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Does BLM helicase unwind nucleosomal DNA?

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Abstract

RecQ helicases maintain chromosome stability by resolving several highly specific DNA structures. BLM, the protein mutated in Bloom's syndrome, is a member of the RecQ helicase family, and possesses both DNA-unwinding and strand-annealing activity. In this study, we have investigated the unwinding activity of BLM on nucleosomal DNA, the natural nuclear substrate for the enzyme. We generated a DNA template including a strong nucleosome positioning sequence flanked by forked DNA, which is reportedly one of the preferred DNA substrates for BLM. BLM did not possess detectable unwinding activity toward the forked DNA substrate. However, the truncated BLM, lacking annealing activity, unwound it partially. In the presence of the single strand DNA binding protein RPA, the unwinding activity of both the full-length and the truncated BLMs was promoted. Next, the histone octamer was reconstituted onto the forked DNA to generate a 'forked mononucleosome'. BLM did not unwind the nucleosomal DNA but the truncated BLM unwound it partially. The unwinding activity for the mononucleosome was not promoted dramatically with RPA. These results indicate that full length BLM may require additional factors to unwind nucleosomal DNA in vivo, and that RPA is, on its own, unable to perform this auxiliary function.

Keywords

BLM; DNA helicase; nucleosome

Introduction

Bloom's syndrome is a human autosomal recessive disorder with increased incidence of cancer (Ellis and German 1996). Cells from patients with Bloom's syndrome display a high degree of genome instability and a drastic increase in the frequency of sister chromatid exchanges mediated by homologous recombination (Ellis et al. 1995a; Singh et al. 2009).

The gene (BLM) defective in Bloom's syndrome encodes for one of the five members of the RecQ family of DNA helicases in humans (Ellis et al. 1995b). The RecQ family includes the WRN and RECQ4 proteins defective in Werner's and Rothmund-Thomson syndromes, respectively (Yu et al. 1996; Kitao et al. 1999). The RecQ family is defined by the presence of a helicase domain, which is a highly conserved amino acid sequence motif in the middle of the polypeptide chain. Most RecQ helicases have two additional characteristic domains C-terminal to the helicase domain, RecQ-Ct (RecQ C-terminal) domain and HRDC (helicase RNaseD C-terminal) domain. The RecQ-Ct domain is implicated in the structural stability of the protein and participates in protein-protein and DNA structure-specific binding via a zinc-binding motif and a winged-helix domain (Killoran and Keck 2006). The HRDC domain is thought to participate in structure-specific DNA binding (Killoran and Keck 2006). Outside of

these domains, there is little sequence homology among family members (Bachrati and Hickson 2003). BLM unwinds DNA with a 3' to 5' polarity and possesses strong DNA substrate specificity, preferring unusual DNA structures such as forks, bubbles, D-loops, Holliday junctions, and G-quadruplexes (Karow et al. 1997; Sun et al. 1998; van Brabant et al. 2000; Mohaghegh et al. 2001).

The monomer of BLM protein comprises 1417 amino acid residues, with predicted molecular mass of 159 kDa (Ellis et al. 1995b). Size-exclusion chromatography and electron-microscopic studies indicate a native molecular mass of ~700–900 kDa and tetrameric or hexameric ring structures (Karow et al. 1999). The RecQ core of BLM, which contains the helicase domain, the RecQ-Ct domain, and the HRDC domain, has unwinding activity in a monomeric form, indicating that the oligomeric structure is not necessary for the unwinding activity (Janscak et al. 2003). Recently, BLM has been shown to possess strand-annealing activity (Cheok et al. 2005; Machwe et al. 2005).

Nuclear DNA in eukaryotes is organized as chromatin, a complex between DNA and small, highly basic proteins, histones (van Holde 1988; Zlatanova and Leuba 2004). The basic repeating structural unit of chromatin is the nucleosome which encompasses the nucleosome core particle (NCP) and the DNA linking consecutive nucleosomes in the chromatin fiber (Zlatanova et al. 1998). The NCP contains 147 bp of DNA wrapped in a left-handed (negative) sense around a histone octamer consisting of two molecules each of histones H2A, H2B, H3, and H4 (Arents and Moudrianakis 1993; Luger et al. 1997; van Holde and Zlatanova 1999; Harp et al. 2000). The octamer is a modular structure possessing two-fold symmetry: the H3/H4 tetramer is at the center, with the two H2A/H2B dimers attached on either side. The tetramer organizes the central portion of nucleosomal DNA, whereas each H2A/H2B dimer contacts ~30 bp on either side. All DNA transactions in the nucleus - DNA replication, recombination, repair, and transcription - take place in the context of chromatin. How all these processes deal with the existence of nucleosomes along the DNA is still unclear. It is obvious that for the majority of these processes, DNA has to be freed of nucleosomes (at least temporarily), so as to release the DNA in a state that can be denatured, thus providing template access by the respective cellular machineries. Helicases are involved in many of these processes, but we still do not know whether and how they unwind nucleosomal DNA. Several DNA helicases have been shown to detach bound proteins during DNA unwinding (Mackintosh and Raney 2006), but whether they can disrupt the stable, highly organized structure of the nucleosome particle remains unclear. Only a few reports have addressed these questions, but they have studied either non-homologous systems, or helicases that only work under limited cellular circumstances (see Discussion).

The nucleosomal DNA unwinding by BLM must occur at the initial steps of events (e.g. recombination repair), because at the later steps, the DNA stretches involved might have been freed of bound proteins or covered with some different proteins, such as Rad51. Recently, it has been reported that BLM contributes not only to the late phases of recombinational DNA repair but also to the initiation of the process by human exonuclease 1 (hExo1) (Gravel et al. 2008; Nimonkar et al. 2008). So, the BLM unwinding activity on the nucleosomal DNA might be important in the initiation of DNA repair in nucleosomal DNA. We expect that BLM encounters nucleosomes in many other processes. In this study, we have analyzed the unwinding of nucleosomal DNA by BLM helicase.

Materials and Methods

Protein Expression and Purification

C-terminally His6-tagged BLM was expressed from pJK1 (gift from Dr. Ian Hickson) using the yeast galactose-inducible BLM expression system and purified using Ni-NTA Agarose

(QIAGEN) according to Karow et al. (Karow et al. 1997). RecQ core fragment encompassing amino acid residues 642–1290 (BLM^{642–1290}) was overexpressed from pJP71 (gift from Dr. Pavel Janscak) in BL21 codon-plus cells using the CBP system (Janscak et al. 2003) and purified using chitin beads (New England BioLabs) and SP Sepharose. All three subunits of hRPA were co-expressed from p11d-tRPA (gift from Dr. Marc Wold) in BL21 codon-plus cells and purified using sequentially Affi-Gel[®] Blue Gel (BioRad), hydroxyapatite gel, and Q Sepharose chromatography, as described previously (Henricksen et al. 1994).

DNA substrates

All oligonucleotides used for the preparation of DNA substrates were purchased from Integrated DNA Technologies, Inc (Coralville, IA). The nucleotide sequences used in this study were as follows:

50-F1, CGAAGGTTTCG TGTTTCATCCT CACACTCACT TAAGCCGAAT
TCTTAGGGTT;

50-F2, GCTATCGTAC ATGATATCCT CACACTCTGA ATAGCCGAAT
TCTTAGGGTT;

50-R, biotin-AACCCTAAGA ATTCGGCTTA AGTGAGTGTG AGGATATCAT
GTACGATAGC;

27lag, GAGTGTGAGG ATATCATGTA CGATAGC;

27lead, GCTATCGTAC ATGATATCCT CACACTC (Machwe et al. 2006);

208-FT, TTTTTTTTTT TTTTCGGGG AATTCGGTAT TCCCAGG;

208-FA, AAAAAAAAAA AAAAACGGGG AATTCGGTAT TCCCAGG;

208-R, biotin-AATTCCAACG AATAACTTCC AGG.

For the 18 bp replication fork mimic, the biotin-labeled 50-R and 27lead, and the 50-F2 and 27lag are incubated pair-wise at 95°C for 5 min, followed by a 15 min incubation at 65°C and an hour incubation at 37°C; then the two annealed pairs were mixed and incubated at 25°C for 12–18 h. For the 35 bp forked-DNA, the biotin-labeled 50-R and 50-F1 were incubated at 95°C for 5 min, followed by a 15 min incubation at 65°C and an hour incubation at 37°C. For the nucleosomal DNA, a 208 bp nucleosome positioning sequence from 5S rDNA of sea urchin was used for the template (Simpson et al. 1985). Two types of DNA were amplified using 208-FT-208-R, and 208-FA-208-R to generate dT₁₅-208 and dA₁₅-208. The non-biotinylated strand of dT₁₅-208 and biotinylated strand of dA₁₅-208 are purified using Dynabeads M-280 Streptavidin (Invitrogen, Dynal AS, Oslo, Norway) as described previously (Jenne and Famulok 1999). The two ssDNAs were mixed in an equal proportion and were incubated at 95°C for 5 min, followed by a gradual (1°C/min) decrease to 37°C to generate dT₁₅ forked-DNA. Nucleosome core histones were prepared from chicken erythrocytes (Tomschik et al. 2001) and reconstituted onto the forked-DNA by the salt stepping method as previously described (Tomschik et al. 2005).

Helicase Assay

For naked DNA, helicase assays were carried out in a 10 µl reaction volume containing reaction buffer (40 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 2 mM ATP, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol), 0.5 nM biotin-labeled DNA substrates, and 24 nM BLM protein, unless indicated otherwise. After incubating at 37°C for 30 min, the reaction was stopped by adding 1/10 volume of helicase stop buffer (10 mg/ml proteinase K, 40% sucrose, 100 mM EDTA, 2% SDS, and 0.2% bromophenol blue). The reaction mixtures were resolved by electrophoresis in 10% or 5% non-denaturing polyacrylamide gels. The DNA was electro-transferred to a nylon

membrane (HybondTM-N+, GE Healthcare). After blocking in 1% Blocking Reagent (Roche), the membranes were incubated with streptavidin-horseradish peroxidase conjugate (1:5000 dilution, GE Healthcare) in maleic acid buffer at 25°C for 1 h. The biotin-labeled DNAs were visualized using SuperSignal[®] West Femto (Pierce). For reconstituted DNA, *MspI* digestion at 37°C for 1 hr was carried out before helicase reaction.

Annealing Assay

Strand-annealing activity of BLM or BLM^{642–1290} was measured in the above reaction buffer without ATP for 20 min at 37°C using biotin-labeled 50-R and unlabeled 50-F2 oligonucleotides. The reaction was stopped by adding 1/10 volume of the stop buffer (40% Ficoll, 50 mM EDTA, 2% SDS, and 0.2% bromophenol blue) and analyzed as above.

Results

Unwinding Activity of Recombinant BLM and its Truncated Version on Naked DNA Substrates

It has been reported that full length BLM cannot unwind the ~200 bp DNA, which is required for nucleosome formation, without additional proteins (Brosh et al 2000). So, full length BLM helicase, its RecQ core fragment (BLM^{642–1290}, lacking the annealing activity), and single strand DNA binding protein RPA (that promotes the unwinding activity) were purified. The recombinant proteins used in this study were analyzed by gel electrophoresis (Fig. 1A). It is well known that BLM helicase has preference for unusual DNA structures (Karow et al. 1997; Sun et al. 1998; van Brabant et al. 2000; Mohaghegh et al. 2001). To verify whether our recombinant proteins were enzymatically active, we tested their unwinding activities on a 18 bp replication fork mimic and 35 bp forked DNA. The 18 bp replication fork mimic DNA substrate contains a 18 bp-long parental region, 5 nt non-complementary sequence, and two homologous 27 bp parental-daughter arms (Fig. 1B). The 35 bp forked DNA substrate contains a 35 bp-long double stranded DNA and 15 nt forked structure (Fig. 1C). To trace the DNA products, both DNA contained a biotin label at the 5' end of the 50-R strand. The forked DNA was treated with BLM proteins, in the presence of ATP with or without RPA (Fig. 1B and C). Notably, full length BLM did not unwind this structure completely, whereas BLM^{642–1290} unwound it completely. As expected, RPA promoted the BLM unwinding activity. The difference between the two BLM forms is obviously due to the presence (in full-length BLM) or absence (in BLM^{642–1290}) of strand-annealing activities (Figs. 1B and D). Thus, the degree of unwinding observed with full length BLM reflects the equilibrium between unwinding and re-annealing. Thus, our recombinant proteins are enzymatically active and show the expected behavior.

RecQ Core Fragment of BLM Unwinds Nucleosomal DNA

To compare the BLM helicase activity on naked and nucleosomal DNA, we constructed a biotin-labeled forked DNA substrate that contains a strong nucleosome positioning signal, the 208 bp fragment from the 5S rRNA gene from sea urchin (Fig. 2A, see Materials and Methods). When reconstituted with histone octamers, this DNA construct places a single nucleosome over the positioning sequence, leaving the forked structure free for BLM landing. For the nucleosome substrate, the histone octamer was reconstituted onto the DNA to generate the forked-mononucleosome (Fig. 2A). The reconstitution product, obtained with an optimized histone/DNA ratio, was analyzed by polyacrylamide gel electrophoresis (Fig. 2B). As is usual with *in vitro* nucleosome reconstitution protocols, there is always some residual free DNA (this free DNA disappears when higher histone/DNA ratios are used; using such high ratios is, however, undesirable, since they give rise to massive aggregation of the reconstituted material). To distinguish the contaminating naked DNA from the reconstituted fraction to be used in the helicase reaction, the reconstitution mixture was pretreated with *MspI*, which cuts in the center

of the nucleosome positioning sequence (Fig. 3, lane 8–14). Nucleosome-organized DNA is protected from the restriction enzyme. In this gel, the ssDNA runs more slowly as compared to the forked-DNA (Fig. 3, lanes 1–2 and 8–9). As shown in Fig. 3, full length BLM could not unwind the naked forked-DNA (~200 bp) (Fig. 3, lane 3). No unwinding of the forked-DNA substrate was observed upon raising the enzyme concentration from 2.4 nM to 72 nM (data not shown). These results were not unexpected in view of the relatively low processivity of the enzyme on partial duplex substrates (Brosh et al. 2000). BLM could not unwind the forked-mononucleosome either (Fig. 3, lane 10). Truncated BLM^{642–1290} did produce ssDNA (Fig. 3, lanes 6–7), indicating its ability to partially unwind the forked-DNA. BLM^{642–1290} also partially unwound the forked-mononucleosomal DNA (Fig. 3, lanes 11–14). BLM^{642–1290} also unwound *MspI*-digested residual naked DNA present in the forked-nucleosome preparation (Fig. 3, lane 14). Increasing the concentration of BLM^{642–1290} from 7.2 nM to 72 nM led to an increase in the amount of unwound DNA on both the naked and the mononucleosomal DNA substrates.

RPA does not Dramatically Alter the BLM Unwinding Activity for Nucleosomal DNA

Next, we tested whether the ssDNA binding protein RPA promotes BLM unwinding activity for nucleosomal DNA; it is well known that RPA interacts with and stimulates BLM unwinding activity on naked DNA substrates (Brosh et al. 2000). RPA was added before BLM helicase reactions. The concentration of RPA (200 nM) used in these experiments did not promote DNA unwinding on its own (Fig. 4A, lanes 2–3, 9–10). Of note, somewhat higher concentrations of RPA have been shown to ‘unwind’ dsDNA, in a non-enzymatic reaction, presumably involving binding to and stabilization of ssDNA created as a result of ‘breathing’ motions in double helical DNA (Treuner et al. 1996).

On the forked-DNA substrate, RPA promoted the unwinding activity of both full length BLM and BLM^{642–1290} (Fig. 4A, compare lanes 4 with 5, and lanes 6 with 7). In both cases the presence of RPA significantly increased the amount of the ssDNA product. In contrast, RPA did not promote the unwinding activity of either enzyme on the forked-nucleosome (Fig. 4A, lanes 11–14). Full length BLM could not unwind the nucleosomal DNA, with or without RPA. Truncated BLM^{642–1290}, on the other hand, partially unwound nucleosomal DNA; however, no significant contribution from RPA was evident (Fig. 4A, lanes 13 and 14, and Fig. 4B).

Discussion

Our results show that the RecQ core of the human RecQ subfamily DNA helicase BLM can unwind nucleosomal DNA. However, under the in vitro reaction conditions used in this study, full length BLM could not unwind DNA packaged into a nucleosome.

Many nucleoside triphosphate (NTP)-dependent enzymes are part of multisubunit protein complexes that alter chromatin structure for processes such as transcription to occur (Saha et al. 2006). The mechanisms of action of these so-called chromatin (or nucleosome) remodelers still remain poorly understood, but it is generally believed that they change nucleosome positioning and structure through the ability of their ATP-dependent subunits to translocate along dsDNA (Flaus and Owen-Hughes 2004). Interestingly, some chromatin remodeling translocases such as SWI/SNF belong to Super family II (SF-II) helicases (Berger 2008). Note, however, that although they possess canonical helicase domains, they seem to be devoid of helicase activity.

Several bona fide helicases (RecBCD, an SF-I helicase and SV40 large T antigen, an SF-III helicase) have been shown to have unwinding activities on nucleosomal DNA (Eggleston et al. 1995; Ramsperger and Stahl 1995). We note, however, that the RecBCD helicase is of prokaryotic origin and it does not encounter nucleosomes in its natural environment. The SV40

T antigen also functions under special circumstances (only in virally infected cells). Recently, the human MCM4/6/7 helicase (an SF-VI helicase that participates in replication) was reported to unwind nucleosomal DNA, only in collaboration with the FACT complex (Tan et al. 2006). Here we demonstrate that the RecQ core of BLM (an SF-II helicase) can also unwind nucleosomal DNA. We expect that the observed behavior is not confined to the specific sequence used for nucleosome reconstitution, since this is a naturally-occurring sequence that possesses a well-defined nucleosome positioning signal (places the histone octamer at one major position on the DNA fragment). The positioning strength is directly related to the affinity of the octamer to the respective sequence (Lowary and Widom, 1998; Thåström et al. 1999); thus, it is expected that less-well positioning sequences would be easier to unwind. Based on previous reports and our data we suggest that most helicases possess nucleosomal DNA unwinding activity.

It is important to note that full length BLM could not unwind nucleosomal DNA under the conditions of our assay, independently of whether RPA, a “helper” protein for helicase action on naked DNA substrates, was present or not. This may indicate that the portions of the molecule that are exterior to the RecQ core (these domains are responsible for DNA annealing, protein binding, and BLM oligomerization) inhibit the structural transitions in the nucleosome particle that make DNA unwinding possible. At present, it is unclear what these structural transitions may be. In principle, one can envisage either a total displacement of the histone core of the nucleosome, or partial histone dissociation from portions of nucleosomal DNA, unwinding of the free portion, and reattachment of the histones [one must note that such scenarios have been envisioned for the process of transcription, e.g. van Holde et al. (1992)]. This second mechanism though will not lead to the release of ssDNA molecules that we observe in our assay. Thus, the present data favor the first mechanism, i.e. total histone dissociation.

Finally, it is important to note that the inhibition of nucleosomal DNA unwinding observed with the full length BLM could not be overcome by RPA; RPA could not enhance the unwinding activity of the truncated protein either. While the RecQ core of the enzyme is active on nucleosomal DNA on its own, full-length BLM (the physiologically-relevant enzyme) may need the assistance of other, yet unidentified, proteins.

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Fig. 1.

Purification of full-length and truncated versions of recombinant human BLM and RPA, and verification of their enzymatic activity. (A) Coomassie Blue-stained SDS-polyacrylamide gel of purified BLM, BLM⁶⁴²⁻¹²⁹⁰, and RPA. The recombinant proteins were expressed and purified as described in Experimental Procedures. Samples were analyzed on 10% (BLM, BLM⁶⁴²⁻¹²⁹⁰) or 15% (RPA) polyacrylamide gels. (B) 0.5 nM biotin-labeled 18 bp replication fork-mimic DNA was incubated with 2.4 nM BLM or BLM⁶⁴²⁻¹²⁹⁰. The products of the reaction were analyzed by 10% non-denaturing PAGE. (C) 0.5 nM biotin-labeled 35bp forked-DNA was incubated with 7.2 nM BLM or BLM⁶⁴²⁻¹²⁹⁰ with or without 50 nM RPA. The products of the reaction were analyzed by 10% non-denaturing PAGE. (D) Strand-annealing

activities of BLM and BLM⁶⁴²⁻¹²⁹⁰. Biotin-labeled 50-mer oligonucleotide (50-R) was incubated with 50-mer complimentary oligonucleotide (50-F2) in the presence of BLM or BLM⁶⁴²⁻¹²⁹⁰ (2.4, 7.2 nM). The products of the reaction were analyzed by 10% non-denaturing PAGE. Asterisks indicate the locations of the biotin labels.

Fig. 2. Forked-mononucleosome. (A) Schematic representation of the forked-DNA and the generation of the forked-mononucleosome. (B) PAGE analysis of the reaction mixture used for reconstitution of nucleosome onto the forked-DNA.

Fig. 4.

Lack of RPA promoting activity of BLM helicase on nucleosomal DNA. (A) 0.5 nM of forked-DNA (lanes 1–7) or forked-mononucleosome (lanes 8–14) were used as a substrate in helicase reactions in the absence (lane 2, 4, 6, 9, 11 and 13) or presence (lane 3, 5, 7, 10, 12 and 14) of RPA (200 nM). (B) Quantitation of the helicase reaction. The percentage of unwound products is plotted against the respective proteins present during the reactions. The data points represent the average values from three independent experiments and error bars indicate the standard deviation (S.D.).