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Interaction between Yeast Sgs1 Helicase and DNA Topoisomerase III*

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The *Saccharomyces cerevisiae* Sgs1 protein is a member of the RecQ family of DNA helicases that includes the human Bloom's syndrome and Werner's syndrome proteins. In this work, we report studies on the interaction between Sgs1 and DNA topoisomerase III *in vitro* and *in vivo*. Affinity chromatography experiments with various fragments of Sgs1, a 1447-amino acid polypeptide, suggested that its N-terminal one-fifth was sufficient for interaction with DNA topoisomerase III. Gel electrophoretic mobility shift assays also indicated that a fragment Sgs1(1–283), containing residues 1–283, inhibited the binding of DNA topoisomerase III to single-stranded DNA. A shorter protein fragment containing residues 1–107 also showed partial inhibition in these assays. Studies of a *sgs1 top1* double mutant lacking both Sgs1 and DNA topoisomerase I showed that the slow growth phenotype of this double mutant is suppressed by expressing full-length Sgs1, but not Sgs1 without the N-terminal 107 amino acid residues. In *sgs1 top3* cells devoid of DNA topoisomerase III, however, expression of full-length Sgs1 or Sgs1 lacking the N-terminal 107 amino acid residues has the same effect of reducing the growth rate of the double mutant. These *in vitro* and *in vivo* data indicate that Sgs1 and DNA topoisomerase III physically interact and that this interaction is physiologically significant.

The RecQ family of DNA helicases is involved in the maintenance of genome stability in organisms ranging from bacteria to human. In *Escherichia coli*, the RecQ protein has been implicated in the recombination and repair of double-stranded DNA breaks (1, 2), in the suppression of illegitimate recombination (3), and in the processing of nascent DNA at blocked replication forks (4). In the budding yeast *Saccharomyces cerevisiae,* mutations in the *SGS1* gene encoding the Sgs1 helicase, a RecQ homologue, result in an increase in both illegitimate and homologous recombination (5–7). Null *sgs1* mutants also exhibit a reduced life span, and this reduction appears to correlate with the accumulation of extrachromosomal rDNA rings and the redistribution of the Sir3 silencing protein from the telomeres to the nucleolus (8). In humans, five RecQ homologues have been identified. Two of these have been shown to be the determinants of Bloom's syndrome (BLM)[1] and Werner's syndrome (WRN); a third, RecQL4, has been implicated in Rothmund-Thomson syndrome (9–11). All three syndromes are rare genetic disorders characterized by genome instability and a predisposition to cancer.

Functionally, the yeast Sgs1 helicase and several of its homologues have been closely tied to the DNA topoisomerases. The yeast *SGS1* gene was originally identified in a screen for extragenic suppressors of the slow growth phenotype of *top3* mutant cells lacking DNA topoisomerase III (5). In addition, application of the two-hybrid assay in yeast for the identification of interacting proteins led to the suggestion that Sgs1 can interact with DNA topoisomerase III (Top3) and probably with DNA topoisomerase II as well (5, 12). DNA topoisomerase II is essential in yeast. It is required in the resolution of intertwined chromosomes during mitosis and meiosis, and chromosome nondisjunction and breakage occur in its absence (13–15). The cellular role of Top3 is less well understood. Mutations in *TOP3* result in a pleiotropic phenotype including slow growth, hyperrecombination, and defective sporulation (5, 16, 17). Null mutations in *SGS1* suppress the slow growth and hyperrecombination characteristics of *top3* mutants and also partially suppress the sporulation defect of the mutants (5, 17). In addition to these findings, a functional link between Sgs1 and DNA topoisomerase I was also suggested. A double mutant *sgs1 top1*, which lacks both Sgs1 and DNA topoisomerase I, was found to exhibit a slow growth phenotype, whereas growth of either *sgs1* or *top1* single mutant appeared normal (18).

Recently, RecQ and Top3 homologues have been identified in the fission yeast *Schizosaccharomyces pombe* (19–22). Unlike *S. cerevisiae*, *S. pombe* cells deficient in Top3 undergo only a limited number of divisions before arresting, owing to aberrant chromosome division (21, 22). Similar to *S. cerevisiae*, however, deletion of the *S. pombe* gene encoding the RecQ/Sgs1 homologue (the gene is termed *rqh1*$^+$, *hus2*$^+$, or *rad12*$^+$; the gene product is herein termed Rqh1) partially suppressed the defects of the *top3* mutant. These results indicate that the functional association of RecQ helicase with DNA topoisomerase III is conserved in the two widely diverged yeasts. The essential role of Top3 in *S. pombe* is also intriguing in view of an earlier finding that disruption of murine *TOP3*α, the gene encoding one of the two known isoforms of DNA topoisomerase III, results in early embryonic lethality (23). Whether mammalian DNA topoisomerase III functionally interacts with the RecQ family of DNA helicases is uncertain. Recently, physical interaction between human DNA topoisomerase IIIα and BLM (24), between human DNA topoisomerase IIIα or IIIβ and the β-isoform of human RecQ5 DNA helicase (25), and between yeast Sgs1 and human topoisomerase IIIβ (26) was reported. These

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[1] The abbreviations used are: BLM, Bloom's syndrome; WRN, Werner's syndrome; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

results suggest that interaction between the RecQ and Top3 families of proteins may be functionally significant in general.

The RecQ family of proteins can be divided into two categories based on their sizes. Yeast Sgs1 and Rqh1 and human BLM, WRN, and RecQ4 proteins are approximately twice the size of *E. coli* RecQ and human RecQ1. It was recently found that human RecQ5 had three splicing variants, two proteins of the smaller size class, and a large protein containing 991 amino acid residues (25). For proteins of the larger size class, additional sequences are present on the N- and C-terminal sides of their conserved central helicase domains. It is plausible that the N- and C-terminal regions of these larger proteins play important cellular roles and augment the helicase activity of the core domains. The human WRN protein, for example, possesses a DNA exonuclease activity within its N-terminal region (27–30). Such differences in regions outside of the conserved helicase domains may be closely tied to the different manifestations of mutations in these genes.

In the case of Sgs1, a protein of 1447 amino acid residues, the region spanning residues 400–1268 is sufficient for its helicase activity (31). Although not required for the helicase activity *in vitro*, the C-terminal 200 amino acid residues of Sgs1 appear to be necessary for some of its functions *in vivo.* Whereas the expression of full-length Sgs1 in *sgs1 top3* mutant cells reduces the growth rate, the expression of a recombinant Sgs1 lacking this C-terminal region has no effect (18). For the N-terminal domain of Sgs1, two-hybrid studies suggested that the region spanning amino acid residues 1–500 physically interacted with Top3 (5). In view of the genetic interaction between *SGS1* and *TOP3*, a physical interaction between their protein products appears significant. To investigate this interaction in more detail, we have used purified proteins to study the formation of the putative Sgs1-Top3 complex. A series of fusion proteins containing glutathione *S*-transferase (GST) at their N termini and various Sgs1 fragments at their C termini was constructed and purified. Using affinity chromatography and other biochemical assays, specific interaction between Sgs1 and Top3 was confirmed. The region of Sgs1 essential for this interaction resides within the first 283 amino acid residues of Sgs1, and the first 107 amino acid residues of the protein appear to be important in this interaction. In addition, we show that the N-terminal 107 amino acid residues of Sgs1 are required for the complete function of Sgs1 *in vivo*. This requirement most likely reflects a functional significance of the specific interaction between Sgs1 and Top3.

EXPERIMENTAL PROCEDURES

Expression and Purification of GST-Sgs1 Fusion Proteins—To obtain fusion proteins joining various fragments of Sgs1 to GST, the plasmid pRB121 containing the full-length Sgs1 sequence (31) was digested with *Bam*HI plus either *Hin*dIII, *Hpa*I, or *Eco*RI restriction endonucleases. The resulting DNA fragments containing codons 1–605, 1–408, and 1–283 of *SGS1* were cloned into pGEX-KG or pGEX-4T-1 (Amersham Pharmacia Biotech) to produce pGST-Sgs1(1–605), pGST-Sgs1(1–408), and pGST-Sgs1(1–283), respectively. In these expression plasmids, codons of *SGS1* specified by the numbers in parentheses are fused to the 3′ side of the GST codons. Plasmids for expressing shorter N-terminal fragments of Sgs1 fused to GST, pGST-Sgs1(1–107), and pGST-Sgs1(107–283) were obtained by digesting pGST-Sgs1(1–283) with *Nco*I restriction enzyme and cloning of the appropriate fragments into pGEX-KG.

Expression plasmids were transformed into *E. coli* BL21 (DE3) pLysS cells and grown at 37 °C in Luria broth containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. At a cell density of about 4×10^8 cells/ml, isopropyl-1-thiogalactopyranoside was added to 0.1 mM to induce synthesis of the GST fusion protein. After 4–6 h, cells were collected by centrifugation, rapidly frozen, and stored at −80 °C.

Cells were thawed and resuspended in ice-cold Buffer A (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3, 140 mM NaCl, 2.7 mM KCl) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of leupeptin and pepstatin, 20 μg/ml benzamidine). The resuspended cells were treated with lysozyme (final concentration, 200 μg/ml) for 30 min on ice and briefly sonicated to complete lysis, and Triton X-100 was then added to 1%. Following a further 30 min of incubation on ice, the lysate was centrifuged at 38,000 revolutions/min, and the supernatant was collected. Purification of the GST-Sgs1 fusion proteins was achieved by affinity chromatography of the cleared supernatant on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), following the protocol of the supplier. For the gel electrophoretic mobility shift experiments, the GST fusion proteins were further purified by gel filtration on a Amersham Pharmacia Biotech S200 column. Proteins were dialyzed and stored at −80 °C in 20 mM Hepes, pH 7.5, 200 mM NaCl, and 40% glycerol. The fusion proteins were greater than 50% pure (by mass), as estimated from the intensities of Coomassie-stained protein bands resolved by SDS-polyacrylamide gel electrophoresis. A major contaminant in these preparations was a protein with an apparent molecular mass of 70 kDa, which was probably the *E. coli* DnaK protein known to bind to glutathione-Sepharose 4B; the amount of this contaminant was less than 6%, except for the GST-Sgs1(1–107) preparation, in which it was present at 28% of the total protein. In addition, small amounts of the degradation products of the fusion proteins were also present, as indicated by the presence of minor bands in Western blots of the fusion proteins.

Expression and Purification of Sgs1(1–283)—To express the first 283 codons of yeast *SGS1* in yeast from the inducible GAL1 gene promoter, the expression vector pGST-Sgs1(1–283) was first digested with *Bam*HI and *Xho*I, and the DNA segment containing the Sgs1 codons was cloned in between the corresponding restriction sites in pG1TT (31). In the resulting construct pRB413, codons for a hexahistidine tag were also placed immediately before the stop codon of Sgs1.

pRB413 was transformed into a protease-deficient strain BCY123 (originally obtained from the laboratory of R. Kornberg, Stanford University) and expression of Sgs1(1–283) was carried out as described for Sgs1(400–1268) (31). Cells were harvested and resuspended in Ni(II) binding buffer (20 mM Tris·HCl, pH 8.0, 0.5 M NaCl, 0.1% Triton X-100). Cell lysis was achieved by blending with acid-washed glass beads (Sigma 425–600 μm) in a Bead-Beater (Biospec Products). Cell debris was removed by centrifugation, and imidazole and zinc acetate were added to the supernatant to 10 mM and 10 μM, respectively. The extract was applied to a Ni(II)-agarose column (His-Bind resin, Novagen) and washed with Ni(II) binding buffer containing 40 mM imidazole and 10 μM zinc acetate. The protein was eluted with Ni(II) binding buffer containing 200 mM imidazole. The eluted protein was then dialyzed into Buffer B (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 10% glycerol) and loaded onto an S-Sepharose column equilibrated with the same buffer. The column was washed with Buffer B, and the Sgs1(1–283) protein was then eluted with Buffer A plus 1 M NaCl. Peak fractions of the eluted protein were pooled and concentrated by vacuum dialysis against 20 mM Hepes, pH 7.5, 200 mM NaCl, and 40% glycerol.

Overexpression of Top3—Top3 was overexpressed using the plasmid pRK500, which contains the wild-type *TOP3* coding sequence under the control of the PHO5 promoter (32). The plasmid pRK500 was transformed into strain JEL1 (33), and colonies were grown at 30 °C in leucine-dropout medium supplemented with 2% (w/v) glucose. Cells from 0.5 liter of culture grown from a selected colony were twice-pelleted and washed with sterile water, and then resuspended and grown in eight 2-liter flasks, each containing 1 liter of YEP-low phosphate medium (34) supplemented with 2% (w/v) glucose. Cells were harvested by centrifugation after 8–16 h at 30 °C.

Cells containing overexpressed Top3 were resuspended in Buffer C (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing 10 mM NaCl and lysed by blending with glass beads as described above. Cell debris was removed by centrifugation, and the lysate was used directly for affinity chromatography.

Affinity Chromatography Experiments—Glutathione-Sepharose 4B beads (approximately 200 μl) were incubated with about 0.5 ml of each of the GST fusion proteins in Buffer C for at least 1 h at 4 °C. Unbound GST-Sgs1 fusion protein was removed by washing the beads three times, each time with 1 ml of Buffer C. The cell-free extract containing overexpressed Top3 was then incubated with Sepharose beads coated with different GST-Sgs1 fusion proteins. In each experiment, extract from approximately 50 ml of culture of Top3-overexpressing cells was used. After incubation at 4 °C for 1 h, the Sepharose beads were washed four times, each time with 1 ml of Buffer C containing 10 mM NaCl. Proteins bound to the fusion protein-coated beads were then successively eluted with Buffer C containing 140 mM and 250 mM NaCl (200 μl each time). Ten-μl fractions were collected, and each fraction

was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting.

Purification of Top3 Protein—Wild-type Top3 was overexpressed and purified from yeast by a modification of a previously described protocol (32). The polymin P and ammonium sulfate precipitation steps of the original procedure were omitted, and an additional chromatography step was performed by loading the eluate from the phosphocellulose column, after dialysis against Buffer A plus 400 mM KCl, onto a heparin column equilibrated with the same buffer. The heparin column was eluted with Buffer A plus 1.5 M KCl, and fractions containing Top3 were pooled, dialyzed against Buffer A plus 400 mM KCl, and used in chromatography on the single-stranded DNA column as described (32). A hexahistidine-tagged Top3 was also expressed in insect cells and purified by chromatography on Ni(II) resin. For expression of the tagged protein, the *TOP3* coding region was amplified by the polymerase chain reaction using a pair of oligonucleotide primers, 5′-GACGGATCCATGAAAGTGCTATGTGTCGCAGAG-3′ and 5′-GTCCCAAGCTTACATGGATGCCTTGACACGGTC-3′. In the product, the *TOP3* sequence was placed between the *Bam*HI and *Hin*dIII restriction sites (underlined). The digested fragment was inserted into the baculovirus vector pHTb (Life Technologies, Inc.) to join the 5′ start of *TOP3* to the 3′ side of six histidine codons in the vector. The pHTb-Top3 construct was then used to create a recombinant "bacmid" DNA for transformation of Sf9 insect cells, as described in the Life Technologies, Inc. protocol. Following several rounds of virus amplification, Sf9 cells were infected with the recombinant baculovirus and harvested after 72 h of culturing in roller bottles. Cells were resuspended in lysis buffer (20 mM Tris·HCl, pH 8.5, 0.5 M NaCl), and CHAPS detergent was added (to 0.04%) to effect cell lysis. Following a 30-min incubation on ice, debris was removed by centrifugation, and the supernatant was dialyzed against the lysis buffer to remove chelating agents. Imidazole was then added to 20 mM, and the cell extract was applied to a Ni(II)-agarose column. The column was washed extensively with lysis buffer containing first 20 mM and then 60 mM imidazole, and the hexahistidine-tagged Top3 was eluted with lysis buffer plus 200 mM imidazole. The eluted protein was dialyzed and concentrated by vacuum dialysis into 50 mM Tris·HCl, pH 8.0, 300 mM KCl, 40% glycerol, 1 mM dithiothreitol, and 1 mM EDTA.

Western Blotting—SDS-polyacrylamide gel electrophoresis was carried out as described (35). Transfer of proteins to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) was done electrophoretically in a Bio-Rad mini-cell run at 150 mA for 90 min in a transfer buffer containing 10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid, pH 11.0. Rabbit antibodies against Top3 were raised against a fusion protein of Top3 and the *E. coli* malE protein, as described previously (32).

DNA Binding Assays—To examine the binding of purified Top3 to single-stranded DNA in the absence and presence of recombinant Sgs1 proteins, a 41-nucleotide long DNA oligomer was synthesized and radiolabeled at its 5′ end by the use of [γ-^{32}P]ATP and polynucleotide kinase. The sequence of the 41-nucleotide oligomer was 5′-GACGTGGGCAAAGGTTCGTCAATGGACTGACAGCTGCATGG-3′. DNA binding reactions were performed in one of two ways. In one series of experiments, Top3 and GST-Sgs1 proteins were preincubated in 20 μl of DNA binding buffer (20 mM triethanolamine, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin) for 15 min at room temperature. Radiolabeled DNA (1 nM in nucleotide) was added, and incubation was continued for a further 15 min. Reactions were assayed by electrophoresis in a 6% polyacrylamide gel (37.5:1, acrylamide:bisacrylamide molar ratio) in 89 mM Tris·borate, pH 8.3, 2 mM EDTA. The gel slabs were dried and analyzed using a phosphorimager (Fuji). In a second series of experiments, DNA-protein complexes were fixed by treatment with glutaraldehyde (36) as follows. Top3 and a GST-Sgs1 fusion protein were preincubated in 20 μl of DNA binding buffer for 5 min at 0 °C. The radiolabeled DNA (final concentration, 1 nM) was then added, and incubation was continued for a further 15 min at 0 °C. Glutaraldehyde was added to the mixture to a final concentration of 0.2% (v/v) to cross-link the protein-DNA complexes. Incubation was continued for 15 min on ice, and then for an additional 15 min at 37 °C. Reactions were assayed by gel electrophoresis and analyzed as described above.

Relaxation of Negatively Supercoiled DNA—Reaction mixtures each containing 50 ng of supercoiled pBluescript DNA in 20 μl of 20 mM Tris·HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM ATP, 2 mM dithiothreitol, and 100 μg/ml bovine serum albumin were incubated with the indicated amounts of Top3 and GST-Sgs1 fusion proteins. After 30 min at 30 °C, reactions were stopped by the addition of 5 μl of a buffer containing 0.5% (w/v) proteinase K, 100 mM Tris·HCl, pH 7.5, 200 mM EDTA, and 2.5% (w/v) SDS to each. After incubation at 30 °C for a further 15 min, products were analyzed by electrophoresis in a 0.8% agarose gel in a buffer containing 1.3 μM chloroquine, 89 mM Tris·borate, pH 8.3, 2 mM EDTA. DNA was visualized by Southern blotting using a ^{32}P-labeled probe prepared by oligomer-primed random labeling of pBluescript DNA.

Complementation Assays—The plasmid pRB136 and its derivatives were used to test the effects of expressing various Sgs1 deletions in yeast *sgs1 top1* and *sgs1 top3* cells. pRB136 contains the full-length Sgs1 coding sequence under the GAL1 promoter in the plasmid pG1TT (31). To construct pSgs1Δ(1–107) for the expression of Sgs1 lacking the N-terminal 107 amino acid residues, a 300-base pair *Nco*I-*Nco*I restriction fragment was first removed from pRB126, a plasmid identical to pRB121 (31) but without the hexahistidine codons in pRB121. The *Bam*HI to *Xho*I segment containing the deleted region was then cloned into pRB136. To construct pSGS1Δ(1–283), an 800-base pair *Nco*I-*Eco*RI fragment within the *SGS1* coding region was deleted. This was accomplished by partial digestion of pRB126 with *Nco*I and complete digestion of the products with *Eco*RI; rejoining the ends of the appropriate fragments after filling-in by DNA polymerase I yielded the desired in-frame deletion. This deletion was then moved into pRB136 as described above.

Plasmids containing the *sgs1* deletions were obtained from *E. coli* transformants, and these were used to transform yeast strains AMR58 (*MATα top1::LEU2 sgs1–3::TRP1 leu2 trp1 ade2 his3 ura3*), a gift of R. Sternglanz (State University of New York at Stony Brook), and RB1 (*MATa top3::TRP1 sgs1::KAN leu2 trp1 his3 ura3*). Strain RB1 was constructed by deleting the *SGS1* gene in strain JCW253, a Δ*top3::TRP1* derivative of strain CH1585 (*MATa leu2-Δ1 trp1-Δ63 ura3–52 his3-Δ200*). A near-complete deletion of the *SGS1* gene in RB1 was achieved by replacing the *SGS1* segment between the *Afl*II and *Acc*I restriction sites with a *KAN* gene marker. Individual colonies harboring the various plasmids were selected and grown at 30 °C on agar plates containing uracil-dropout medium supplemented with 2% (w/v) glucose. To compare the growth of transformants, individual colonies were picked, streaked, and grown on YPD plates (AMR58 strain) or uracil-dropout medium supplemented with 2% (w/v) glucose (RB1 strain) at 30 °C for 2–3 days. The growth rates of various transformants of strain AMR58 in liquid culture were also measured by inoculating 5 ml of YPD and monitoring the absorbance of the culture at a wavelength of 600 nm.

RESULTS

Interaction between Sgs1 and Top3—Previous yeast two-hybrid assays suggest that Sgs1 and Top3 physically interact and that the region of Sgs1 responsible for this interaction lies within the first 500 amino acid residues of the protein (5). To test biochemically whether the two proteins directly contact each other, a set of plasmids was constructed for the expression of fusion proteins in which various N-terminal fragments of Sgs1 are joined to glutathione *S*-transferase (see Fig. 1).

Each of the overexpressed GST-Sgs1 fusion proteins was first bound to glutathione-Sepharose 4B beads, and the coated beads were then used in affinity chromatography of Top3. Initially, an extract of yeast cells expressing a normal level of Top3 was used. The amount of Top3 in the cell extract was apparently rather low, however, and no detectable amount of the protein was found in any of the fractions by Western blotting using anti-Top3 antibodies (data not shown). Extracts of yeast cells overexpressing a plasmid-borne *TOP3* gene (32) were therefore used in subsequent experiments. As shown in Fig. 2*A,* Top3 in these extracts was retained by glutathione-Sepharose 4B beads coated with the fusion protein GST-Sgs1(1–605) and was present in eluates containing 140 and 250 mM NaCl (Fig. 2*A, lanes g* and *h,* respectively). No Top3 was detectable in these eluates, however, when the same extracts were chromatographed on beads coated with GST (compare *lanes c* and *d* and to *lanes g* and *h*).

Significantly, although the particular preparation of rabbit antibodies raised against a yeast Top3 and *E. coli* malE fusion protein cross-reacted with a number of other proteins in the *S. cerevisiae* cell extracts (see Fig. 2*A, lanes a* and *e,* and Fig. 2*B, lanes a, d,* and *g*), only the band corresponding to Top3 was detectable after chromatography of the cell extracts over im-

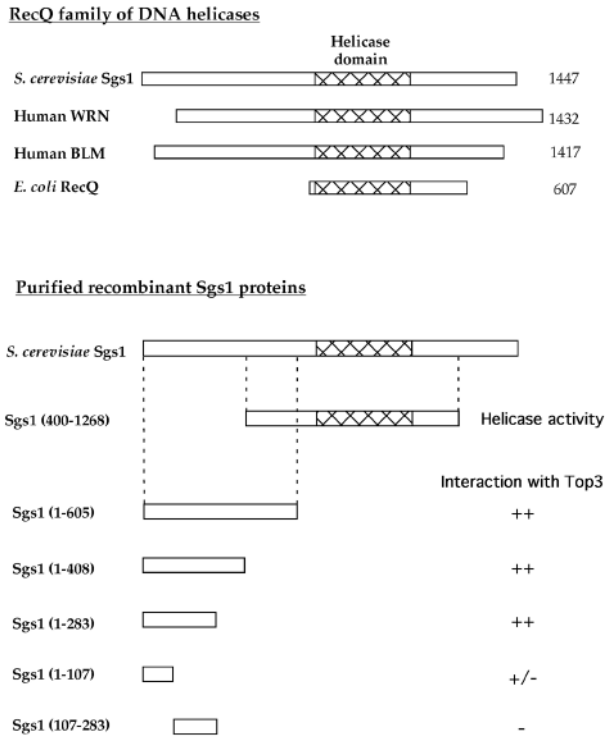

FIG. 1. **Summary of the various Sgs1 constructs used in this study.** In the *top panel,* several representative members of the RecQ helicase family are aligned. The *bottom panel* depicts fragment Sgs1(400–1268), which was previously used to study the helicase activity of Sgs1 (31), and various N-terminal fragments of Sgs1 used in the present study of interaction between Sgs1 and DNA topoisomerase III. The ability of Sgs1 fragments to interact with Top3 is indicated by ++ (interaction observed both in affinity chromatography and Top3-mediated DNA binding and relaxation assays), +/– (weak interaction not detected by affinity chromatography but detected by the other biochemical assays), or – (no interaction detected). The *cross-hatched* segments comprise the helicase domain, which includes the seven conserved helicase motifs of the proteins.

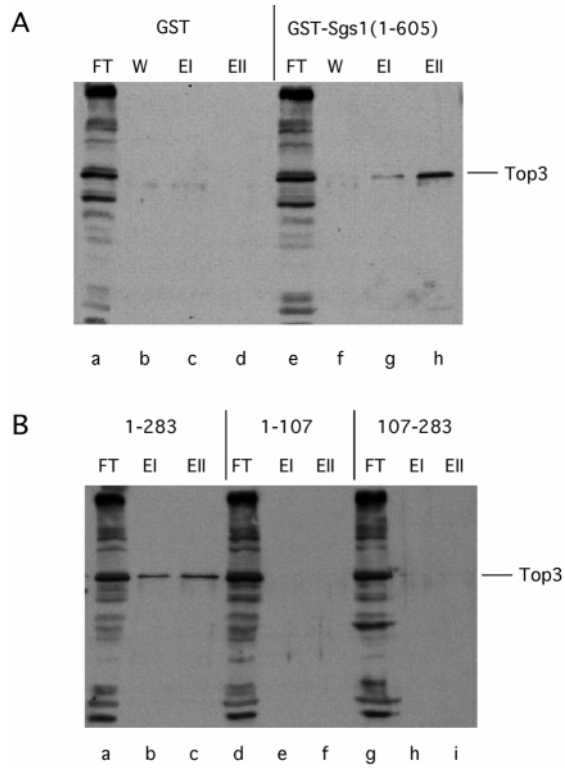

FIG. 2. **Affinity chromatography experiments demonstrating that the N-terminal region of Sgs1 interacts with Top3.** Extracts of *S. cerevisiae* cells overexpressing Top3 were passed over GST-Sgs1 fusion proteins immobilized on glutathione-Sepharose 4B beads, as described under "Experimental Procedures." Bound proteins were eluted off the beads in 200-μl washes, and 10 μl of each fraction was analyzed by Western blotting using anti-Top3 antibodies. *A,* fractions from chromatography on GST or GST-Sgs1(1–605) columns. *B,* fractions from chromatography on immobilized GST-Sgs1(1–283), GST-Sgs1(1–107), or GST-Sgs1(107–283). *FT,* flow-through; *W,* wash; *EI,* 140 mM NaCl eluate; *EII,* 250 mM NaCl eluate.

mobilized GST-Sgs1(1–605) (Fig. 2*A*). This observation suggests that the retention of Top3 on the GST-Sgs1(1–605) coated beads is specific. Thus, the affinity chromatography results are entirely consistent with previous data from yeast two-hybrid analysis and provide further evidence that the N-terminal region of Sgs1 directly contacts Top3.

To more precisely map the region of Sgs1 involved in this interaction, similar experiments were carried out with GST-Sgs1 fusion proteins containing shorter segments of Sgs1. The fusion proteins containing Sgs1(1–283) (Fig. 2*B, lanes a–c*) and Sgs1(1–408) (data not shown) interacted with Top3. However, when Sgs1(1–283) was further subdivided into fragments spanning amino acids 1–107 and 107–283, the resulting products GST-Sgs1(1–107) and GST-Sgs1(107–283) were less efficient than their parent in Top3 retention (Fig. 2*B, lanes e, f, h,* and *i*). These results suggest that interaction between yeast Sgs1 and Top3 involves mainly the N-terminal 1–283 amino acid residues of the helicase and that both the 1–107 and 107–283 regions probably contribute to this interaction.

Inhibition of Top3 Binding to DNA by N-terminal Fragments of Sgs1—Interaction between the N-terminal region of Sgs1 and DNA topoisomerase III was further demonstrated by the effects of various Sgs1 fragments on Top3 binding to single-stranded DNA. Electrophoretic mobility shift measurements were carried out to monitor the binding of Top3 to a radiolabeled DNA oligonucleotide 41 nucleotides in length. In the gel autoradiogram shown in Fig. 3*A,* a new band of the radiolabeled oligonucleotide, with a much reduced electrophoretic mobility relative to that of the free oligonucleotide, was detected in the presence of Top3 (compare the pattern shown in *lane a* with those shown in *lanes b* and *c*). At higher Top3 concentrations, an additional band with even lower mobility was also seen (Fig. 3*A, lane d*). These results are consistent with previous observations that Top3 has a high affinity for single-stranded DNA (32) and that the two radiolabeled species of reduced mobilities relative to the free oligomer presumably correspond to the oligonucleotide with 1 and 2 bound Top3 molecules. In contrast to Top3, GST-Sgs1 fusion proteins containing various Sgs1 N-terminal fragments effected no band shift in these assays (data not shown). The presence of GST-Sgs1(1–283) was found to be inhibitory, however, to the binding of Top3 to the oligonucleotide (see Fig. 3*A, lane j*). GST-Sgs1(1–600) was similar to GST-Sgs1(1–283) in reducing the binding of Top3 to the oligonucleotide (data not shown), but GST-Sgs1(1–107) or GST-Sgs1(107–283) was much less effective in this inhibition (Fig. 3*A, lanes e–h*; see also the results described below).

The effects of various GST-Sgs1 fusion proteins on the binding of Top3 to the oligonucleotide were also examined by gel electrophoresis after cross-linking the protein-oligonucleotide complexes with glutaraldehyde (36). In Fig. 3*B,* the dependence of the relative amount of Top3-bound oligonucleotide on the concentration of GST or a GST-Sgs1 fusion protein is displayed. GST-Sgs1(1–283) and the longer GST-Sgs1(1–605) inhibited Top3 binding to the oligonucleotide to a comparable degree, but GST and GST-Sgs1(107–283) showed little inhibitory effect. Significantly, GST-Sgs1(1–107) showed a moderate, but reproducible, inhibition of Top3 binding to the oligonucleotide. At a

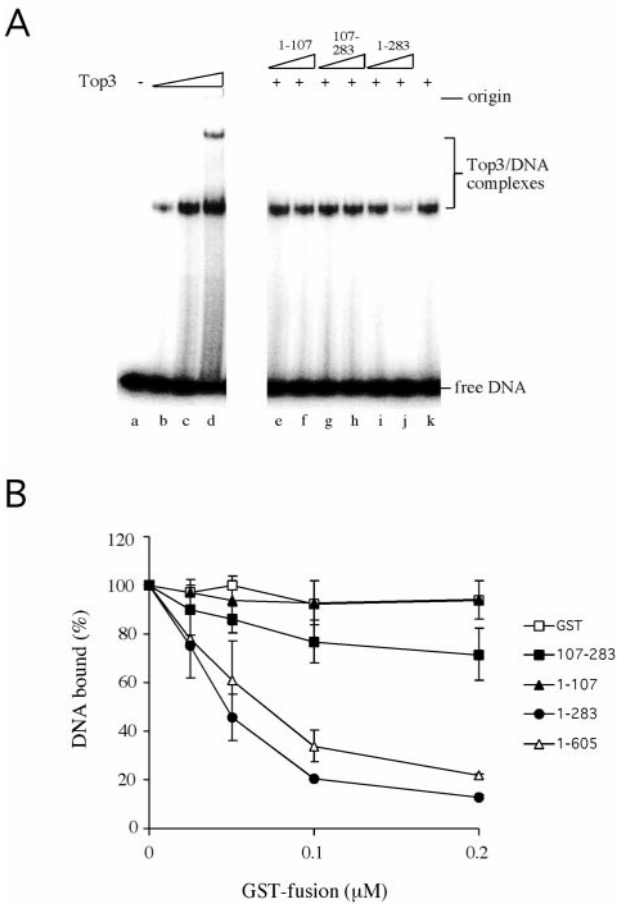

FIG. 3. **Inhibition of Top3 binding to single-stranded DNA by the GST-Sgs1 fusion proteins.** *A, lane a,* oligonucleotide only. In *lanes b–d,* increasing amounts of Top3 (15, 75, and 150 nM, respectively) were incubated with the oligonucleotide for 15 min, and the reaction mixtures were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel. In reaction mixtures analyzed in *lanes e–j,* 75 nM Top3 was preincubated with the fusion protein GST-Sgs1(1–107), GST-Sgs1(107–283), or GST-Sgs1(1–283), as indicated at the *top,* with each fusion protein present at either 20 nM (*lanes e, g,* and *i*) or 200 nM (*lanes f, h,* and *j*). 5′-^{32}P-Labeled oligonucleotide was then added, and the reaction mixtures were analyzed by electrophoresis after a 15-min incubation at room temperature. The *lane k* sample contained Top3 but no fusion protein. *B*, Top3 and GST-Sgs1 fusion proteins were incubated with radiolabeled oligonucleotide and then treated with glutaraldehyde to fix the protein-DNA complexes. Reactions were analyzed by electrophoresis in polyacrylamide gel, and the binding of Top3 to the oligonucleotide was quantitated by measuring the radioactivity of the slower migrating protein-DNA complexes. The amount of protein-bound DNA in the absence of any GST or GST-Sgs1 fusion protein is taken as 100% (approximately 10% of the oligonucleotide was bound by Top3 under these conditions). Each reaction mixture contained 20 nM Top3 protein and the indicated amount of GST or GST-Sgs1 fusion protein. Each *data point* shows the average of three experiments, and *error bars* indicate the standard deviation of the mean.

0.2 μM concentration of this fusion protein, for example, Top3 binding was reduced by 28%, compared with a 6% reduction for the same molar concentration of either GST-Sgs1(107–293) or GST alone. At a higher protein concentrations of 0.4 μM, GST-Sgs1(1–107) effected a 65% inhibition of DNA binding by Top3, whereas GST-Sgs1(107–293) and GST alone inhibited 26 and 23% of binding, respectively (data not shown). Thus the N-terminal domain of 107 residues of Sgs1 apparently retains the ability to interact with Top3, albeit less strongly relative to Sgs1(1–283).

To test whether the inhibition of Top3 binding to single-stranded DNA by a GST-Sgs1 fusion protein might reflect a property of the fusion protein rather than that of the Sgs1

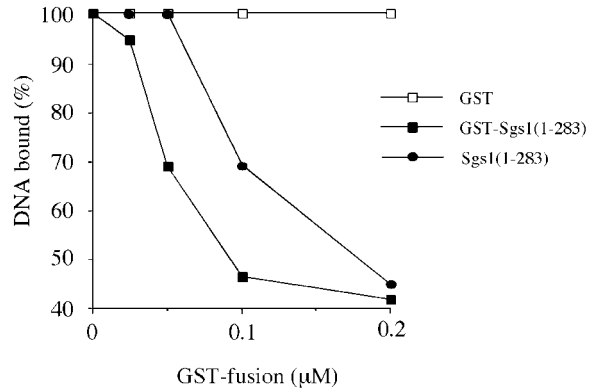

FIG. 4. **Comparison of the effects of Sgs1(1–283) and GST-Sgs1(1–283) fragments on Top3 binding to single-stranded DNA.** Reaction mixtures containing Top3 (20 nM) and various amounts of GST, GST-Sgs1(1–283), or Sgs1(1–283) were preincubated at 0 °C for 5 min prior to addition of a 41-nucleotide-long single-stranded DNA. After further incubation at 0 °C for 15 min, Top3-DNA complexes were fixed by cross-linking with glutaraldehyde and analyzed by electrophoresis in 6% polyacrylamide gel. The *data points* shown were from a single experiment.

domain in it, we overexpressed and purified a hexahistidine-tagged Sgs1(1–283) fragment without the GST domain. Fig. 4 shows that the presence of Sgs1(1–283) with a hexahistidine tag at its C terminus was also inhibitory to the binding of Top3 to the DNA oligomer. These results indicate that inhibition of Top3 binding to single-stranded DNA is mostly determined by direct interaction between Top3 and the N-terminal region of Sgs1.

Inhibition of Top3-catalyzed Relaxation of Negatively Supercoiled DNA by an N-terminal Fragment of Sgs1—The results described above indicate that fragments of Sgs1 containing the N-terminal 107 amino acid residues can interfere with Top3 binding to single-stranded DNA through a direct interaction between the Sgs1 fragments and Top3. To examine whether the same Sgs1 fragments also affect the catalytic activity of Top3, the relaxation of a negatively supercoiled DNA plasmid by Top3 in the presence and absence of the Sgs1 fragments was examined. As described previously (32), Top3 exhibited a weak relaxation activity with negatively supercoiled DNA (Fig. 5, *lane b*). However, in the presence of GST-Sgs1(1–283), this relaxation activity was inhibited (Fig. 5, compare *lane b* with *lanes c* and *d*). In contrast, GST-Sgs1(107–283) did not significantly affect the activity of Top3 (*lanes g* and *h*), whereas the presence of the GST-Sgs1(1–107) fragment produced only a marginal decrease in Top3 activity. These results indicate that the first 283 amino acid residues of Sgs1 can inhibit the Top3-mediated relaxation of a negatively supercoiled DNA molecule. Presumably, the weaker effect of the 1–107 domain of Sgs1 is due to its weaker interaction with Top3.

The N-terminal Region of Sgs1 Is Required for Sgs1 Function in Vivo—To test the functionality of the N-terminal region of Sgs1 *in vivo*, expression of various Sgs1 fragments was tested for their ability to complement *sgs1* in either a *top1* or a *top3* background. Previously, it was shown that a *sgs1 top1* strain grew slowly unless a functional *SGS1* gene was introduced (18). As shown in Fig. 6*A,* the presence of a plasmid expressing full-length *SGS1* gene from a plasmid-borne GAL1 promoter improved the growth of a *sgs1 top1* strain, whereas the introduction of a control plasmid without the *SGS1* insert did not. Significantly, deletion of the N-terminal region of the plasmid-borne *SGS1* abolished the ability of the plasmid to complement *sgs1* in the *top1* background. Thus, *sgs1 top1* cells harboring a plasmid expressing Sgs1 lacking the N-terminal 107 or 283 amino acid residues grew more slowly than cells expressing

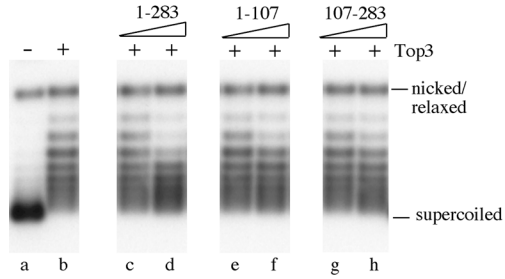

FIG. 5. **Relaxation of negatively supercoiled plasmid by Top3 in the presence of different GST-Sgs1 fusion proteins.** Top3 was present at 100 nM in all samples except the control run in *lane a*. The *lane b* sample contained Top3 but no fusion protein. In each of the remaining three pairs of lanes, the GST-Sgs1 fusion protein specified at the *top* was present at 25 nM (*left lane* of each pair) or 100 nM (*right lane* of each pair). Incubation with plasmid DNA was carried out at 30 °C for 30 min. Reactions (20 μl) were stopped and analyzed by electrophoresis in 0.8% agarose gel as described under "Experimental Procedures," and the resolved DNA bands were visualized by autoradiography after blot hybridization with a ^{32}P-labeled probe.

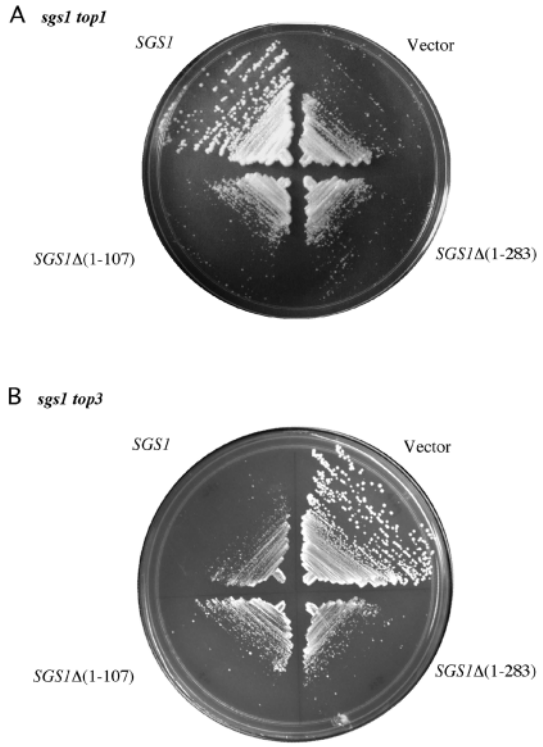

FIG. 6. **Role of the N-terminal region of Sgs1 *in vivo*.** Different deletions in the *SGS1* gene were constructed, and these were individually expressed from a plasmid-borne GAL1 gene promoter. *A,* individual colonies of transformants in a *sgs1 top1* strain AMR58 were picked and streaked on YPD plates, grown at 30 °C for 2 days, and photographed. *B,* colonies of transformants in a *sgs1 top3* strain RB1 were picked and streaked on uracil-dropout plates. Cells were grown and photographed after 2 days at 30 °C.

full-length Sgs1 (Fig. 6*A*). The growth rates of *sgs1 top1* cells harboring different plasmids were also compared by measuring their doubling times in liquid medium. Cells expressing a plasmid-borne full-length *SGS1* gene had a doubling time of 153 min, compared with 205 min for those harboring the control vector. Cells transformed with the plasmid expressing *SGS1*Δ(1–107) or *SGS1*Δ(1–283) also showed a long doubling time, of 200 or 203 min, respectively. These measurements demonstrate that deleting the N-terminal region of Sgs1 prevents its normal function in the *top1* background.

The effects of expressing a truncated Sgs1 protein on the growth of *sgs1 top3* cells were also examined. Previous studies showed that *top3* strains grew more slowly than their *TOP3*$^+$ parents but that this slow growth was suppressed by deletion of the *SGS1* gene (5). As expected, expression of full-length Sgs1 in *sgs1 top3* cells resulted in slower growth of the colonies (Fig. 6*B*). Deletion of the N-terminal region of Sgs1 did not affect the function of Sgs1 in this strain, as the growth of the double mutant was also slowed down by the expression of either *SGS1*Δ(1–107) or *SGS1*Δ(1–283) (Fig. 6*B*).

Taken together, the above results show that the N-terminal domain of Sgs1 protein is essential for the normal function of the protein in a *top1* but not *top3* mutant background. This finding is consistent with the notion that the N-terminal region of Sgs1 is important for its interaction with the Top3 protein; in the absence of Top3, deletion of this region would have no effect on Sgs1 function.

DISCUSSION

Several experimental approaches reported here demonstrate that the Sgs1 helicase directly interacts with yeast DNA topoisomerase III via the N-terminal domain of the helicase. Our results thus confirm and extend the initial observation from two-hybrid studies that Sgs1 and Top3 physically interact *in vivo* (5). Using affinity chromatography, we mapped the region of Sgs1 required for interaction with Top3 to within the first 283 amino acid residues of the helicase. Biochemical assays on the effects of various Sgs1 fragments on DNA binding and the relaxation of negatively supercoiled DNA by Top3 also revealed a significant interaction between Top3 and the first 107 amino acid residues of Sgs1. These results suggest that the N-terminal 107 amino acid residues of Sgs1 contain an important region for interaction with Top3.

To test whether physical interaction between Sgs1 and Top3 is significant for the function of these proteins in yeast, we expressed various N-terminal deletions of Sgs1 in cells of two genetic backgrounds. Deletions within the first 400 amino acid residues of Sgs1 are not expected to affect its helicase activity, because a recombinant Sgs1 protein containing amino acid residues 400–1268 exhibits an active helicase activity *in vitro* (31). A functional test of Sgs1Δ(1–107) was performed in *top3* mutant cells. Whereas deletion of the entire *SGS1* gene suppresses the slow growth and hyperrecombination phenotype of *top3* cells (5), deletion of just the N-terminal 107 amino acid residues of Sgs1 had no effect on the growth of *top3* cells. Thus, in a *top3* null genetic background, Sgs1 lacking the N-terminal 107 amino acid residues is functionally similar to intact Sgs1. This finding is consistent with the idea that the primary role of the N-terminal part of Sgs1 may be its interaction with DNA topoisomerase III.

A second series of experiments was performed in which various N-terminal deletions of Sgs1 were expressed in *top1 sgs1* cells. The ability of Sgs1 to suppress the slow growth phenotype of the double mutant appears to correlate with the presence of the N-terminal part of Sgs1. Thus, expressing full-length Sgs1 improved growth, whereas expressing Sgs1 lacking residues 1–107 did not. These results suggest that interaction between Sgs1 and Top3 is important in the suppression of the slow growth of *top1 sgs1* cells. In combination, the results of expressing Sgs1 truncations in both the *top1 sgs1* and *top3 sgs1* backgrounds are consistent with the biochemical data implicating a role for the first 107 amino acid residues of Sgs1 in its interaction with yeast DNA topoisomerase III. Weinstein and Rothstein[2] have also observed that deleting the N-terminal 82 amino acids of Sgs1 does not suppress the slow growth phenotype of *top3* cells but reduces the growth rate of *top1* cells. Interestingly, they also found that deleting this Top3-interact-

[2] J. Weinstein and R. Rothstein, personal communication.

ing domain of Sgs1 in an otherwise wild-type genetic background led to a slow growth phenotype similar to that produced by inactivating Top3.

Recently, Mullen *et al.* (37) examined the roles of both the N-terminal domain and the helicase activity of Sgs1 in the complementation of various *sgs1* phenotypes. They concluded that Sgs1 encodes an important function within its N-terminal region in addition to its helicase activity. Deletion of the N-terminal 158 amino acid residues of Sgs1, for example, was found to show hyper-recombination and slow growth phenotypes in excess of the null *sgs1* allele (37). Our, studies as well as those of Weinstein and Rothstein,[2] suggest that the primary role of the N-terminal region of Sgs1 is its interaction with Top3.

Genetic studies in the budding yeast *S. cerevisiae* (5, 17) and the fission yeast *S. pombe* (21, 22) indicate that the functional interaction between the RecQ helicase Sgs1/Rqh1 and DNA topoisomerase III has been conserved in the two widely diverged organisms. A functional conservation among RecQ helicases of yeast and multicellular eukaryotes has also been suggested. Expression of either human BLM or human WRN protein in the budding yeast, for example, was found to suppress the hyperrecombination phenotype of *sgs1* null mutations (7). However, the physiological effects of expressing BLM and WRN in mutant yeast cells are not identical. Only expression of the BLM protein slowed down the growth of a *sgs1 top3* strain and rescued the hypersensitivity of *sgs1* cells to hydroxyurea (7). Similarly, the BLM protein, but not the WRN protein, was recently shown to suppress the premature aging observed in yeast *sgs1* cells (38).

Physical interaction between BLM and human DNA topoisomerase IIIα has recently been reported (24). Two independent domains on BLM were shown to interact with the topoisomerase; one domain resides within the first 212 N-terminal amino acid residues of BLM, and a second domain at the extreme C-terminal region of BLM, composed of amino acid residues 1266–1416 (24). For the yeast Sgs1 and Top3 pair, interaction between Top3 and the C-terminal region of Sgs1 was not detected.[3] When both the N- and C-terminal Top3-interaction domains in BLM were deleted, expression of the resulting recombinant BLM protein in yeast *sgs1* cells induced slow growth (24). In light of the similarity between yeast *SGS1* and human BLM, it would be interesting to see whether inactivation of the murine BLM homologue might modulate the early embryonic lethality of a murine *top3*α null mutation (23).

The mechanism by which Sgs1 and Top3 act to suppress hyper-recombination remains unclear. Based on the genetic evidence, Sgs1 appeared to act upstream of Top3, and it is plausible that Sgs1 may be involved in the formation of a DNA substrate that requires Top3 for its processing (5). The particular substrates of Sgs1 and Top3 *in vivo* have not been identified, however. Based on the presence of a type IA DNA topoisomerase domain and a putative DNA helicase domain in the reverse gyrase of thermophiles that catalyzes the positive supercoiling of DNA, the possibility that an Sgs1-Top3 complex might act as a reverse gyrase was raised (5). Recent studies indicate, however, that reverse gyrase of thermophiles does not exhibit DNA helicase activity (39, 40). Furthermore, measurements of the linking numbers of plasmids in various yeast topoisomerase and *sgs1* mutants do not support the notion that Sgs1 and Top3 could effect DNA positive supercoiling.[4] Surprisingly, the helicase activity of Sgs1 also appears to be dispensable for some of its functions, as evidenced by the ability of truncated Sgs1 proteins lacking the helicase domain to complement the slow growth of *sgs1 top1* cells (37). The biochemical experiments described in the present work indicate that the N-terminal fragment of Sgs1 can inhibit Top3 activity. Both DNA binding and relaxation of a negatively supercoiled DNA substrate by Top3 were inhibited. Although inhibition of Top3 is consistent with the genetic data suggesting that Sgs1 acts upstream of Top3, experiments utilizing full-length Sgs1 protein are needed to determine whether intact Sgs1 functions to stimulate or inhibit Top3 activity.

Finally, despite the evidence linking Sgs1 and Top3 activities, there are a number of observations that suggest Sgs1 and Top3 can also function independently of one another. First, although deletion of *SGS1* suppresses all of the mitotic defects of *top3* mutants, it cannot completely suppress the sporulation defect of *top3* cells (17). In *S. pombe*, deletion of *rqh1* only partially suppressed the defect observed in *top3* cells (22). These findings suggest that at least in some circumstances, DNA topoisomerase III can function independently of Sgs1/Rqh1. Second, the overexpression of *E. coli* DNA topoisomerase I, a type IA enzyme homologous to yeast DNA topoisomerase III, has been shown to suppress the slow growth but not the hyperrecombination phenotype of *top3* cells (16). The sporulation defect of *S. cerevisiae top3* cells is also partially suppressed by the expression of the *E. coli* enzyme (17). It seems unlikely that *E. coli* DNA topoisomerase I acts via interaction with Sgs1, but rather, at least when overexpressed, a type IA DNA topoisomerase can function independently of Sgs1 in certain cellular processes.

In summary, there is now substantial evidence that Sgs1 and Top3 proteins interact physically and that this interaction is significant for a subset of their functions *in vivo*. The identification of the particular DNA and/or another molecular target(s) of these enzymes in the cell and the mechanism(s) by which they act to maintain genome integrity await further studies.

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