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Collaborating functions of BLM and DNA topoisomerase I in regulating human *rDNA* transcription

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Abstract

Bloom's syndrome (BS) is an inherited disorder caused by loss of function of the recQ-like BLM helicase. It is characterized clinically by severe growth retardation and cancer predisposition. BLM localizes to PML nuclear bodies and to the nucleolus; its deficiency results in increased intra- and inter-chromosomal recombination, including hyper-recombination of *rDNA* repeats. Our previous work has shown that BLM facilitates RNA polymerase I-mediated *rRNA* transcription in the nucleolus (Grierson *et al.*, 2012). This study uses protein co-immunoprecipitation and *in vitro* transcription/translation (IVTT) to identify a direct interaction of DNA topoisomerase I with the C-terminus of BLM in the nucleolus. *In vitro* helicase assays demonstrate that DNA topoisomerase I stimulates BLM helicase activity on a nucleolar-relevant RNA:DNA hybrid, but has an insignificant effect on BLM helicase activity on a control DNA:DNA duplex substrate. Reciprocally, BLM enhances the DNA relaxation activity of DNA topoisomerase I on supercoiled DNA substrates. Our study suggests that BLM and DNA topoisomerase I function coordinately to modulate RNA:DNA hybrid formation as well as relaxation of DNA supercoils in the context of nucleolar transcription.

Keywords

BLM helicase; DNA topoisomerase I; *rRNA* transcription; Bloom's syndrome; nucleolus; RNA polymerase I

1. Introduction

Human cells in interphase contain several nucleoli, sub-nuclear structures that contain the highly repetitive ribosomal DNA (*rDNA*) genes mapping to the satellite regions of the acrocentric chromosomes. Nucleolar *rDNA* associates with the nucleolar-dedicated RNA polymerase I and numerous other proteins required for ribosome biogenesis. The predominant function of nucleoli is the transcription of ribosomal RNA (*rRNA*) from *rDNA*, occurring during S- and G2-phases of the cell cycle [1,2]. Nascent *rRNAs* generated during RNA polymerase I-mediated *rRNA* transcription have a tendency to re-associate with template *rDNA* and form *rRNA:rDNA* hybrids that can inhibit *rRNA* transcription and

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facilitate *rDNA* recombination (reviewed in [3]). DNA topoisomerase I, a component of the RNA polymerase I transcription complex, relaxes the negative and positive supercoiling associated with *rRNA* transcription and prevents the formation of inhibitory *rRNA:rDNA* hybrids [4-8].

Bloom's syndrome (BS), an inherited disorder characterized by a high predisposition to cancer and severe growth retardation, is caused by loss of function of the BLM helicase [9]. BLM belongs to the conserved recQ subfamily of ATP-dependent 3'-5' helicases [10,11]. It localizes to the nucleolus and binds *rDNA* [12-14]. The C-terminus of BLM is required for its nucleolar retention and *rDNA* binding within the *18S*-coding region and the intergenic spacers (IGS) [13,14]. BLM deficiency leads to hyper-recombination within *rDNA* [15,16] and a reduction of overall *rDNA* repeat numbers in comparison to wild-type cells [13,14]. Hyper-recombination within *rDNA* generates extra-chromosomal *rDNA* circles (ERC), the accumulation of which is associated with aging in *S. cerevisiae* [17]. BLM-deficient cells display *rDNA* hyper-recombination [15,16], while some of the clinical characteristics of BS are suggestive of aging. These observations first suggested that nucleolar BLM maintains the stability of *rDNA* via direct binding to *rDNA* and implicate it in *rDNA* metabolism.

Our previous work demonstrated that BLM is a component of the RNA polymerase I transcription complex and unwinds RNA:DNA hybrids with 3' overhangs of DNA [18]. It also suggested that BLM and DNA topoisomerase I may cooperatively function to limit the accumulation of *rRNA:rDNA* hybrids in the nucleolus. Here, we report that BLM interacts directly with DNA topoisomerase I. Protein co-immunoprecipitation from nuclear extracts and sub-fractionated nuclei from cultured cells demonstrate that this interaction occurs in nucleoli. Purified recombinant proteins co-immunoprecipitate *in vitro*, while *in vitro* transcription/translation (IVTT) coupled to immunoprecipitation demonstrates that the interaction is mediated by a domain within the C-terminus of BLM. We show using helicase assays that DNA topoisomerase I stimulates BLM helicase activity on a GC-rich *rDNA*-like RNA₂₀:DNA₃₃ duplex substrate that models a co-transcriptionally formed *rRNA:rDNA* hybrid, but does not do so on a DNA₂₀:DNA₃₃ substrate. Finally, we show that BLM stimulates the DNA relaxation activity of topoisomerase I. Our data suggest that BLM and DNA topoisomerase I interact and cooperate to promote efficient *rRNA* transcription by RNA polymerase I.

2. Materials and Methods

2.1 Cell lines

MCF7 and HEK 293T cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% fetal bovine serum (Hyclone). All cells were cultured at 37°C and 5% CO₂.

2.2 Nucleolar isolation

Nucleoli were isolated from 293T cells according to the protocol of the Lamond Lab (www.lamondlab.com). Briefly, proliferating 293T cells were harvested by trypsinization, washed in PBS, re-suspended in buffer A (10mM HEPES, pH7.9, 10mM KCl, 1.5mM MgCl₂, 0.5mM DTT) and incubated on ice for 5 min. Cell suspensions were homogenized until approximately 90% of the cells were disrupted to produce intact nuclei. Lysis was monitored by light microscopy. Homogenized suspensions were centrifuged at 218g for 5 min at 4°C, nuclear pellets re-suspended in 3ml of S1 solution (0.25M sucrose, 10mM MgCl₂), layered over 3ml of S2 solution (0.38M sucrose, 0.5mM MgCl₂), and centrifuged at 1430g for 5 min at 4°C. Resultant nuclear pellets were re-suspended in 3ml of S2 solution and sonicated at 4°C (Fisher Scientific Sonic Dismembrator model 500). Liberation of

nucleoli was monitored by light microscopy. Resultant nucleolar suspensions were layered over 3ml of S3 solution (0.88M sucrose, 0.5mM MgCl₂), centrifuged at 3000g for 10 min at 4°C and re-suspended in 500ul of S2 solution.

2.3 Protein co-immunoprecipitation

Protein co-immunoprecipitations used 293T nuclear lysates prepared according to published protocols [19] or nucleolar and nucleoplasmic lysates prepared as described above. Antibodies used in co-immunoprecipitation included α BLM (Santa Cruz Biotech, sc-7790) and α DNA topoisomerase I (Bethyl, A302-589A). Protein-antibody complexes were captured with Dynabeads Protein G (Invitrogen, 100-04D), washed, eluted and separated by 8% SDS-PAGE, and detected using standard western blotting procedures using α BLM (Bethyl Laboratories, A300-110A), α RPA194 (Santa Cruz Biotech, sc-48385) and α RNA polymerase II (Abcam, ab817).

2.4 Protein purification

pYES-BLM expression vector (*pJK1*) was kindly provided by I. Hickson [11]. BLM was purified as previously described [19], with an additional heparin-sepharose purification step. Briefly, hexa-histidine (6X-His)-tagged BLM was over-expressed in *Saccharomyces cerevisiae*. Yeast were lysed at 20k psi using a French Press Cell Disrupter (Thermo) and lysates were separated by ultracentrifugation at 65,000g for 1hr at 4°C. Cleared lysates were purified by FPLC using Ni-NTA Superflow (Qiagen), followed by Heparin-Sepharose 6 Fast Flow (Amersham Biosciences) and finally Q-Sepharose (Sigma). Purity of the resultant BLM was determined by 8% SDS-PAGE, staining of gels with SYPRO Ruby Protein Gel Stain (Sigma) and analysis using ImageQuant software. The helicase-dead mutant BLM^{K695E} was purified by batch purification [19,20].

2.5 In vitro transcription/ translation (IVTT)

IVTT reactions were performed according to Lillard-Wetherell *et al.*, 2004 [21]. Briefly, *pET24D-BLM-N*, *pET24D-BLM-H* and *pET24D-BLM-C* were used in IVTT according to manufacturer's instructions (TNT Rabbit Reticulocyte Lysate kit, Promega). IVTT products were mixed with full-length wild-type human DNA topoisomerase I (Topogen) according to published protocols [22] and incubated for 2hrs at 4°C with rotation. Subsequently, α DNA topoisomerase I (Bethyl, A302-589A) was added with an additional 2hr incubation at 4°C. Finally, Dynabeads Protein G (Invitrogen, 100-04D) were added for 2hrs at 4°C, washed 5 times with binding buffer, eluted with 1X SDS-PAGE sample buffer, separated on 10% SDS-PAGE, dried and imaged using ImageQuant software.

2.6 Helicase assays

Oligonucleotides were purchased from Invitrogen. Oligonucleotide sequences (5'-3' orientation) are as follows: DNA₂₀-CGCTAGCAATATTCTGCAGC, DNA₃₃-GCTGCAGAATATTGCTAGCGGGAATTCGGCGCG and RNA₂₀-CGCUAGCAAUAUUCUGCAGC. RNA₂₀ and DNA were 32 P end-labeled using polynucleotide kinase (PNK; NEB) according to manufacturer's instructions. Duplex substrates were generated by heating to 95°C for 5 min and slow cooling. Helicase assays were performed as previously described [18]. Helicase products were separated on 12% non-denaturing polyacrylamide gels, dried and analyzed using ImageQuant software.

2.7 Topoisomerase I assays

DNA relaxation assays mediated by human DNA topoisomerase I (Topogen) were performed as described [23] in reaction buffer supplied by the manufacturer (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 150mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol).

200ng replicative form I (RF I) DNA [24] was used in 20ul reactions containing various dilutions of human DNA topoisomerase I in the presence or absence of purified BLM or helicase-dead mutant BLM^{K695E} [20]. The only measure of activity for BLM after purification is helicase unwinding. In the case of wild-type protein a loss of unwinding activity was observed upon dilution. Since there is no direct measure of the activity of the mutant as it is helicase-dead, for the topoisomerase I stimulation assays, higher concentrations of the mutant were used to overcome any loss of activity occurring upon dilution of protein as the reason for a potential negative result (supplementary figure 3). The mutant was diluted to the same extent as wild-type BLM. Reactions were stopped at various intervals of time (10-30 minutes) in stop buffer and analyzed on a 1.3 % agarose gel in TAE buffer as described [23]. Gels were stained by ethidium bromide and the DNA bands were quantitated by ImageJ software. The relative fold-stimulation of topoisomerase I activity by BLM was estimated by dividing the ratio of products (topoisomers) to substrate (RF I) in the presence of BLM by that in its absence for each reaction.

3. Results

3.1 BLM interacts with DNA topoisomerase I from nuclear and nucleolar-enriched extracts

Our recent study revealed a novel role for BLM in facilitating RNA polymerase I-mediated *rRNA* transcription via interaction with RNA polymerase I and resolution of RNA-DNA hybrid structures formed during transcription [18]. This role is consistent with localization of BLM in the nucleolus [12] and suggests its participation in nucleolar DNA metabolism and subsequent effects on ribosome biogenesis and protein production. DNA topoisomerase I is a component of the RNA polymerase I transcription complex and facilitates efficient *rRNA* transcription in bacteria, yeast and human cells [7,8,25] by relaxation of DNA supercoils and prevention of RNA-DNA hybrids generated during transcription. Therefore, we asked whether BLM and DNA topoisomerase I interact with each other (Fig 1A, Supplementary Figure 1a). Nuclear extracts from two cell lines, MCF7 and 293T, were used to test for co-immunoprecipitation using anti-BLM (α BLM) or anti-DNA topoisomerase I (α DNA topoisomerase I) antibodies. Figure 1A shows that each antibody immunoprecipitated both BLM and DNA topoisomerase I. Control immunoprecipitation experiments using α BLM or α DNA topoisomerase I antibodies and nuclear extracts from Bloom's syndrome cell line GM8505 did not immunoprecipitate topoisomerase I or BLM, respectively (data not shown). These results are consistent with BLM and DNA topoisomerase I interactions and their role in a common RNA polymerase I-associated complex (see Figure 1C below)[18].

As BLM and DNA topoisomerase I localize to the nucleolus and the nucleoplasm, we asked whether the BLM-DNA topoisomerase I interaction occurs specifically in the nucleolus. Nuclei were fractionated into nucleoli and nucleoplasm (Figure 1B) and the resultant sub-nuclear fractions used in co-immunoprecipitation experiments with α BLM antibodies. The RNA polymerase I-specific subunit RPA194 was used as a nucleolar marker, RNA polymerase II as a nucleoplasmic marker and α -actin as a cytoplasmic marker (Figure 1B). Results from co-immunoprecipitation experiments using the sub-nuclear fractions showed specific interactions of BLM with DNA topoisomerase I and RNA polymerase I subunit RPA194 in fractions from nucleoli (Figure 1C; Supplementary Figure 1c). These results suggest that their function may be specific to nucleolar metabolism and are consistent with the presence of a majority of BLM in the nucleolar fraction (Fig 1B).

Purified full-length human proteins were used for *in vitro* protein co-immunoprecipitations to determine whether the interaction between BLM and DNA topoisomerase I is direct or indirect. Recombinant BLM was expressed in *S. cerevisiae* and FPLC-purified; DNA topoisomerase I was purchased from Topogen (cat # TG2005H-RC1). Purified proteins are

shown in Figure 2A. Immunoprecipitation with either α BLM or α DNA topoisomerase I antibodies recovered both proteins demonstrating a direct protein-protein interaction (Figure 2B, Supplementary Figure 2).

3.2 BLM and DNA topoisomerase I interact directly *in vitro* via the BLM C-terminus

BLM is a modular protein with distinct domains dictating specific functions and localization to sub-nuclear regions. The C-terminus of BLM (amino acids 1116-1331) is required for localization to nucleoli and binding to *rDNA* [13,14]. BLM binds nucleolar *rDNA* both in the *rDNA* transcription unit as well as in the intergenic spacer region (IGS) [14]. The N-terminus of BLM is required for its accumulation in PML bodies and is also primarily used for interaction with DNA topoisomerases II α and III α [13,19,26].

In vitro transcription/translation (IVTT) of BLM using ³⁵S-methionine was used to generate the N-terminal (N: amino acids 25-612), helicase (H: amino acids 613-1025) and C-terminal (C: amino acids 1021-1417) segments of BLM. Co-immunoprecipitation of these segments with full-length human DNA topoisomerase I showed that the C-terminal region of BLM interacts with DNA topoisomerase I (Figure 3). These data suggest that this region of BLM may not only mediate BLM nucleolar localization and *rDNA* binding [13,14], but also direct protein-protein interactions with other nucleolar proteins.

3.3 DNA topoisomerase I stimulates BLM helicase activity on GC-rich RNA₂₀:DNA₃₃ substrate designed to model rRNA/rDNA hybrids, but not on DNA₂₀:DNA₃₃

The direct interaction of DNA topoisomerase I and BLM and their functions as components of an RNA polymerase I-associated transcription complex [7,18] suggested that BLM and DNA topoisomerase I may cooperate to unwind hybrid secondary structures presumed to form in the nucleolus during *rRNA* transcription (RNA-DNA hybrids) and *rDNA* replication (DNA-DNA hybrids). *In vitro* helicase assays used FPLC-purified BLM and equimolar human DNA topoisomerase I (Topogen) with the GC-rich RNA₂₀:DNA₃₃ duplex substrate (modeling co-transcriptionally formed *rRNA*:*rDNA* hybrids) or a DNA₂₀:DNA₃₃ duplex substrate (modeling *rDNA*:*rDNA* hybrids formed during replication). Helicase assays evaluated different BLM concentrations ranging from 0.15nM-0.3nM at time points from 15 sec-6 min in the presence or absence of equimolar amounts of DNA topoisomerase I. Reactions were stopped at the indicated time point and analyzed on native PAGE; bands were quantified to calculate the amount of substrate unwound for each time point.

Figure 4A shows a representative experiment performed with 0.25nM BLM on a RNA-DNA hybrid duplex substrate with a 3' DNA overhang. DNA topoisomerase I did not display unwinding activity by itself on the RNA-DNA hybrid substrate (Figure 4A) or the DNA-DNA substrate (data not shown). BLM by itself or BLM with equimolar DNA topoisomerase I unwound a GC-rich RNA₂₀:DNA₃₃ hybrid duplex following Michaelis-Menten kinetics (Figure 4B). Equimolar DNA topoisomerase I stimulated BLM unwinding activity at all protein concentrations studied for the RNA:DNA hybrid duplex (Figure 4B). Such stimulation was not observed on the corresponding GC-rich DNA₂₀:DNA₃₃ hybrid duplex (Figure 4C), suggesting substrate specificity for the stimulation observed with RNA₂₀:DNA₃₃ hybrid duplex. The kinetics of BLM unwinding activity using RNA-DNA or DNA-DNA hybrids were used to calculate the change in specific activity of BLM in the presence or absence of equimolar DNA topoisomerase I. BLM was pre-complexed with equimolar DNA topoisomerase I in these experiments. With a DNA₂₀:DNA₃₃ hybrid duplex, equimolar DNA topoisomerase I had a modest inhibitory effect on BLM activity at lower BLM concentrations that was insignificant with increased BLM concentrations (Figure 4C, data not shown). In contrast, with a RNA-DNA hybrid duplex, DNA topoisomerase I increased the specific activity of BLM unwinding from 2.75 fmol unwound/

min/nM BLM (BLM alone) to 3.72 fmol unwound/min/nM BLM (in the presence of DNA topoisomerase I) (Figure 5). Our data are analogous to those of Hu *et al.* [26], Bhattacharyya *et al.*, [27] and Russell *et al.* [19], which demonstrate that DNA topoisomerase III α and DNA topoisomerase II α , respectively, modulate BLM helicase activity. These results suggest a productive interaction of the two proteins specifically in the context of *rRNA* transcription.

3.4 BLM stimulates DNA topoisomerase I activity on supercoiled DNA

DNA topoisomerase I plays crucial roles in replication and transcription by relieving torsional stress generated during these processes [5,28-31]. It is intricately involved in *rDNA* transcription by RNA polymerase I and facilitates its movement by relaxation of supercoils generated on both sides of the RNA polymerase complex [4,31,32]. Our earlier studies revealed a role of BLM in facilitating RNA polymerase I-mediated *rDNA* transcription via its interaction with RNA pol I [18]. In addition to the resolution of RNA-DNA hybrids presented in the previous section, we tested the effect of BLM on relaxation of supercoiled DNA by DNA topoisomerase I. A 2.9kbp supercoiled plasmid was used to assess the relaxation activity of purified recombinant topoisomerase I in the presence or absence of purified BLM. DNA topoisomers were separated on agarose gels and quantified. A representative gel is shown in Figure 6A. Topoisomerase I activity was judged by the conversion of the negatively supercoiled substrate (RFI) to its topoisomers; more relaxed topoisomers migrate slower than the substrate. BLM alone did not alter the supercoiled state of the plasmid (Figure 6A, lane 6). In contrast, the activity of topoisomerase I at two concentrations (Figure 6A: 0.5 Units in lane 2 and 3, or 0.05 Units in lane 4 and 5) was enhanced in the presence of BLM (Figure 6A: compare lanes 2 and 3 or lanes 4 and 5). In order to estimate the fold-stimulation of topoisomerase activity by BLM, the amount of topoisomerase in the reaction was adjusted to get a maximum of 20% conversion of substrate to topoisomers in 30 minutes. Reactions were performed in the presence or absence of BLM or its helicase-dead mutant, BLM^{K695E}, and analyzed. The fold stimulation of topoisomerase activity by BLM or its mutant was calculated by comparing the ratio of products (topoisomers) to substrate (RF I) in the presence of BLM to that in its absence. The results indicated that BLM stimulated topoisomerase activity nearly 4-fold (Figure 6B). Similar stimulation of DNA topoisomerase I was observed using wild-type BLM or the helicase-dead mutant, BLM^{K695E}, suggesting that the stimulation may be independent of the BLM helicase activity (Figure 6B, Supplementary Figure 3). Our results are consistent with the conclusion that the interaction of BLM and topoisomerase I is reciprocally productive and suggests an active participation of BLM in *rRNA* transcription.

4. Discussion

Transcription of *rDNA* repeats by RNA polymerase I is crucial for ribosome biogenesis and cellular growth. Our previous study identifying a role for BLM in facilitating *rRNA* transcription suggests limited *rRNA* biogenesis as a potential mechanism underlying the ubiquitous growth retardation observed in Bloom's syndrome [18]. Regulation of *rRNA* transcription occurs at both transcription initiation and elongation. Due to the highly repetitive nature of *rDNA* loci, nascent *rRNA* generated during on-going transcription have the propensity to form stable *rRNA:rDNA* hybrids that cause RNA polymerase I stalling, inhibit transcription elongation and slow the overall *rRNA* transcriptional output. In bacteria, yeast and human cells, DNA topoisomerase I functions as a component of the RNA polymerase I transcription complex to relax *rDNA* template supercoiling to disfavor the formation of *rRNA:rDNA* hybrids and facilitate *rRNA* transcription [7,8,33]. The role of BLM in facilitating *rRNA* transcription and its ability to unwind RNA:DNA hybrids [18]

suggested that BLM and DNA topoisomerase I may interact directly and cooperate to unwind these *rRNA* transcription hybrids.

These studies demonstrate that BLM and DNA topoisomerase I interact in nucleolar-enriched extracts of MCF7 and 293T cells, consistent with both proteins being components of the RNA polymerase I transcription complex. The C-terminal domain of BLM, the same region required for BLM nucleolar localization, mediates this direct interaction. Previously characterized interactions of BLM with other DNA topoisomerases, for example III α and II α , occur primarily via the N-terminal region of BLM [19,26]. Our studies show that more than one region of BLM mediates interaction with all topoisomerases, suggesting that specific interactions modulate specific functions of BLM and that these interactions may be mediated independently.

BLM interactions with DNA topoisomerase II α and DNA topoisomerase III α modify BLM helicase activity on unique nucleic acid substrates [19,26,27]. We asked whether the direct interaction between BLM and DNA topoisomerase I similarly influences BLM as well as DNA topoisomerase I activity. Using *in vitro* helicase assays, we were unable to demonstrate a significant functional effect of DNA topoisomerase I on BLM-mediated unwinding of DNA₂₀:DNA₃₃ duplex substrate. However, DNA topoisomerase I stimulated BLM unwinding activity on RNA₂₀:DNA₃₃ duplex substrate, which models a co-transcriptionally formed *rRNA*:*rDNA* hybrid. The role of BLM in facilitating *rRNA* transcription is also supported by its stimulatory effect on DNA topoisomerase I function in relaxation of supercoiled DNA independent of helicase activity. Since DNA topoisomerase I activity is independent of ATP, the results suggest a direct modulation of topoisomerase I activity by BLM through protein-protein interactions. Such a role is consistent with the increased sensitivity of BLM-deficient cells to topoisomerase I inhibitors like camptothecin as well as increased formation of topoisomerase I-DNA cleavage complexes in treated cells [34,35]. Topoisomerase I also plays a significant role in preventing replication stress from the collapse between transcription and replication forks by reducing the formation of RNA-DNA hybrids [36]. Our data suggest a coordinated role for BLM and DNA topoisomerase I in removing or preventing these hybrids at the *rDNA* loci. Whether BLM and topoisomerase I play redundant or cooperative roles in removal of RNA-DNA hybrids outside of *rRNA* transcription, as well as relaxing torsional stress during replication, is not clear. However, our results pose an attractive possibility for such functions in preventing increased replication stress in both BLM- and DNA topoisomerase I-deficient cells.

In the context of nucleolar *rRNA* transcription, data presented in this study and our earlier study [18] suggest a model in which BLM and DNA topoisomerase I are components of the RNA polymerase I transcription complex and serve to exclude *rRNA*:*rDNA* hybrids from the *rDNA* as well as relieve the torsional stress generated during transcription. Prevention of RNA:DNA hybrid formation by DNA topoisomerase I is imperfect, as some RNA:DNA hybrids form in DNA topoisomerase I-proficient cells [8]. The presence of BLM within the RNA polymerase I transcription complex may allow those RNA:DNA hybrids that form to be unwound, thereby preventing interference with transcription and replication. The ability of DNA topoisomerase I to stimulate this ability of BLM may further ensure exclusion of RNA:DNA hybrids from the *rDNA*. Our studies have also uncovered a unique role for BLM in facilitating the relaxation of supercoiled DNA by topoisomerase I. This observation is consistent with the decreased overall *rRNA* transcription rate seen in BLM-deficient cells [18] and suggests active participation of BLM in RNA polymerase I transcriptional efficiency. Our results are consistent with the inhibition of RNA polymerase I transcription in yeast deficient for the BLM homologue *SGS1* and another 3'-5' helicase, *SRS2*, suggesting a conserved role for RecQ-like helicases in nucleolar *rRNA* transcription [37].

The collaborative functioning of BLM and DNA topoisomerase I to promote efficient *rRNA* transcription and thus cell growth has implications for cancer therapeutics. Cells deficient in either BLM or DNA topoisomerase I display slower growth rates [7,38]. Drugs targeting DNA topoisomerase I are used chemotherapeutically, such as topotecan to treat small cell lung cancer and irinotecan to treat colon cancer (www.uptodate.com). Understanding the nucleolar mechanisms by which BLM functions would provide the opportunity to inhibit selectively its growth-promoting activity in nucleolar metabolism while preserving its role in maintaining extra-nucleolar genomic stability. Thus, chemotherapeutic dual targeting of DNA topoisomerase I and BLM nucleolar function may synergistically inhibit tumor cell growth.

5. Conclusion

The BLM helicase actively participates in nucleolar metabolism by facilitating *rRNA* transcription by preventing *rRNA-rDNA* hybrid formation and relieving torsional stress through its interactions with DNA topoisomerase I. Such a nucleolar role implicates BLM in promoting ribosome biogenesis and cell growth, and provides a basis for understanding the growth defects and camptothecin sensitivity associated with BLM-deficient cells and in those affected by Bloom's syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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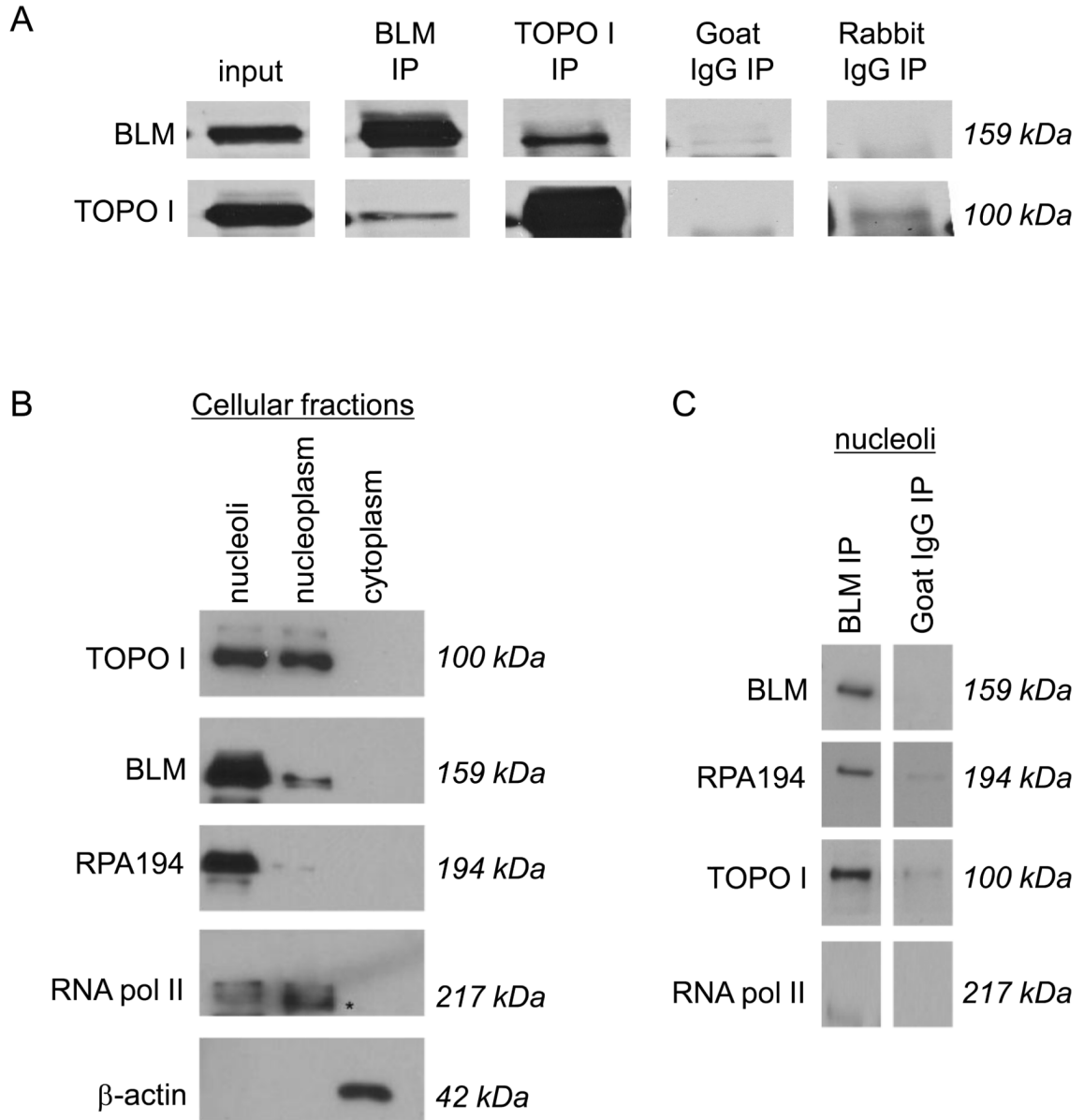


Figure 1. BLM and DNA topoisomerase I associate in mammalian cells

(A) Co-immunoprecipitations used MCF7 and 293T nuclear extracts, and either anti-BLM or anti-DNA topoisomerase I antibodies (referred as α BLM or α DNA topoisomerase I respectively). Immunoprecipitated proteins were separated using 8% SDS-PAGE and detected by western blotting using α BLM or α DNA topoisomerase I. Goat IgG was chosen as an isotype-matched negative control for α BLM; rabbit IgG was an isotype-matched negative control for α DNA topoisomerase I. (B) 293T nuclei were sub-fractionated into nucleoli and nucleoplasm. The purity of the sub-cellular fractions is shown using the RNA polymerase I-specific subunit RPA194 as a nucleolar marker; RNA polymerase II as a nucleoplasmic marker (indicated with an *); and α -actin as a cytoplasmic marker. Proteins were separated using 8% SDS-PAGE and detected by western blotting using the respective antibodies as indicated. (C) Nucleolar fractions were used in protein co-immunoprecipitation with α BLM. Immunoprecipitated proteins were separated using 8% SDS-PAGE and

detected by western blotting using α BLM, α DNA topoisomerase I, α RPA194 and α RNA polymerase II. Goat IgG was used as an isotype-matched negative control for α BLM.

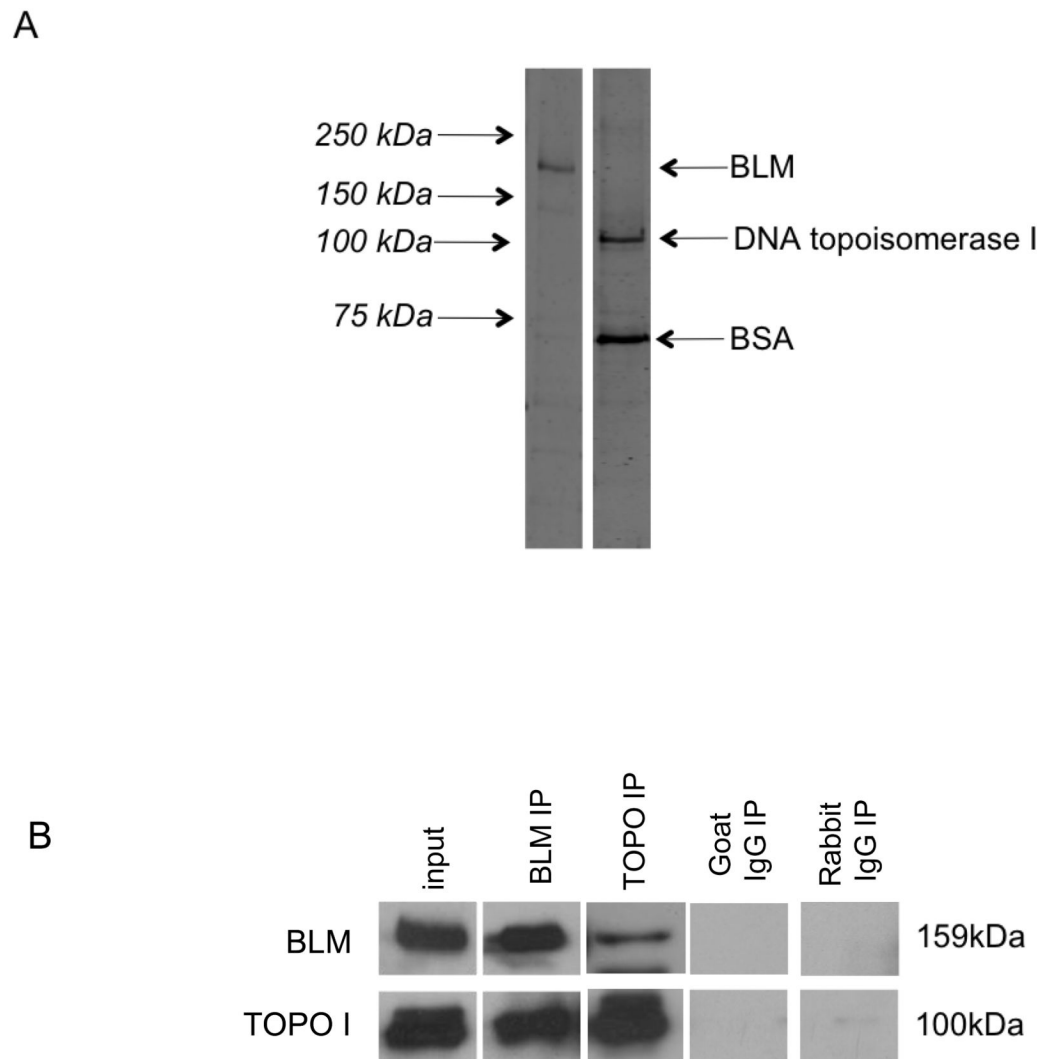


Figure 2. BLM and DNA topoisomerase I directly interact *in vitro*

(A) The purity of recombinant FPLC-purified BLM and purchased DNA topoisomerase I (Topogen) was determined using 8% SDS-PAGE and staining with Sypro Ruby Protein Gel Stain (Sigma). DNA topoisomerase I is supplied with 50ng/ul of bovine serum albumin (BSA), as indicated. (B) BLM and DNA topoisomerase I were used in *in vitro* protein co-immunoprecipitation using α BLM or α DNA topoisomerase I. Immunoprecipitated proteins were separated using 8% SDS-PAGE and detected by western blotting using α BLM and α DNA topoisomerase I. Goat IgG was used as an isotype-matched negative control for α BLM; rabbit IgG was used as an isotype-matched negative control for α DNA topoisomerase I.

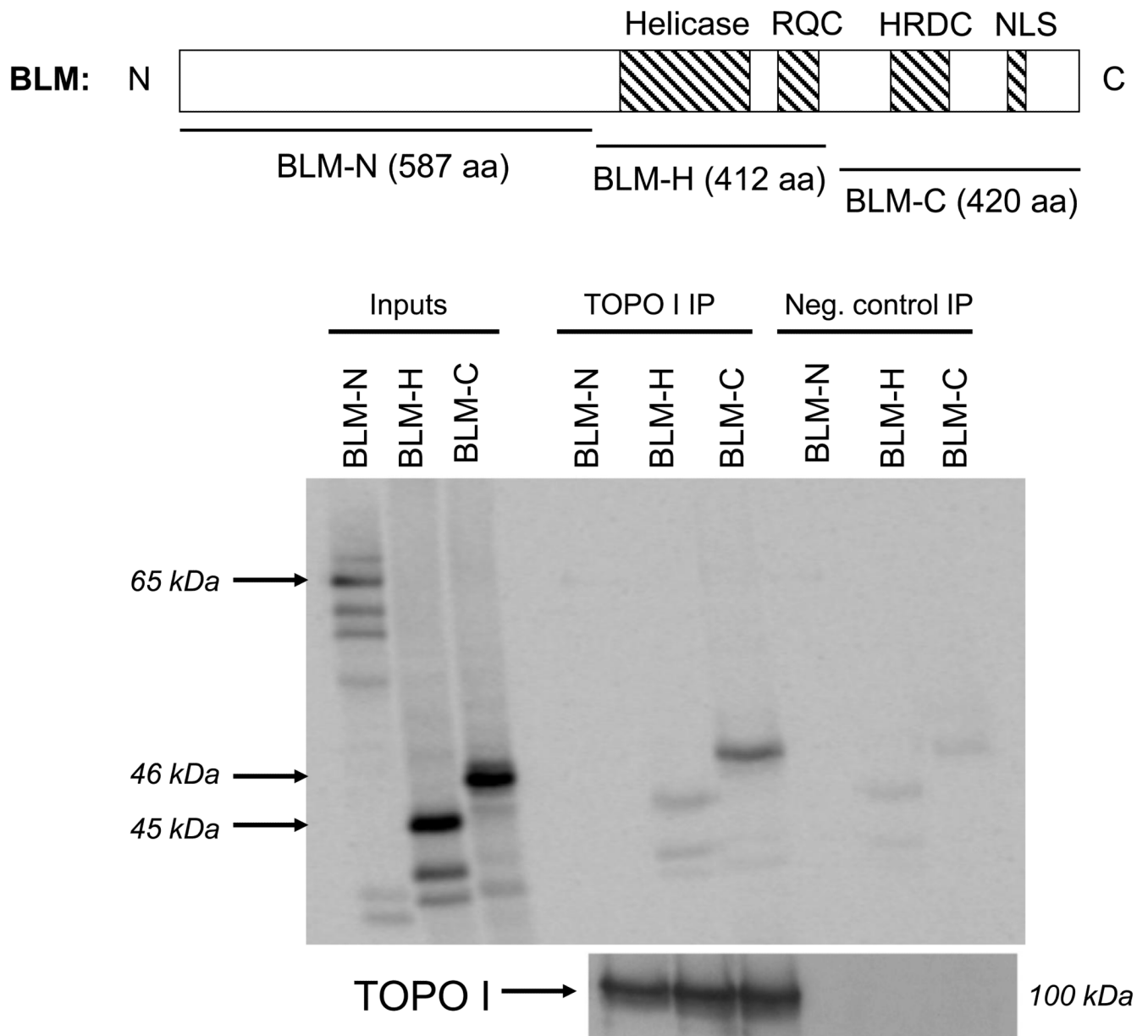


Figure 3. DNA topoisomerase I interacts *in vitro* with the C-terminus of BLM

In vitro transcription/translation (IVTT) was performed using the TNT Rabbit Reticulocyte Lysate kit (Promega) using *pET24D-BLM-N*, *pET24D-BLM-H* and *pET24D-BLM-C*. The resultant ^{35}S -methionine-labeled input BLM fragments are shown on the left. N-, H- and C-fragments were used in co-immunoprecipitation with purified DNA topoisomerase I protein (Topogen) and α DNA topoisomerase I. Following capture with Protein G Dynabeads (Invitrogen), immunoprecipitated proteins were separated using 8% SDS-PAGE; western blotting identified DNA topoisomerase I; autoradiography identified BLM fragments. Negative control immunoprecipitations were carried out by omitting DNA topoisomerase I to control for non-specific binding of BLM fragments to α DNA topoisomerase I or to the beads.

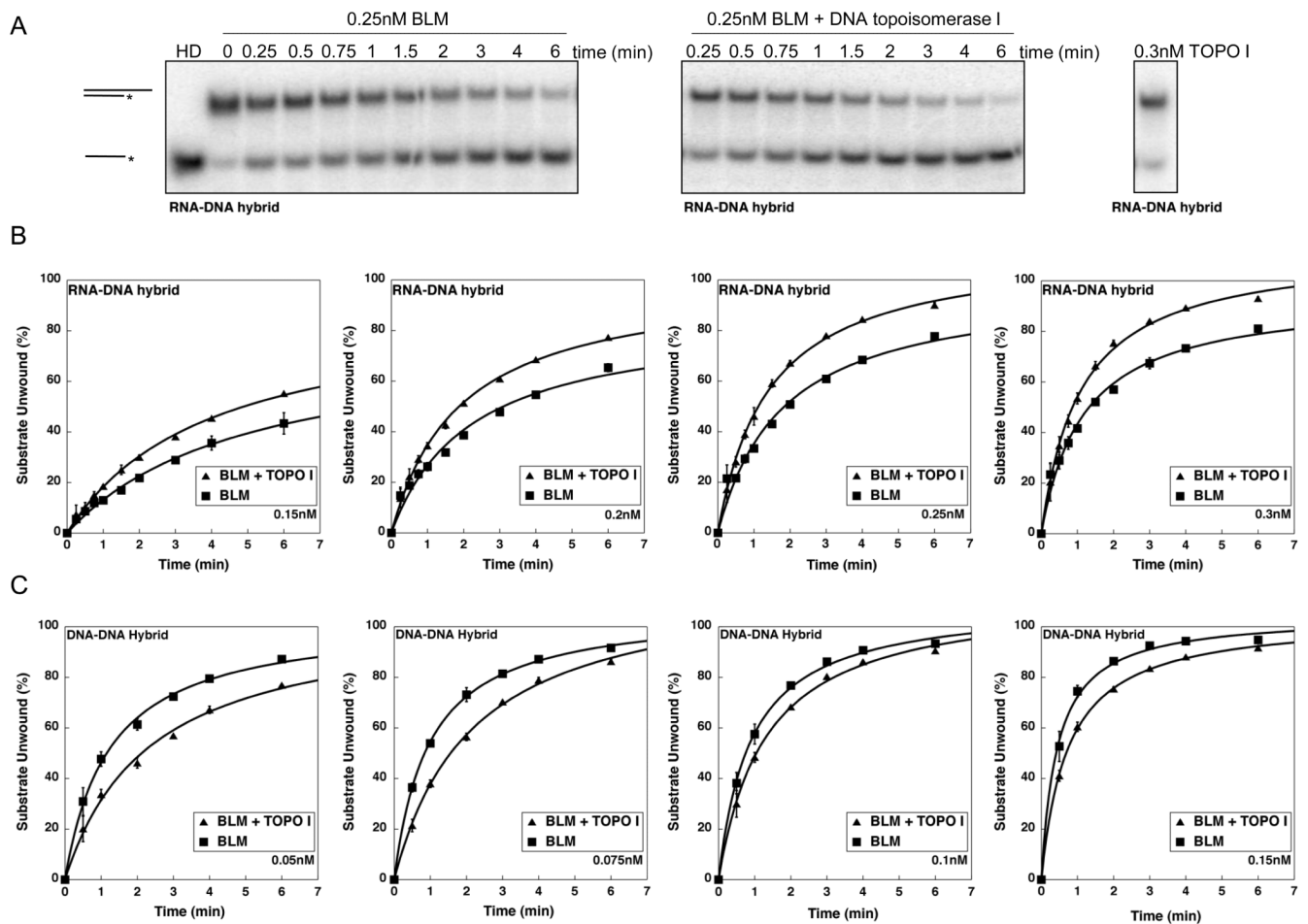


Figure 4. Equimolar DNA topoisomerase I stimulates the helicase activity of BLM using RNA₂₀:DNA₃₃ hybrid duplex substrates

A. Representative gels demonstrate the unwinding activity of BLM. RNA₂₀:DNA₃₃ hybrid duplex substrate was 0.25nM was incubated with BLM in the absence or presence of equimolar DNA topoisomerase I for 15 sec, 30 sec, 45 sec, 1 min, 1.5 min, 2 min, 3 min, 4 min and 6 min as described in Materials and Methods. Reaction products were separated using 12% non-denaturing acrylamide gels, dried and imaged using ImageQuant software. Unwinding is evaluated by conversion of the double-stranded substrate to single-stranded product. Heat-denatured substrate (HD) is generated by heating to 95°C for 5 min followed by rapid cooling. DNA topoisomerase I (topo I) does not display unwinding activity by itself. B and C. Kinetics of BLM-catalyzed unwinding of RNA₂₀:DNA₃₃ (panel B) or DNA₂₀:DNA₃₃ (panel C) hybrid duplex substrates in the presence or absence of equimolar DNA topoisomerase I. Reactions were performed as in A, with different amounts of proteins as indicated in each panel. Unwinding was calculated by quantifying the amount of single-stranded product relative to the total amount of substrate in the reaction. The percent substrate unwound was plotted against time. All unwinding reactions followed Michaelis-Menten kinetics. Kinetics of the BLM-catalyzed reactions in the absence of DNA topoisomerase I is represented by closed squares and that in the presence of DNA topoisomerase I is represented by closed triangles.

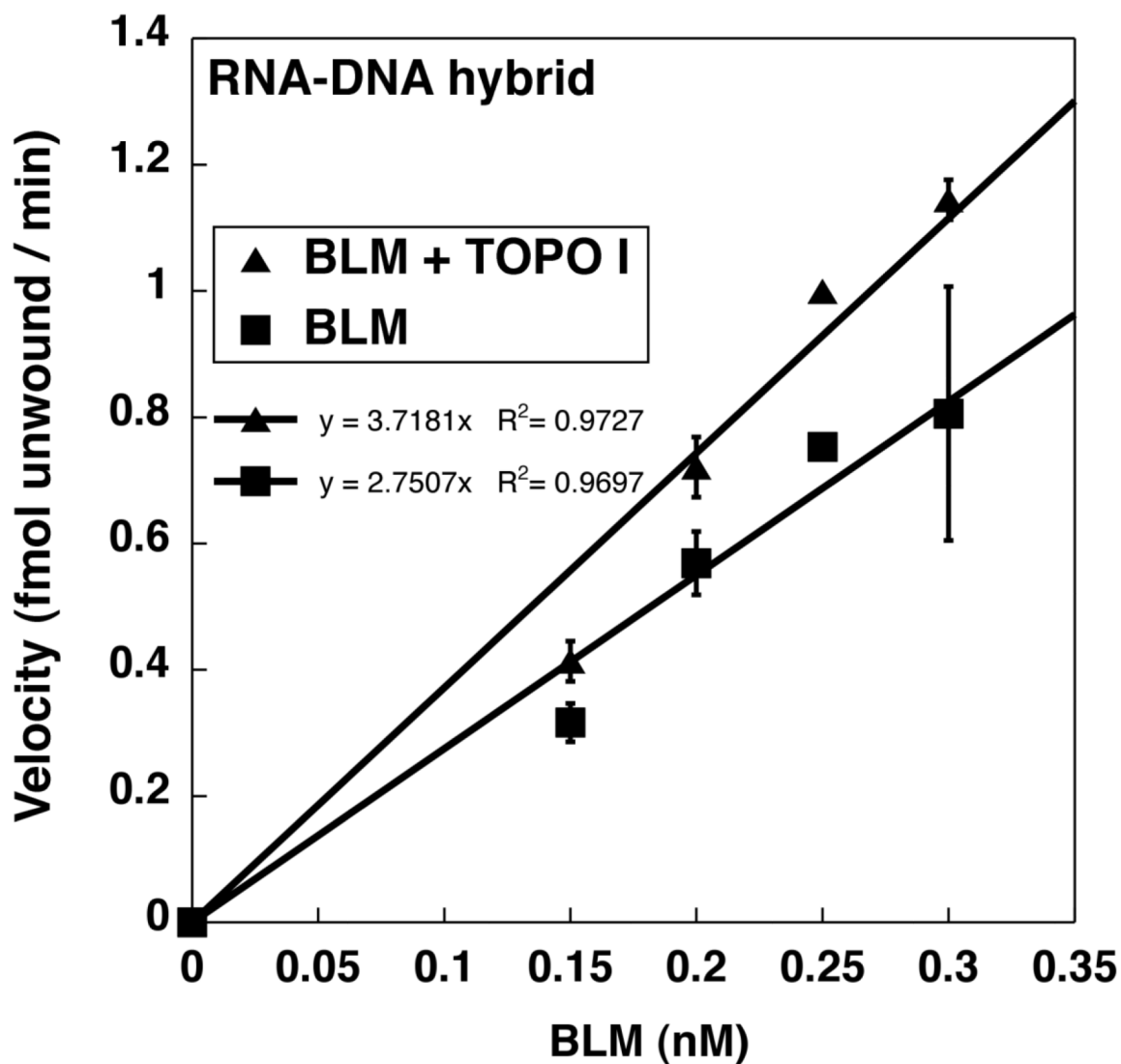


Figure 5. DNA topoisomerase I stimulates BLM unwinding activity using RNA₂₀:DNA₃₃ hybrid duplex substrates

The specific activity of BLM on RNA₂₀:DNA₃₃ hybrid duplex substrates in the presence or absence of DNA topoisomerase I was calculated from curves in Fig 4B by plotting the initial velocity of the reaction at 1 minute against protein concentration used. The slope of the line indicates the specific activity and is indicated in the graph (BLM alone: 2.75 fmol unwound/ min/ nM BLM); BLM with DNA topoisomerase I: 3.72 fmol unwound/ min/ nM BLM).

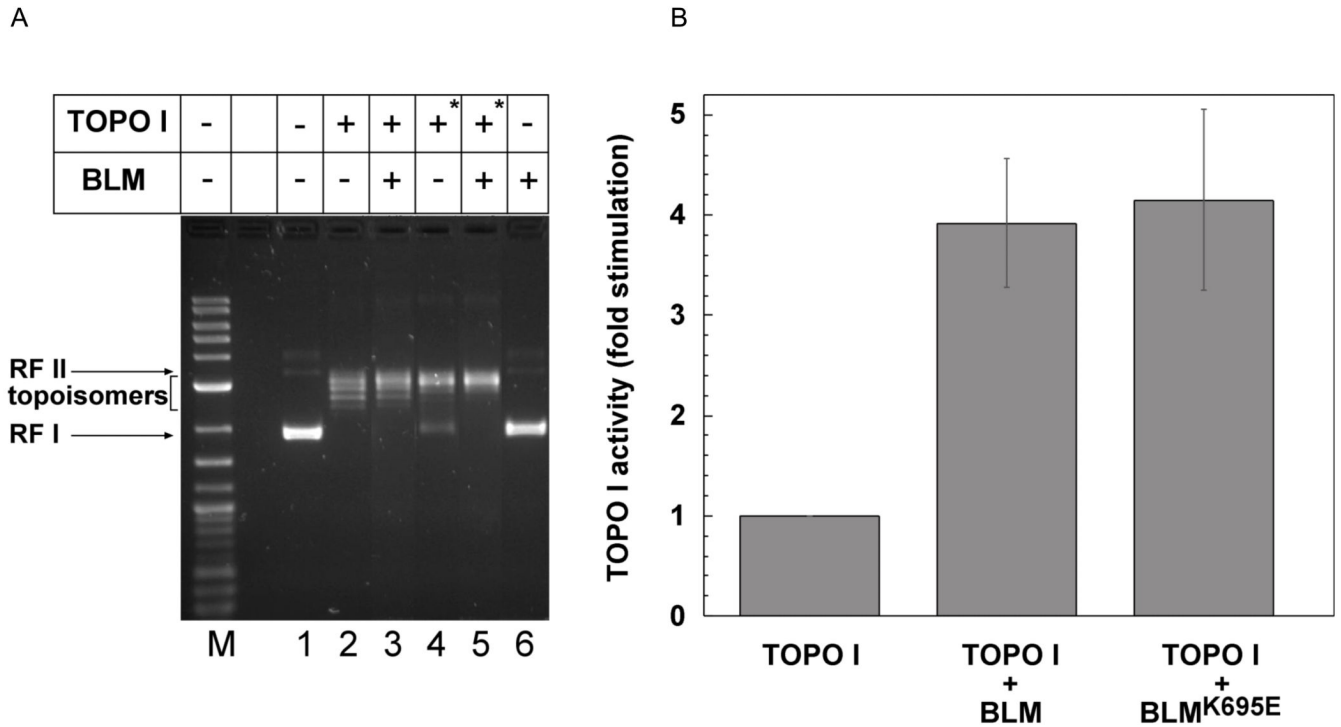


Figure 6. BLM stimulates DNA topoisomerase I activity

(A). Human DNA topoisomerase I (Topogen) was incubated with a 2.9 kb RF I DNA in the presence or absence of 0.65nM BLM, as indicated, for 30 minutes. Reactions were stopped with stop buffer containing SDS and analyzed on a 1.3% agarose gel as described in Material and Methods. Two concentrations of topoisomerase I were used: **0.5** units (lanes 2 and 3) and **0.05** units (lanes 4 and 5, indicated by asterisk); Lane 1: no protein; Lane 6: no topoisomerase. (B). Graph representing the relative fold-stimulation of topoisomerase activity in the presence of BLM. Reactions were performed as described in A except that the DNA topoisomerase I concentration was adjusted in order to get a maximum of 20% conversion of RF I DNA to relaxed DNA in 30 minutes. Reactions were performed for 10-30 minutes and the ratio of product (topoisomers) to substrate (RF I) for each reaction measured as described in Materials and Methods. The fold stimulation was obtained by dividing the ratio obtained in the presence of BLM or its helicase-dead mutant, BLM^{K695E} by that in the absence of BLM. The graph is a representation of at least 5 independent experiments. The only measure of activity for BLM after purification is helicase unwinding. In the case of wild-type protein a loss of unwinding activity was observed upon dilution. Since there is no direct measure of the activity of the mutant as it is helicase-dead, for the topoisomerase I stimulation assays, higher concentrations of the mutant (9nM, supplementary figure 3) were used to overcome any loss of activity occurring upon dilution of protein as the reason for a potential negative result. The mutant was diluted to the same extent as wild-type BLM.