The Bloom’s Syndrome Helicase Can Promote the Regression of a Model Replication Fork

Christine Ralf, Ian D. Hickson, and Leonard Wu

From the Cancer Research UK Laboratories, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, United Kingdom

Homozygous inactivation of BLM gives rise to Bloom’s syndrome, a disorder associated with genomic instability and cancer predisposition. BLM encodes a member of the RecQ DNA helicase family that is required for the maintenance of genome stability and the suppression of sister-chromatid exchanges. BLM has been proposed to function in the rescue of replication forks that have collapsed or stalled as a result of encountering lesions that block fork progression. One proposed mechanism of fork rescue involves regression in which the nascent leading and lagging strands anneal to create a so-called “chicken foot” structure. Here we have developed an in vitro system for analysis of fork regression and show that BLM, but not Escherichia coli RecQ, can promote the regression of a model replication fork. BLM-mediated fork regression is ATP-dependent and occurs processively, generating regressed arms of >250 bp in length. These data establish the existence of a eukaryotic protein that could promote replication fork regression in vivo and suggest a novel pathway through which BLM might suppress genetic exchanges.

In every cell cycle, the replication apparatus encounters numerous chemical and protein blocks that can perturb fork progression (1). The consequences of such encounters and the subsequent replication re-start processes employed will depend on both the type of lesion and on the template strand on which the damage resides. Lesions on the lagging strand template can, theoretically, be by-passed via the priming of an Okazaki fragment downstream of the lesion resulting in a gap in the nascent lagging strand. These gaps can then be filled, post-replicatively, by error-prone translesion polymerases or repaired in an error-free manner by homologous recombination (1, 2). Lesions on the leading strand template, however, may cause the replisome to stall. On such occasions, lagging strand synthesis can become uncoupled from leading strand synthesis, with the result that the lagging strand is extended beyond the site of the lesion (3–6). This apparently futile extension of the nascent lagging strand may actually serve an important role in lesion bypass. If, as proposed, the fork were to regress via annealing of the nascent strands, the longer lagging strand can provide a template for the prematurely terminated leading strand to be extended. In this way, by a process of template switching, leading strand synthesis can be continued beyond the point of the block on the parental template, effectively “bypassing” the lesion. The fork can then be re-established to allow leading strand synthesis to re-commence through either reversal of the regressed fork or recombination-dependent invasion of the regressed arm into the parental template at a point downstream of the lesion (7, 8). In Escherichia coli, a number of proteins have been shown to promote the regression of stalled forks, which include the DNA helicase RecG and the recombinase protein RecA (9, 10). These proteins are unrelated functionally, which suggests that multiple mechanisms may exist to promote fork regression that could be utilized under different circumstances of replication fork repair (11). In eukaryotes, the mechanisms for processing stalled forks is less well characterized. Nevertheless, several lines of evidence indicate that fork regression might occur at eukaryotic replication forks (12, 13). However, how the process of fork regression is facilitated in eukaryotes is, as yet, unclear.

The Bloom’s syndrome (BS) helicase, BLM, has been implicated in the repair of damaged replication forks (14). BLM is a member of the highly conserved RecQ family of DNA helicases. The family is named after the prototypical member RecQ, and members are defined by the presence of a conserved catalytic helicase domain (15). Several RecQ helicases also contain additional identifiable motifs, known as the RQC and HRDC domains (15). The precise function of these domains is unknown, although they have been implicated in the binding of proteins and/or DNA structures (16–22). In all organisms, RecQ helicases are essential for the maintenance of genome stability with many RecQ helicase mutants displaying replication defects and elevated levels of recombination events (14). Indeed, one of the defining characteristics of BS cells is an increased frequency of sister chromatid exchanges (SCEs) (23), which are thought to arise from the aberrant repair of damaged replication forks (14). The BLM protein has been shown to contain three distinct biochemical activities as follows: a 3′–5′ DNA unwinding activity, a Holliday junction branch migration activity, and a single-stranded DNA annealing activity (24–27). In what role and in which cellular context these activities function remain to be determined. Given that the process of fork regression involves the annealing of the nascent leading and lagging strands, the BLM helicase may promote this process through its dual activities.

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BLM Can Regress Replication Forks

A

Joint molecule (5704bp)

Anneal

Topoisomerase I

RF

Interlinked pG46-B5' and pG68-A

B

Fork Regression

C

RF/RFII

AvrII

36-mer

D

Fork (Rus A insensitive)

Or

RF or RFII

RusA

Holliday junction (Rus A sensitive)

E

RecG

RF

1036-12216

bp

1017

208

220

134

75

1

2

3

4

5

6

7

8
BLM Can Regress Replication Forks

Helicase Assays—The splayed arm helicase substrate, prepared as described previously (28), was incubated with either BLM or RecQ, at the indicated concentrations, in buffer H at 37 °C for 30 min. Reaction products were separated on native 10% polyacrylamide gels. Gels were dried and exposed to PhosphorImaging screens for quantitative analysis using a Storm scanner and ImageQuant software (Amersham Biosciences).

RESULTS

Preparation of a Model Stalled Replication Fork—To analyze events that occur during replication fork repair, we developed a strategy for the preparation of structural mimics of a stalled replication fork, as outlined in Fig. 1A. This protocol involves the annealing of a plasmid (pG68-A) to a near identical plasmid (pG46-B5') via a 68-nt single-stranded gap in pG68-A and a complementary 46-nt, 5’ end-labeled, single-stranded gap in pG46-B5' (Fig. 1A). The annealing creates a pararmic joint, which can be converted to a plectonemic joint by treatment of the DNA with topoisomerase I. This concomitantly results in the catenation of the two plasmids (Fig. 1A). The resulting joint molecule, termed RF, contains a structural mimic of a replication fork in which the 5’ end of the “lagging strand” is labeled. RF contains a number of features that make it particularly amenable for analyzing the process of replication fork regression. First, the sequences of the arms representing the daughter chromosomes are homologous, allowing fork regression to occur unimpeded by sequence heterology. Second, the absence of free DNA ends, other than those at the fork, eliminates potential artifactual loading sites onto the substrate that would not exist in vivo. Third, the ability to individually alter the length of the single-stranded gaps on each of the constituent plasmids of RF enables analysis of the effect that different configurations of nascent strands at the fork might have on fork regression (see below). Further details of the preparation of RF are contained in supplemental Methods and supplemental Figs. 1–4.

Regression of the labeled fork in RF would result in the transfer of restriction endonuclease recognition sites from those arms representing the daughter chromosomes into the regressed arm of the fork (Fig. 1B). Therefore, regression can be assessed by the restriction endonuclease–dependent release of the 5' label of pG46-B5' as a linear duplex fragment of a predefined length that is dependent on the particular restriction enzyme used (Fig. 1B). In the nonregressed state, the label in RF remains associated with the joint molecule following digestion. Because of the differentially sized gaps in pG68-A and pG46-B, the labeled fork in substrate RF resembles a

| Protein | Source |
|---------|--------|
| BLM | expressed and purified as described previously (25). RecQ, RecQ, and RusA were kindly gifts from Drs. Peter McGlynn, James L. Keck, and Robert G. Lloyd, respectively. Topoisomerase I was purchased from Promega. N.BbvC IA, N.BbvC IB, and restriction enzymes were purchased from New England Biolabs. |

Fork Regression Assays—The replication fork substrate (10 pm) was incubated with the indicated concentration of BLM, RecG, or RecQ in buffer H (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM MgCl2, 2 mM ATP, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin). Where indicated 1 μl (2–10 units) of wheat germ topoisomerase I was also included in the reactions. Unless otherwise indicated, reactions were incubated for 60 min at 37 °C and then quenched with 10 mM ATP and 125 ng of M13 ssDNA prior to the addition of 8 mM MgCl2 and 1 μl (2–20 units depending on the enzyme) of the indicated restriction enzyme. Reactions were incubated for a further 60 min to ensure products were digested to completion, and the reaction products were then separated on a 10% polyacrylamide gel.

Gels were dried and exposed to PhosphorImaging screens for quantitative analysis using a Storm scanner and ImageQuant software (Amersham Biosciences).

RusA Cleavage Assays—The X12 four-way junction substrate, prepared as described previously (28), RF/HindIII, or RFII/HindIII were incubated with the indicated concentrations of RusA in buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 10 mM MgCl2, 10% glycerol at 37 °C for 30 min. Reactions were stopped by the addition of 1% SDS and 50 mM EDTA and de-proteinized with 1 mg/ml proteinase K for a further 10 min at 37 °C. Reaction products were run on a 0.7% agarose gel. Gels were dried and exposed to PhosphorImaging screens for quantitative analysis using a Storm scanner and ImageQuant software (Amersham Biosciences).

FIGURE 1. Preparation of a model replication fork. A, strategy for the preparation of joint molecules containing replication forks that can regress. The free 5’-phosphate of pG46-B5’ was labeled with 32P, as indicated by the asterisk. The region of the joint molecule boxed by a dotted line has been enlarged below to show the interlinking of pG46-B5’ and pG68-A following topoisomerase I treatment. See supplemental Methods and text for details. B, predicted products of fork regression of substrates RF or RFII, resulting in the incorporation of the 5’ label of pG46-B5’ and restriction sites into the regressed arm (A, AvrII; B, BamHI; E, EcoRI; P, PvuII; X, XbaII). Dotted lines indicate the length in base pairs of the labeled fragment predicted to be released from the regressed arm by digestion with the indicated enzymes. The length of the single-stranded gap present ahead of the “lagging strand” in substrates RF and RFII is indicated. C, PAGE analysis of products generated by AvrII digestion of substrate RF and RFII as indicated above the lanes. The positions of the substrates and product and molecular size markers are indicated on the left and right of the gel, respectively. Because of the circular and branched nature of RF and RFII molecules, they remain in the wells when resolved by PAGE. D, schematic diagram showing the product(s) of linearizing RF or RFII with HindIII. The position of the 5’ label is indicated by an asterisk. Shown also are the products of RusA cleavage of the four-way junction arising during fork regression (for clarity, only one orientation of cleavage is presented). Lower panel, incubation of X12, RFII/HindIII, or RF/HindIII in the absence or presence of RusA 24 or 73 nm as indicated above the gels. The position of X12, the nicked duplex generated by RusA cleavage of X12, or linear pG46-B5’ is indicated on the right of the respective gels. E, PAGE analysis of fork regression products detected with different restriction enzymes (U, Uncut; A, AvrII; B, BamHI; E, EcoRI) following treatment of RF with 14 nM RecQ as indicated above the lanes. The sizes and positions of the products released by restriction digestion following RecQ treatment are indicated on the right.
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As predicted, RusA was found to cleave X12 to completion, whereas ~45% of RFII was cleaved by RusA to release linearized pG46-B5’ (Fig. 1D). This indicated that nearly half of the labeled forks in RFII were in a Holliday junction configuration, a finding consistent with the fraction of label released as the 36-bp fragment following digestion of RFII with AvrII (Fig. 1C). In contrast, a similar sized fragment was not released following AvrII digestion of RF (Fig. 1C). This indicates that the 14-nt gap on RF suppresses spontaneous fork regression and that the majority (>90%) of the labeled end in RF is therefore in a fork configuration. Predictably, digestion of RF with either EcoRI or BamHI, whose sites are more distal to the 5’-labeled end of pG46-B5’, also did not release any appreciable amount of label as fragments predicted to arise from digestion of regressed arms (Fig. 1, B and E, lanes 1–4). To confirm that the release of the 36-bp product by AvrII digestion of RFII was because of spontaneous conversion of the labeled fork into a Holliday junction, we treated RFII with the Holliday junction-specific endonuclease, RusA (29). To do this, we pre-digested RFII with HindIII, which cuts at a unique site in both circular halves of RFII to generate a labeled replication fork that contains “replicated” arms of ~2.8 kb (Fig. 1D). This allowed us to analyze specifically the labeled branch structure in RFII, because resolution of the labeled regressed fork by RusA in the HindIII linearized substrate would be expected to release a labeled linear fragment of 2.8 kb (Fig. 1D). As a control we also incubated RusA with a Holliday junction (X12) that was constructed from oligonucleotides and consisted of four 25-bp duplex arms. The junction was mobile because of a 12-bp homologous core, which contained a single CC dinucleotide, the preferred cleavage site for RusA (29).

stalled replication fork in that there is a single-stranded gap (of 14 nt) on the leading strand template, a situation that would arise following the uncoupling of leading and lagging strand synthesis (Fig. 1, A and B). The 14-nt gap also served to prevent spontaneous fork regression. To confirm this, we generated a related joint molecule, RFII, in which the single-stranded gap on the leading strand template is only 3 nt as opposed to the 14 nt in the RF joint molecule (supplemental Methods and Fig. 1B). Because of the close proximity of the ends of the “nascent” strands at the fork of RFII, these strands would be more likely to anneal and result in spontaneous fork regression. RF and RFII were digested with AvrII to detect the presence of a labeled regressed arm. Digestion of RFII with AvrII released ~45% of the label as a 36-bp fragment indicating that nearly half of the labeled forks in substrate RFII had undergone spontaneous fork regression (Fig. 1C). In contrast, a similar sized fragment was not released following AvrII digestion of RF (Fig. 1C). This indicates that the 14-nt gap on RF suppresses spontaneous fork regression and that the majority (>90%) of the labeled end in RF is therefore in a fork configuration. Predictably, digestion of RF with either EcoRI or BamHI, whose sites are more distal to the 5’-labeled end of pG46-B5’, also did not release any appreciable amount of label as fragments predicted to arise from digestion of regressed arms (Fig. 1, B and E, lanes 1–4). To confirm that the release of the 36-bp product by AvrII digestion of RFII was because of spontaneous conversion of the labeled fork into a Holliday junction, we treated RFII with the Holliday junction-specific endonuclease, RusA (29). To do this, we pre-digested RFII with HindIII, which cuts at a unique site in both circular halves of RFII to generate a labeled replication fork that contains “replicated” arms of ~2.8 kb (Fig. 1D). This allowed us to analyze specifically the labeled branch structure in RFII, because resolution of the labeled regressed fork by RusA in the HindIII linearized substrate would be expected to release a labeled linear fragment of 2.8 kb (Fig. 1D). As a control we also incubated RusA with a Holliday junction (X12) that was constructed from oligonucleotides and consisted of four 25-bp duplex arms. The junction was mobile because of a 12-bp homologous core, which contained a single CC dinucleotide, the preferred cleavage site for RusA (29).

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BLM Can Regress Replication Forks

BLM Can Reverse a Stalled Replication Fork to Generate a Regressed Arm—We next investigated if BLM could regress the labeled fork in RF into a Holliday junction. To do this, BLM was incubated with substrate RF. Reaction products were then digested with AvrII, BamHI, or EcoRI to detect the presence of regressed arms. BLM was found, in a concentration-dependent manner, to convert forks in the RF substrate into Holliday junctions as evidenced by the restriction enzyme-dependent release of labeled fragments whose sizes were identical to those generated by RecG (Fig. 2A and data not shown). The generation of the released linear product was dependent on the presence of ATP, indicating a requirement for the helicase and/or branch migration activity of BLM (Fig. 2B, compare lanes 3 and 4). At the highest concentration of BLM tested, ~40% of RF molecules contained a regressed arm (Fig. 2A). Given that <10% of the starting substrate RF contained pre-existing Holliday junctions (Fig. 1), we conclude that BLM can generate regressed arms de novo from a replication fork structure.

Because the detection of fork regression required the sequential digestion of the BLM-treated substrate with restriction enzymes, it was possible that the linear released product arose as a secondary action of BLM on the linearized substrate by a process unrelated to fork regression. To eliminate this possibility, reactions were quenched by addition of 10 mM ATP and 125 ng of M13 single-stranded DNA to competitively inhibit further action of BLM prior to the addition of EcoRI. EcoRI-released products could only be detected if ATP was and M13 ssDNA were added together with EcoRI (i.e. following BLM incubation) but not if added at the start of the reaction together with BLM (Fig. 2B, compare lanes 2 and 4). This result confirms that ATP and M13 ssDNA acted as effective competitive inhibitors of BLM and that the released labeled product arose as a result of EcoRI digestion of BLM-generated products and not the action of BLM on EcoRI-digested pG46-B5’. To further establish that the EcoRI-released product arose as a result of fork regression, the product was confirmed to be double-stranded and to contain the restriction sites for BamHI and AvrII, which are located between the EcoRI site and the 5’ end of the gap in pG46-B5’ (Fig. 1B). Digestion of the EcoRI-released product with either BamHI or AvrII gave rise to labeled products of 61 or 36 bp in length, respectively, consistent with the EcoRI-generated product being double-stranded and arising as a result of fork regression (Fig. 2C, lanes 8–10).

BLM Acts Progressively to Generate Regressed Arms >250 bp in Length—Next, we analyzed the extent to which BLM could promote fork regression on RF. Using PvuII, regressed arms of 266 bp could be detected following BLM incubation (Figs. 1B and 3A, lanes 11 and 17). However, few forks regressed far enough to transfer a BsaXI site into the regressed arm, which is 39 bp distal to the PvuII site and would be predicted to release a 305-bp fragment when cut with BsaXI (Figs. 1B and 3A, lanes 12 and 18).

Fork regression is predicted to be a progressive process in which the length of the regressed arm would be proportional to protein concentration and/or time. Interestingly, however, although the fraction of molecules that contained regressed...
cates that, as predicted, fork regression catalyzed by BLM is a progressive phenomenon that initiates at the fork.

**BLM-mediated Fork Regression Occurs Independently of the Generation of DNA Supercoiling**—Positive supercoiling can induce spontaneous fork regression in partially replicated plasmids (30). Therefore, it was possible that BLM does not act directly at the replication fork in substrate RF to promote regression. Rather, because of the circular nature of RF, BLM, through its ability to track along DNA, could induce positive supercoils within the substrate that indirectly cause fork regression. To investigate if supercoiling is required for BLM to promote fork regression, we tested BLM on RF that had been linearized by HindIII to prevent the formation of any DNA supercoiling that might arise because of translocation of BLM along the uncut substrate (Fig. 1D). BLM was found to efficiently regress the labeled fork in RF/HindIII (Fig. 4A). This indicates that any supercoiling that might arise within intact circular RF is not the driving force for BLM-mediated fork regression and that the generation of positive supercoils is therefore not the mechanism by which BLM promotes fork regression. Further confirmation that supercoiling is not required for BLM-mediated fork regression was provided by the fact that the inclusion of wheat germ topoisomerase I, which relaxes both positive and negative supercoils, did not inhibit the ability of BLM to regress forks within RF (Fig. 4B).

**Fork Regression Activity Is Not a General Feature of RecQ Helicases**—BLM can unwind or process a wide variety of branched and non-B-form DNA structures, which are also substrates for certain other helicases (15, 26, 28, 31). Among these is *E. coli* RecQ, which contains the three functional domains (the helicase, RQC, and HRDC domains), that are found in many RecQ family helicases, including BLM (15). RecQ has also been implicated in the unwinding of the lagging strand of stalled replication forks in vivo (32, 33). However, *E. coli* RecQ was not able to catalyze fork regression on RF at concentrations at which BLM promoted robust fork regression (Fig. 5, C and D, compare lanes 1–4 and 10–12). Moreover, even at vast molar
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excesses of RecQ (1 μM) over BLM (20 nM), RecQ was still unable to catalyze fork regression (data not shown). Analysis of helicase activity using a conventional splayed armed duplex substrate indicated that, at equimolar concentrations, RecQ had an ∼50-fold higher helicase activity than did BLM (Fig. 5, A and B). To eliminate the possibility that excessive RecQ helicase activity might be inhibitory to fork regression, we used concentrations of RecQ and BLM that gave equivalent helicase activity, as determined by analyses using the splayed arm substrate. At these lower concentrations, RecQ was still unable to catalyze fork regression (Fig. 5, compare lanes 1–4 with lanes 5–9).

**DISCUSSION**

The disruption of replication forks is a common occurrence in all actively dividing cells, and several pathways have been proposed to explain how replication might be re-initiated from a stalled fork (Fig. 6). Here we have shown that BLM is capable of promoting the regression of model stalled replication forks *in vitro*. This novel activity of BLM acts directly at the replication fork and regresses the fork in a processive manner for distances of >250 bp. Regression to this extent is consistent with the size of chicken foot structures present in replication intermediates purified from yeast cells exposed to replicative stress (12, 34).

The mechanism by which BLM catalyzes fork regression of RF requires further investigation to determine which of the helicase, branch migration, and strand annealing activities of BLM is required and how they coordinate to facilitate fork regression. A simple model, in which the nascent strands are unwound by the helicase activity of BLM, followed by their subsequent spontaneous annealing to each other, is unlikely, because fork regression could not be induced with *E. coli* RecQ, which can efficiently unwind lagging strands at replication forks (33). The inability of RecQ to catalyze fork regression suggests that this activity might be a specific activity of BLM and not an ancestral function of RecQ helicases. The simplest interpretation of the comparison between BLM and RecQ is that the nonconserved regions of BLM are required for promoting fork regression. However, the HRDC domain, which is conserved in BLM, RecQ, and several other RecQ family helicases, has been shown to modulate the structure-specific binding and unwinding activities of the protein in which it resides and has been implicated in functionally distinguishing members of the RecQ helicase family (16, 22). Ongoing studies aim to identify which conserved and nonconserved regions of BLM are important for catalyzing fork regression.

The cellular diagnostic feature of BS cells is an elevated frequency of SCEs, which are generated by homologous recombination (35). One proposed mechanism by which BLM suppresses SCEs is through the dissolution of double Holliday junctions into non-crossover products (36). BLM-mediated dissolution is dependent on the type IA topoisomerase hTOPO IIIα, which requires a physical interaction with BLM for its correct cellular localization (36, 37). Despite this requirement, a mutant BLM protein that lacks the evolutionarily conserved hTOPO IIIα interaction domain is still able to partially suppress the elevated SCEs seen in BS cells (38). Furthermore, under some circumstances, BLM and hTOPO IIIα dissociate in response to replicative stress (39). These observations suggest that BLM may function in both hTOPO IIIα-dependent and hTOPO IIIα-independent pathways to prevent SCEs. One possibility is that fork regression by BLM generates the substrates utilized in both of these pathways. Following template switching (Fig. 6, c and d), the regressed fork may be reversed to reset the fork by the branch migration activity of BLM (Fig. 6, d and e), as suggested previously (26). Alternatively, the regressed arm may be utilized by the recombinase repair machinery to re-initiate replication downstream of the fork arresting lesion, generating a second Holliday junction (Fig. 6, f and g). This is a particularly attractive model because BLM will already be at the site of the stalled fork and will therefore be poised to dissolve the double Holliday junction before other junction resolvases can resolve the Holliday junctions into crossover products giving rise to SCEs (Fig. 6, g and h). The presence of BLM may also prevent resolvases from cleaving the first Holliday junction arising from fork regression and thus generating a double strand break. BLM-mediated fork regression may therefore be important in keeping stalled forks intact. In the absence of BLM, stalled forks may be converted to double strand breaks by endonucleases, such as MUS81,
stimulating the inter-sister and inter-homologue recombination seen in BS cells (Fig. 6, I–l) (23, 40).

In humans, four other RecQ helicases exist in addition to BLM. Where studied, the phenotypes of metazoan RecQ helicase mutants indicate both overlapping and nonredundant roles for RecQ helicases (41, 42). In common with BS cells, elevated SCEs are also a feature of RECQ5-deficient human cells (41, 42). Therefore, although fork regression might be an activity specific for BLM, it remains to be determined whether other human RecQ helicases could also promote fork regression that might act in either partially redundant or parallel pathways to BLM to prevent SCEs.

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