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In humans, homologous recombination (HR) represents a major pathway for the repair of DNA double strand breaks (DSBs), harmful lesions that can lead to genomic instability, cell death, or tumorigenesis. There is an emerging role in the HR pathway for the RecQ family of helicases. Mutation of the BLM protein, one of five RecQ helicases in humans, causes Bloom's syndrome, a rare autosomal recessive disease in which patients are predisposed to cancer, including breast cancers. Cells isolated from these patients display genomic instability and hyper-recombination. BLM protein contains a robust ATP hydrolysis activity and the ability to unwind several complex DNA structures believed to arise during HR repair, and it interacts with components of the recombination machinery, including the Rad51 recombinase. These findings suggest that BLM functions to regulate the HR pathway; however, its mechanism of action remains elusive. In the current study, we characterize the effects of purified BLM protein on the Rad51 presynpatic filament, a key intermediate in the HR repair pathway. We also address the importance of ATP hydrolysis in the role of BLM. This study begins to elucidate how BLM functions in the HR pathway, and through this role it may act to maintain genome stability and prevent cancer formation.

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INTRODUCTION

Cells are continually exposed to high energy radiation and mutagenic chemicals that result in DNA damage. Among the most lethal DNA lesions are double-strand breaks (DSBs), which if left unrepaired can lead to mutations, cellular apoptosis, or even tumorigenesis. To maintain the genome, eukaryotic cells have evolved multiple pathways for repairing damaged DNA. A prominent pathway for the repair of DSBs in mammalian cells is homologous recombination (HR). This pathway maintains genomic integrity by using a homologous piece of DNA as a template for restoring any genetic information that was lost or damaged at the break site. In the HR pathway, the 5' strands at the break site are nucleolytically resected leaving 3' ssDNA tails exposed. Many protein factors, including the Rad51 recombinase and other members of the *RAD52* epistasis group, nucleate upon these tails to form an active nucleoprotein filament termed the presynaptic filament. Once assembled, the presynaptic filament can locate and invade a homologous DNA duplex, either a sister chromatid or homologous chromosome, to form a DNA intermediate called D-loop (displacement loop). D-loop formation is critical for subsequent steps in the recombination reaction, including DNA synthesis, branch migration, resolution of DNA intermediates, and ligation, which serve to complete the repair process (Reviewed in Paques and Haber, 1999; Sung et al., 2000). While repair of specific DSBs by recombination is beneficial to the cell, uncontrolled recombination leads to chromosome rearrangements, cell-cycle checkpoint abnormalities, and genomic instability. For this reason, cells have evolved specific mechanisms to prevent untimely recombination, and a number of factors essential to this process have been described. One such factor is the human RecQ-like helicase BLM, whose inactivation is responsible for the autosomal recessive disease Bloom's syndrome (BS)(Ellis et al., 1995). Patients afflicted with Bloom's syndrome are characterized by short stature, facial erythema, immunodeficiency, impaired fertility, and early onset of a wide array of cancers. The variety and number of cancers seen in Bloom's patients reflects the frequency and array of cancers that develop at a later age in the general population (German, 1993), suggesting that individuals lacking the BLM helicase are not specifically susceptible to certain cancers, but rather their overall carcinogenic process is accelerated. BLM is one of five members of the RecQ helicase family identified thus far in humans. Interestingly, mutations in two other human RecQ helicases, WRN and RecQ4, are also associated with human diseases, Werner's and Rothmund-Thomson syndromes, respectively. Although patients afflicted with these diseases display somewhat different phenotypes than BLM, they all possess a predisposition to cancer (Reviewed in Mohaghegh and Hickson, 2001).

BODY

Cells isolated from BS patients display a high degree of genomic instability (German et al., 1974) and a massive increase in sister chromatid exchanges (SCEs), a phenomenon dependent upon HR (Kuhn and Therman, 1986; Wang et al., 2000a). BLM binds to Rad51 *in vitro* (Wu et al., 2001), and *in vivo* evidence suggests that BLM colocalizes with Rad51 and other HR factors, including the breast tumor suppressor BRCA1 (Bischof et al., 2001; Wang et al., 2000b). In BS cells, the frequency of Rad51 foci, corresponding to sites of recombination, following irradiation is greatly increased (Wu et al., 2001). Taken together, available evidence suggests that in the absence of BLM, Rad51-mediated HR becomes much more frequent, thus implicating BLM in the negative regulation of HR. In accordance with this idea, the genomic instability observed in BS cells could be explained by unregulated recombination. Since BLM protein has DNA helicase activity and can dissociate DNA structures thought to arise during recombination (Karow et al., 2000; van Brabant et al., 2000), it has been proposed that recombination modulation by BLM stems from its ability to disrupt DNA joints made by Rad51.
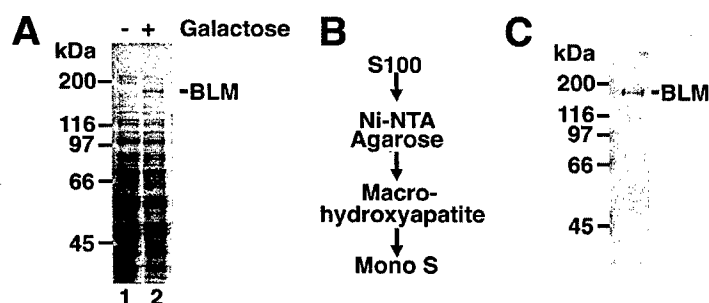

Figure 1. Expression of BLM. (A) BLM (lane 2) was expressed from the pYES vector (a gift from Dr. Ian Hickson at Cancer Research UK) in the JEL1 strain of *S. cerevisiae* by galactose induction. Cell extracts were separated on a 7.5% denaturing gel. Lane 1 contains JEL1 cell extract without galactose induction. **(B)** Purification scheme for obtaining nuclease-free BLM protein. **(C)** Purified BLM protein (800 ng) was run in a 7.5% denaturing gel and stained with Coomassie blue.

Expression and purification of BLM and HR proteins- Human BLM protein was expressed in yeast as previously described (Karow et al, 1997; Fig. 1A). However, published protocols for purification were insufficient in that the resulting protein contained a contaminating nuclease. A new purification scheme was devised for BLM (Fig. 1B), and milligram quantities of nearly homogenous, nuclease-free protein were obtained for subsequent experiments. Purified BLM was verified to be active in ATP hydrolysis and helicase assays (see Figs. 4&5) in accordance with published data (Karow et al., 1997). In order to establish *in vitro* assays for studying HR, additional protein factors were needed; hRad51 and hRPA were expressed and purified as previously described (Sigurdsson et al., 2001).

BLM Disrupts the Rad51 presynaptic filament- Unlike humans, lower eukaryotes, such as *S. cerevisiae* (commonly known as baker's yeast), contain only a single member of the RecQ helicase family, Sgs1. Similar to BS cells, yeast *sgs1* mutants display a hyper-recombination phenotype (Watt et al., 1996). Interestingly, deletion of another yeast helicase Srs2, which is a member of the SF1 family, also displays a hyper-recombination phenotype (Klein et al., 2000). *sgs1Δsrs2Δ* double mutants are synthetic lethal, and this lethality can be rescued by mutating proteins essential for HR, including Rad51 and Rad52 (Gangloff et al., 2000). Our laboratory has previously shown that Srs2 attenuates HR reactions *in vitro* by disrupting the Rad51 presynaptic filament (Krejci et al., 2003). Given the premise that BLM appears to downregulate recombination and it binds Rad51, it is logical to think that it may act in a similar fashion to Srs2; namely, BLM could prevent recombination by disrupting the Rad51 presynaptic filament. To address this, I turned to an assay previously developed in the lab for studying yeast Rad51 filament disruption by Srs2 (Krejci et al., 2003). When Rad51 binds to a topologically relaxed dsDNA substrate, it causes lengthening of the DNA. Subjecting the reaction mixture to treatment with calf thymus topoisomerase will cause a change in the DNA linking number if Rad51 is bound to the dsDNA (Fig. 2A). The product of this reaction is an underwound DNA species called Form U. Thus, formation of Form U DNA can be used to gauge the presence of free Rad51 molecules generated as a result of disruption of the presynaptic filament. When Rad51 and ssDNA were first incubated together, forming the presynaptic filament, and then relaxed dsDNA was added to the reaction, no change in dsDNA topology was detected because Rad51 remained bound to ssDNA. However, when BLM was added to the reaction, Form U DNA was seen (Fig. 2B). Control experiments showed that BLM alone was not capable of making Form U DNA, nor did it affect the ability of Rad51 to make Form U DNA.
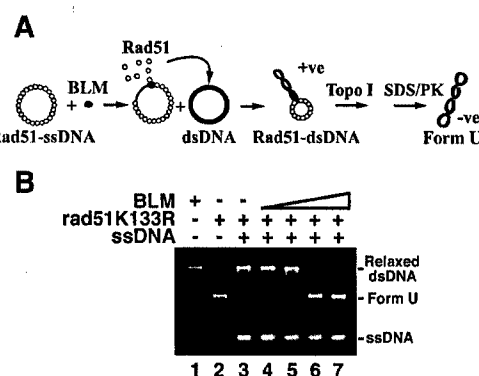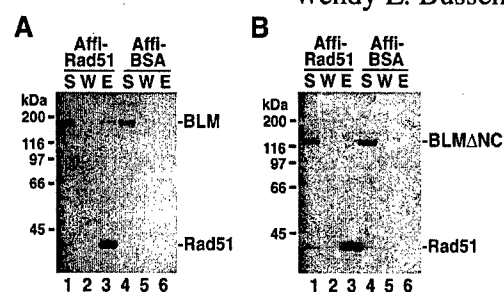

Figure 2. Disruption of the Rad51 presynaptic filament. (A) Schematic of the reaction for removal of Rad51 from ssDNA. Rad51 is removed from the ssDNA by BLM and becomes trapped on the topologically relaxed duplex DNA. Rad51 binding to the duplex induces positive supercoils in the DNA that are subsequently relaxed by calf thymus topoisomerase I (Topo I). Treatment with SDS and proteinase K removes Rad51 from the dsDNA, resulting in the formation of an underwound DNA species (Form U). (B) The Rad51-ssDNA presynaptic filament was incubated with increasing amounts of BLM (100-400 nM in lanes 4-7) followed by the addition of topologically relaxed duplex DNA and calf thymus topoisomerase I. The DNA species were deproteinized, resolved by agarose gel electrophoresis, and visualized with ethidium bromide. Lane 2 contains Rad51 incubated with dsDNA alone as a positive control. Lane 3 contains Rad51 incubated with the DNA substrates in the absence of BLM as a negative control. BLM alone (lane 1) is incapable of generating Form U DNA.

*Physical interaction of BLM and Rad51-*Since preliminary data have begun to show that BLM may disrupt the Rad51 presynaptic filament, I wish to address whether physical interaction between Rad51 and BLM is necessary for Rad51 displacement. Our collaborator, Ian Hickson, at Cancer Research UK has constructed an N- and C-terminal truncation of BLM (BLMΔNC) that retains helicase activity, but is unable to bind to Rad51 (Wu et al., 2001). We obtained the expression construct from him and expressed and purified the protein following the same protocol as above. To confirm that BLMΔNC did not interact with Rad51, I coupled either purified Rad51 or BSA to Affi-gel beads (Bio-Rad) and used them in pull-down experiments. As seen in figure 3, BLM, but not BLMΔNC, was retained specifically on the Rad51-coupled beads, confirming that BLMΔNC does not interact with Rad51. I am currently in the process of using BLMΔNC protein in the assay described above to address the significance of protein-protein interaction in disruption of the Rad51 presynaptic filament.

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Figure 3. Physical interaction of BLM and Rad51. (A) Purified BLM protein (2.5 ug) was mixed with Affi-gel 15 beads conjugated to either Rad51 (10 ug) or BSA (10 ug). The reactions were incubated on ice, washed with buffer, and proteins retained on the beads were eluted with SDS loading buffer. Equal amounts of the supernatant (S), wash (W), and elution (E) from each reaction were separated on a 7.5% denaturing gel and stained with Coomassie blue. **(B)** The same reaction as above, except carried out with purified BLMΔNC protein.

The role of ATP hydrolysis in BLM functions- BLM contains a canonical Walker A motif and hydrolyzes ATP quickly in the presence of ssDNA (Karow et al., 1997). In order to address the significance of ATP hydrolysis by BLM, I mutated the conserved lysine residue to either alanine or arginine (Fig. 4A). Work with other ATPases has demonstrated that mutating the conserved lysine to alanine abolishes both ATP binding and hydrolysis activity, while mutating it to arginine allows the protein to bind DNA but not hydrolyze it (Sung et al., 1988 & 1996). This latter mutation is useful for distinguishing whether ATP binding is sufficient for protein function, or if ATP hydrolysis is also required. The mutations were confirmed by DNA sequencing and the proteins were expressed in *S. cerevisiae* and purified to near homogeneity following the same protocol outlined above (Fig. 4B). As expected, the Walker mutants were devoid of ATP hydrolysis activity in the presence of ssDNA (Fig. 4C). To eliminate the possibility that the lack of ATP hydrolysis activity was due to an inability of the mutant proteins to bind to DNA, I tested their ability to bind ^{32}P-labeled forked DNA structures *in vitro*. As seen in figure 5, panel A, the Walker mutants bound DNA with the same affinity as wild-type BLM, demonstrating that lack of ATP hydrolysis was not due to a deficiency in DNA binding.
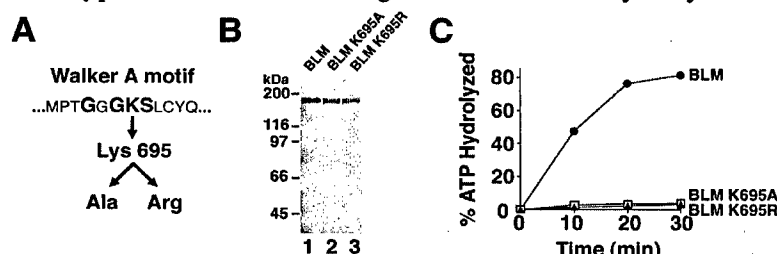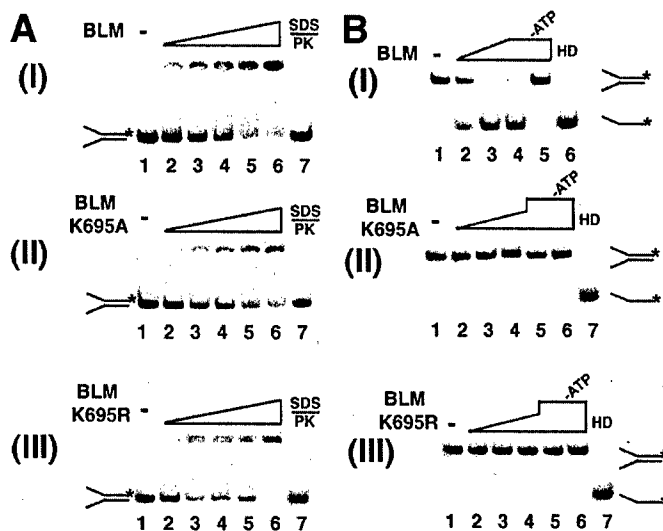

Figure 4. Purification and Characterization of BLM Walker mutants. (A) The Walker A motif in the BLM sequence is shown. Conserved residues are highlighted. To ablate ATP hydrolysis activity, the conserved lysine was mutated to alanine or arginine. **(B)** Purified BLM, BLM K695A, and BLM K695R proteins (800 ng each) were separated in a 7.5% denaturing gel and stained with Coomassie blue. **(C)** BLM, BLM K695A, or BLM K695R (100 nM) was incubated with ^{32}P-γ-ATP in the presence of φX174 ss virion DNA at 37°C. At the indicated times a portion of the reaction was removed and separated by thin-layer chromatography. Results were visualized by phosphorimage analysis.

Helicases are thought to use the energy from ATP hydrolysis to traverse along DNA. Thus, it is reasonable to predict that BLM Walker mutants will be devoid of helicase activity. I tested the ability of either wild-type BLM or the Walker mutants to unwind a ^{32}P-labeled forked DNA substrate *in vitro*. As expected, the Walker mutants were unable to unwind the structure, even at twice the molar concentration of wild-type protein (Fig. 5B, panel I, lane 4, panels II & III, lane 5). I am currently testing the Walker mutants in the filament disruption assay described above. Preliminary experiments show they are unable to displace Rad51 from ssDNA, suggesting that ATP hydrolysis is necessary for presynaptic filament disruption (data not shown).

Figure 5. BLM Walker mutants retain normal DNA binding activity but lack helicase activity. (A) Increasing concentrations of BLM (I), BLM K695A (II), or BLM K695R (III) were incubated with a ^{32}P-labeled forked DNA substrate (20 bases of duplex, 20 bases noncomplementary) for 20 minutes at 37°C, and the reaction mixtures were analyzed in 10% native polyacrylamide gels. In lanes 1, DNA substrate was incubated without protein. In lanes 7, the highest amount of protein was incubated with DNA, but the reaction mixture was treated with SDS and proteinase K (SDS/PK) prior to gel analysis. **(B)** Increasing amounts of BLM (I), BLM K695A (II), or BLM K695R (III) were incubated with a ^{32}P-labeled forked DNA substrate in the presence of ATP for 30 minutes at 37°C. Reactions were deproteinized with SDS and proteinase K and separated on 10% native polyacrylamide gels. In panel I, lane 5, and panels II and III, lane 6, ATP was omitted from the reactions. In panel I, lane 6, and panels II and III, lane 7, the highest amount of protein was incubated with DNA in the presence of ATP, but reaction mixtures were heat denatured before gel analysis.

KEY ACCOMPLISHMENTS

- BLM, Rad51, and RPA proteins have been purified to near homogeneity.
- Demonstrated that BLM protein disrupts the Rad51 nucleoprotein filament in the *in vitro* assay described here.
- BLM Walker mutants (K695A and K695R) have been constructed, expressed, and purified to near homogeneity.
- Demonstrated that the BLM Walker mutants are devoid of ATP hydrolysis activity.
- Demonstrated that the BLM Walker mutants bind to forked DNA substrates, but are unable to unwind them.
- Preliminary data shows that ATP hydrolysis by BLM is important for removing Rad51 from ssDNA in the system tested here.

REPORTABLE OUTCOMES

- Poster Presentation at MB&B Annual Retreat at Wood's Hole Massachusetts, October, 2004, hosted by the Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine. Poster Title "Role of the BLM Helicase in Homogous Recombination."

- Abstract sent for poster presentation at the Era of Hope Department of Defense Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania, July, 2005. Abstract title "Role of the BLM Helicase in Homologous Recombination."

CONCLUSIONS

As new evidence linking dysfunctional HR to tumor formation emerges, the need for understanding this process at the mechanistic level becomes more urgent. The work presented here begins to elucidate the mechanism by which BLM functions to regulate HR. Through its ability to interact with Rad51, BLM may disrupt the presynaptic filament, thus attenuating undesirable recombination. This idea is supported by the biochemical evidence presented here in which BLM removes Rad51 from ssDNA. In addition, mutation in the conserved Walker A motif of BLM abolishes the protein's ATPase and helicase activities. Using these mutants, I will now be able to address the importance of ATP hydrolysis by BLM in the function of disrupting the Rad51 presynaptic filament. In addition, I have constructed the tools for examining how the physical interaction with Rad51 dictates the functions of BLM in regulating HR reactions. By dissecting the functions of BLM in HR, we may be able to rationalize how the absence of BLM accelerates the onset of cancers in BS patients, and in doing so begin to understand how regulation of DNA repair pathways suppresses tumorigenesis in general.

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APPENDICES
None