Association and regulation of the BLM helicase by the telomere proteins TRF1 and TRF2

Kate Lillard-Wetherell¹, Amrita Machwe², Gregory T. Langland¹, Kelly A. Combs¹, Gregory K. Behbehani¹, Steven A. Schonberg³,⁴, James German⁵, John J. Turchi⁶, David K. Orren² and Joanna Groden¹,*

¹Department of Molecular Genetics, Biochemistry and Microbiology, Howard Hughes Medical Institute, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA, ²Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536, USA, ³Department of Pediatrics, George Washington University School of Medicine, Washington DC 20052, USA, ⁴American Quest Laboratories, Inc., Chantilly, VA 20153, USA, ⁵Department of Pediatrics, Weill Medical College of Cornell University, New York, NY 10021, USA and ⁶Department of Biochemistry and Molecular Biology, Wright State University School of Medicine, Dayton, OH 45435, USA

Received April 28, 2004; Revised June 3, 2004; Accepted June 15, 2004

In addition to increased DNA-strand exchange, a cytogenetic feature of cells lacking the RecQ-like BLM helicase is a tendency for telomeres to associate. We also report additional cellular and biochemical evidence for the role of BLM in telomere maintenance. BLM co-localizes and complexes with the telomere repeat protein TRF2 in cells that employ the recombination-mediated mechanism of telomere lengthening known as ALT (alternative lengthening of telomeres). BLM co-localizes with TRF2 in foci actively synthesizing DNA during late S and G2/M; co-localization increases in late S and G2/M when ALT is thought to occur. Additionally, TRF1 and TRF2 interact directly with BLM and regulate BLM unwinding activity in vitro.

Whereas TRF2 stimulates BLM unwinding of telomeric and non-telomeric substrates, TRF1 inhibits BLM unwinding of telomeric substrates only. Finally, TRF2 stimulates BLM unwinding with equimolar concentrations of TRF1, but not when TRF1 is added in molar excess. These data suggest a function for BLM in recombination-mediated telomere lengthening and support a model for the coordinated regulation of BLM activity at telomeres by TRF1 and TRF2.

INTRODUCTION

Several proteins are important for telomere maintenance, including TRF1 and TRF2, both of which bind to duplex telomeric repeats via a MYB-binding domain within the C-terminus of each protein (1). TRF1 is vital for controlling telomere elongation by inhibiting telomerase at chromosome ends and perhaps by inhibiting C-strand synthesis (2). Furthermore, TRF1 promotes parallel pairing of duplexed telomeric tracts in vitro suggesting a role in packaging telomeres (3). TRF2 plays a key role in telomere maintenance and stability, as its alteration has profound effects on telomere length, the frequency of chromosome end-to-end fusions and cellular senescence (4–6). TRF2 is sufficient for the formation in vitro of a protective telomeric loop, or T-loop, in which the single-stranded 3' overhang is inserted into the antecedent duplexed telomere tracts (7,8). This loop structure is thought to sequester chromosomal ends in order to prevent DNA end-joining and the activation of DNA damage checkpoint signaling (4,7). Additionally, telomeric loops may be intermediates in recombination-mediated pathways of telomere lengthening (9). Clearly, TRF1 and TRF2 are central players in telomere elongation, protection and structure.

Telomerase is required for the primary mechanism of telomere length maintenance in mammalian cells and is expressed in embryonic tissues, germ cells and stem cells, but not
in normal somatic cells with a finite lifespan (10,11). Telomeres can also be lengthened by telomerase-independent pathways, referred to as ALT (alternative lengthening of telomeres), that are mediated by recombination (12–15). In Saccharomyces cerevisiae, two ALT-related pathways have been identified that require either Rad50 or Rad51 (16,17). Sgs1, the sole S. cerevisiae RecQ helicase, is required for RAD50-dependent recombination-mediated telomere lengthening (18–20). In immortalized human cells that use ALT, RAD50 with associated factors NBS1 and MRE11, RAD51 and RAD52 co-localize with telomeric DNA, TRF1 and TRF2 in specialized ALT-associated promyelocytic leukemia (PML) nuclear bodies (APBs) (21–24). APBs are enriched in cells during G2/M (23) and incorporate bromodeoxyuridine (BrdU) (24), suggesting that telomere synthesis occurs within APBs after most DNA replication is complete.

Somatic cells from persons with the inherited chromosome breakage syndrome, Bloom syndrome (BS), feature excessive chromosome breakage and intra- and interchromosomal homologous exchanges (25). The gene mutated in BS, BLM, encodes a RecQ-like ATP-dependent 3’ → 5’ helicase that presumably functions in some types of DNA transactions (26). As the absence of BLM is associated with excessive recombination (27), in vitro experiments have tested the ability of BLM to suppress recombination and/or resolve recombination intermediates. In vitro, BLM promotes branch migration of Holliday junctions, resolves D-loops and unwinds G-quadruplex DNA (28–31). A function of BLM in maintaining telomeres is suggested by the latter, as D-loops and G-quadruplex structures are thought to be present at telomeres (7,32). In addition, co-localization of BLM with telomeres, TRF1 and TRF2 has been described in cells utilizing ALT (33,34) and a functional in vitro interaction with TRF2 was recently demonstrated (35). Intriguingly, Schwalder et al. (36) report a telomere-specific association domain at the N-terminus of the BLM protein. Primary cells from BS persons demonstrate a normal distribution of telomere length as compared with age-matched controls; however, some hyper-variability in telomere length has been noted for BS cells in culture (33,36).

In the present study, the function of BLM at telomeres was further investigated. We report an increase in the frequency of associations between chromosome ends in BS cells suggesting an inability to resolve telomere associations in the absence of BLM. Additionally, we find that BLM associates with TRF2 in immortalized cells using ALT but not in telomerase-positive cells, consistent with previous reports (34). BLM co-localizes with TRF2 in foci actively synthesizing DNA during late S and G2/M; co-localization is enriched during these phases of the cell cycle when ALT is thought to occur. Additionally, we report that TRF1 and TRF2 physically and functionally interact with BLM in vitro. TRF2 stimulates BLM unwinding of telomeric and non-telomeric substrates. Conversely, TRF1 inhibits BLM unwinding of telomeric substrates only. Finally, BLM helicase activity is stimulated by TRF2 with equimolar concentrations of TRF1, but not when TRF1 is present in molar excess. These experiments demonstrate that BLM is a component of a telomere-associated complex in cells using ALT and suggest a unique mechanism for regulation of BLM helicase activity at telomeres by TRF1 and TRF2.

RESULTS

Cytogenetic evidence for telomere dysfunction in BS cells

Cytogenetic evaluation of BS cells reveals an excess of various chromatid lesions, including the quadriradial configuration (QR) signifying the hyper-recombination associated with BLM mutation (25). In addition, telomere associations (TAs) between homologous chromosome arms at metaphase are observed in BS cells by G-banding (Fig. 1A). TAs may also be found in cells from normal persons; although in cultured cells, TAs are most frequently found in cells defective in telomere maintenance, presumably due to recombination and end-joining between shortened telomeres (37,38). A clear (non-staining) area is generally present between associated telomeres in BS cells indicating that these TAs are not end-to-end fusions [Fig. 1A(b) and (c)]. When this clear area is lacking, the configuration becomes indistinguishable from a dicentric chromosome formed by end fusion [Fig. 1A(a)]. TAs are present in 0.57% of metaphases from BS blood lymphocytes in short-term culture as compared with 0.1% of non-BS controls, a statistically significant difference by chi-square analysis ($P = 0.05$) (Table 1). TAs between homologous chromosome arms are also observed in metaphases prepared from immortalized BS fibroblast cells (GM08505) analyzed by fluorescence in situ hybridization (FISH) with sub-telomeric and teleric repeat probes (Fig. 1B and C). Telomeric sequences are present at the junction of most TAs observed by FISH analysis (Fig. 1C). The latter observation demonstrates that TAs in BS cells do not occur exclusively as a result of end fusion between chromosomes lacking telomeric repeat DNA. These cytogenetic data suggest that TAs are elevated significantly in primary and transformed BS cells. This increase in TAs may be the result of increased telomere recombination or intertwined chromosome ends that remain unresolved in the absence of the BLM helicase.

BLM and TRF2 co-localize and associate in telomerase-negative cells

To determine if BLM co-localizes with telomeres, dual immunostaining was performed with antibodies specific for BLM and TRF2. As the increased frequency of TAs in BS cells suggested a function for BLM in telomere recombination, telomerase-negative immortalized cells using ALT (Saos2) were compared with telomerase-positive immortalized cells (MCF7) and primary cells (WI-38). BLM co-localizes with TRF2 in Saos2 cells, but does not co-localize with TRF2 in either MCF7 or WI-38 cell lines (Fig. 2A). Co-localization of BLM and TRF2 was also observed in another cell line using ALT, VA13/2RA, and is absent in HeLa cells, a telomerase-positive cell line (data not shown). BLM and TRF2 additionally co-localize with PML in cells using ALT demonstrating that co-localization occurs within APBs (data not shown) in agreement with previous results (34). These observations demonstrate that co-localization of BLM and TRF2 is markedly enhanced in cells using ALT and further suggest a role for BLM in telomere recombination.
BLM and TRF2 foci are enriched during G2/M and undergo DNA synthesis

Cells that use the ALT pathway are enriched with APBs in G2/M, suggesting that recombination-mediated telomere lengthening is cell cycle regulated (23). If BLM is involved in ALT, its association with TRF2 is predicted to increase as cells progress into G2/M. To test this, VA13/2RA and Saos2 cells were blocked at the G1/S boundary and analyzed at 4 and 10 h post-release to compare co-localization of BLM with TRF2 during G1/early S or late S/G2/M, respectively (Fig. 2B). Cell cycle distribution was confirmed by flow cytometry (Supplementary Material). BLM co-localizes with TRF2 throughout the cell cycle in both cell lines; however, the number of cells with overlapping foci increases ~2–3-fold as cells progress into late S and G2/M. During G1 and early S, foci in which BLM and TRF2 co-localize are present in 15% of VA13/2RA cells and 21% of Saos2 cells; during late S and G2/M, the number of cells with BLM/TRF2 foci increases to 38 and 54%, respectively. Co-localization of BLM with PML increases in a similar manner confirming that co-localization occurs in APBs (data not shown). Increased localization in APBs could be either due to translocation of BLM to APBs or due to an increase in the number of APBs during late S and G2/M.

Proteins implicated in ALT, including NBS1 and TRF1, co-localize with foci of BrdU-incorporation during late S and G2 (24). Because APBs are enriched during G2/M (23), it is probable that these foci represent centers of telomere lengthening in cells using ALT. Thus, immunofluorescence was used to determine whether BLM and TRF2 similarly co-localize with active centers of DNA incorporation during late S and G2/M. VA13/2RA cells (ALT) were blocked in G1/S and pulse-labeled with BrdU at 10 h post-release from block at which time cells were in late S and G2/M as determined by flow cytometry (Supplementary Material). Cells were evaluated with antibodies specific for BrdU as well as for BLM or TRF2. During late S and G2/M, both BLM and TRF2 immunostaining overlap with BrdU at distinct foci (Fig. 2C). In contrast, few distinct BrdU foci were distinguishable in telomerase-positive cells (MCF7) during G2/M; co-localization with BLM or TRF2 was not observed. Our results indicate that BLM, TRF2 and PML co-localize with sites of ongoing DNA synthesis in APBs during late S and G2/M. This suggests that BLM is involved in ALT-associated telomere lengthening that occurs after completion of most DNA synthesis.

BLM and TRF2 associate in vivo

Other proteins implicated in ALT, including the RAD50/MRE11/NBS1 complex, associate with telomere proteins in vivo (24,39). Immunoprecipitations were performed to
Table 1. Telomeric associations (TAs) in BS and non-BS lymphocytes

<table>
<thead>
<tr>
<th>Source of metaphases</th>
<th>BSL-1*</th>
<th>BSL-3</th>
<th>BSL-4</th>
<th>BSL-5</th>
<th>BSL-6</th>
<th>BSL-8</th>
<th>BSL-9</th>
<th>CL-1*</th>
<th>CL-2</th>
<th>Average BSLs</th>
<th>Average CLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of TAs</td>
<td>3</td>
<td>20</td>
<td>10</td>
<td>22</td>
<td>27</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>92</td>
<td>1</td>
</tr>
<tr>
<td>Metaphases scored</td>
<td>103</td>
<td>1031</td>
<td>2941</td>
<td>6667</td>
<td>3462</td>
<td>1471</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>16174</td>
<td>1000</td>
</tr>
<tr>
<td>Frequency</td>
<td>2.91</td>
<td>1.94</td>
<td>0.34</td>
<td>0.33</td>
<td>0.78</td>
<td>0.34</td>
<td>1.00</td>
<td>0.00</td>
<td>0.20</td>
<td>0.57</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*a* Metaphases prepared from BS lymphocytes (BSL) from seven patients.

* Metaphases prepared from control lymphocytes (CL) from two non-BS donors.

* Frequencies calculated as configurations per 100 metaphases scored.

determine whether BLM and TRF2 are components of the same telomere-specific protein complex. BLM immunoprecipitations from nuclear extracts prepared from cells using ALT (Saos2 and VA13/2RA) and telomerase (MCF7 and HeLa) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis using anti-TRF2 antibodies. TRF2 co-immunoprecipitates with BLM in nuclear extracts from cells using ALT but not from telomerase-positive cells (Fig. 2D). DNase treatment does not interfere with the association of BLM with TRF2, suggesting that the interaction is independent of DNA tethering (data not shown). These data confirm an in vivo, ALT-specific association between BLM and TRF2 and suggest these proteins may be part of a larger telomere-associated protein complex that facilitates and regulates ALT.

**TRF1 and TRF2 interact with BLM in vitro**

TRF1 and TRF2 induce the formation of divergent higher order DNA structures from telomere sequences in vitro (3,7,8,40). TRF2 is postulated to protect telomere ends within T-loops, and can promote formation of looped telomeric structures in vitro (7,8). Although TRF1 cannot mediate loop formation, it can promote bending of DNA and parallel pairing of telomere sequences (3,40). In addition to TRF2, BLM is reported to co-localize in vivo with TRF1 (33,34). Therefore, we tested whether there is a direct interaction between BLM and TRF1 in vitro using enzyme linked immunosorbent assay (ELISA). (Fig. 3A). BLM protein was bound to wells of microtiter plates, and after blocking, purified recombinant TRF1 was added in increasing concentrations. Bound TRF1 was detected using an anti-TRF1 antibody, HRP-conjugated secondary and colorimetric substrate. TRF1 bound to wells coated with BLM in a dose-dependent manner; DNase treatment did not interfere with this interaction. TRF1 did not bind significantly to wells coated with bovine serum albumin (BSA), indicating that TRF1 binding is mediated by specific interaction with BLM. To confirm this data, in vitro immunoprecipitations were performed with purified recombinant BLM protein and [35S]-labeled TRF1 made by in vitro transcription translation (IVTT) (Fig. 3B). Immunoprecipitations with anti-TRF1 antibody were analyzed by western blotting with anti-BLM antibody and by autoradiography to detect [35S]-labeled TRF1. In the absence of TRF1, no BLM is immunoprecipitated with the anti-TRF1 antibody. In contrast, a significant amount of BLM is immunoprecipitated when TRF1 is present in the reaction. These results demonstrate that BLM and TRF1 interact directly.

Our in vivo experiments indicate that TRF2 and BLM associate in cells using ALT, but do not address whether this is a physical interaction. In order to test whether BLM and TRF2 interact directly, immunoprecipitations with purified recombinant BLM and TRF2 were performed (Fig. 3C). Immunoprecipitations with anti-TRF2 antibody were analyzed by western blotting with anti-BLM and anti-TRF2 antibodies. Both pellet and supernatant from each reaction were analyzed, representing bound and unbound protein, respectively. In the absence of TRF2, the majority of BLM protein remains in the supernatant. In contrast, a significant amount of BLM is immunoprecipitated when TRF2 is present in the reaction. These results demonstrate that BLM and TRF2 interact directly and confirm ELISA results previously published (35).

To identify the regions of BLM responsible for interaction with TRF1 and TRF2, three separate [35S]-labeled segments of BLM were synthesized by IVTT, representing the N-terminus, the helicase domain and the C-terminus (Fig. 3D). [35S]-Labeled TRF1 and BLM segments were incubated together, immunoprecipitated with anti-TRF1 antibody and analyzed by autoradiography (Fig. 3E). The BLM helicase segment co-immunoprecipitates with TRF1. No proteins were non-specifically immunoprecipitated with IgG or with anti-TRF1 when TRF1 was not added (data not shown). Likewise, BLM protein segments from IVTT and purified TRF2 were incubated together, immunoprecipitated with anti-TRF2 antibodies and analyzed by autoradiography (Fig. 3F). The BLM C-terminal segment and the BLM helicase segment co-immunoprecipitate with TRF2. The interaction of TRF2 with the BLM C-terminal segment appears to be more significant than its interaction with the helicase segment. No BLM segments were non-specifically immunoprecipitated with IgG or with anti-TRF2 when TRF2 was not added (data not shown). These results demonstrate direct interactions between BLM and TRF1 mediated by the BLM helicase domain, and between BLM and TRF2 mediated by the BLM C-terminus and helicase domains. Additionally, these data suggest that co-localization of BLM with TRF1 and TRF2 in APBs is due to their direct interactions.
BLM unwinds substrates that resemble native telomere conformations

Telomeric DNA isolated from cells demonstrates the existence of a 3'-overhang of the G-rich strand. To investigate a role for BLM in maintaining such a structure, we constructed a DNA duplex that resembles a native linearized telomere with a 3'-single-stranded region composed of five 5'-TTAGGG-3' repeats and a 38 bp duplexed region containing 23 bp of telomeric repeats for TRF1 and TRF2 binding (41) (Fig. 4A). BLM unwinds this substrate efficiently, reaching 60% unwinding of total substrate at the highest concentrations of BLM tested (Fig. 4B and E).

In mammalian cells, telomeric 3'-overhangs may be folded back and inserted into the duplex telomeric sequences to form ‘T-loops’ in order to protect chromosome ends from degradation and end-joining (7,8). At the site of insertion, the single-stranded overhang displaces the G-rich strand of the duplex to form a D-loop structure that is stabilized by TRF2 binding. Additionally, telomere D-loops may be present as intermediates in recombination during ALT (9). T-Loops must be resolved in order for chromosome ends to be replicated, thus helicases to unwind these structures are likely essential for proper DNA replication. To investigate the role of BLM in telomere loop metabolism, a model substrate was created in vitro that approximates a D-loop...
Our substrate was constructed by annealing partially complementary oligomers to form a duplex with bubble, then annealing a third oligomer complementary to the bubble. This substrate contains 21 bp of perfect telomeric repeats within the D-loop and imperfect telomeric repeat sequences on both duplex arms for TRF1 and TRF2 binding. DNase I footprinting analysis demonstrates that BLM protects a small area on the C-rich strand within the D-loop region near the point where the invading strand exits the duplex; this suggests that BLM binds specifically to the D-loop structure (Supplementary Material). Helicase assays demonstrate that BLM efficiently displaces the invading strand of the telomeric D-loop substrate and converts it to a ‘bubble-containing’ duplex (Fig. 4D and E). This displacement occurs in a dose-dependent manner and is nearly complete at a BLM concentration of 10 nM.

TRF1 and TRF2 oppositely regulate BLM activity on telomeric substrates

As both TRF1 and TRF2 interact directly with BLM, we tested whether these proteins affect BLM unwinding
activity on telomeric substrates. Substrates were pre-incubated with varying amounts of TRF1 or TRF2, before starting the helicase reaction with a fixed concentration of BLM. In a dose-dependent manner, TRF2 stimulated BLM unwinding of the telomeric 3'-overhang at concentrations between 6 and 60 nM (Fig. 5B and C) and the telomere D-loop at concentrations between 3 and 15 nM (Fig. 5E and F). Conversely, TRF1 inhibited BLM unwinding of the telomeric 3'-overhang at concentrations between 6 and 60 nM (Fig. 5B and C) and the telomere D-loop at concentrations between 3 and 15 nM (Fig. 5E and F). When these experiments were performed utilizing the 3'-5'-helicase UvrD, TRF1 and TRF2 had no significant effect on unwinding (Supplementary Material). Thus, TRF1 and TRF2 effects on unwinding are not universally applicable to DNA helicases.
TRF2 but not TRF1 affects BLM unwinding of a non-telomeric 3'-overhang

We next set out to determine whether the regulation of BLM unwinding activity by TRF1 and TRF2 was related to their ability to stably bind DNA-containing telomeric repeats. A non-telomeric 3'-overhang substrate with a 38 bp duplexed region was generated, which is the same length and structure as the telomeric 3'-overhang substrate (Fig. 4A), but without telomeric repeats. BLM unwinds the 3'-overhang substrate in a dose-dependent manner at concentrations between 1 and 10 nM (Fig. 6A). The non-telomeric and telomeric 3'-overhang substrates are unwound with similar efficiency, reaching near 60% unwinding of total substrate at the highest concentrations of BLM tested (Fig. 6B). Gel shift assays confirmed that TRF1 and TRF2 bind stably to the telomeric substrate, but not to non-telomeric substrate (Supplementary Material). We next tested the ability of BLM to unwind the non-telomeric 3'-overhang substrate in the presence of TRF1 and TRF2. When TRF2 is added to helicase reactions with non-telomeric substrate, TRF2 (at concentrations between 4 and 40 nM) stimulates BLM unwinding of this substrate (Fig. 6C and E). In contrast, TRF1 (at concentrations between 6 and 60 nM) has no affect on BLM unwinding of this substrate (Fig. 6D and E). These results indicate that TRF1 inhibition of BLM unwinding on telomeric substrate is due to specific binding to
telomere repeat sequences. On the other hand, TRF2 stimulation of BLM unwinding is not specific for telomeric substrates, suggesting that stable binding to DNA is not required for this stimulatory effect.

**Effects of TRF1 and TRF2 in combination on BLM helicase activity vary with relative concentrations**

Our results indicate that singly, TRF1 and TRF2 act in opposition to one another to regulate BLM helicase activity on telomere substrates. To address the mechanism coordinating BLM activity, we performed helicase assays using the telomeric 3'-overhang substrate with both TRF1 and TRF2 in varying molar ratios (Fig. 7). When equimolar concentrations of TRF1 and TRF2 (4 nM each) are pre-incubated with the substrate, TRF2 stimulates BLM unwinding. TRF2 stimulates BLM unwinding when it is present in excess of TRF1. However, when TRF1 is present in excess of TRF2, the stimulatory effect of TRF2 on BLM unwinding is inhibited. These data argue that regulation of BLM unwinding by TRF1 and TRF2 is dependent on the relative molar ratio of these proteins at the telomere.

**DISCUSSION**

BS cells are characterized by high frequencies of SCE and QRs. In addition to these well-documented chromosomal abnormalities, we show that cells lacking BLM have elevated levels of TAs. A clear, non-staining area is generally present between associated telomeres visualized by G-banding, indicating that TAs are not end-to-end fusions, but more likely represent unresolved recombination events or entanglements between telomeres in BS cells. This observation stimulated our investigation of the role of BLM in telomere maintenance, particularly in cells that utilize recombinant pathways for telomere lengthening, termed ALT.

We report that BLM and TRF2 co-localize in ALT-associated PML bodies (APBs) and can be co-immunoprecipitated from cells using ALT, but not from telomerase-positive cells. Association of BLM with TRF2 has been reported by other groups (34, 35); however, our work is the first to

**Figure 6.** Effects of TRF1 and TRF2 on BLM unwinding of non-telomeric substrate. (A) BLM unwinds the non-telomeric 3'-overhang substrate. Reactions were performed with BLM (0, 1, 2, 4, 6, 8 and 10 nM; lanes 1–7) as described in Figure 4B. Reaction with BLM, without addition of ATP is shown in lane 8. The migration of substrates are shown on the left by drawings. (B) BLM unwinding of non-telomeric 3'-overhang substrate, calculated as described in Figure 4E. Unwinding of non-telomeric 3'-overhang is represented by a solid line. Unwinding of the telomeric 3'-overhang is shown for comparison (data derived from Fig. 4B), represented by a dashed line. (C) TRF2 stimulates unwinding of non-telomeric substrate by BLM. TRF2 (0, 4, 8, 20 and 40 nM; lanes 1–5) was pre-incubated with DNA substrate followed by addition of BLM (4 nM). Reactions with TRF2 only and no protein are shown in lanes 6 and 7, respectively. (D) TRF1 has no effect on unwinding of non-telomeric substrate by BLM. TRF1 (0, 6, 12, 24 and 60 nM; lanes 1–5) was pre-incubated with DNA substrate followed by addition of BLM (4 nM). Reactions with TRF1 only and no protein are shown in lanes 6 and 7, respectively. (E) BLM unwinding in the presence of TRF1 (dotted line) and TRF2 (solid line) derived from data shown in (C) and (D), quantitated as described in Figure 4E.
demonstrate co-immunoprecipitation of endogenous BLM with TRF2 specifically from cells using ALT. Further, we have pinpointed the timing of this association during the cell cycle. Co-localization of BLM with TRF2 and PML increases to a maximum during late S and G2/M, consistent with the reported enrichment of APBs during G2/M when ALT is thought to occur (23,24). Furthermore, BLM and TRF2 co-localize with foci of DNA synthesis during late S and G2/M. These data clarify the spatial and temporal association between BLM and telomere synthesis in cells using ALT, not established in previous experiments, and suggest that BLM has a role in the ALT mechanism for telomere lengthening. In this regard, the role of BLM in ALT likely parallels that of its RecQ homolog Sgs1 during RAD50-dependent recombination-mediated telomere lengthening in *S. cerevisiae* (18–20).

The co-localization of BLM with TRF1 and TRF2, reported here and elsewhere (33,34), prompted us to examine whether these proteins interact directly, and the effects of such interactions on BLM activity. We find that BLM interacts directly with TRF2 *in vitro* in agreement with a previous report (35). Our experiments have mapped this interaction primarily to the BLM C-terminus and secondarily to the helicase domain. Interestingly, TRF2 interacts with another RecQ helicase, WRN, through a portion of the WRN helicase and RecQ C-terminal domains, protein regions conserved between BLM and WRN (35). Additionally, we report that TRF2 stimulates BLM unwinding of two telomere substrates in vitro, a 3'-overhang and a telomere D-loop structure. TRF2 also stimulates BLM unwinding of a non-telomeric substrate, suggesting that physical interaction between BLM and TRF2 primarily mediates this stimulatory effect. Importantly, TRF2 binds stably to both of our telomere substrates indicating that stable DNA binding, although not required, does not hinder the ability of TRF2 to stimulate BLM activity. This result is significant as TRF2 is bound to telomeric DNA *in vivo*. Additionally, we find that TRF2 stimulates BLM unwinding of substrates with a short duplex (21 bp; telomeric D-loop), as well as a long duplex (38 bp; 3'-overhang substrates), suggesting that TRF2 stimulates BLM enzymatic activity and processivity. A dual mechanism of stimulation by TRF2 may be required to promote BLM unwinding of long telomeric DNA tracts, such as those found in cells using ALT. These findings contrast with those of Opresko et al. (35) who reports that TRF2 stimulates BLM unwinding of short duplexes (22 bp) that do not bind TRF2, but does not stimulate BLM unwinding of long duplexes (34 bp) that bind TRF2. Our substrates were designed to approximate more closely native telomere conformations and are distinct from the forked substrates used by Opresko et al.; therefore, substrate composition and structure may be a critical factor for analyzing TRF2 stimulation of BLM activity.

This work also demonstrates a direct interaction between BLM and TRF1, mediated specifically by the BLM helicase domain. In contrast to TRF2, we find that TRF1 inhibits BLM unwinding of two types of telomeric substrates, but does not affect BLM unwinding of a non-telomeric substrate. These data suggest that TRF1 binding to telomeric DNA is necessary for its inhibition of BLM helicase activity. One possibility is that TRF1 binding to telomeric substrate makes the DNA more difficult to unwind; however, TRF1 does not inhibit unwinding of telomeric substrates by the UvrD helicase. Taken together, these results suggest that TRF1 binding to both BLM and telomeric DNA might sterically hinder the ability of BLM to unwind telomeric substrates. Although co-localization of BLM and TRF1 has been reported by others (33,34), we were unable to confirm this *in vivo* association by immunoprecipitation owing to lack of suitable TRF1 antibodies for western blotting.

Our data demonstrate that TRF2 stimulates BLM unwinding in the presence of an equimolar concentration of TRF1, and suggest that TRF2 is the dominant regulator of BLM activity. However, the stimulatory effect of TRF2 on BLM unwinding is blocked when TRF1 is present in excess. Without TRF2 stimulation, BLM may be unable to traverse and unwind efficiently long, GC-rich telomeric tracts found in cells using ALT. Therefore, regulation of BLM activity at the telomere may be dependent on the relative concentrations of TRF1 and TRF2. It remains to be determined how TRF1 and TRF2 function to coordinate BLM activity in cells using ALT. One mechanism is suggested by the recent finding that poly(ADP)-ribosylation of TRF1 by tankyrase directs ubiquitin-dependent

---

**Figure 7.** Effects of combined TRF1 and TRF2 on BLM unwinding of telomeric substrate. Helicase assays were performed using the telomeric 3’-overhang substrate. TRF1 and/or TRF2 was pre-incubated with DNA substrate followed by addition of BLM (4 nM fixed). Reaction are as follows: lane 1: BLM only; lane 2: BLM and TRF2 (4 nM); lane 3: BLM and TRF1 (4 nM); lane 4: BLM, TRF1 (4 nM) and TRF2 (4 nM); lane 5: BLM, TRF1 (4 nM) and TRF2 (8 nM); lane 6: BLM, TRF1 (4 nM) and TRF2 (12 nM); lane 7: BLM, TRF2 (4 nM) and TRF1 (8 nM); lane 8: BLM, TRF2 (4 nM) and TRF1 (12 nM); lane 9: no protein control. Percent unwinding is calculated as described in Materials and Methods; graph bars represent average percent unwinding and error bars represent standard deviation for three separate experiments.
degradation of TRF1 and telomere lengthening (42,43). Degradation of TRF1 would then relieve TRF1-mediated inhibition and allow stimulation of BLM by TRF2 during telomere lengthening when BLM activity might be required.

Although the exact function of BLM at telomeres is unknown, the biochemical attributes of BLM suggest that it functions to regulate D-loop formation following strand invasion and/or resolve recombinant or entangled telomeres in cells using ALT. BLM may additionally resolve G-quadruplex structures (29) that may occur in G-rich telomeric DNA; persistence of these structures would disrupt telomere lengthening and integrity. Importantly, our data do not exclude a function for BLM at telomeres in telomerase-positive or non-immortalized cells. In fact, the increased frequency of TAs in primary and telomerase-positive, immortalized BS cultures suggest a role for BLM in resolving recombination events at telomeres in non-ALT cells. The low frequency of such events in non-ALT cells would make the association of BLM with telomeres difficult to detect using current methodologies. Nevertheless, our findings demonstrate an association of BLM with ALT and suggest that TRF1 and TRF2 play key roles in regulating BLM activity on telomeric structures.

MATERIALS AND METHODS

Cell lines

Two immortalized and telomerase-negative human cell lines, Saos2 and WI-38-VA13/2RA, two immortalized and telomerase-positive human cell lines, MCF7 and HeLa, and one non-immortalized primary cell line of diploid human fibroblasts, WI-38, were obtained from ATCC (21). The SV40-transformed BS fibroblast cell line GM08505 was obtained from the Coriell Cell Repository. MCF7 and Saos2 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco-BRL) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), and GM08505, WI-38 and WI-38-VA13/2RA in minimal essential media (Gibco-BRL) containing 10% FBS; all cell lines were grown at 37°C and in 5% CO2. Lymphoblasts were isolated from whole blood using standard separation methods, and were maintained in Roswell Park Memorial Institute (RPMI) 1640 media containing 20% FBS.

Cytogenetics

Metaphase spreads were prepared from phytohemagglutinin-stimulated lymphoblasts according to standard procedure and were analyzed by Giemsa staining. Telomeric fluorescence in situ hybridization (FISH) was performed to analyze telomeric associations in BS cells as follows. Prior to hybridization, metaphase spreads prepared on glass slides were fixed in 4% paraformaldehyde, treated with 1 mg/ml pepsin in 20 mM glycine (pH 2) and dehydrated in a serial ethanol series (70, 90 and 100%). FISH mixture [10 mM Tris, 70% formamide, 0.5% blocking agent (Roche), 0.5 µg/ml biotin-labeled (CCCTAA), probe] was applied to the slide, heated to 80°C for 3 min and hybridized overnight in a wet chamber at room temperature. Excess hybridization mixture was removed by washing twice in 70% formamide in 10 mM Tris–HCl (pH 7.2) and Tris-buffered saline (TBS) with 0.1% Tween-20, and slides were then blocked for 30 min in PBS [phosphate-buffered saline (PBS) with 0.2% fish gelatin (Sigma) and 0.5% BSA]. Slides were incubated for 30 min at 37°C in fluorescein avidin D (Vector Laboratories) diluted 1:100 in PBS followed by three washes at 45°C in PBS with 0.1% Tween-20. Slides were next incubated for 30 min at 37°C with FITC-conjugated anti-avidin antibody (Vector Laboratories) diluted 1:100 in PBS followed by three washes at 45°C in PBS with 0.1% Tween-20. DAPI was added to final wash to stain DNA. Slides were mounted in Fluoromount and coverslips were sealed with nail polish. Subtelomeric FISH was performed as indicated by manufacturer (Cytocell). Images were captured on a Hamamatsu digital camera using QED imaging software.

Cell cycle synchronization, BrdU pulse-labeling and flow cytometry

Cells were blocked at G1/S by aphidicolin treatment for 16–18 h (Sigma, cat. A0781) (1 µg/ml). Cells were released by removal of the aphidicolin-containing medium and fixed at indicated time points post-release for either immunofluorescence (1:1 methanol–acetone fixative) or flow cytometry (70% ethanol). BrdU pulse-labeling was performed as reported by Wu et al. (24). Cell cycle analyses were performed on propidium iodide-labeled cells using a Coulter Epics XL flow cytometer.

Immunostaining

The rabbit anti-BLM antibody was generated, purified and used for immunofluorescence as described by Yankiwi et al. (33). An anti-PML monoclonal antibody (Santa Cruz, cat. PG-M3), an anti-TRF2 monoclonal antibody (Oncogene, cat. OP129) and a rat monoclonal anti-BrdU antibody (Accurate Scientific, cat. OBT0030) were used for immunofluorescence. Secondary antibodies included FITC-conjugated donkey anti-rabbit, FITC-conjugated goat anti-mouse, rhodamine-conjugated goat anti-mouse and rhodamine-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories). Fluorescence microscopy was performed using an Axiosoplan 2 Zeiss microscope. Images were captured on a Hamamatsu digital camera using QED imaging software.

In vivo protein co-immunoprecipitations

In vivo immunoprecipitations from nuclear extracts using goat anti-BLM antibodies (Santa Cruz, cat. sc-7789 and 7790) were performed as previously described (44). Monoclonal anti-TRF2 antibodies (Oncogene, cat. OP129) were used for western blot analysis. Nuclear extracts were treated with DNase I (10 µg/ml) prior to immunoprecipitation. 50–100 µg of nuclear extract was loaded for input lanes on each gel. Immunoprecipitations were confirmed by re-probing western blots with anti-MLH1 antibodies (PharMingen, cat. 13271A) and anti-BLM antibodies (Novus, cat. NB 100-161).
**Protein expression and purification**

pYES-BLM expression vector (pJK1) was provided by I. Hickson (University of Oxford, Oxford, UK) (45). For expression and purification, pJK1 was transfected into the *S. cerevisiae* strain JEL1 provided by J. Wang (Harvard University, Cambridge, MA, USA) (46). Yeast were inoculated into −Ura DO+ minimal base containing 2% glucose (Clontech, cat. 8607-1, 8602-1) and grown to an OD$_{600}$ of 0.6 in an orbital shaker at 30°C. Yeast were pelleted and resuspended in an equal volume of −Ura DO+ minimal base containing 2% galactose (Clontech, cat. 8607-1, 8611-1) and grown for an additional 20–24 h. Yeast were resuspended in cold Buffer A (50 mM KPO$_4$ pH 7.0, 500 mM KCl, 10% glycerol and 1:500 mammalian protease inhibitors (Sigma, cat. P-834) at a volume of 35 ml/l of culture and lysed using a French press cell. Yeast lysates were bound to a charged NiNTA resin (Novagen, cat. 69670) in 1× binding buffer (Buffer A + 50 mM imidazole) at 4°C for at least 2 h. Resin was applied to the column and washed with 1× binding buffer at least five times. BLM was eluted with 500 mM imidazole and dialyzed into Buffer Z [60 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol] for 4 h at 4°C. Small aliquots were frozen and stored at −80°C. Purity was confirmed by Coomassie and silver stain (Supplementary Material). UvrD helicase was provided by S. Matson (University of North Carolina, Chapel Hill, NC, USA).

High titer baculoviral stocks containing constructs for H-tagged TRF1 and TRF2 were provided by T. de Lange (The Rockefeller University). Insect cells (Sf9 strain) were infected with TRF2 baculovirus (multiplicity of infection of 5–10) and harvested after 48–72 h. After lysis by sonication in buffer containing 2% galactose (Clontech, cat. 8607-1, 8611-1) and grown for an additional 20–24 h. Yeast were resuspended in cold Buffer A (50 mM KPO$_4$ pH 7.0, 500 mM KCl, 10% glycerol and 1:500 mammalian protease inhibitors (Sigma, cat. P-834) at a volume of 35 ml/l of culture and lysed using a French press cell. Yeast lysates were bound to a charged NiNTA resin (Novagen, cat. 69670) in 1× binding buffer (Buffer A + 50 mM imidazole) at 4°C for at least 2 h. Resin was applied to the column and washed with 1× binding buffer at least five times. BLM was eluted with 500 mM imidazole and dialyzed into Buffer Z [60 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol] for 4 h at 4°C. Small aliquots were frozen and stored at −80°C. Purity was confirmed by Coomassie and silver stain (Supplementary Material). UvrD helicase was provided by S. Matson (University of North Carolina, Chapel Hill, NC, USA).

**Expression constructs, in vitro transcription and translation (IVTT), and interaction site mapping by in vitro immunoprecipitation**

IVTT was performed according to manufacturer instructions (Promega) with [35S]-methionine (Amersham). BLM–pET in vitro immunoprecipitation translation (IVTT), and interaction site mapping by in vitro transcription and translation (PCR) with a 5′ primer with a T7 promoter site. IVTT-generated proteins were verified for size by western analysis. In vitro immunoprecipitations with [35S]-methionine-labeled BLM segments were performed as previously described (44) using goat anti-TRF1 antibody (Santa Cruz, cat. sc-6165) and mouse anti-TRF2 antibody (Oncogene, cat. OP129).

Normal goat IgG and normal mouse IgG were used as negative controls in the respective experiments.

**In vitro immunoprecipitation of full-length proteins**

Purified BLM (500 ng) and/or TRF2 (450 ng) were incubated in binding buffer (40 mM Tris pH 8.0, 4 mM MgCl$_2$, 0.1 mg/ml BSA, 5 mM DTT and 0.1% Nonidet-P40) for 1 h at 4°C, followed by an additional 1 h incubation with 2 µg of anti-TRF2 mouse monoclonal antibody (Oncogene, cat. OP129). Equilibrated protein A–sepharose suspension was added to each sample, followed by incubation at 4°C for 1 h. Protein A–sepharose beads were collected by centrifugation and the supernatants removed for SDS–PAGE. After the pellet was washed three times with binding buffer, bound proteins were eluted by boiling the beads in binding buffer plus 2% SDS, 10% glycerol, 0.1% bromophenol blue (0.1%), and 350 mM β-mercaptoethanol for 5 min. Proteins in the supernatant and pellet were separated by SDS–PAGE and detected by western blotting using rabbit anti-TRF2 (Santa Cruz, cat. Sc-9143), rabbit anti-BLM (Novus Biologicals, cat. NB100-161) and donkey anti-rabbit-HRP conjugated antibodies followed by ECL detection (Amersham Pharmacia). In vitro immunoprecipitation with goat anti-TRF1 antibody (Santa Cruz, cat. sc-6165) was performed as mentioned earlier, utilizing purified BLM (500 ng) and [35S]-labeled TRF1 made by IVTT (20 µl of a 50 µl IVTT reaction). BLM was detected by western blotting as described earlier. The portion of the SDS–PAGE gel containing the [35S]-labeled TRF1 was dried and analyzed by autoradiography.

**ELISA assay for detecting TRF1–BLM interaction**

This assay was performed using standard techniques (35,47) with the following modifications. Briefly, purified recombinant BLM was diluted to 2.5 ng/µl in carbonate binding-buffer (0.016 mM Na$_2$CO$_3$, 0.034 mM NaHCO$_3$ pH 9.6). An aliquot of 50 µl (125 ng) was added to individual wells of a 96-well microtiter plate and allowed to bind overnight at 4°C. In the same manner, BSA was bound to wells as a negative control, and TRF1 (0.25 ng/µl) was bound as a positive control. Plates were washed three times with PBST (PBS with 0.5% Tween-20) and blocked in blocking buffer (PBS with 0.5% Tween-20 and 3% BSA) for 2 h at 30°C. Plates were washed once more in blocking buffer prior to addition of TRF1 (varying concentrations 0.25 to 1.5 ng/µl) diluted in binding buffer (50 mM Tris–HCl pH 7.4, 5 mM MgCl$_2$, 5 mM ATP, 100 µg/ml BSA, 50 NaCl). TRF1 was incubated in plates for 1 h at 30°C. Where indicated, 10 U DNase I (Roche, cat. 766 785) were added to wells with TRF1. Plates were vigorously washed with wash buffer five times to remove unbound TRF1 from wells. TRF1 (bound fraction) was detected using anti-TRF1 antibody (Santa Cruz, cat. sc-6165) which was diluted 1:1000 in blocking buffer and incubated at room temperature for 1 h. Unbound primary antibody was removed by three washes with wash buffer. Horseradish peroxidase-conjugated rabbit anti-goat antibody (Calbiochem, cat. 4015014) diluted 1:10 000 was added to each well and incubated at room temperature for 1 h. Unbound secondary antibody was removed by three washes with wash buffer. Excess liquid was removed.
completely by tapping plates upside down before addition of 50 μL TACS-sapphire detection reagent (Trevigen). After incubation for 10 min in the dark, reactions were stopped by equal volume of 0.2 N HCl. Absorbance was read at 490 nm and all values were corrected based on background found in reactions containing no primary and secondary antibody.

**DNA substrates**

Telomeric D-loop substrate was prepared by a two-step method as described previously (48). Oligonucleotides were purchased from Integrated DNA Technologies (Corvalle, IA, USA) and Operon (Alameda, CA, USA). Oligonucleotides (sequences in 5′–3′ orientation) used to generate the D-loop-like substrate were the partially complementary G80BUB21 (AGCTCTCCTTTCCCTCTTCCTGATACGGCTGCTTC) and C80 (GCTGATCAACCCTCACTAGTGAAGCTTGTAGGTAACCCCTAACCTAACCCTAAGGACCAACTAAGCTTCACTAGGTAGGGTTGATCAGC) AGGGTTACAAGCTTCACTAGGGTTGTCCAGTCACAGTC

**Helicase assays**

DNA substrate (3.5 fmol) was incubated with BLM in helicase buffer (final 1× buffer concentration of 20 mM Tris–HCl pH 7.5, 2 mM MgCl2, 0.1 mg/ml BSA and 1 mM DTT) in 50 μL volume for 15 min at 37°C in the presence of ATP (2 mM). In reactions with both BLM and TRF1 or TRF2, substrate was first incubated either with TRF1 or TRF2 for 5 min on ice followed by addition of BLM and an additional incubation at 37°C for 15 min in 50 μL of helicase buffer. Reactions were stopped with one-sixth volume of helicase stop dye (30% glycerol, 50 mM EDTA, 0.9% SDS, 0.25% bromophenol blue and 0.25% xylene cyanol). The DNA products were separated on non-denaturing polyacrylamide (8 or 12%) gels and stained with ethidium bromide. The DNA products were separated on non-denaturing polyacrylamide (8 or 12%) gels and stained with ethidium bromide.

**REFERENCES**


**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**ACKNOWLEDGEMENTS**

We thank Drs Titia de Lange, Ian Hickson, Eric Knudsen, Richard Kolodner, Andrew Lowy, Yolanda Sanchez and James Wang for experimental reagents, and Dr George Babcock and Sandy Schwamberger for help in flow cytometry experiments. We also thank Jonathon Henning and Alison Best for laboratory assistance, and Drs Kathy Heppner Goss and Carolyn Price for insightful discussion during manuscript preparation. J.G. is an Investigator with the Howard Hughes Medical Institute. This work was supported by the National Institutes of Health (G.L.) CA59268-06A1, the Center for Environmental Genetics (J.G.) ES06096, the Ellison Medical Foundation (D.O.) NS-008900 and the Albert J. Ryan Foundation (K.L.-W.).


