BLM, the protein mutated in Bloom’s syndrome, possesses a helicase activity that can dissociate DNA structures, including the Holliday junction, expected to arise during homologous recombination. BLM is stably associated with topoisomerase IIIα (Topo IIIα) and the BLAP75 protein. The BLM-Topo IIIα-BLAP75 (BTB) complex can efficiently resolve a DNA substrate that harbors two Holliday junctions (the double Holliday junction) in a non-crossover manner. Here we show that the Holliday junction unwinding activity of BLM is greatly enhanced as a result of its association with Topo IIIα and BLAP75. Enhancement of this BLM activity requires both Topo IIIα and BLAP75. Importantly, Topo IIIα cannot be substituted by *Escherichia coli* Top3, and the Holliday junction unwinding activity of BLM-related helicases WRN and RecQ is likewise impervious to Topo IIIα and BLAP75. However, the topoisomerase activity of Topo IIIα is dispensable for the enhancement of the DNA unwinding reaction. We have also ascertained the requirement for the BLM ATPase activity in double Holliday junction dissolution and DNA unwinding by constructing, purifying, and characterizing specific mutant variants that lack this activity. These results provide valuable information concerning how the functional integrity of the BTB complex is governed by specific protein-protein interactions among the components of this complex and the enzymatic activities of BLM and Topo IIIα.

Homologous recombination (HR) is important for several nuclear processes, including the repair of damaged DNA, rescue of stalled DNA replication forks, and pairing of homologous chromosomes during meiosis. HR can produce recombinant chromosomes that harbor a crossover of the chromosomal arms. Although these crossovers are critical for the proper segregation of homologous chromosomes in meiosis I, inadvertent crossover formation in mitotic cells can lead to chromosome translocation and loss of heterozygosity, which are potentially oncogenic. For this reason, eukaryotes have developed specific mechanisms for suppressing the formation of DNA crossovers in mitotic cells.

Several members of the RecQ helicase family have been implicated in mitotic crossover suppression (reviewed in 10–12). BLM, the protein mutated in the autosomal recessive cancer-predisposing disorder Bloom’s syndrome (BS), is one of the five members of the RecQ helicase family in humans. Cells from the patients with BS display a high degree of genomic instability and a dramatic increase in the frequency of sister chromatid exchanges mediated by HR (16, 17). BLM is thought to prevent crossover formation by (i) promoting the synthesis-dependent single strand annealing pathway of HR by dissociating the D-loop structure, an intermediate formed early in the HR reaction, and (ii) acting in conjunction with additional protein factors to dissolve the double-Holliday junction (DHJ), a late HR intermediate, to generate solely non-crossover recombinants (18–20). BLM has also been proposed to function in the rescue of stalled replication forks, perhaps by promoting fork regression to form a Holliday junction (HJ) intermediate (21). The regressed fork can then be dissociated by branch migration of the HJ to allow bypass of the blocking lesion through template switching or be utilized as a substrate for HR to mediate replication restart (22). In its DHJ dissolution role, BLM acts specifically with the type IA topoisomerase Topo IIIα (20). The BLM-Topo IIIα pair is tightly associated with a third protein called BLAP75 (23). Attenuation of BLAP75 levels by RNA interference destabilizes both BLM and Topo IIIα (24). Biochemical analyses have revealed specific and direct interactions of BLAP75 with BLM and Topo IIIα and a strong enhancement of the BLM-Topo IIIα-mediated DHJ dissolution reaction by this novel protein (25, 26).

Parallel studies in other eukaryotes have led to the notion that the mechanism of BLM-mediated crossover suppression is evolutionarily conserved (11, 27). In particular, the absence of Sgs1, the sole RecQ-like helicase in *Saccharomyces cerevisiae*, increases crossover formation in double-strand break-induced HR (9) and causes HR-dependent aberrant DNA structures to accumulate during replication in response to DNA damage (28). SGS1 was initially identified as a suppressor of the slow growth phenotype of strains mutated for the *TOP3* gene, the orthologue of human Topo IIIα (29). Mutations in *TOP3* phenotype the sgs1 mutant in both increased crossover formation.
and accumulation of recombination-dependent aberrant DNA structures (9, 28), reinforcing the idea that Sgs1 and Top3 function together to resolve HR intermediates in a non-crossover manner. Furthermore, Rml1, the putative yeast ortholog of BLAP75 (30, 31), co-immunoprecipitates with the Sgs1-Top3 complex, and deletion of either Top3 or Rml1 destabilizes the heteromeric complex (31). Rml1 mutants exhibit hyper-recombination and increased gross chromosomal rearrangements (30, 31).

The mechanistic details that underlie the action of the BTB complex and its equivalent in other organisms remain poorly characterized. Here we demonstrate that BLAP75 in conjunction with Topo IIIα greatly stimulates the HJ unwinding activity of BLM. This functional interaction is highly specific, as the BLAP75-Topo IIIα pair has no effect on either WRN (a RecQ helicase) or *Escherichia coli* RecQ helicase activity, nor can *E. coli* Top3 substitute for Topo IIIα in the enhancement of the BLM helicase activity. We provide evidence that the stimulation of the BLM helicase activity is independent of the catalytic activity of Topo IIIα. Lastly, we address the role of ATP hydrolysis by BLM in the BTB complex functions by mutating the conserved lysine residue in its Walker A motif needed for ATP binding and hydrolysis. The BLM K695A and K695R mutant proteins are both devoid of ATPase and helicase activities and the ability to catalyze DHJ dissolution within the context of the BTB complex.

**Experimental Procedures**

**Protein Expression and Purification**—See the supplemental data for details.

**DNA Substrates**—The φX174 viral (+) strand and replicative form I DNAs were purchased from Invitrogen. The oligonucleotides used for this study were synthesized by Integrated DNA Technologies and are listed in Table 1. All the oligonucleotides were purified from 12% denaturing polyacrylamide gels containing 8 M urea prior to use. Oligos P1 and P2 were annealed by passage through a Biospin-6 column (Bio-Rad). Annealing reactions were carried out by heating equimolar amounts of the indicated oligonucleotides at 95 °C for 10 min in annealing buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT) followed by slow cooling to room temperature. Subsequent steps were carried out at 4 °C to prevent dissociation of the annealed DNA strands. The DNA substrates were separated from the unhybridized oligonucleotides in 12% polyacrylamide gels run in TAE buffer (30 mM Tris acetate, pH 7.4, 0.5 mM EDTA). The relevant portions of the gel were excised and the annealed DNA substrates eluted from the gel slices by electro-elution (30 mA overnight) into TAE buffer in dialysis tubing. The substrates were concentrated in a YM-30 Centricon device and filter dialyzed into TE buffer (10 mM Tris, pH 8, 1 mM EDTA). Radiolabeled oligo B1 and unlabeled oligo R1 were annealed and ligated to create a DHJ substrate as described (20, 26, 34).

**Co-immunoprecipitation**—Purified BLM, BLM K695A, or BLM K695R and Topo IIIα or Topo IIIα Y337F, 3 μg each, were incubated in 30 μl of buffer K (20 mM K₂HPO₄, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, and 1 mM DTT) containing 150 mM KCl at 4 °C for 10 min. Rabbit polyclonal anti-BLM antibody (0.4 μl of a 1:5 dilution of ab476; Abcam Ltd.) was added, followed by a 30-min incubation at 4 °C. The reactions were incubated for 30 min at 4 °C with 10 μl of Protein-G coupled magnetic beads (Dynal Biotech) to capture BLM and associated Topo IIIα. The beads were isolated using a magnet, washed twice with 30 μl of buffer K containing 150 mM KCl, and the bound proteins were eluted with 30 μl of SDS-PAGE sample loading buffer. The supernatant (S) that contained unbound proteins, the wash (W), and the SDS eluate (E), 10 μl each, was subjected to 7.5% SDS-PAGE, and the proteins were stained with Coomassie Blue.

**GST-pulldown Assay**—For GST-pulldown experiments, GST-tagged BLAP75 (3 μg) was incubated with purified BLM, BLM K695A, BLM K695R, Topo IIIα, or Topo IIIα Y337F (3 μg each) in 30 μl of buffer K containing 200 mM KCl at 4 °C for 10 min and then mixed with 5 μl of glutathione-Sepharose beads (GE Healthcare) for an additional 30 min at 4 °C. After washing the beads twice with 30 μl of buffer K containing 200 mM KCl, the bound proteins were eluted with 30 μl of SDS-PAGE sample loading buffer. The supernatant (S), wash (W), and SDS eluate (E), 10 μl each, were resolved by 10% SDS-PAGE, and proteins were stained with Coomassie Blue. His₆-tagged Topo IIIα or Topo IIIα Y337F was detected by Western blotting using horseradish peroxidase-conjugated anti-histidine antibodies (Sigma).

**ATPase assay**—In Fig. 3A, combinations of BLM (10 nm), BLAP75 (50 nm), and Topo IIIα (120 nm) were incubated at 37 °C with φX174 viral (+) strand DNA (70 μM nucleotides), 1 mM ATP, and 1 μCi [γ-³²P]ATP in 10 μl of buffer R (50 mM Tris-HCl, pH 7.8, 250 μg/ml bovine serum albumin, 25 mM KCl, 4 mM MgCl₂, and 1 mM DTT). In Fig. 7C, ATP hydrolysis by BLM, BLM K695A, or BLM K695R protein (80 nm each) was

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**Table 1**

| Oligo name | DNA sequence (5’ to 3’) |
|-----------|------------------------|
| P1        | GAAGCTCGGGCAATTGCTGTTAGAGATATCGGATTGCCCTACATTGACAGCGTTTA |
| P2        | ACGAATCTTAGGGCGAATTCCATCCAGCAGAGACTCACAGTCGTTAG |
| 2         | TGCTGACGCCGCTACTAGTACAGTGACCCGTAATGGCTAGCCTG |
| 5         | TCGGAATTCTACAGTGGTACAGTACACTCGGTACAGTGAGATCC |
| 6         | GTCGAACCTCCTAGAGCAGCTCCATGATCACTGGAGTACCTGGTAATTCG |
| 7         | CAGGCTCACTAGAGCTCCACTGTTAGTACAGTGACCCGTAATGGCTAGCCTG |
| B1        | GTATTATCGATTACGGGTATCTCGATGATCGTCGGATCCTCTAGACAGCTCCATGATCACTGGAGTACCTGGTAATTCG |
| R1        | CTTATGCGATTTGCGGTTTTGCACGAAATCTCTGATTACATGTCGTAATGGCTAGCCTG |

**BTB Complex Characterization**

The mechanistic details that underlie the action of the BTB complex and its equivalent in other organisms remain poorly characterized. Here we demonstrate that BLAP75 in conjunction with Topo IIIα greatly stimulates the HJ unwinding activity of BLM. This functional interaction is highly specific, as the BLAP75-Topo IIIα pair has no effect on either WRN (a RecQ helicase) or *Escherichia coli* RecQ helicase activity, nor can *E. coli* Top3 substitute for Topo IIIα in the enhancement of the BLM helicase activity. We provide evidence that the stimulation of the BLM helicase activity is independent of the catalytic activity of Topo IIIα. Lastly, we address the role of ATP hydrolysis by BLM in the BTB complex functions by mutating the conserved lysine residue in its Walker A motif needed for ATP binding and hydrolysis. The BLM K695A and K695R mutant proteins are both devoid of ATPase and helicase activities and the ability to catalyze DHJ dissolution within the context of the BTB complex.
examined in 10 μl of buffer R containing φX174 viral (+) strand DNA (70 μM nucleotides), 2 mM ATP, and 1 μCi [γ-32P]ATP. At the indicated times, a 2.5 μl aliquot of the reaction was removed and mixed with an equal volume of 1% SDS. The level of ATP hydrolysis was measured by thin layer chromatography in polyethyleneimine cellulose sheets (35) coupled with phosphorimaging analysis (Quantity One software from Bio-Rad).

**DNA Binding**—BLM, BLM K695A, and BLM K695R (amount as indicated) were incubated at 37 °C with the radiolabeled forked substrate (0.5 nm) in 10 μl of reaction buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM ATP, 0.8 mM MgCl2, 200 μg/ml bovine serum albumin, and an ATP regenerating system consisting of 20 mM creatine phosphate and 20 μg/ml creatine kinase) containing either 100 mM or the indicated concentration, and analyzed as above.

**HJ Unwinding**—Combinations of BLM (10 nm), Topo IIIα (120 nm), and BLAP75 (50 nm or amount as indicated) were incubated on ice for 10 minutes in 9.5 μl of buffer H (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM ATP, 0.8 mM MgCl2, 200 μg/ml bovine serum albumin, and an ATP regenerating system consisting of 20 mM creatine phosphate and 20 μg/ml creatine kinase) containing either 100 mM or the indicated concentration of KCl. Following the addition of the radiolabeled HJ substrate (0.5 nm) in 0.5 μl of TE buffer, the reactions were incubated for an additional 10 min (or the indicated times) at 37 °C. After treatment with SDS and proteinase K as above, the deproteinized reaction mixtures were resolved in 10% native polyacrylamide gels in TAE buffer. Where indicated, reactions were deproteinized by treatment with SDS and proteinase K (0.2% and 0.5 mg/ml, respectively) at 37 °C for 3 min prior to gel analysis. The gels were dried onto Whatman DE81 paper and subjected to analysis in a Bio-Rad Personal FX phosphorimager.

**DNA Fork Unwinding**—BLM, BLM K695A, or BLM K695R (amount as indicated) protein was incubated at 37 °C with the radiolabeled forked DNA (0.5 nm) in 10 μl of buffer H containing 40 mM KCl. The reaction was stopped after 30 min, deproteinized, and analyzed as above.

**DHJ Dissolution Assay**—BLM, BLM K695A, or BLM K695R (10 nm) was incubated with Topo IIIα or Topo IIIα Y337F (120 nm) and BLAP75 (50 nm) for 10 min on ice in 11.5 μl of reaction buffer (50 mM Tris-HCl, pH 7.8, 2 mM ATP, 0.8 mM MgCl2, 80 mM KCl, 1 mM DTT, 100 μg/ml bovine serum albumin, and the ATP-regenerating system described above) followed by the addition of the DHJ substrate (1.2 nm) in 1 μl of TE buffer. After a 5-min incubation at 37 °C, the reaction mixtures were deproteinized as above, mixed with 10 μl of sample loading buffer (20 mM Tris-HCl, pH 7.5, 50% glycerol, and 0.08% Orange G) containing 50% urea, incubated at 95 °C for 3 min, and resolved in an 8% polyacrylamide gel containing 20% formamide and 8% urea in TAE buffer at 55 °C. In Fig. 9A, ATP was omitted or substituted with ATPγS, AMP-PNP, or ADP (2 mM each) as indicated. The ATP regeneration system was omitted from the reactions that used ADP or the non-hydrolyzable ATP analogues. In Fig. 9B, WRN (15 nm) or RECQ5 (15 nm) was included where indicated.

**RESULTS**

**Expression and Purification of the BTB Components**—C-terminally His6-tagged BLM was expressed using the yeast galactose-inducible BLM expression system of Karow et al. (36) (Fig. 1A). N-terminally His6-tagged Topo IIIα was expressed in E. coli Rosetta (DE3) pLysS cells (Novagen) from an isopropyl β-D-thiogalactopyranoside-inducible T7 promoter. BLM and Topo IIIα were purified to >95% homogeneity by multi-step purification schemes that we developed (Fig. 1, B–E). The purified Topo IIIα is catalytically active as evidenced by its ability to relax negatively supercoiled φX174 DNA (data not shown) and to function in the DHJ dissolution reaction (26; see below).

BLAP75 was expressed and purified from E. coli using our published procedure (26). As controls in the biochemical analyses, we obtained the E. coli RecQ and Top3 proteins from the Kowalczykowski group (University of California) and the RECQ5 protein from the Janscak group (IMCR, University of Zurich).

**Effects of BLAP75 and Topo IIIα on BLM-mediated ATP Hydrolysis and HJ Unwinding**—We tested the effect of BLAP75 and Topo IIIα on BLM-mediated unwinding of a 32P-labeled synthetic HJ. Although neither BLAP75 nor Topo IIIα alone had a significant effect on the extent of HJ unwinding (Fig. 2A, lanes 3 and 4), pre-incubation of all three proteins led to a marked enhancement of the reaction (lanes 5–7). Time course experiments showed dissociation of ~50% and >80% of the HJ substrate by the BTB complex in 2 and 10 min, respectively (Fig. 2B). In comparison, BLM unwound <2 and 14% of the HJ substrate within the same timeframes, respectively (Fig. 2B).
Unwinding of the HJ substrate required ATP hydrolysis, as the products were not seen when ATP was omitted or when ATP was replaced by ADP, AMP-PNP, or ATPγS (data not shown). In agreement with this, BLM variants harboring mutations in the conserved lysine residue of the Walker A motif that ablate ATPase activity are also devoid of HJ unwinding activity (see below). As expected, neither BLAP75 nor Topo IIIα alone or in combination could dissociate the HJ substrate in the absence of BLM (Fig. 2A, lane 8 and data not shown). We examined whether Topo IIIα and BLAP75 increased the rate of ATP hydrolysis by BLM, but saw no stimulation by these proteins when they were used either alone or in combination (Fig. 3A).

The above HJ unwinding experiments were performed in the presence of 100 mM KCl. We previously showed that under more physiological salt conditions, the DHJ dissolution activity of BLM and Topo IIIα becomes almost completely dependent on the presence of BLAP75 (26). In concordance with this, elevating the salt concentration to 140 mM reduced HJ unwinding by BLM, whereas the level of unwinding catalyzed by the BTB complex was not affected significantly (Fig. 3B).

Specificity of Enhancement of BLM Activities by Topo IIIα and BLAP75—To address the specificity of the functional interactions documented in the last section, we substituted BLM with two other members of the RecQ helicase family, namely, the human WRN and _E. coli_ RecQ helicases. Both WRN and RecQ unwind the HJ structure (37, 38), but do not interact with BLAP75 in vitro (26) or catalyze DHJ dissolution with Topo IIIα, either in the presence or absence of BLAP75 (26, 39). As shown in Fig. 4A, under conditions wherein a strong enhancement of the BLM HJ unwinding activity by BLAP75 and Topo IIIα was seen, the addition of the latter two proteins had no effect on HJ unwinding by either WRN or RecQ.

Previously published studies have shown that the _E. coli_ Top3 protein, which is also a type IA topoisomerase, can function with BLM in DHJ dissolution (25, 39). We asked whether Top3 protein could co-operate with BLAP75 to enhance the
DNA unwinding activity of BLM. As shown in Fig. 4B, HJ unwinding by BLM was refractory to Top3, regardless of whether BLAP75 was present or not. Affinity pull-down assays provide evidence that Top3 does not interact with either BLM or BLAP75 (data not shown). Taken together, the results indicate that the enhancement of the BLM activities by Topo IIIα and BLAP75 occurs in a highly specific fashion, likely reflective of the specific protein-protein interactions that occur within the context of the BTB complex.

The DNA Relaxation Activity of Topo IIIα Is Dispensable for Stimulation of HJ Unwinding—To determine the role of the DNA relaxation activity of Topo IIIα in BTB complex functions, we used site-directed mutagenesis to generate a catalytically dead Topo IIIα mutant by changing the active site tyrosine residue (amino acid 337) to phenylalanine (40). The Topo IIIα Y337F mutant could be expressed to the same level as the wild type protein and was purified to near homogeneity using the procedure that we devised for the wild type protein (Fig. 5A).

A prolonged incubation of the Y337F mutant Topo IIIα protein with φX174 RF1 DNA did not result in any topological change in the DNA, confirming that the mutant protein was devoid of DNA relaxation activity (data not shown). In accordance with previously published data (20), the catalytically inactive Topo IIIα Y337F mutant is incapable of catalyzing DHJ dissolution with BLM, with or without BLAP75 (Fig. 5D). The lack of dissolution activity in the BTB complex that harbors Topo IIIα Y337F mutant protein is not because of a defect in complex assembly, as Topo IIIα Y337F retains the ability to bind both BLM and BLAP75 (Fig. 5, B and C) and stimulates DNA unwinding by BLM in conjunction with BLAP75 (see below).

The results presented earlier demonstrate that BLAP75 and Topo IIIα together enhance the HJ unwinding activity of BLM. Because dissociation of the synthetic HJ substrate is not expected to be dependent on a DNA relaxation activity, we
predicted that the Topo IIIα Y337F protein would retain the ability to up-regulate the BLM HJ unwinding activity in conjunction with BLAP75. As shown in Fig. 6, Topo IIIα Y337F is just as proficient as its wild type counterpart in enhancing the dissociation of the HJ structure with a dependence on BLAP75.

Expression and Purification of BLM K695A and K695R Mutants—For addressing the role of ATP binding and hydrolysis in BLM and BTB complex functions, we substituted the conserved lysine residue (lysine 695) in the Walker type A motif with either alanine or arginine using site-directed mutagenesis (Fig. 7A). Based on previous studies done with the equivalent Walker mutants of other ATPases (41, 42), both the BLM K695A and K695R mutants are expected to be defective in ATP hydrolysis. The use of these BLM mutants would therefore allow us to ascertain the role of ATP hydrolysis in reactions that are catalyzed by BLM and the BTB complex. The two mutant BLM proteins can be expressed at a level similar to that of wild type BLM in yeast cells (data not shown) and were purified to near homogeneity using the chromatographic procedure devised for the wild type protein (Fig. 7B).

Role of ATP Binding and Hydrolysis by BLM in DHJ Dissolution—BLM possesses a robust single-stranded DNA-dependent ATPase activity ($k_{cat} = 1500$ min$^{-1}$; 36), whereas neither of the two BLM mutant proteins shows more than 1% of the wild type level of ATP hydrolysis (Fig. 7C). Importantly, both BLM mutant variants retain the ability to associate with Topo IIIα and BLAP75 (Fig. 7D and E) and appear to be just as proficient as wild type BLM in DNA binding (Fig. 8A). The BLM mutants are, as expected, defective in DNA unwinding, as assayed with a forked helicase substrate (Fig. 8B) and also the HJ substrate (C).

A previously published study has shown that ATP is needed for the BLM-Topo IIIα pair to dissolve the DHJ, and that ATP cannot be substituted by the non-hydrolyzable analogue AMP-PNP (20). We verified that DHJ dissolution by the BTB complex likewise requires ATP, as little or no product was seen upon the omission of ATP (Fig. 9A, lane 9). Furthermore, neither ADP nor AMP-PNP or ATPγS was effective in the DHJ dissolution reaction (Fig. 9A). To eliminate the possibility that the ATP hydrolysis dependence of DHJ dissolution might stem from a requirement for ATP by either Topo IIIα or BLAP75, we performed DHJ dissolution experiments with ATP but using mutant variants of the BTB complex that harbored either the BLM K695A or BLM K695R protein. The results revealed that neither of the mutant BTB variants was effective in mediating significant dissolution of the DHJ substrate (Fig. 9B). Taken together, the results indicate that ATP hydrolysis by BLM is indispensable for DHJ dissolution by the BTB complex.

In the BTB-mediated DHJ dissolution reaction, it is thought that the BLM helicase activity migrates the double Holliday structure inward to yield a hemicatenane structure, which is then resolved by Topo IIIα via DNA strand passage (14, 22, 27). We considered the possibility that a BTB complex that harbors a helicase-dead BLM variant might nonetheless be efficacious in DHJ dissolution if another RecQ helicase is present to process the DNA substrate into a hemicatenane structure. Because WRN appears to interact with BLM and can efficiently unwind the Holliday structure (37, 43), we asked whether it could promote DHJ dissolution with mutant BTB complexes assembled.
with either BLM K695A or BLM K695R. WRN does not affect the DHJ dissolution activity of the wild type BTB complex (data not shown), nor does it function with either of the mutant BTB variants in this regard (Fig. 9B). The human RECQ5 helicase, which is also capable of unwinding the Holliday structure (44; data not shown), is likewise unable to confer DHJ unwinding activity to either of the mutant BTB complexes (Fig. 9B) or influence the activity of the wild type BTB complex (data not shown).

**DISCUSSION**

Elegant studies by Wu and Hickson (20) have shown a unique ability of the BLM-Topo IIIα/H9251 pair to mediate the dissolution of the DHJ to yield non-crossover recombinants, an activity that likely accounts for the role of this protein complex in the suppression of crossover formation and chromosome rearrangements during HR (12, 20, 27). The BLAP75 protein associates with BLM-Topo IIIα, and attenuation of the BLAP75 level by RNA interference destabilizes both BLM and Topo IIIα (23, 24). Biochemical analyses have verified that BLAP75 directly interacts with both BLM and Topo IIIα. Importantly, BLAP75 exerts a dramatic enhancement of the BLM-Topo IIIα-mediated DHJ dissolution reaction (25, 26). This finding has provided the first evidence that BLAP75, in addition to being indispensable for the structural integrity of the BTB complex (hence the stability of BLM and Topo IIIα), is also important for the functional attributes of this complex.

In the current study, we have addressed several outstanding issues regarding the contributions of BLM, Topo IIIα, and BLAP75 to the functions of the BTB complex. First, we demonstrated that the ability of BLM to unwind the HJ is greatly enhanced by the combination of Topo IIIα and BLAP75 (Fig. 2, A and B). Up-regulation of the HJ unwinding ability of BLM within the context of the BTB complex may facilitate (i) the regression and subsequent processing of stalled or damaged DNA replication forks (21, 22) and (ii) the branch migration of distant HJs to form a hemicatenane structure that can be decatenated by the strand passage activity of Topo IIIα (45). Interestingly, although *E. coli* Top3 protein can co-operate with BLM in the DHJ dissolution reaction, the results presented herein show that it is ineffective at enhancing the HJ unwinding ability of BLM within the context of the BTB complex may facilitate (i) the regression and subsequent processing of stalled or damaged DNA replication forks (21, 22) and (ii) the branch migration of distant HJs to form a hemicatenane structure that can be decatenated by the strand passage activity of Topo IIIα (45). Interestingly, although *E. coli* Top3 protein can co-operate with BLM in the DHJ dissolution reaction, the results presented herein show that it is ineffective at enhancing the HJ unwinding ability of BLM with or without BLAP75. The observation that Topo IIIα works in conjunction with BLAP75 to stimulate the BLM helicase activity on a non-topologically constrained substrate suggests that Topo IIIα regulates the BLM helicase activity independently of its DNA relaxation function. Consistent with this premise, the catalytically null Topo IIIα Y337F mutant

**FIGURE 8.** DNA Binding and unwinding by BLM K695A and BLM K695R mutant proteins. A, binding of a 32P-labeled forked DNA substrate by BLM, BLM K695A, and BLM K695R was examined by a DNA mobility shift assay. DNA unwinding of the 32P-labeled forked DNA substrate by BLM, BLM K695A, and BLM K695R with or without ATP. HD, heat-denatured substrate. C, combinations of BLM, BLM K695A, BLM K695R, Topo IIIα, and BLAP75 were examined for HJ unwinding activity. The results are presented in the histogram.

**FIGURE 9.** Dependence of DHJ dissolution on ATP hydrolysis. A, DHJ dissolution by combinations of BLM, Topo IIIα, and BLAP75 was examined in the presence of the indicated nucleotide. PNP, AMP-PNP; γS, ATPγS. The results are presented in the histogram. B, DHJ dissolution by combinations of BLM, Topo IIIα, and BLAP75 was examined with ATP as the nucleotide cofactor. The results are presented in the histogram.
protein is just as effective as the wild type counterpart in the enhancement of HJ unwinding by BLM in conjunction with BLAP75 (Fig. 6). These results thus provide evidence that Topo IIIα plays a role in BTB function independent of its catalytic activity.

The DHJ dissolution reaction mediated by the BLM-Topo IIIα complex requires ATP hydrolysis (20). Here, we have presented evidence that the DHJ dissolution by the BTB complex is similarly dependent on ATP hydrolysis. To further explore the ATP hydrolysis dependence of this reaction, we have constructed, purified, and characterized two BLM mutant variants, K695A and K695R, that are defective in ATP hydrolysis. These mutant BLM proteins retain the ability to bind DNA and the Topo IIIα and BLAP75 proteins. Importantly, these mutants lack DNA unwinding activity with or without Topo IIIα and BLAP75 and are devoid of DHJ dissolution activity within the context of the BTB complex. Taken together, it is clear that both the DNA unwinding and DHJ dissolution activities of the BTB complex are linked to ATP hydrolysis by the BLM protein. The requirement for ATP hydrolysis in these BTB complex functions is consistent with the results from genetic studies in yeast demonstrating that the Sgs1 ATPase/helicase activity is indispensable for conferring cellular resistance to the DNA damaging agent methyl methanesulfonate (46), stabilizing DNA polymerases at stalled replication forks (47), ensuring viability in the srsΔ background (46), and suppressing the hyper-recombination phenotype of sgsΔ cells (48–50). It has been proposed that the Sgs1 helicase activity is necessary during DNA replication for restarting stalled replication forks (46).

Interestingly, genetic studies have provided evidence for ATPase/helicase-independent functions of Sgs1 as well. For instance, ATPase/helicase dead alleles of SGS1 appear to be functional in limiting crossovers and the length of gene conversion tracts induced by a site-specific double-strand break in mitotic cells and are able to restore sporation efficiency in the sgsΔ background (46, 48, 49). These observations raise the possibility that the biological functions of the RecQ class of helicases may not always require ATP hydrolysis by these proteins. Accordingly, it will be important to determine whether or not BLM also possesses ATPase/helicase-independent functions in cells.

Aside from BS, the BLM protein has also been functionally linked to other cancer-predisposing diseases. For instance, BLM has been associated with components of the Fanconi anemia pathway of DNA damage response and repair. Specifically, the FANCA protein associates with the BTB complex in HeLa cell extracts (23). DT40 chicken cells deleted for FANC C display elevated sister chromatid exchange levels, reminiscent of BS cells, and double mutant analysis has revealed epistasis between BLM and FANC C in this regard (51). Moreover, FANC C mutant cells show defective BLM focus formation in response to mitomycin C treatment, suggesting that Fanconi anemia proteins are needed for the proper cellular localization of BLM (51). The results and research material described herein should facilitate future efforts directed at delineating the functional role of the BTB complex in various genome maintenance pathways.

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BTB Complex Characterization