The Bloom's Syndrome Gene Product Interacts with Topoisomerase III*

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Bloom’s syndrome is a rare genetic disorder associated with loss of genomic integrity and a large increase in the incidence of many types of cancer at an early age. The Bloom’s syndrome gene product, BLM, belongs to the RecQ family of DNA helicases, which also includes the human Werner’s and Rothmund-Thomson syndrome gene products and the Sgs1 protein of Saccharomyces cerevisiae. This family shows strong evolutionary conservation of protein structure and function. Previous studies have shown that Sgs1p interacts both physically and genetically with topoisomerase III. Here, we have investigated whether this interaction has been conserved in human cells. We show that BLM and hTOPO IIIα, one of two human topoisomerase III homologues, co-localize in the nucleus of human cells and can be co-immunoprecipitated from human cell extracts. Moreover, the purified BLM and hTOPO IIIα proteins are able to bind specifically to each other in vitro, indicating that the interaction is direct. We have mapped two independent domains on BLM that are important for mediating the interaction with hTOPO IIIα. Furthermore, through characterizing a genetic interaction between BLM and TOP3 in S. cerevisiae, we have identified a functional role for the hTOPO IIIα interaction domains in BLM.

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The Bloom’s syndrome gene product interacts with topoisomerase III through characterizing a genetic interaction between BLM and hTOPO IIIα, indicating that the interaction is direct. We have mapped two independent domains on BLM that are important for mediating the interaction with hTOPO IIIα. Moreover, the purified BLM and hTOPO IIIα proteins are able to bind specifically to each other in vitro, indicating that the interaction is direct. We have mapped two independent domains on BLM that are important for mediating the interaction with hTOPO IIIα. Furthermore, through characterizing a genetic interaction between BLM and TOP3 in S. cerevisiae, we have identified a functional role for the hTOPO IIIα interaction domains in BLM.

Bloom’s syndrome is a rare genetic disorder characterized by retarded growth, sun sensitivity, immunodeficiency, and a predisposition to a wide variety of cancers (1). Cells from affected individuals show genomic instability, the hallmark feature being hyperrecombination between sister chromatids. The gene mutated in Bloom’s syndrome, BLM, encodes a member of the RecQ family of DEXH box DNA helicases (2). This family of helicases has been conserved from bacteria to humans, and all members share a central core domain comprising seven highly conserved motifs found in all known DNA helicases (3). In addition to BLM, there are at least four other human RecQ homologs. Defects in two of these, WRN and RECLQ4, are also associated with disease conditions (4, 5). WRN is the gene mutated in the premature aging disorder Werner’s syndrome, and RECLQ4 is mutated in Rothmund-Thomson syndrome, a rare condition associated with skin and skeletal abnormalities, as well as some features of premature aging (6). Like Bloom’s syndrome, both of these diseases also give rise to an elevated incidence of cancer, although to a lesser extent than is seen in Bloom’s syndrome.

In eukaryotes, the RecQ helicase family can be divided into two subfamilies: those that consist of essentially the helicase domain and those in which the helicase domain is flanked by large, poorly conserved N- and C-terminal domains (3). BLM falls into the latter class, along with WRN and RECLQ4, as well as the Schizosaccharomyces pombe Rqh1 (7) and Saccharomyces cerevisiae Sgs1 proteins (8, 9). In addition to structural homology, there exists a certain degree of conservation of function among the various eukaryotic RecQ helicases. For example, Sgs1p, BLM and WRN display 3’–5’ DNA helicase activity, suggesting that they share a similar mechanism of action (10–12). Mutations in BLM, WRN, SGS1, or rqh1 result in genomic instability (3), and more specifically, BLM, sgs1, and rqh1 mutants display elevated levels of homologous recombination (7, 8, 13–15). Moreover, the hyperrecombination phenotype of sgs1 mutants can be partially suppressed by ectopic expression of either BLM or WRN (16).

The torsional stress introduced into DNA when the complementary strands are separated by helicases is relieved by a class of enzymes known as topoisomerases. These enzymes catalyze the passage of intact DNA strands across transient DNA breaks and fall into one of two classes, designated type I and type II, depending upon their mechanism of action. Type I topoisomerases generate single-stranded DNA nicks, whereas type II topoisomerases make double-stranded DNA breaks (17, 18). There is increasing evidence to suggest that the RecQ helicases act in concert with one member of the type I class, topoisomerase III. S. cerevisiae expresses a single topoisomerase III enzyme encoded by the TOP3 gene (19). top3Δ mutants grow very slowly, display hyperrecombination between repetitive sequences throughout the genome, and have defects in meiotic recombination (8, 20). Mutations in SGS1 partially suppress the pleiotropic effects of top3 mutations, suggesting that Sgs1p and Top3p act in the same pathway (8). Moreover, Sgs1p and Top3p interact physically and may, therefore, act in a coordinated manner as a heterodimeric or larger complex (8). What role such a complex plays in the cell is unclear. Harmon et al. (21) have shown that E. coli RecQ and topo III together can catalyze the linking and unlinking of covalently closed circular DNA molecules, and it has been suggested that such an activity may function in suppressing recombination.

In mammals, two topoisomerase III homologues, TOPO IIIα and TOPO IIIβ, have been identified (22–24). Both of these enzymes can relax negatively supercoiled DNA, and TOPO IIIα has been shown to be essential for embryonic development in mice (22, 24, 25). Human TOPO IIIβ (hTOPO IIIβ) is able to...
interact physically with Sgs1p when expressed in *S. cerevisiae* (26), suggesting that the interaction between RecQ helicases and topoisomerase III enzymes has been conserved between eukaryotic species.

In this study, we have examined the possibility that BLM acts in concert with one of the human TOPO III isozymes. Purified BLM and hTOPO IIIa proteins were found to interact directly, and this association was mediated by two regions located in the N- and C-terminal domains of BLM that can independently bind hTOPO IIIa. BLM was also found to genetically interact with yeast TOP3, and this interaction was shown to require the presence of either one of the two hTOPO IIIa interaction domains.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The SV40-transformed normal human fibroblast cell line, WI-38/NA-13, which was obtained from the ATCC, was used as a representative of a human cell line from a normal individual. The GM08505 cell line is an SV40-transformed fibroblast cell line from a Bloom's syndrome patient (obtained from NIGMS, National Institutes of Health) and contains a BLM homologous frameshift mutation resulting in premature truncation of the protein at position 739 (2). PSNB2 cells were derived from a clone of GM08505 cells stably transfected with pcDNA3/BLM, an expression construct containing the full-length BLM cDNA downstream of the CMV promoter. The derivation and characterization of these cells will be described in detail elsewhere. 1 All three cell lines were routinely cultured in α-minimum Eagle's medium supplemented with 10% fetal bovine serum. HeLa S3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. *Escherichia coli* and *S. cerevisiae* Strains—The *E. coli* BL21(DE3) strain was obtained from NEB. Gene disruptions were done in the *S. cerevisiae* YP-1 strain (his3A1 leu2A matα ura3-A MATa ura3-ad2-1 ade2-101 lys2). The SGS1 open reading frame was replaced with *LYS2* as described previously (15). The TOP3 open reading frame was replaced with a *kanMX* cassette using the method of Wach et al. (27). Transforms were selected on plates containing G418, and the disruption of TOP3 was confirmed using polymerase chain reaction (PCR).

**Plasmids**—The entire BLM open reading frame was cloned between the BamHI and XhoI sites of pcDNA3 (Invitrogen) to generate pcDNA3/BLM. pMAL-C2 (New England Biolabs) and pGEX-4T-1 (Amer-shers Pharmacia Biotech) plasmids were used for the generation of maltose-binding protein (MBP) and glutathione S-transferase (GST) fusion peptides, respectively. Portions of the BLM cDNA that encoded various N- and C-terminal regions of BLM were amplified by PCR. Sense and antisense primers contained the unique XhoI site in the open reading frame and a 3' antisense codons, respectively, of each desired fragment. The antisense primer had an additional in-frame antisense stop codon. EcoRI and XhoI sites were also engineered into the sense and antisense primers, respectively, to allow in-frame cloning of the PCR fragments into pMAL-C2 and pGEX-4T-1. pBluescript II, pBluescript, and pBluescript-NC contained residues 213–1417, 1–1265, and 213–1265 of BLM, respectively.

**In Vitro Transcription/Translation**—pcDNA3 containing the entire open reading frame of either hTOPO IIIa or hTOPO IIIb was used in the TNT coupled reticulocyte system containing [35S]methionine, following the manufacturer's recommendations (Promega). Half of each reaction was then boiled and separated by SDS-PAGE before being subjected to either autoradiography or Western blotting.

**Indirect Immunofluorescence Analysis**—Cells were grown on coverslips and fixed with 4% paraformaldehyde in 250 mM HEPEs, pH 7.4, 0.1% Triton X-100, for 20 min. After 3–5 washes over 20 min in PBSA, cells were permeabilized in 0.5% Triton X-100 for 20 min and then washed as before. Blocking in 5% fetal bovine serum in PBSA was followed by 16 h incubation with the primary antibodies BFL-103, IHC33, or D6, which were used at a 1:50, 1:200, and 1:200 dilution, respectively. 3–5 PBSA washes over 20 min were followed by incubating for a further 30 min with anti-mouse Cy3 (Sigma) or anti-rabbit fluoroscein isothiocyanate (Dako) secondary antibody diluted 1:800 and 1:200 respectively. Cells were washed five times in PBSA, and the DNA was stained using Hoechst 33258 at 50 ng/ml. Stained slides were mounted in 90% glycerol, 20 mM Tris-HCl, pH 8.0, and 50 μg/ml para-phenylenediamine. Slides were viewed at ×100 magnification on a Zeiss Axioskop microscope. Image acquisition and analysis were performed using Kromascan (Kinetic Imaging), and the images were pseudocoloured and merged in Adobe Photoshop.

**Whole Cell and Nuclear Extracts**—Nuclear extracts were prepared from exponentially growing HeLa S3 cells. Approximately 106 cells were washed in PBSA and the pellet lysed in 5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl2, 10 mM NaCl, 1% Nonidet P-40 and 1 mM PMSF) on ice for 45 min. The nuclei were then harvested at 5000 × g for 5 min, and the supernatant was discarded. The pellet was then resuspended in 0.15 ml of TKM buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 25 mM KCl, and 1 mM PMSF) to which 15 μl of 0.2 mM EDTA, pH 8.0, and 2 volumes of Buffer D (80 mM Tris, pH 7.5, 2 mM EDTA, 0.53 mM NaCl, 20% glycerol supplemented with 1 mM DTT, 1 mM PMSF, and Complete protease inhibitor mixture (Roche Molecular Biochemicals), used at the manufacturer's recommended concentration), were added, and the mixture was incubated on ice for 30 min. The nuclear extract was then cleared by centrifugation at 14,000 rpm in a microcentrifuge at 4 °C, and the supernatant stored at −70 °C. Whole cell extracts were typically prepared from 2 liters of exponentially growing HeLa S3 cultures. After rinsing of cells in cold PBSA, the cell pellet was resuspended in 3 ml of lysis buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM NaCl, 2 mM MgCl2, 2 mM EDTA, and 1 mM PMSF) supplemented with 0.5% Triton X-100, 1 mM DTT, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM glucose 1-phosphate, 10 mM microcystin, 0.1 mM para-nitrophenylphosphate, 1 mM PMSF, and Complete protease inhibitor mixture (Roche Molecular Bio-

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1 P. S. North et al., manuscript in preparation.

2 The abbreviations used are: PCR, polymerase chain reaction; BSA, bovine serum albumin; DTT, dithiothreitol; kb, kilobase(s); GST, glutathione S-transferase; MBP, maltose-binding protein; PBSA, phospho-buffered saline; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonlfy fluoride; TBS, Tris-buffered saline.

3 H. Turley et al., manuscript in preparation.
milk, 0.3% Tween 20, 1 mM DTT, and 1 mM PMSF for 60 min. Filters were washed four times before being boiled in sample loading buffer (50 μl Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.2% bromphenol blue, 20% glycerol) for 5 min at 4 °C. The lysate was then cleared by centrifugation at 10,000 rpm in a Beckman JA10 rotor, the cell pellet was washed four times before being boiled in 1 ml of cold PBSA. The resin was washed a further 200 of 0.5–0.6. Isopropyl-1-thio-D-galactopyranoside was then added to a final concentration of 0.4 mM, and the cultures were allowed to grow for a further 2–3 h before being chilled on ice for 30 min. After centrifugation at 10,000 rpm in a Beckman JA10 rotor, the cell pellet was resuspended in 10 ml of PBSA supplemented with 1 mM PMSF and Complete protease inhibitor mixture (Roche Molecular Biochemicals) at the manufacturer’s recommended concentration. Cells were then lysed by sonication, and the lysate was cleared by centrifugation at 42,000 rpm in a Beckman 70 Ti rotor for 30 min at 4 °C. 1 ml of 50% slurries of amylose-agarose (NEB) or GSH-agarose resin (Sigma) were then added to the lysates to capture MBP or GST fusion proteins, respectively, and rotated end-over-end for 30 min at 4 °C. The agarose resin was then pelleted in a benchtop microcentrifuge for 5 s at 14,000 rpm and washed in 1 ml of cold whole cell extract lysis buffer. Samples were then rotated end-over-end for 30 min at 4 °C. Immunoprecipitates were pelleted in a benchtop microcentrifuge for 5 s and washed in 1 ml of cold whole cell extract lysis buffer.

The pellet was washed a further four times before being boiled in sample loading buffer (50 μl Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.2% bromphenol blue, 20% glycerol) for 5 min. 5 min. 5 min. 5 min. 5 min.

Far Western Analysis—Typically, 0.2–1.0 μg of each polypeptide was subjected to SDS-PAGE and transferred to Hybond-ECL filters (Amersham Pharmacia Biotech) using a TE 70 semi-dry transfer unit (Amersham Pharmacia Biotech). BLM and hTOPO IIIα were detected by conventional Western analysis using 1,500 dilutions of IHIC33 or D6 as primary antibody, respectively. Anti-rabbit IgG/horse radish peroxidase conjugates (Sigma) were used as secondary antibody at a 1:10,000 dilution and detected using ECL (Amersham Pharmacia Biotech) following the manufacturer’s instructions.

Expression and Purification of Recombinant Proteins—Recombinant BLM and hTOPO IIIα were expressed and purified as described previously (12, 28, 30). MBP and GST fusion peptides were expressed and purified from BL21 (DE3) cells (New England Biolabs) transformed with the pMAL-C2 or pGEX-4T-1 expression plasmids containing various portions of the BLM cDNA. Overnight cultures of transformants inoculated into 400 ml of LB at a 1:100 dilution were grown at 37 °C to an 

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RESULTS

Generation of Anti-BLM and Anti-hTOPO IIIα Antibodies—Anti-BLM antibodies were raised in rabbits against a chimeric protein consisting of MBP fused to residues 1–449 of BLM (rBLM) or a HeLa nuclear extract, as indicated above the lanes. The sizes of molecular mass standards (in kDa) run in parallel are shown on the left. The position of BLM is indicated on the right. Right panel, Western blot using the IHIC33 antibodies against a whole cell extract of GM08505 or PSNB2 cells, as indicated above the lanes. B. Western blot with the BFL-103 antibody against recombinant BLM and purified recombinant proteins comprising MBP alone or MBP fused to the N-terminal (residues 1–447) or C-terminal (residues 966–1417) domains of BLM, as indicated above the lanes.

FIG. 1. Characterization of anti-BLM. A, left panel, Western blot using the IHIC33 antibodies against either purified recombinant BLM (rBLM) or a HeLa nuclear extract, as indicated above the lanes. The position of BLM is indicated on the right. Right panel, Western blot using the IHIC33 antibodies against a whole cell extract of GM08505 or PSNB2 cells, as indicated above the lanes. B, Western blot with the BFL-103 antibody against recombinant BLM and purified recombinant proteins comprising MBP alone or MBP fused to the N-terminal (residues 1–447) or C-terminal (residues 966–1417) domains of BLM, as indicated above the lanes.

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by BFL-103 resides in the nonconserved, N-terminal domain of BLM (Fig. 1B). Additional validation of this antisera is described below, in Fig. 4.

Antibodies were also raised using purified recombinant hTOPO IIIα as antigen. Serum from immunized rabbits, designated D6, contained antibodies that recognized the purified recombinant hTOPO IIIα (30) (Fig. 2A). Two degradation products of the hTOPO IIIα protein present in the preparation were also recognized by this antisem. In HeLa cell extracts, D6 antibodies detected two proteins of approximately 97 and 95 kDa (Fig. 2A). The same two proteins were also detectable in extracts from WI-38/VA-13 cells, a normal human fibroblast cell line, and from GM08505 cells (data not shown and see Fig. 4D, below). Because hTOPO IIIα and hTOPO IIIβ share 36% homology and the D6 antisemur was generated using full-length recombinant hTOPO IIIα, it was possible that the reason for the presence of two immunoreactive bands on the Western blot was that the D6 antisemur can cross-react with hTOPO IIIβ. To eliminate this possibility, expression vectors containing the hTOPO IIIα and IIIβ cDNAs were used to synthesize the two human isozymes in an in vitro transcription/ translation system. Using this system, both proteins were produced at comparable levels, as judged by [35S]methionine incorporation, but only hTOPO IIIα was recognized by D6 antisemur (Fig. 2B), confirming that the D6 antisemur is specific for hTOPO IIIα. It is possible, therefore, that the two proteins recognized by the D6 antisemur in cell extracts represent either the full-length and a proteolytically cleaved version of hTOPO IIIα or different posttranslational modified forms of the protein that exist in human cells.

**BLM and hTOPO IIIα Exist as a Complex in Human Cells**—Given that Sgs1p and Top3p interact physically in yeast (8), we tested the possibility that BLM may form a complex with topoisomerase III in human cells. Initially we sought evidence that BLM and hTOPO IIIα could be co-immunoprecipitated from HeLa cell extracts. For this, immunoprecipitates were prepared using either IHIC33 (anti-BLM) or D6 (anti-hTOPO IIIα) antibodies. These precipitates were then separated by SDS-PAGE and Western blotted for the presence of hTOPO IIIα and BLM. In D6 immunoprecipitates, two proteins of 97 and 95 kDa that reacted with the D6 antisemur could be detected (Fig. 3, upper panel). The slower migrating form could also be detected in IHIC33 immunoprecipitates, indicating that BLM and at least one of the hTOPO IIIα forms is tightly associated in HeLa cells. This interaction appeared to be specific because the faster migrating D6-immunoreactive species was not present in IHIC33 immunoprecipitates. As expected, Western blotting with IHIC33 revealed BLM to be present in the hTOPO IIIα immunoprecipitate (Fig. 3, lower panel). However, BLM was also detected in the D6 immunoprecipitate (Fig. 3, lower panel), indicating that the complex of BLM and hTOPO IIIα can be captured using either antibody.

The co-immunoprecipitation of BLM and hTOPO IIIα from human cell extracts is consistent with these proteins forming a complex. To provide additional evidence for this, we analyzed whether BLM and hTOPO IIIα co-localize in the nucleus of intact human cells. In order for co-staining studies to be undertaken without the requirement to directly conjugate antibodies to fluorochromes, the BFL-103 mouse monoclonal antibody to BLM and the D6 rabbit polyclonal antibody to hTOPO IIIα were used for this part of the study. As a first step, however, we wanted to confirm that the staining patterns obtained with the IHIC33 and BFL-103 anti-BLM antibodies were the same. Indirect immunofluorescence of exponentially growing WI-38/VA-13 cells using either IHIC33 or BFL-103 revealed BLM to be localized to prominent nuclear foci in many of the cells (Fig. 4A). This was in accordance with the results of Neff et al. (32). Merging the fluorescent signals for BLM obtained with the BFL-103 and IHIC33 antisera revealed a very strong concordance (Fig. 4A).

Analysis of the subcellular localization of hTOPO IIIα also revealed a punctate pattern of nuclear staining (Fig. 4B). Merging the signals for BLM and hTOPO IIIα proteins also indicated a strong concordance, indicating that BLM and hTOPO IIIα co-localize in human cell nuclei. As expected from the Western blotting data described in Fig. 1, BLM staining using the BFL-103 antisemur was absent from Bloom’s cell line, GM08505 (Fig. 4C), providing additional evidence that BFL-103 is specific for BLM. In contrast, staining of PSNB2 cells (GM08505 containing the BLM cDNA) revealed that BLM resides in nuclear foci that co-localized with hTOPO IIIα. BLM was also found distributed in the nucleoli of PSNB2 cells, a staining pattern that was also seen in nontransfected cells, albeit to a quantitatively lesser extent than in PSNB2 cells.

**FIG. 2.** Characterization of anti-hTOPO IIIα antibodies. A, Western blot using the D6 antibodies against purified recombinant hTOPO IIIα (left panel) or a HeLa whole cell extract (right panel). The position of hTOPO IIIα is indicated on the right. B, in vitro transcription/translation reactions containing no DNA (lane 1), or pcDNA3-hTOPO IIIα (lane 2), or pcDNA3-hTOPO IIIβ (lane 3). Left panel, autoradiograph of reaction products. Right panel, Western blot using the D6 antisemur. The positions of the hTOPO IIIα and hTOPO IIIβ bands are indicated on the right.

**FIG. 3.** BLM and hTOPO IIIα can be co-immunoprecipitated from HeLa cell extracts. Immunoprecipitates were prepared using either anti-BLM (IHIC33) or anti-hTOPO IIIα (D6) antibodies as indicated above the lanes. After separation by SDS-PAGE and transfer to nitrocellulose filters, immunoprecipitates were probed for the presence of either hTOPO IIIα, using the D6 antibody (upper panel), or BLM, using the IHIC33 antibody (lower panel).
This striking nucleolar staining in PSNB2 cells was presumably due to the overexpression of BLM from the CMV promoter in this line. Interestingly, although hTOPO IIIα staining was still evident in the Bloom's syndrome mutant cell line, the number of nuclear foci detected using the D6 antibody was consistently and substantially reduced compared with that seen in PSNB2 cells (Fig. 4C). This could have been due to one of two reasons: either hTOPO IIIα is mislocalized in GM08505 cells, or hTOPO IIIα is stabilized in PSNB2 cells, perhaps through its association with BLM. This latter possibility was excluded, however, because Western analysis using the D6 antiserum on whole cell extracts from GM08505 and PSNB2 cells revealed similar levels of hTOPO IIIα (Fig. 4D). This suggests that the correct localization of hTOPO IIIα to nuclear foci is at least partially dependent upon the presence of BLM. Together, these data strongly suggest that BLM and hTOPO IIIα exist as a complex in vivo.

**BLM and hTOPO IIIα Interact Directly in Vitro**—We next wanted to determine whether the interaction between BLM and hTOPO IIIα was a direct one, because in vivo the interaction may be mediated via an accessory protein. Far Western analysis was therefore performed to determine whether purified recombinant BLM and hTOPO IIIα could interact directly in vitro. Essentially, this procedure involves the immobilization of one of the proteins of interest to a nitrocellulose filter, which is then incubated in buffer containing the second protein. The filter is then washed to remove any unbound material, and the presence of the second protein is detected by
BLM Contains Two hTOPO IIIα Interaction Domains—We next investigated which region of the BLM protein was responsible for mediating interactions with hTOPO IIIα. The BLM protein essentially consists of three domains: a central conserved helicase domain found in all RecQ helicases, flanked by divergent N- and C-terminal regions (2, 3). In yeast, Top3p has been shown to interact with the N-terminal domain of Sgs1p (8). We therefore analyzed whether the N-terminal domain of BLM was required for interaction with hTOPO IIIα. Recombinant fusion peptides were generated that consisted of portions of the N-terminal domain of BLM fused to the C terminus of MBP to aid affinity purification. These fusion peptides were then transferred to nitrocellulose and tested for their ability to bind hTOPO IIIα using far Western analysis. hTOPO IIIα was found to bind to a peptide containing residues 1–447 of BLM (Fig. 6A). Analysis of further truncation derivatives of BLM revealed that residues 1–212 were sufficient to direct binding of hTOPO IIIα (Fig. 6, A and B, upper panel). To eliminate the possibility of an artifact caused by the binding of hTOPO IIIα to the junction where MBP is fused to the BLM N-terminal domain, a second peptide was generated consisting of GST fused to residues 1–212 of BLM. Switching the tag to GST in this way had no effect on the extent of hTOPO IIIα binding, confirming that the hTOPO IIIα interaction domain resides solely in those residues derived from the BLM protein (Fig. 6B, lower panel). An MBP fusion peptide that contained residues 1–142 of BLM did not bind to hTOPO IIIα, indicating that residues 143–212 of BLM are important for the formation of the hTOPO IIIα binding site (Fig. 6B, upper panel).

To examine the possibility of additional sites of interaction between BLM and hTOPO IIIα, far Western analysis was performed using the C-terminal domain of BLM. A GST fusion peptide containing residues 966–1417 of BLM was found to interact with hTOPO IIIα (Fig. 6A). The location of this second site of interaction was further mapped by analysis of various truncated derivatives of the C-terminal domain and was found to reside between residues 1266–1417 (Figs. 6, A and B, lower panel). Hence, two independent interaction domains for hTOPO IIIα are present in the BLM protein.

BLM and Yeast TOP3 Genetically Interact—Mutation of the SGS1 gene, encoding the sole S. cerevisiae RecQ homologue, suppresses the slow growth and hyperrecombination phenotype of top3 mutants (8). This genetic interaction indicates that Sgs1p and Top3p act in the same biochemical pathway and suggests that Sgs1p generates a DNA structure that if not resolved by Top3p leads to slow growth (8). We therefore analyzed whether BLM could also genetically interact in this way with yeast TOP3. In an sgs1Δ top3Δ double mutant, complementation of sgs1 by plasmid-encoded Sgs1p should result in the appearance of a slow growth phenotype, reminiscent of a top3Δ mutant. This effect has indeed been demonstrated previously by others following ectopic expression of not only Sgs1p, but also BLM (16, 32, 33). In our study, we also found that expression of the BLM protein from a galactose-inducible promoter induced slow growth in sgs1Δ top3Δ cells (Fig. 7A). However, expression of BLM in an sgs1Δ mutant (Fig. 6A) or in wild-type cells (Fig. 7B) had little effect on growth, indicating that this BLM-induced effect was dependent on the absence of functional Top3p.

The hTOPO IIIα Interaction Domains Are Required for the Genetic Interaction between BLM and Yeast TOP3—We next tested the possibility that the genetic interaction between BLM and yeast TOP3 requires the hTOPO IIIα interaction domains on BLM. Galactose-inducible expression constructs were generated containing cDNAs encoding mutant BLM proteins that lack the N-terminal (BLM-N), C-terminal (BLM-C), or both (BLM-NC) hTOPO IIIα interaction domains. Because these modifications to BLM involved truncations that could have
global effects on protein folding, it was important to confirm that the mutant proteins were still functionally active. It has been shown previously that expression of BLM cDNAs containing missense, helicase-inactivating mutations are not functional when expressed in sgs1Dtop3D cells (32). Similarly, in our study, the ability to induce slow growth in sgs1Dtop3D cells was used as an indicator of BLM function. As with wild-type BLM, we found that all three of the truncated BLM proteins induced slow growth when expressed in sgs1Dtop3D cells (Fig. 7A), demonstrating that loss of either or both of the hTOPO IIIα interaction domains on BLM does not adversely affect BLM activity in yeast. However, although expression of wild-type BLM was tolerated in wild-type and sgs1Δ strains, expression of the BLM-NC mutant protein was still able to induce slow growth in sgs1Δ cells, albeit to a slightly lesser extent than it was in sgs1Δ top3Δ cells (Fig. 7A). Hence, the deleterious effect induced by BLM-NC is largely independent of TOP3 status, indicating that BLM-NC and TOP3 do not genetically interact. In contrast, expression of the mutant BLM-N and BLM-C proteins, which each lack only one of the hTOPO IIIα interaction domains, had little effect in sgs1Δ cells (Fig. 7A). Therefore, as with wild-type BLM, BLM-N and BLM-C induce slow growth that is dependent upon the absence of Top3p.
indicating that either interaction domain is sufficient for establishing a genetic interaction between BLM and yeast Top3.

Taken together, these data suggest that the hTOPO IIIα interaction domains target yeast Top3p to a BLM-induced DNA structure (which, if not resolved, is detrimental to the cell), probably via a direct interaction with BLM itself. However, there are two alternative explanations for the results described above. First, the BLM-NC-induced slow growth in sgs1Δ cells is not due to uncoupling of the activities of BLM and Top3p; rather, the mutant BLM-NC protein has an additional, dominant-negative effect that acts via a mechanism independent of the pathway in which Sgs1p and Top3p act. Second, loss of both hTOPO IIIα interaction domains has a stabilizing effect on the protein, resulting in the accumulation of higher levels of active BLM helicase and hence an increase in the number of BLM-generated deleterious DNA structures. These two alternative explanations were excluded, however, by the following observations: (i) expression of BLM-NC had little effect in wild-type cells (Fig. 7B), indicating that the BLM-NC protein only induces slow growth in the absence of Sgs1p and is unlikely, therefore, to be acting through a process independent of the pathway in which Sgs1p and Top3p act; and (ii) BLM-NC was expressed at a comparable level to that of full-length wild-type BLM (and possibly at an even lower level, given that approximately 50% of the wild-type protein in the extracts was found to be partially degraded) (Fig. 7C).

DISCUSSION

We have shown that the Bloom’s syndrome gene product, BLM, forms a direct physical association with the human topoisomerase IIIα isozyme, and we have identified two independent sites of interaction on BLM. Deletion of both sites was found to severely compromise the ability of BLM to functionally interact with topoisomerase III in S. cerevisiae. Increasing evidence suggests that the biochemical functions of RecQ helicases in different species are intimately associated with those of topoisomerase III enzymes (3), because the genetic interaction between Sgs1p and Top3p, first revealed in S. cerevisiae (8), has been shown to be conserved in the distantly related fission yeast S. pombe. We have described elsewhere that mutations in the gene encoding the only known fission yeast RecQ homologue, rqh1, can rescue the lethality caused by deletion of top3Δ (34). Here, we have shown a similar genetic interaction exists between one of the human RecQ homologs, BLM, and budding yeast Top3p. In common with Sgs1p and Top3p, which form a complex in budding yeast (8), BLM also directly interacts with hTOPO IIIα, and a complex containing both proteins can be detected in vivo.

The interaction between Top3p and Sgs1p is mediated by the N-terminal domain of Sgs1p (8). Similarly, hTOPO IIIα was also found to interact with the N-terminal domain of BLM, suggesting that the functional domain organization of these two helicases may be similar, despite the lack of extensive primary sequence conservation between their respective N-terminal domains. A second hTOPO IIIα interaction domain was also identified in the extreme C-terminal portion of BLM. Interestingly, although both hTOPO IIIα interaction domains map to the nonconserved regions of BLM, either one was sufficient for BLM to genetically interact with S. cerevisiae Top3p. There may, therefore, be conserved features of the secondary and tertiary structure of the topoisomerase III interaction domains of BLM and Sgs1p that are not evident by analysis of their amino acid sequences. It is not known whether the C-terminal domain of Sgs1p is also involved in binding to Top3p.

The ability of mutations in SGSI to suppress the slow growth phenotype of top3 mutants has been interpreted previously to suggest that the Sgs1 helicase generates a DNA structure that must be acted upon by Top3p in order to prevent accumulation of an as yet unidentified, toxic intermediate (8). The requirement for either of the hTOPO IIIα interaction domains to be present on BLM in order for the genetic interaction between BLM and yeast Top3p to be maintained suggests that Top3p is recruited to its sites of action via a direct interaction with the BLM protein itself. The fact that hTOPO IIIα appeared to be mislocalized in GM08505 cells is also consistent with a role for BLM in targeting hTOPO IIIα to its correct site of action.

The presence of two independent hTOPO IIIα interaction domains on BLM raises the possibility that BLM and hTOPO IIIα interact with a 1:2 stoichiometry, which has mechanistic implications for how topoisomerase III might resolve a RecQ helicase-generated DNA structure. Topoisomerase III is a type I topoisomerase and therefore only makes single-stranded DNA nicks. However, the recruitment of two topoisomerase III molecules to a DNA structure by a RecQ helicase could, in principle, be used to generate a double-stranded DNA break, with each topoisomerase III molecule making a nick in close proximity on opposite strands of the duplex. The possibility that topoisomerase III can, under certain circumstances, act coordinately to cleave both strands of a DNA duplex is consistent with the recent observations of Harmon et al. (21). These authors showed that together, the E. coli RecQ and Top3 proteins can catalyze the passage of double-stranded DNA through a break in a second, covalently closed double-stranded DNA molecule. It has been suggested that such an activity could act either to decatenate newly replicated daughter DNA molecules prior to cell division or to disrupt early recombination intermediates between inappropriately paired DNA molecules (21, 35).

Bloom’s, Werner’s, and Rothmund-Thomson syndromes are clinically distinct entities indicating that the human RecQ homologues perform at least some nonoverlapping functions within the cell. sgs1 mutants are to a certain extent a pheno- copy of both Bloom’s and Werner’s syndromes. BLM mutants display hyperrecombination and have DNA replication abnormalities (1, 3). Similarly, sgs1 mutants also display hyperrecombination (8, 15) and are sensitive to the ribonucleotide-reductase inhibitor hydroxyurea (16), suggesting that they have some defect in replication. In common with the phenotype of WRN mutants, sgs1 mutants have a reduced replicative life span and display, prematurely, several markers of aging, such as sterility and redistribution of Sir proteins from telomeres to the nucleolus (36). The Sgs1p and WRN proteins also both localize to the nucleolus (36–38), suggesting a conservation of function in rDNA metabolism. Furthermore, expression of either BLM or WRN can partially suppress the hyperrecombination phenotype of sgs1 mutants (16). It is possible, therefore, that the Sgs1/Top3 interaction has been conserved not only between BLM and hTOPO IIIα but also between other RecQ family helicases in humans and one of the topoisomerase III isozymes. It is likely that the evolutionarily divergent N- and C-terminal domains play a key role in functionally distinguishing the mammalian RecQ helicases through either directing additional enzymatic functions or mediating specific protein-protein interactions. For example, a DNA exonuclease function resides in the N-terminal domain of WRN (39, 40), an activity that is apparently absent from the other human RecQ homologs. The fact that the two hTOPO IIIα interaction domains map to nonconserved regions in BLM might suggest that the interaction between BLM and hTOPO IIIα is specific for this pair of proteins. However, hTOPO IIIβ has been shown to interact with Sgs1p when expressed in yeast (26), suggesting that in human cells hTOPO IIIβ is also likely to interact with one or more of the RecQ helicases. Therefore, it is possible that
although the ancestral recQ gene has undergone several duplication and functional diversification events during evolution, the RecQ helicases may all share a common mechanism of action in that they act in concert with a topoisomerase III partner. Hence, the phenotype of RecQ helicase mutants may be at least partially the consequence of a functional impairment of topoisomerase III. The fact that mutations in at least three of the known human RecQ helicases give rise to cancer-prone disorders therefore raises the possibility that the gene encoding hTOPO IIIα may also be a tumor suppressor gene.

Ongoing work is aimed at addressing this possibility.

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