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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 [18]). 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.

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 *rRNA*s 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 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 *Saccharomyces 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 RNA_{20}:DNA_{33} duplex substrate that models a co-transcriptionally formed *rRNA:rDNA*

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hybrid, but does not do so on a DNA_{20}:DNA_{33} 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% CO_2.

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 (10 mM HEPES, pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM 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 218 × *g* for 5 min at 4 °C, nuclear pellets re-suspended in 3 ml of S1 solution (0.25 M sucrose, 10 mM $MgCl_2$), layered over 3 ml of S2 solution (0.38 M sucrose, 0.5 mM $MgCl_2$), and centrifuged at 1430 × *g* for 5 min at 4 °C. Resultant nuclear pellets were re-suspended in 3 ml 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 3 ml of S3 solution (0.88 M sucrose, 0.5 mM $MgCl_2$), centrifuged at 3000 × *g* for 10 min at 4 °C and re-suspended in 500 μl 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 αBLM (Santa Cruz Biotech, sc-7790) and αDNA 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 αBLM (Bethyl Laboratories, A300-110A), αRPA194 (Santa Cruz Biotech, sc-48385) and αRNA polymerase II (Abcam, ab817).

2.4. Protein purification

pYES-BLM expression vector (*pJK1*) was kindly provided by Ian 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 *S. cerevisiae*. Yeast were lysed at 20k psi using a French Press Cell Disrupter (Thermo) and lysates were separated by ultracentrifugation at 65,000 × *g* for 1 h 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. [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 2 h at 4 °C with rotation. Subsequently, αDNA topoisomerase I (Bethyl, A302-589A) was added with an additional 2 h incubation at 4 °C. Finally, Dynabeads Protein G (Invitrogen, 100-04D) were added for 2 h at 4 °C, washed 5 times with binding buffer, eluted with 1× 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: DNA_{20}-CGCTAGCAATATTCTGCAGC, DNA_{33}-GCTGCAGAATATTGCTAGCGGGAATTCGGCGCG and RNA_{20}-CGCUAGCAAUAUUCUGCAGC. RNA_{20} and DNA_{20} 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. Toposiomerase 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, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol). 200 ng replicative form I (RF I) DNA [24] was used in 20 μl 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 min) 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 (αBLM) or anti-DNA topoisomerase I (αDNA topoisomerase I) antibodies. Fig. 1A shows that each antibody immunoprecipitated both BLM and DNA topoisomerase I. Control immunoprecipitation experiments using αBLM or αDNA 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 Fig. 1C) [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 (Fig. 1B) and the resultant sub-nuclear fractions used in co-immunoprecipitation experiments with αBLM antibodies. The RNA polymerase I-specific subunit RPA194 was used as a nucleolar marker, RNA polymerase II as a nucleoplasmic marker and β-actin as a cytoplasmic marker (Fig. 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 (Fig. 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).