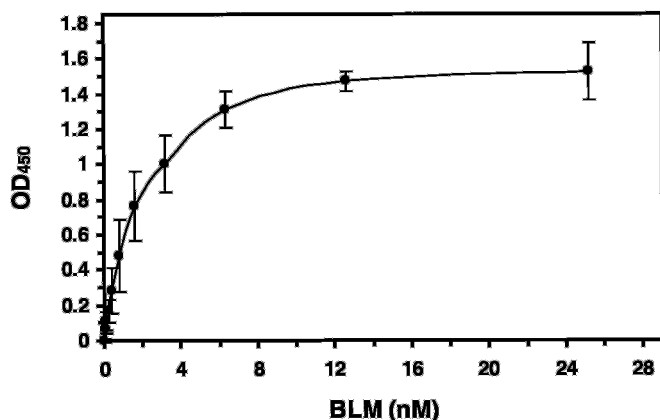


FIG. 5. Detection of a BLM-hRPA complex by ELISA. hRPA-coated wells (15 nM, heterotrimer, application) were incubated with increasing amounts of BLM protein for 30 min at 24 °C. Wells were aspirated and washed three times, and bound BLM protein was detected by ELISA using a rabbit polyclonal antibody against BLM protein. Absorbance readings at each point were corrected by subtracting a background A_{450} reading generated with BSA-coated wells.

does not seem likely since the effect does not occur in the presence of $(dT)_{-263}$.

BLM Forms a Direct Complex with hRPA ELISA Studies—The specific functional interaction between hRPA and BLM helicase suggested to us that the two proteins physically interact with one another. ELISAs were used to test for a protein-protein interaction. Increasing amounts of BLM protein were incubated in helicase reaction buffer containing 50 mM NaCl and 100 μ g/ml BSA with hRPA that had been immobilized on polystyrene microtiter wells. Bound BLM protein was detected immunologically. As shown in Fig. 5, the colorimetric signal was both dose-dependent and saturable. The specificity of this interaction was demonstrated by the absence of color in wells that had been precoated with BSA rather than hRPA (data not shown). In control experiments, the colorimetric signal from the BLM-hRPA interaction was resistant to pretreatment of both BLM protein and hRPA with DNase I suggesting that a contaminating DNA bridge is not responsible for the signal (data not shown). The specific binding of BLM protein to the hRPA-coated wells was analyzed according to Scatchard binding theory. The data were analyzed by a Hill plot as described under "Materials and Methods." The transformed data were linear indicating a single set of binding sites for hRPA with BLM protein. The apparent dissociation constant (K_d) was determined to be 1.3 nM.

We next examined the salt dependence of the BLM-hRPA interaction (Fig. 6). 100 ng of BLM protein (6.3 nM, monomer) was incubated in helicase reaction buffer containing the indicated concentrations of sodium chloride with hRPA that had been precoated on the microtiter wells. The BLM-hRPA interaction was nearly completely resistant to sodium chloride concentrations up to 150 mM. The protein interaction was reduced by 15% at a sodium chloride concentration of 200 mM. Further reduction in the BLM-hRPA interaction was detected as the salt concentration was increased up to 400 mM. However, a significant fraction (30%) of the BLM-hRPA complexes was resistant to dissociation at sodium chloride concentrations up to 600 mM.

Far Western Studies—In order to confirm the results of the ELISA studies, and to identify the subunit(s) of hRPA that mediates the interaction with BLM, Far Western analysis was performed (see "Materials and Methods"). For this, hRPA was immobilized on a nitrocellulose filter, which was then incubated with purified BLM protein. The filter was then washed to

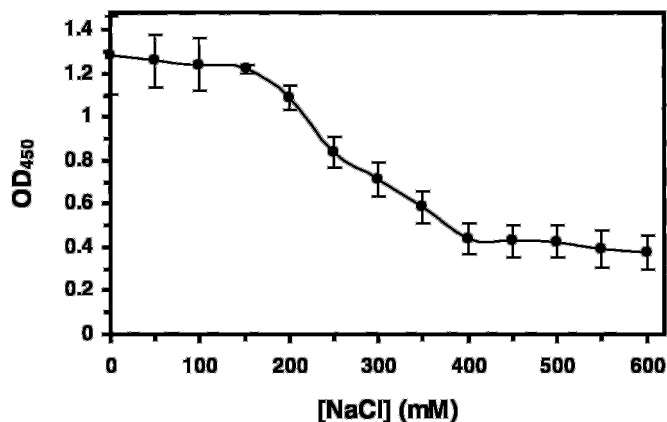


FIG. 6. The effect of NaCl concentration on the binding of BLM protein to immobilized hRPA. Various concentrations of NaCl (0–600 mM) were included in the initial solution used for incubation of BLM protein with hRPA bound to the microtiter well. ELISAs were conducted as described under "Materials and Methods."

remove unbound protein, and the presence of BLM was detected by conventional Western blotting. As controls, the membrane also contained topoisomerase III α , which was very recently shown to bind BLM (35), BSA, and BLM itself. Moreover, a second filter was prepared containing the same proteins, which was incubated in buffer alone. Fig. 7 shows that the anti-BLM antibody detected a band at the position of the 70-kDa subunit of the hRPA preparation (as well as some minor degradation products of this subunit), as well as at the position of the topoisomerase III α -positive control. No band was seen at the positions of either the BSA-negative control or the 32- and 14-kDa subunits of RPA. The immunoreactivity at the position of the 70-kDa hRPA subunit was not due to cross-reactivity of the anti-BLM antiserum with hRPA, because this band was absent from the control blot that had been incubated with buffer alone. The BLM present on the control membrane confirmed that the Western blotting procedure was successful for each membrane. From these studies, we conclude that BLM binds to the 70-kDa subunit of hRPA.

DISCUSSION

The cellular and molecular roles of the BLM gene product remain to be defined and characterized. Recently, it was shown that missense alleles of Bloom's syndrome abolish the ATPase and helicase activities of the BLM protein and fail to correct the genomic instability of Bloom's syndrome cells (26, 27). These findings indicate that the catalytic activities of the BLM protein play an essential role in the maintenance of genomic integrity. However, the precise defects in DNA metabolic pathways that give rise to the cellular and clinical phenotypes of BS remain to be elucidated. To understand better the functional roles of the BLM protein, we have examined the catalytic activities of the BLM protein and the effect of hRPA on its activities. The single-stranded DNA-binding protein RPA has been well characterized and implicated in the processes of replication, recombination, and repair. The results presented here suggest that BLM and hRPA are likely to function together in one of these fundamental processes.

In this study, we demonstrate a functional interaction between BLM and RPA. hRPA stimulates BLM helicase activity on DNA duplexes ranging from 30 to 259 bp. scRPA was also shown to stimulate BLM helicase activity on the 259-bp partial duplex substrate. A specific interaction between BLM helicase and RPA is supported by the absolute requirement for RPA in the BLM-catalyzed unwinding of the 259-bp duplex. Two heterologous SSBs, ESSB and T4 gp32, both failed to stimulate

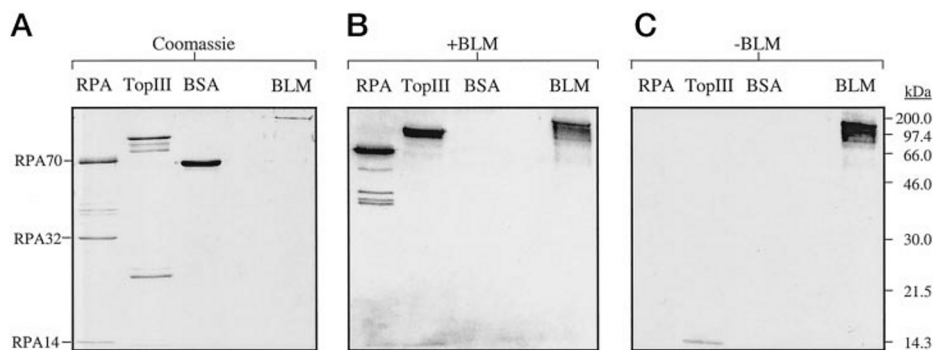


FIG. 7. **BLM and hRPA interact via the 70-kDa subunit of hRPA.** Purified hRPA, topoisomerase III α , BSA and BLM (as indicated above the lanes) were subjected to SDS-polyacrylamide gel electrophoresis on three identical gels. *a*, the proteins were stained with Coomassie Blue. *b* and *c*, the proteins were transferred to nitrocellulose membrane and then incubated with either purified BLM (*b*, +BLM) or buffer alone (*c*, -BLM). Western blotting using anti-BLM antibody was then used to detect the presence of BLM on each membrane. The positions of the 70-, 32-, and 14-kDa subunits of hRPA are indicated on the left. The positions of the molecular mass standards running parallel are shown on the right.

BLM-catalyzed unwinding of the 259-bp partial duplex as well as a 71-bp duplex (data not shown). These results suggest that an additional role of SSB in the BLM-catalyzed helicase reaction is performed by RPA other than simply coating the single strands generated during DNA duplex unwinding and preventing reannealing of separated strands.

An important property of DNA helicases is their dependence of unwinding on duplex length. Many helicases exhibit a limited reaction mechanism in which the fraction of DNA duplex molecules unwound decreases substantially as the duplex length increases (36). Our analysis of helicase activity as a function of BLM protein concentration on increasing length DNA duplexes reveals that BLM helicase belongs to this class of enzymes that catalyze a limited unwinding reaction. Very little unwinding of the 259- or 851-bp duplexes is detected at BLM protein concentrations that unwind nearly all of the 30- or 71-bp substrate molecules. Thus BLM helicase, acting alone, unwinds only relatively short DNA duplexes.

The limited unwinding reaction of BLM helicase is overcome by the participation of RPA. Both human and yeast RPA, which display a high level of homology (28), are able to stimulate the BLM-catalyzed unwinding reaction. It is possible, therefore, that RPA interacts functionally with the Sgs1 helicase in yeast. The effect of hRPA on the BLM helicase reaction is demonstrated by comparing BLM unwinding activity on different length DNA duplex substrates in the absence and presence of hRPA as a function of BLM concentration (Figs. 1 and 4). On the short 30-bp partial duplex substrate, a small (1.6-fold) stimulatory effect of hRPA on the BLM helicase reaction was detected at the lowest concentration of BLM tested, 2 nM monomer. At higher concentrations of BLM, the difference between reactions in the presence and absence of hRPA was reduced as the substrate unwound reached a maximum (approximately 90%). On the 71-bp partial duplex, a difference was only detected at higher concentrations of BLM (32 and 64 nM monomer). In the hRPA-supported reaction, the extent of unwinding the 71-bp duplex reaches >90% substrate unwound compared with approximately 60% of the substrate unwound in reactions lacking hRPA. A number of explanations are possible. Based on the high affinity of hRPA for ssDNA, the difference may partly reflect inhibition of reannealing of the displaced strand during unwinding. However, an additional effect of hRPA on BLM unwinding is evident based on the inability of heterologous SSBs to stimulate BLM helicase activity on the 71-bp partial duplex or the 259-bp partial duplex (data not shown).

The most obvious effect of hRPA on BLM helicase activity was observed with the 259-bp partial duplex. A 17-fold increase in duplex unwinding was detected at a BLM concentration of 16 nM monomer when hRPA (144 nM, heterotrimer) was pres-

ent, and a 38-fold difference between helicase reactions conducted in the presence versus absence of hRPA was detected at a BLM concentration of 32 nM monomer. Previously, the longest DNA duplex reported to be unwound by BLM helicase was 91 bp. These results demonstrate a functional requirement of hRPA for BLM-catalyzed unwinding of the 259-bp DNA duplex substrate. However, hRPA does not stimulate BLM helicase to unwind effectively the 851-bp partial duplex substrate, even over a prolonged 3-h incubation. We conclude that the stimulatory effect of hRPA on the BLM unwinding activity is limited to partial duplex substrates of ≤ 259 bp, at least under the reaction conditions described here.

If BLM helicase unwinds substantially long duplex tracts (≥ 851 -bp) *in vivo*, an additional factor is likely to be necessary for efficient unwinding. The inability of RPA to stimulate BLM-catalyzed unwinding of the long 851-bp duplex suggests a difference from the previously demonstrated WRN-RPA interaction. In the WRN-RPA interaction, a significant percentage of the 849-bp M13 partial duplex was unwound (up to 30% substrate unwound in 2 h) (29). However, the conditions for the WRN and BLM helicase reactions are different from one another, and we cannot rule out that BLM helicase may be able to unwind the 851-bp duplex under other reaction conditions.

Specific stimulation of BLM helicase activity by RPA suggests that the two proteins functionally interact. However, hRPA does not increase the specific ATPase activity of BLM protein suggesting that the observed increase in helicase activity is not due to a greater rate of ATP hydrolysis. We did observe a 4-fold inhibition of BLM ATP hydrolysis in the presence of the M13 ssDNA circle effector at the highest concentration of hRPA tested (256 nM heterotrimer) (Table II). At an hRPA concentration of 256 nM heterotrimer, the ratio of ssDNA binding equivalents over ssDNA-binding sites (R) in the ATPase reaction is 0.24. Despite the fact that 75% of the ssDNA-binding sites would be vacant, it is possible that a direct competition between hRPA and BLM helicase for binding sites on the M13mp18 ssDNA circle contributes to the inhibition of BLM ATPase activity at the very high concentration of hRPA. The 6–8-fold stimulation of ATP hydrolysis in the presence of long dT tracts or circular M13 ssDNA molecules compared with (dT)₁₆ (see Table I) may be explained by the longer time that BLM protein exists in the DNA-bound state. The results may be interpreted to suggest that BLM protein translocates processively along an ssDNA effector. The translocation of BLM enzyme along ssDNA may be hindered by the presence of bound hRPA molecules. However, the inhibitory effect is specific to the M13 ssDNA circle as it was not observed with (dT)₂₆₃. The basis for this difference is not known but

may reflect different binding properties of hRPA for the two DNA effectors.

The unwinding activity of WRN helicase (29) and human helicase ϵ (39), like BLM, is stimulated by hRPA. However, the ssDNA-stimulated ATPase activity of these helicases is not stimulated by hRPA. Although hRPA does not appear to increase the processivity of these enzymes during translocation along ssDNA, hRPA may play a role in the recruitment of the helicase to the ssDNA–double-stranded DNA junction of the ongoing helicase reaction. Consistent with this notion, we found that the unwinding activity of BLM helicase was not stimulated by hRPA on a 102-bp blunt duplex DNA substrate (data not shown). These data would suggest that an ssDNA loading dock is a requirement for the functional interaction between BLM helicase and hRPA. In addition, hRPA may tether BLM helicase to the DNA substrate at the unwinding fork to facilitate progression of the helicase through relatively long DNA duplex tracts.

A functional interaction between BLM and hRPA is strongly supported by our demonstration of a physical interaction between the two proteins that is mediated via the 70-kDa subunit of hRPA. ELISA experiments demonstrate that the physical interaction is saturable and shows a high affinity interaction between BLM protein and hRPA ($K_d = 1.3$ nM). The BLM–hRPA complex is resistant to salt concentrations up to 200 mM, indicating a fairly stable interaction. The physical interaction between BLM and hRPA presumably mediates the specific stimulatory effect of hRPA on BLM helicase activity. This is the first reported functional interaction between BLM helicase and another protein. WRN protein also exhibits a functional and physical interaction with hRPA (29), raising the possibility that the two helicases, defective in two distinct genomic instability disorders, compete for hRPA in DNA metabolic pathways. Further studies are necessary to address the importance of the WRN/BLM helicase interaction with hRPA and other molecular partners in pathways defective in these syndromes.

The demonstration of a physical and functional interaction between BLM and hRPA suggests that the two proteins function together in some aspect of DNA metabolism *in vivo*. Recently, BLM protein was shown to colocalize with RPA in meiotic prophase nuclei of mouse spermatocytes (30). RPA has been previously shown to play a role in both homologous synapsis and recombination. The appearance of BLM protein is delayed relative to RPA at the synaptonemal complex, suggesting the involvement of BLM in a late stage of zygote DNA replication or meiotic synapsis. The colocalization of BLM protein and RPA supports the notion that these proteins may functionally interact to generate ssDNA during meiotic synapsis. This suggestion is consistent with the results reported here that characterize a specific functional and physical interaction between BLM and hRPA.

The elevated sister chromatid exchange and hyper-recombination associated with BS suggest a defect in recombination. However, a replication defect may be primarily responsible for the genomic instability. BS fibroblasts display reduced replication fork progression and the accumulation of abnormal replication intermediates (10). BLM helicase (and other RecQ family helicases) have been proposed to be important to overcome structural abnormalities that arise during replication (2). It is conceivable that BLM helicase and RPA act together to unwind various types of nucleic acid structures at the replication fork, thereby facilitating efficient DNA replication or recombination. The specific interaction between BLM and hRPA suggests that

these proteins are likely to function coordinately *in vivo*. The characteristic cellular and clinical phenotypes of BS suggest that unique interactions of BLM protein with DNA and cellular proteins such as RPA are critical to the biological function of the BLM-catalyzed unwinding reaction necessary to maintain genomic integrity *in vivo*.

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REFERENCES

- German, J. (1993) *Medicine (Baltimore)* **72**, 393–406
- Chakraverty, R. K., and Hickson, I. D. (1999) *BioEssays* **21**, 286–294
- Ellis, N. A., and German, J. (1996) *Hum. Mol. Genet.* **5**, 1457–1463
- Watt, P. M., Hickson, I. D., Borts, R. H., and Louis, E. J. (1996) *Genetics* **144**, 935–945
- Langlois, R. G., Bigbee, W. L., Jensen, R. H., and German, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 670–674
- Cheng, R. Z., Murano, S., Kurz, B., and Shmookler, R. R. (1990) *Mutat. Res.* **237**, 259–269
- Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocci, S., Proytcheva, M., and German, J. (1995) *Cell* **83**, 655–666
- Gianneli, F., Benson, P. F., Pawsey, S. A., and Polani, P. E. (1977) *Nature* **265**, 466–469
- Hanaoka, F., Yamada, M., Takeuchi, F., Goto, M., Miyamoto, T., and Hori, T. (1985) *Exp. Med. Biol.* **190**, 439–457
- Lonn, U., Lonn, S., Nylen, U., Winblad, G., and German, J. (1990) *Cancer Res.* **50**, 3141–3145
- Nakayama, K., Irino, N., and Nakayama, H. (1985) *Mol. Gen. Genet.* **200**, 266–271
- Hanada, K., Ukita, T., Kohno, Y., Saito, K., Kato, J., and Ikeda, H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3860–3865
- Gangloff, S., McDonald, J. P., Bendixen, C., Arthur, L., and Rothstein, R. (1994) *Mol. Cell. Biol.* **14**, 8391–8398
- Watt, P. M., Louis, E. J., Borts, R. H., and Hickson, I. D. (1995) *Cell* **81**, 253–260
- Stewart, E., Chapman, C. R., Al-Khodairy, F., Carr, A. M., and Enoch, T. (1997) *EMBO J.* **16**, 2682–2692
- Yu, C. E., Oshima, J., Fu, Y. H., Wijsman, E. M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G. M., Mulligan, J., and Schellenberg, G. D. (1996) *Science* **272**, 258–262
- Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., Yamaoka, T., Hoshino, S., Ozawa, K., Eki, T., Nogami, M., and Okumura, K. (1994) *Nucleic Acids Res.* **22**, 4566–4573
- Kitao, S., Ohsugi, I., Ichikawa, K., Goto, M., Furuichi, Y., and Shimamoto, A. (1998) *Genomics* **54**, 443–452
- Kitao, S., Shimamoto, A., Goto, M., Miller, R. W., Smithson, W. A., Lindor, N. M., and Furuichi, Y. (1999) *Nat. Genet.* **22**, 82–84
- Salk, D., Bryant, E., Hoehn, H., Johnston, P., and Martin, G. M. (1985) *Adv. Exp. Med. Biol.* **190**, 305–311
- Salk, D., Au, K., Hoehn, H., and Martin, G. M. (1981) *Cytogenet. Cell Genet.* **30**, 92–107
- Lindor, N. M., Devries, E. M., Michels, V. V., Schad, C. R., Jalal, S. M., Donovan, K. M., Smithson, W. A., Kvolis, L. K., Thibodeau, S. N., and Dewald, G. W. (1996) *Clin. Genet.* **49**, 124–129
- Karow, J. K., Chakraverty, R. K., and Hickson, I. D. (1997) *J. Biol. Chem.* **272**, 30611–30614
- Sun, H., Karow, J. K., Hickson, I. D., and Maizels, N. (1998) *J. Biol. Chem.* **273**, 27587–27592
- Karow, J. K., Newman, R. H., Freemont, P. S., and Hickson, I. D. (1999) *Curr. Biol.* **9**, 597–600
- Neff, N. F., Ellis, N. A., Ye, T. Z., Noonan, J., Huang, K., Sanz, M., and Proytcheva, M. (1999) *Mol. Biol. Cell* **10**, 665–676
- Bahr, A., De Graeve, F., Keding, C., and Chatton, B. (1998) *Oncogene* **17**, 2565–2571
- Wold, M. S. (1997) *Annu. Rev. Biochem.* **66**, 61–92
- Brosh, R. M., Jr., Orren, D. K., Nehlin, J. O., Ravn, P. H., Kenny, M. K., Machwe, A., and Bohr, V. A. (1999) *J. Biol. Chem.* **274**, 18341–18350
- Walpita, D., Plug, A. W., Neff, N. F., German, J., and Ashley, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5622–5627
- Kenny, M. K., Schlegel, U., Furneaux, H., and Hurwitz, J. (1990) *J. Biol. Chem.* **265**, 7693–7700
- Lechner, R. L., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11185–11196
- Matson, S. W., and George, J. W. (1987) *J. Biol. Chem.* **262**, 2066–2076
- Matson, S. W., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 14009–14016
- Wu, L., Davies, S. L., North, P. S., Goulaouic, H., Riou, J.-F., Turley, H., Gatter, K. C., and Hickson, I. D. (2000) *J. Biol. Chem.* **275**, 9636–9646
- Matson, S. W., Bean, D. W., and George, J. W. (1994) *BioEssays* **16**, 13–22
- Lohman, T. M., and Ferrari, M. E. (1994) *Annu. Rev. Biochem.* **63**, 527–570
- Sibenaller, Z. A., Sorensen, B. R., and Wold, M. S. (1998) *Biochemistry* **37**, 12496–12506
- Seo, Y. S., Lee, S. H., and Hurwitz, J. (1991) *J. Biol. Chem.* **266**, 13161–13170