The BLM Helicase Is Necessary for Normal DNA Double-Strand Break Repair

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Abstract

Experiments with the supF20 mutagenesis system demonstrate that extracts from Bloom’s syndrome (BS) cells are unable to use microhomology elements within the supF20 gene to restore supF function after the induction of a double-strand break (DSB). Additional experiments with the pUC18 mutagenesis system demonstrate that although the efficiency and fidelity of DSB repair by BS extracts are comparable with those of normal extracts when ligatable ends are present, a significant 5-fold increase in mutation rate with BS extracts is observed when terminal phosphates are removed from the DNA substrate that needs repair. Mutant plasmids recovered after DSB repair by BS extracts contain smaller deletions within the lacZ gene not commonly recovered from normal extracts. This work demonstrates that BS cells, lacking the BLM helicase, process DSBs differently than normal cells and strongly suggests a role for the BLM helicase in aligning microhomology elements during recombinational events in DSB repair.

Introduction

BS is a rare recessive disorder characterized by growth retardation, male infertility, sun-sensitive facial erythema, and predisposition to neoplasia. Cells derived from individuals with BS display a high incidence of chromosomal abnormalities and a high rate of spontaneous mutation. The gene mutated in BS encodes a RecQ-like helicase known as BLM. In general, DNA helicases function in many aspects of DNA metabolism including DNA repair, replication, recombination, transcription, and RNA processing, although the exact function of the BLM helicase remains uncharacterized. Additionally, the biochemical basis for the increased sun sensitivity of BS patients is unknown but suggests an abnormality in the ability to respond to DNA damage.

Previous evaluations of several DNA repair mechanisms in BS cells have revealed no dramatic defect. The activity of repair enzymes involved in the response to DNA damage and in DNA mismatch repair, nucleotide excision repair, base-excision repair and removal of O6-methylguanine are comparable with normal levels, although there may be alterations in the temporal regulation of some of these enzymes. Rung and Kraemer were the first to suggest that there may be an alteration in the ability of BS cells to repair DSBs in DNA, because the ability of BS cells in culture to rejoin linearized vector DNA was reduced and more error-prone than normal cells. However, these experiments could not differentiate the effects of alterations in DNA replication from those of DNA repair.

DSBs in DNA occur under normal physiological conditions, most predominantly during somatic recombination and repair of postreplicative gaps. DSBs also occur after exposure to ionizing radiation and oxidative stress. These lesions can be potentially lethal to the cell, blocking replication and/or transcription. Two major mechanisms of DSB repair exist: homologous recombination and nonhomologous end-joining. In homologous recombination, a DSB on one chromosome is repaired using the genetic information on the undamaged homologous chromosome via DNA strand exchange mediated in part by the rad52 epistasis group. In contrast, no sequence homology or extremely limited homology is required for nonhomologous end joining of DSBs. Several models have proposed that the free DNA ends at the DSB site are bound by Ku and DNA-protein kinase, which then recruit a Rad50-, Mre11-, and NBS1-containing repair complex to process the ends by endonuclease digestion and alignment of microhomology elements. Finally, the break is joined by the action of a DNA ligase-containing protein complex (reviewed in Refs. 8, 9).

The role of the BLM helicase in DNA DSB repair has been examined directly using cell-free nuclear extracts prepared from BS, corrected BS, and normal, SV40-transformed cells in vitro assays. These experiments demonstrate that the efficiency of DSB repair is similar in nuclear extracts from BS and normal cells but that the fidelity of this type of repair is decreased when nonhomologous end joining is required. Additionally, microhomology elements were less likely to be used to repair DSBs in BS extracts as compared with control extracts. We speculate that the BLM helicase is required to facilitate unwinding of DNA near the DSB for the alignment of microhomology elements used by the nonhomologous end-joining repair pathway in DSB repair.

Materials and Methods

Cell Lines. The SV40-transformed BS fibroblast cell line, GM08505C, was purchased from Coriell Laboratories (Camden, NJ). This cell line was derived from an Ashkenazi Jewish female, homozygous for the BLM allele (a 6-bp deletion/7-bp insertion at nucleotide 2281). Cells were grown in Dulbecco’s Modified Eagle Medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (Hyclone). The corrected BS cell line is the GM08505C transfected with the pop-BLM (full-length human BLM cDNA driven by a RSV-LTR promoter) and grown under selection of 250 μg/ml G418 (Life Technologies, Inc.). Western blotting confirmed expression of BLM at the expected size (data not shown). The SV40-transformed AT fibroblast cell line, AT5BIV, was purchased from the Human Genetics Mutant Cell Repository (Camden, NJ). The SV40-transformed normal human fetal lung fibroblast cell line (W138VA13) was purchased from American Type Culture Collection (Rockville, MD) and used as a control. Both AT and normal cells were grown in Eagle’s Minimal Essential Medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Nuclear Extracts. Nuclear extracts were prepared from cell lines based on the modified method of Lopez and Coppey (10) as described in Li et al. (11) with all of the steps performed at 4°C. Briefly, 1 × 10⁸ cells were harvested, washed in PBS, and resuspended in 4 ml hypotonic buffer [20 mM HEPES (pH 7.6]...
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7.5), 5 mM KCl, 1.5 mM MgCl₂, and 1 mM DTT. Cells were gently lysed using a Dounce homogenizer, and the released intact nuclei were recovered by centrifugation at 2000 × g for 1 min. The nuclei were extensively washed before the nuclear envelope was broken by three cycles of freezing and thawing. Debris was pelleted and soluble nuclear protein recovered from the supernatant by ammonium sulfate precipitation. The precipitate was resuspended in 50 mM Tris (pH 7.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol, and dialyzed overnight against the same buffer. Nuclear extracts were snap frozen and stored in aliquots at −80°C.

**supF20 DSB Repair Assay.** supF20 was a generous gift from Michael Seidman and Michael Lin (National Institute on Aging, National Institutes of Health, Baltimore, MD). supF20 is a derivative of the pU189 plasmid and carries an ampicillin resistance gene and a modified Escherichia coli supF suppressor tRNA gene that serves as a mutagenesis marker. An insertion, including a BssHII restriction endonuclease site, in the 5’ region of the tRNA sequence destroys supF function. Function can only be restored when a recombinational event across a duplicated microhomology patch results in a specific 11 base-pair deletion (12). In this study, a DSBR was generated in purified supF20 DNA at the BssHII restriction endonuclease site. Linear DNA was separated from circular plasmid by electrophoresis and purified from agarose by phenol/chloroform extraction. To assess the DSBR repair efficiency of nuclear extracts, 50 μg of protein from each extract were incubated for 1 h at 30°C with 1 μg of linear supF20 in 50 μL of DSBR repair buffer (65.5 mM Tris (pH 7.5), 10 mM MgSO₄, 1 mM ATP, 91 mM EDTA, and 9.1% glycerol). The reaction was stopped by the addition of EDTA to a final concentration of 20 mM and by incubation in RNase A (Life Technologies, Inc.) and proteinase K (Roche). DNA was purified by phenol/chloroform extraction and 1–10 ng were used to transform E. coli/MBM7070, a strain carrying an amber mutation in the lacZ gene. This mutation can be overcome by functional supF resulting in β-galactosidase synthesis. Transformants were selected on LB plates containing 100 μg/ml ampicillin and 40 μg/ml X-gal. Transformation efficiency, reflecting DSBR repair efficiency, was expressed as the total number of colonies per μg DNA. Fidelity of repair was determined by calculating the frequency of supF20 mutants over the total number of transformed colonies. supF20 mutants with restored supF function were identified as blue colonies on X-gal-containing plates.

**pUC18 DSB Repair Assay.** pUC18 carries an ampicillin resistance gene and the lacZ gene. A DSBR was generated in purified pUC18 DNA at the EcoRI restriction endonuclease site, disrupting the lacZ gene. Linear DNA was separated from circular plasmid by electrophoresis and purified from agarose by phenol/chloroform extraction. For experiments removing the terminal phosphate at the DSBR site, DNA was incubated with 1U alkaline calf intestinal phosphatase (Life Technologies, Inc.) for 1 h at 37°C. To assess the DSBR repair efficiency of nuclear extracts, 50 μg of protein from each extract were incubated for 1 h at 30°C with 1 μg of linear pUC18 in a 50 μL reaction mixture of DSBR repair buffer. The reaction was stopped and DNA purified as above. DNA (1–10 ng) was used to transform E. coli DH5α. Transformants were selected on LB plates containing 100 μg/ml ampicillin and 40 μg/ml X-gal. Transformation efficiency, reflecting DSBR repair efficiency, was expressed as the total number of colonies per μg DNA. Fidelity of repair was determined by calculating the frequency of lacZ mutants over the total number of transformed colonies. lacZ mutants were identified as white or light blue colonies on X-gal plates. Statistical significance was determined using the student’s t test. Mutant colonies were picked and restreaked onto plates containing g/ml ampicillin and g/ml X-gal. Transformation efficiency, reflecting DSBR repair efficiency, was expressed as the total number of colonies per μg DNA. Fidelity of repair was determined by calculating the frequency of lacZ mutants over the total number of transformed colonies. lacZ mutants were identified as white or light blue colonies on X-gal plates. Statistical significance was determined using the student’s t test. Mutant colonies were picked and restreaked onto fresh LB plates containing 100 μg/ml ampicillin and 40 μg/ml X-gal. DNA was purified from overnight cultures using a Qiagen miniprep kit and sequenced using an 18-bp primer (TGAGAGTGACCACATATGC) located at nucleotide 172 of the pUC18 lacZ plasmid. Sequencing was performed by the University DNA Core Laboratory at the College of Medicine using an Applied Biosystem Inc. 377 automated sequencer and dye terminator sequencing chemistry. Sequencer was used to align and compare DNA sequences.

**Results and Discussion**

In a preliminary experiment, the in vitro DSBR repair efficiency of BS nuclear extracts was assessed by examining the transformation capacity of BssHII-digested supF20 vector (see Fig. 1A). Restoration of supF function in the supF20 plasmid requires a specific 11-bp deletion event. Transformation efficiency of linear supF20 was the same order of magnitude for plasmids incubated in BS, corrected BS, and control nuclear extracts (Table 1), suggesting that the plasmid was recircularized by nuclear extracts in vitro and that BS nuclear extracts were indeed capable of repairing DSBR. No restoration of supF function was observed during DSBR repair using BS nuclear extracts compared with 0.256% using control extracts and 0.176% using corrected cell extracts (Table 1), strongly suggesting that BLM may be required for the alignment of the microhomology elements and subsequent 11-bp deletion event. Therefore, we decided to examine the DSBR repair ability and fidelity of BS nuclear extracts using a pUC18/lacZ reporter plasmid (Fig. 1B). Similar to the supF20 repair assay, DSBR repair efficiency using pUC18/lacZ is determined by restoration of the transformation capacity of EcoRI-digested plasmid. As controls for the effectiveness of the assay, nuclear extracts from a normal, a corrected BS and an AT cell line were used. A 3-fold higher mutation rate than normal has been demonstrated recently for AT extracts incubated with pUC18 lacking the 5’ phosphate group at the EcoRI-DSBR site (11).

Successful transformation of bacteria following incubation of linear pUC18 in nuclear extracts of control, corrected BS, and AT cells occurred at a rate of ~10⁶ colonies/μg DNA for each extract. Thus,
BS nuclear extracts were capable of repairing DSBs that retained ligatable ends at an efficiency similar to control, corrected BS, and AT extracts, confirming the observations using the supF20 plasmid.

The DNA termini generated by restriction endonuclease digestion differ from those generated by oxidative damage in that the DNA ends of the former are directly ligatable. To examine the effectiveness of DSB repair when no ligatable ends are present, terminal phosphates were removed from the digested pUC18 substrate by incubation in calf intestinal phosphatase before incubation in nuclear extracts. Without the terminal phosphate at the DSB site, transformation efficiency dropped by approximately an order of magnitude for all three of the extracts. No difference in the relative transformation efficiencies was observed when pUC18 with (Fig. 2A) or without terminal phosphates (Fig. 2B) was used as a substrate. Relative transformation efficiencies were determined in relation to controls in which ATP was omitted from the nuclear extract mix. Thus, DSB repair efficiency of the four cell types was similar.

The frequency of lacZ mutation was then examined to determine whether the contribution of error-prone repair pathways was similar for extracts from each cell type. The relative frequencies of lacZ mutants were significantly higher ($P < 0.05$) in experiments where linear pUC18 without the terminal phosphate group was incubated in BS or AT nuclear extract than the frequency of lacZ mutants from incubations in control or corrected BS nuclear extract (Fig. 2D). There were no significant differences in mutation frequencies when the DNA DSB substrate retained the terminal phosphate (Fig. 2C). Thus, repair of DSBs that did not have a terminal phosphate group was more error-prone in BS extracts than in extracts from normal or corrected BS cells.

Sequence analysis of some of the pUC18 mutants recovered in this study is shown in Fig. 3. The majority of mutations generated by the normal, corrected BS, BS, and AT nuclear extracts were deletions, most of which spanned the EcoRI-induced DSB site. The majority of repair catalyzed in normal extracts and all of the repair catalyzed by AT extracts use small, single or double nucleotide regions of homology at the ends of the deletion, a mechanism that is observed using similar assays (13). Conversely, such short repeat-mediated repair was rarely observed in the mutants generated by the BS extracts. A 6-bp, CGAATT, deletion was frequently observed in BS extracts, occurring in 14 of 29 mutants generated by BS extracts repairing pUC18 without

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<th>Exp. No.</th>
<th>Nuclear extract</th>
<th>ATP</th>
<th>Blue/total no. colonies</th>
<th>Transformation efficiency (colonies/μg DNA)</th>
<th>Relative transformation efficiency</th>
<th>Mutation frequency (%)</th>
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* Relative transformation efficiencies were determined in relation to parallel controls in which ATP was omitted from the nuclear extract mix.

** Mutation frequency was calculated as the number of blue colonies over the total number of colonies × 100%.

Combined average represents the total number of colonies from experiments 1 and 2. The transformation efficiency, relative transformation efficiency, and mutation frequency are the average of the two experiments.
terminal phosphates. In contrast, this deletion was not observed in mutants generated by normal or corrected BS extracts. This 6-bp deletion did not arise via a microhomology-mediated repair mechanism. Additionally, the distribution of deletion size changes with or without BLM in the extracts. Deletions from the control or corrected BS extracts show many sizes ranging from 1 to 250 bp in length. Deletions from BS extracts were generally smaller than the other cell types (averaging 6–10 bp); AT deletions were on average 25 bp in length. These data suggest that the BLM helicase may be responsible for unwinding the termini at DSBs to facilitate the access of the break to other proteins involved in DSB repair or to search for microhomologies.

In this study, we have demonstrated no reduction in the efficiency with which BS nuclear extracts repair a restriction enzyme-induced DSB in two different plasmid substrates. This result is in contrast to the decrease in the efficiency of DSB repair of BS cells reported by Runger and Kraemer (7). In their experiments, linear DNA was transfected into BS fibroblasts, which were then cultured for 2–3 days before DNA recovery and bacterial transformation. It is possible that the efficiency of plasmid replication rather than repair was being measured. Because BS cells exhibit a tremendously increased rate of sister chromatid exchange and an altered progression of the replication fork (1, 2), a defect in DNA replication may explain these experimental differences.

Although the DSB repair efficiency of BS extracts in our experiments was comparable with the efficiency of normal extracts, a significant decrease in the fidelity of repair was observed. This finding suggests that the absence of the BLM protein, a 3′-H11032 helicase, may impede the unwinding of the duplex DNA near the DSB. Indeed, the predominance of short deletions in the mutants obtained after incubation in BS extracts, in contrast to the longer deletions obtained after incubation in normal and corrected BS nuclear extracts, supports this hypothesis.

Two major pathways are responsible for the repair of DSBs in mammalian cells, nonhomologous end joining, and DSB repair via homologous recombination. Interestingly, current reports suggest the BLM helicase may play functional roles in both types of DSB repair. Mutations in Dmblm, which lead to partial male sterility and complete female sterility, can be partially complemented by overexpression of Ku-70, a protein well known for its role in nonhomologous end joining (14). Additionally, WRN, the helicase deficient in Werner’s syndrome, directly interacts with Ku-70 to regulate its intrinsic exonuclease activity (15). These findings suggest mammalian RecQ helicases have functional roles in nonhomologous end joining. BLM is
a member of the BRCA1-associated surveillance complex (16) and may also play a role in DSB repair by homologous recombination because it interacts directly with Rad51, a protein known to function in homologous recombination (17). A greater number of DSBs occur in chicken DT40 cells with homozygous mutations in BLM and Rad54, compared with control cells (18), whereas in response to agents that induce DSBs, BLM colocalizes with the Mre11, p95, and Rad50 complex (16).

In conclusion, it is interesting to note that our results account for some of the cellular phenotypes characteristic of BS. First, BS cells are not hypersensitive to ionization irradiation. We have shown that DSBs, the primary lesion generated by ionizing radiation, are efficiently repaired. Second, the rate of mutation of BS cells is greatly elevated as compared with normal cells, and we have shown DSB repair tends to be error-prone in BS. Although the precise biochemical function of the BLM helicase still remains unclear, the accumulating cytogenetic, biochemical, and cellular experimental evidence support a role for BLM in DSB repair.

References

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