Association of yeast DNA topoisomerase III and Sgs1 DNA helicase: Studies of fusion proteins

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The Sgs1 protein of the budding yeast *Saccharomyces cerevisiae* is a member of the RecQ DNA helicase family that includes the human Bloom, Werner, and Rothmund–Thompson syndrome proteins. The N-terminal region outside the central DNA helicase core of Sgs1, particularly the part containing the first 100 amino acid resiudes of the 1,447-residue protein, is known to be functionally important and has been implicated in Sgs1–DNA topoisomerase III (Top3) interaction. We show in this work that the functionality of a truncated Sgs1 lacking its N-terminal 106 residues can be restored by replacing the truncated region with Top3. Fusion of Top3 to a mutant Sgs1 with a Val-29 to Glu substitution, which interferes with Sgs1–Top3 interaction, similarly restores the functionality of the mutant Sgs1(V29E) protein. The Top3-Sgs1(Δ1–106) and Top3-Sgs1(V29E) fusion proteins behave like wild-type Sgs1 in complementing several aspects of the *sgs1* phenotype, including the hypersensitivity of *sgs1* cells to methyl methanesulfonate and hydroxyurea. Complementation by the fusion proteins required both the topoisomerase activity of Top3 and the helicase activity of the Sgs1 polypeptide. These results suggest that the sole function of the N-terminal 106 amino acid residues of Sgs1 is for Top3 binding, and that the coordinated actions of Sgs1 and Top3 are important in cellular processes such as the processing of DNA after exposure of cells to DNA-damaging agents.

RecQ helicases | methyl methanesulfonate | hydroxyurea hypersensitivity | *top3* (Y355F) mutant

The RecQ family of DNA helicases has been implicated in the maintenance of genome stability (for reviews see refs. 1–4). Five RecQ homologues are known in humans. Mutations in two of the five have been linked to Bloom and Werner syndromes, and hence they were named the Blm and Wrn proteins, respectively (5, 6). A third RecQ homologue, RecQL4, has been implicated in the Rothmund–Thomson syndrome (7). All three syndromes exhibit chromosome instability and a predisposition to cancer; Werner and the Rothmund–Thomson syndromes are known also for signs of premature aging (for reviews see refs. 8 and 9). In the budding yeast *Saccharomyces cerevisiae*, there is only one member of the RecQ family, namely, the Sgs1 helicase encoded by the *SGS1* gene. Yeast cells lacking Sgs1 show an increase in mitotic recombination between repetitive sequences and sequences of imperfect homology (1, 10–13), elevated chromosome missegregation during mitosis and meiosis (14), and sensitivity to a number of DNA-damaging agents (1–3, 15–18). These findings suggest that Sgs1 is involved in processing DNA structures that are formed during recombination, particularly recombinational repair of heteroallelic sequences (1, 10, 13, 17, 18).

The involvement of Sgs1 in replication and transcription by RNA polymerase I, but not by RNA polymerases II and III, also was suggested by studies of a *sgs1* thermal-sensitive mutant in cells lacking a helicase encoded by the *SRS2* gene (19). DNA replication is severely impaired upon inactivation of both Sgs1 and Srs2 helicases, but this effect is alleviated by the introduction of an additional mutation in one of the genes that control homologous recombination, which include *RAD51*, *-52*, *-55*, and *-57* (18). A plausible explanation of these findings is that Sgs1 is involved in processing stalled replication forks through homologous recombination (18, 20–22): the restart of stalled forks may generate Holliday structures which are substrates for unwinding by Sgs1 (23).

Results obtained in both the budding yeast and the fission yeast *Schizosaccharomyces pombe* also have led to the suggestion that Sgs1 and its fission yeast homologue Rqh1 may be involved in S-phase checkpoint response (15, 24–26). Studies of Sgs1 synthetic lethals indicate that some of the functions of Sgs1 may overlap with other proteins. In addition to the Srs2 helicase (19, 27) and DNA-mismatch repair proteins (13), proteins encoded by six genes are essential in strains deficient in Sgs1 (28).

A number of proteins, including DNA topoisomerase III (Top3; ref. 10), DNA topoisomerase II (14), Rad16 (17), and Rad51 (29), are thought to physically interact with Sgs1. Interaction between Sgs1 and Rad53 is also implicated by their colocalization (24). Among these, the interaction between Sgs1 and Top3 seems particularly significant. The *SGS1* gene was identified initially in a screen for extragenic suppressors of the slow-growth phenotype of *top3* mutants (10). Mutations in *SGS1* also suppress the hyperrecombination phenotype of *top3* nulls, and partially suppress the sporulation defect of *top3* diploids (10, 30). By using a two-hybrid screen, Top3 was shown to interact with Sgs1 fragments containing the N-terminal 500 residues of the 1,447-residue full-length protein (10), of which the segment spanning residues 652–996 constitutes a functional DNA helicase *in vitro* (31). Subsequent analyses with the yeast two-hybrid assay, as well as biochemical experiments, have provided strong evidence that the N-terminal domain of Sgs1, especially the part comprising the first 100 amino acid residues, directly interacts with Top3 (21, 27, 32, 33; J. D. Weinstein and R. Rothstein, personal communications). The association of a RecQ-type helicase and a type IA DNA topoisomerase seems to be conserved among eukaryotic organisms. In *S. pombe,* mutant cells lacking Top3 undergo only a few cell divisions before dying, and this lethality is partially suppressed by inactivating Rqh1 (34, 35). In addition, human Blm was shown to physically interact with Top3α (36), one of the two known human Top3 isozymes, and human RecQ5 was reported to coimmunoprecipitate with DNA topoisomerase IIIα as well as IIIβ (37).

There is strong evidence that the physical association between Sgs1 and Top3 is functionally important. For example, the sensitivity of *sgs1* cells to the alkylating agent methyl methanesulfonate (MMS) and the synthetic lethality of *sgs1 slx4* cells are compensated for by expressing full-length Sgs1 but not by expressing an N-terminal truncation of it that retains the helicase activity of the protein but not its affinity for Top3 (32). The physiological effects of deleting the putative Top3-interacting domain are often complex, however, in cells of different genetic backgrounds (21, 27, 32, 33). Whereas expressing a plasmid-borne intact *SGS1* gene in slow-growing *sgs1 top1* double mutant

Abbreviations: MMS, methyl methanesulfonate; HU, hydroxyurea; Top3, DNA topoisomerase III.

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cells restored growth to a normal rate, the same cells expressing an Sgs1 truncation lacking the N-terminal 158 residues grew more poorly than those harboring the empty vector (32). Such a hypermorphic or toxic effect of the Sgs1(Δ1–158) truncation also was observed in an *sgs1 top3* genetic background (32). It has been suggested that the N-terminal domain of Sgs1 may play roles in addition to its interaction with Top3 (32).

To study the role of the N-terminal region of Sgs1 further, we have examined the effects of expressing a chimeric protein in which Top3 is fused to a truncated Sgs1 lacking the N-terminal 106 amino acid residues. We find that the fusion protein behaves like full-length Sgs1 in several physiological assays. Similarly, fusion of Top3 to a mutant Sgs1 with a Val-29 to Glu substitution, which interferes with Sgs1–Top3 interaction, restores the functionality of the mutant Sgs1(V29E) protein. These results suggest that the sole function of the N-terminal region comprising the first 106 residues of Sgs1 is for the binding of Top3, and that the requirement of this domain is alleviated by the fusion of the two polypeptides.

Materials and Methods

Yeast Strains. The Δ*sgs1,* Δ*sgs1* Δ*top1*, and Δ*sgs1* Δ*top3* strains used in this work were RB103 (*MAT***a** *sgs1-3*::*TRP1 leu2-3,112 trp1-1 ade2-1 his3-11,15 ura3-1*), AMR58 (*MATα top1*::*LEU2 sgs1-3*::*TRP1 leu2-3,112 trp1-1 ade2-1 his3-11,15 ura3*), and RB1 (*MAT***a** *top3*::*TRP1 sgs1*::*KAN leu2-3,112 trp1-1 his3-11,15 ura3-1*), respectively. RB1 has been reported (21), and AMR58 was the kind gift of R. Sternglanz (State Univ. of New York at Stony Brook). Strain RB103 was derived by crossing AMR58 and W303-1A (*MAT***a** *leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 can1-100*), a strain kindly provided by R. Rothstein (Columbia Univ., New York); tetrads were dissected and a Trp$^+$ and Leu$^-$ colony was selected. The *top3* and *top1 top3* strains used were JCW253 (*MAT***a** *top3*::*TRP1 leu2 trp1Δ63, ura3-52, his3Δ200*) (21) and W2084-6C (*MAT***a** *top1*:*HIS3 top3*:*TRP1 leu2-3,112, his3-11,15 ura3-1, trp1-1, ade2-1, can1-100*), respectively; the latter strain also was provided by R. Rothstein.

Expression Plasmids. A YEplac195 (38) derivative pRB655 was constructed by multiple cycles of cloning, using restriction fragments derived from pRB121 (31) and a PCR-amplified fragment containing the promoter and upstream region of *SGS1*, for the expression of Sgs1 from its native promoter. In pRB655, nucleotides 1–4341 of wild-type *SGS1* were placed in between an upstream *Xba*I site (added to the *SGS1* sequence by PCR), and *Xho*I, *Sma*I, and *Kpn*I sites downstream (introduced during various cloning steps). Several pRB655 derivatives expressing mutant Sgs1 proteins also were constructed: pRB655(K706A) expresses Sgs1 with a mutation K706A known to inactivate its helicase activity (39), and pRB655(Δ11–105) expresses Sgs1 missing amino acid residues 11–105 of the wild-type protein. Both mutations were introduced by PCR. For the K706A mutation, the primer pair 5′-GAGTC-TACGGGATCCGTAACCATGGTGACGAAGCCGTC-3′ and 5′-CACCACTGCAGGAAGTTGATAGCAAAG(GCTAG-C)ACCACCCCCTGTTGG-3′ were used. The underlined hexameric sequences in these primers mark a *Hpa*I and a *Pst*I site, and the sequence in parentheses indicates an *Nhe*I site created by changing the lysine codon 706 (AAA) to an alanine codon (GCT). The *Hpa*I to *Pst*I fragment from the PCR products was first used to replace the corresponding fragment in pRB121, and a *Hin*dIII to *Xho*I fragment from the resulting plasmid was moved into pRB655 to give the final product pRB655(K706A). To delete codons 11–105 of *SGS1*, a 5′ primer 5′-CCGCGGC-TTA(9)-AGA(10)-CCC(106)-ATG(107)-GTT(108)-GAT(109)-ATA(110)-CC-3′ and a 3′ primer 5′-GGCGGCGTCGACTAACTCATCTTCTTCTCT-3′ were used. The underlined sequence CTTAAG in the 5′ primer marks an *Afl*II site, and the numbers in parentheses indicate the codon numbers in wild-type *SGS1*; the sequence of the 3′ primer corresponds to a region downstream of an *Aat*II site in *SGS1*. The *Afl*II to *Ata*II fragment from the PCR product was subcloned and the region containing the deletion was moved into pRB655 to give pRB655(Δ11–105).

The construction of pRK500 that expresses Top3 from a phosphate-responsive promoter *PHO5* was described (40). A derivative that expresses Top3(Y355F), in which the active-site Tyr-Y355 of wild-type Top3 is changed to a Phe, was constructed by site-directed mutagenesis with reagents from a commercial supplier (CLONTECH) and an oligonucleotide 5′-ATC-AGT-CTC-TGT-TCT-TGG-TTT-CGA-TAT-GAA-CCC-CTT-TTG-3′ for the desired codon change (the phenylalanine codon in the primer is underlined).

Plasmids expressing Top3-Sgs1 fusion proteins from the PHO5 promoter were constructed as follows. The *PHO5*-linked *TOP3* coding region was derived from pRK500 as a *Bam*HI to *Nco*I fragment, after converting the last three *TOP3* codons TCC-ATG-TAA to TCC-ATG-GAA to eliminate the stop codon and create a *Nco*I site (underlined). Two restriction fragments, one containing the entire *SGS1* coding region and the other lacking the first 106 codons of it, were obtained from pRB121 by partial digestion with *Nco*I and complete digestion with *Xho*I. Tandem insertion of the *Bam*HI–*Nco*I Top3 fragment and one of the *Nco*I to *Xho*I fragments in between the *Bam*HI and *Sal*I sites of YEplac195 (38) yielded pRB701, which expresses Top3-Sgs1 (full-length Sgs1 fused to the C terminus of Top3), or pRB702, which expresses Top3-Sgs1(Δ1–106) (Sgs1 lacking residues 1–106 fused to the C terminus of Top3). The plasmid pRB703, which expresses Top3-Sgs1(V29E) with a mutation changing Val-29 of Sgs1 to a Glu, was similarly constructed by the use of a restriction fragment derived from a plasmid-borne *sgs1* segment carrying the mutation (see the section below). From the pRB701, pRB702, and pRB703 trio, two sets of plasmids were derived by replacing appropriate restriction fragments of the trio with the corresponding ones from mutant *top3* or *sgs1* clones. Plasmids pRB711, pRB712, and pRB713 corresponded respectively to pRB701, pRB702, and pRB703 with the Y355F mutation in the *TOP3* coding region, and pRB721, pRB722, and pRB723 corresponded respectively to pRB701, pRB702, and pRB703 with the K706A mutation in the *SGS1* coding region.

Screening for Sgs1 Mutants Defective in Interaction with Top3. A yeast two-hybrid system was used to screen for mutated Sgs1 with reduced affinity for Top3. The entire TOP3 coding region was copied by PCR by using the primers 5′-GGGCCCGAATTCAT-GAAAGTGCTATGTGTC-3′ and 5′-GGGCCGGGATCCT-TACATGGATGCCTTGAC-3′, placing an *Eco*RI site immediately before the ATG initiation codon and a *Bam*HI site immediately after the TAA termination codon. Insertion of the PCR product into the two-hybrid vector pGAD424 (CLONTECH) as an *Eco*RI–*Bam*HI fragment yielded pGAD424-Top3, in which Top3 is fused to the C terminus of the yeast Gal4 protein transactivator domain. Because previous experiments indicated that the N-terminal 108 amino acid residues of Sgs1 play an important role in the interaction between Sgs1 and Top3 (21), mutagenic PCR was carried out to generate a mutant pool within the first 108 codons of *SGS1*. A mutagenesis kit (CLONTECH) and the primer pair 5′-GGCCGGCGGATCCATATGGTT-GAAGCCGTC-3′ and 5′-GGCGGCGTCGACAACCAT-GGGTACGTCAGC-3′ were used, under reaction conditions for the introduction of approximately one base pair change in each DNA molecule. The PCR product was digested with *Bam*HI and *Sal*I and inserted into pFBL23 (41) to give a library expressing a pool of mutagenized Sgs1(1–108), each fused to the N terminus of the DNA-binding domain of the *Escherichia coli* protein LexA. This library was used to transform yeast strain L40 (*MAT***a**/*α ade2 his3 trp1 leu2 URA3*::*(lexAop)*$_8$*-lacZ LYS2*::*(lexAop)*$_4$*-HIS3* (42) bearing pGAD424-Top3, and colonies on Leu- and Trp-dropout plates were replica-plated onto Ura$^-$, Leu$^-$, Trp$^-$, and His$^-$ agar with and without 15 mM

3-aminotriazole. Colonies that failed to grow on the replica plates after 2–3 days at 30°C were picked from the master plates, and DNA samples were prepared from individual colonies. The Sgs1 inserts in selected samples were each amplified by PCR with primers 5′-GACCTGCAATTATTAATCTTTTGTTTCC-3′ and 5′-GCCAGCGCCTTCAGATGTTCTTCAGCC-3′ and were sequenced to identify the individual mutations.

Approximately 1,500 Sgs1 mutants were screened for their ability to interact with Top3, and 40 potential candidates were sequenced to identify the mutations. Four different point mutations, W15R, L16S, D25V, and V29E, were identified; the rest were nonsense or frameshift mutations. These point mutations were introduced into pRB655 and the resulting constructs were used to transform Δ*sgs1,* Δ*sgs1* Δ*top1*, and Δ*sgs1* Δ*top3* strains. Expressing three of the four mutant proteins in these cells showed phenotypic changes similar to those elicited by the expression of wild-type Sgs1 in the same cells. Expression of Sgs1(V29E) in these cells effected, however, phenotypic changes nearly identical to those effected by expressing Sgs1(Δ1–106) in the same cells. Thus, only the V29E point mutation of Sgs1 seemed to inhibit its interaction with Top3 strongly, and this point mutation was selected for use in subsequent experiments.

MMS and Hydroxyurea (HU) Sensitivity. MMS or HU was added to yeast extract/peptone/dextrose (YPD) agar shortly before pouring. The final concentrations of MMS and HU were 1.4 mM and 100 mM, respectively. Yeast cells were collected from individual colonies on plates and resuspended in water. The resuspended samples were adjusted to give the same apparent optical density readings, and serial dilutions of each were spotted onto YPD, MMS, and HU plates and grown at 30°C for 2 days.

Results

Top3-Sgs1 Fusion Proteins. To test whether the sole function of the N-terminal region of yeast Sgs1 helicase comprising the first 100 residues of the protein is for the binding of Top3, we resorted to the study of fusion proteins in which the Sgs1 and Top3 polypeptides are joined in tandem. We reasoned that if joining the two polypeptides created an active Sgs1/Top3 complex, then the N-terminal 100 residues of Sgs1 might be dispensable in a fusion protein if their only function is for Top3 binding.

In the sections below, we report studies of three fusion proteins: Top3-Sgs1, in which the N terminus of Sgs1 is joined to the C terminus of Top3, Top3-Sgs1(Δ1–106), a derivative of Top3-Sgs1 lacking amino acid residues 1–106 of Sgs1, and Top3-Sgs1(V29E), another derivative with a point mutation V29E in Sgs1 (see the sketches in Fig. 1). The V29E mutation replacing Val-29 of Sgs1 by a Glu was originally identified in a yeast two-hybrid screen for single amino acid changes (within the first 106 residues of Sgs1) that interfered with Sgs1/Top3 interaction (see *Materials and Methods*). Expression of Sgs1(V29E) in *sgs1*, *sgs1 top1*, and *sgs1 top3* strains was subsequently shown to effect phenotypical changes that were similar to the expression of Sgs1(Δ1–106), a deletion mutant incapable of interacting with Top3 (see below).

Fusion Proteins With or Without the Top3-Interacting Domain of Sgs1 Can Complement the Hypersensitivity of *sgs1* Cells to DNA-Damaging Agents. Several tests were carried out to assess the functionality of the fusion proteins. Yeast *sgs1* nulls are hypersensitive to MMS and to HU (15, 32). As shown in Fig. 2*A Left*, the MMS hypersensitivity of *sgs1* cells is complemented by the expression of full-length Sgs1 (row 1), but not by the expression of Sgs1(K706A) (row 3), which lacks the helicase activity (38), or Sgs1(Δ1–106) (row 4), which lacks the domain necessary for Top3 binding (21, 33). The presence of the expression vector without insert similarly had no effect (Fig. 2*A*, row 2). These results are in complete agreement with the known requirement of the helicase activity as well as the Top3-interacting domain of Sgs1 for MMS tolerance (32). The expression of Sgs1(V29E) also failed to complement MMS hypersensitivity (Fig. 2*B*, row 3), demonstrating again the importance of the interaction between Sgs1 and Top3 in MMS tolerance.

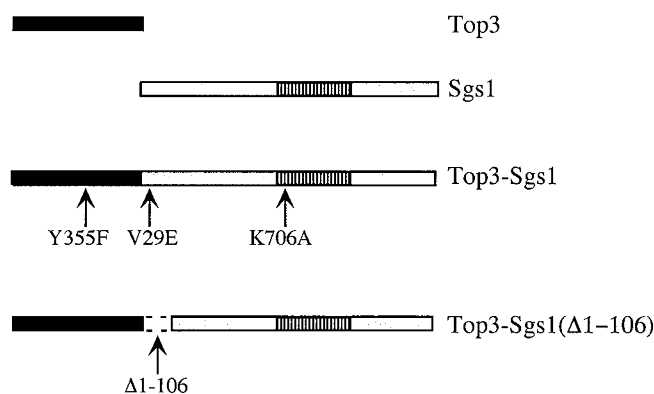


Fig. 1. Schematic representations of Top3, Sgs1, and the Top3-Sgs1 fusion proteins used in this study. Arrows indicate the positions of point mutations Top3(Y355F), Sgs1(V29E), and Sgs1(K706A) that abolish Top3 activity, inhibit interaction between Sgs1 and Top3, and inactivate the helicase activity of Sgs1, respectively. The helicase domain in Sgs1 is indicated by a hatched box. In the fusion protein Top3-Sgs1(Δ1–106), the C terminus of Top3 is joined to residue 107 of Sgs1.

Significantly, whereas the N-terminal segment containing the first 106 residues of Sgs1 is normally required for MMS tolerance, it is dispensable in the Top3-Sgs1 fusion protein. Mutant *sgs1* cells expressing Top3-Sgs1(Δ1–106) showed a level of MMS sensitivity similar to the same cells expressing full-length Sgs1 (compare rows 1 and 5 in Fig. 2*A Left*). The observed complementation by expression of the fusion protein cannot be attributed to the increase in DNA topoisomerase activity associated with the fusion protein; similar expression of Top3 in *sgs1* cells had little effect on MMS hypersensitivity (Fig. 2*B*, row 7). Expression of Top3 fused to the N terminus of intact Sgs1 also increased the resistance of *sgs1* cells to MMS, but to a level below that elicited by the expression of Sgs1 or Top3-Sgs1(Δ1–106) (compare row 3 in Fig. 2*C Left* with rows 1 and 5 in Fig. 2*A Left*). This reduced effect probably reflects a larger distortion in the Top3/Sgs1 complex when the C terminus of Top3 is joined to Sgs1 with an N-terminal domain interacting with Top3.

Similar to MMS hypersensitivity, the hypersensitivity of *sgs1* cells to the S-phase inhibitor HU is alleviated also by the expression of either full-length Sgs1 or Top3-Sgs1(Δ1–106), but not by the expression of Sgs1(Δ1–106), Top3, or Sgs1(K706A) (Fig. 2*A Right*). Expression of Top3-Sgs1, in which Top3 is fused to the N terminus of full-length Sgs1, was again found to complement the HU hypersensitivity of *sgs1* cells, although not as well as the expression of Top3-Sgs1(Δ1–106) (Fig. 2*C Right*, row 3).

The above experiments suggest that the formation of a functional complex between Sgs1 and Top3 is important for resistance to MMS and HU, and that the formation of this complex normally involves the N-terminal region of Sgs1. Deleting the N-terminal 106 residues of Sgs1, or introducing a V29E point mutation into it, weakens the interaction with Top3, and thus interferes with complex formation between the two proteins *in vivo*. Physically joining the N terminus of Sgs1 to the C terminus of Top3 presumably overcomes the translational entropy loss associated with complex formation between two separate proteins, thus making the N-terminal 106 residues of Sgs1 dispensable.

Both the Helicase and Topoisomerase Activities of the Top3-Sgs1 Fusion Proteins Are Required for MMS and HU Tolerance in *sgs1* Cells. The notion that the Top3-Sgs1(Δ1–106) fusion protein acts as an integral entity to complement the MMS and HU hypersensitivity

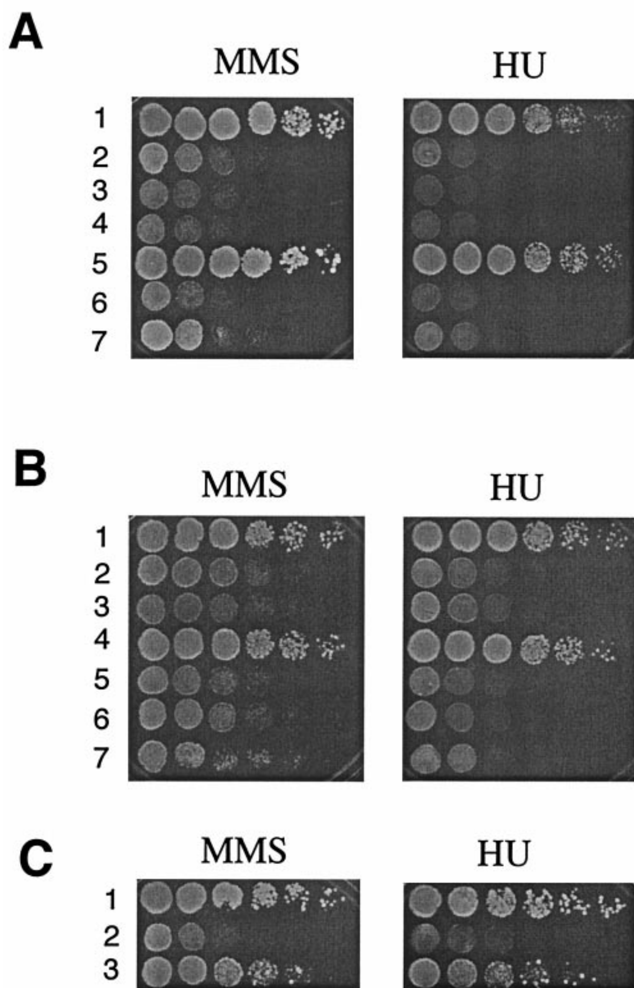


Fig. 2. Complementation of the hypersensitivity of *sgs1* cells to MMS and HU by the expression of various proteins. A strain RB103 (*MATα sgs1-3*::*TRP1 leu2 trp1 ade2 his3 ura3*) deficient in Sgs1 was transformed with various plasmids, and individual transformants were picked, resuspended at equal concentrations, and plated at 5-fold serial dilutions on YPD plates containing either 1.4 mM MMS or 100 mM HU. Cells were grown for 2 days at 30°C and photographed. (*A*) Row 2 shows serial dilutions of cells harboring the empty vector, and the other rows show cells expressing plasmid-borne genes encoding the following proteins: row 1, Sgs1; row 3, Sgs1(K706A); row 4, Sgs1(Δ1–106); row 5, Top3-Sgs1(Δ1–106); row 6, Top3(Y355F)-Sgs1(Δ1–106); row 7, Top3-Sgs1(Δ1–106; K706A). (*B*) The proteins expressed from the plasmid-borne genes were as follows: row 1, full-length Sgs1; row 3, Sgs1(V29E); row 4, Top3-Sgs1(V29E); row 5, Top3(Y355F)-Sgs1(V29E); row 6, Top3-Sgs1(V29E; K706A); row 7, Top3. Row 2 again shows serial dilutions of cells harboring the empty vector. (*C*) The first two rows correspond to the first two rows of *A* and *B*, and the third row shows serial dilutions of cells expressing Top3 fused to the N terminus of full-length Sgs1.

of *sgs1* cells is supported by the finding that both the helicase and topoisomerase activity of the fusion protein are required, although the cells are *TOP3*$^{+}$ and, thus, are expected to express a normal level of wild-type Top3. Introduction of the helicase mutation K706A into Top3-Sgs1(Δ1–106) abolishes the ability of the fusion protein to complement the MMS and HU sensitivity of *sgs1* cells (Fig. 2*A*, compare rows 5 and 7). Similarly, when the active-site residue Tyr-355 of yeast Top3 was replaced by a Phe, the resulting fusion protein Top3(Y355F)-Sgs1(Δ1–106) could no longer complement the MMS and HU hypersensitivity of *sgs1* cells (compare rows 5 and 6 of Fig. 2*A*). Introducing the helicase mutation K706A or the topoisomerase mutation Y355F into the fusion protein Top3-Sgs1(V29E) similarly abolished the ability of the fusion protein to restore MMS and HU tolerance (compare row 4 with rows 5 and 6 in Fig. 2*B*).

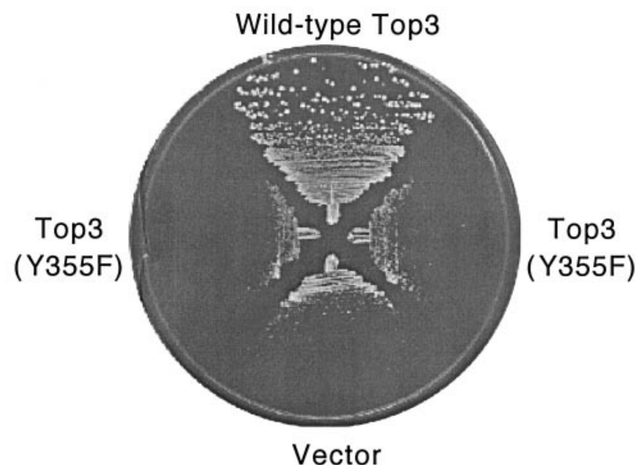
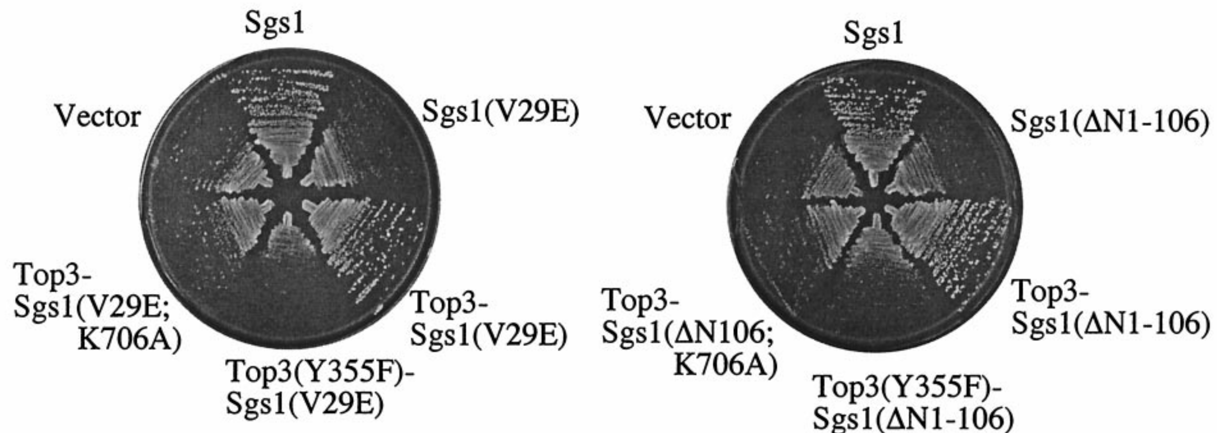


Fig. 3. The topoisomerase activity of Top3 is required for complementation of the slow-growth phenotype of *top1 top3* cells. Mutant cells were transformed with constructs expressing wild-type Top3, Top3 containing a point mutation at the active site (Y355F), or the empty vector control. Colonies were picked and restreaked on YPD plates, and plates were incubated at 30°C for 3 days.

Generally, it has been assumed that complementation of the phenotype of *top3* nulls by the expression of Top3 requires the topoisomerase activity of the enzyme, but a direct experimental test of this notion had been lacking. Therefore, expression of Top3 or Top3(Y355F) in *top3* and *top1 top3* cells was carried out. In either case, expression of Top3, but not Top3(Y355F), complemented the slow-growth phenotype; the results with *top1 top3* cells are shown in Fig. 3. Whereas *top1 top3* cells expressing a plasmid-borne *TOP3* exhibited normal growth, the same cells expressing a plasmid-borne *top3*(Y355F) grew poorly; growth seemed to be even slower than the same cells harboring the expression vector without an insert, suggesting that expression of the mutant Top3 might be toxic to the cell.

Effects of Top3-Sgs1(Δ1–106) or Top3-Sgs1(V29E) Expression on Growth of *top1 sgs1* and *sgs1* Cells. In agreement with previous results (32), the expression of Sgs1(Δ1–106) in *sgs1 top1* or *sgs1* cells was found to impede rather than to help growth (Fig. 4). In *sgs1 top1* cells, which grew more slowly than *SGS1*$^{+}$ *top1* cells, growth was reduced further by the presence of a plasmid expressing Sgs1(Δ1–106) or Sgs1(V29E), but not by the presence of the empty vector (Fig. 4*A*). The result was even more striking in *sgs1* cells. Whereas *sgs1* cells grew at about the same rate as *SGS1*$^{+}$ cells, the same cells expressing Sgs1(Δ1–106) or Sgs1(V29E) grew very poorly (Fig. 4*B*).

In contrast to the above results, the Top3-interacting domain of Sgs1 in a Top3-Sgs1 fusion protein was no longer critical for cell growth in *sgs1 top1* or *sgs1* cells expressing the fusion protein. Rather than hindering cell growth, expression of either Top3-Sgs1(Δ1–106) or Top3-Sgs1(V29E) fully complemented the slow-growth phenotype of *sgs1 top1* cells (Fig. 4*A*). Significantly, both the topoisomerase and the helicase activity of the fusion proteins are important in this complementation. Replacing either Tyr-355 of the Top3 polypeptide by a Phe or Lys-706 of the Sgs1 polypeptide by an Ala inactivated the fusion proteins. In particular, the growth of *sgs1 top1* cells expressing Top3(Y355F)-Sgs1(Δ1–106) or Top3(Y355F)-Sgs1(V29E) was reduced rather than increased relative to that of the same cells harboring the empty vector (Fig. 4*A*). In an *sgs1* single mutant, the hypermorphic effect of Sgs1(Δ1–106) or Sgs1(V29E) also was masked in the corresponding fusion protein, such that the expression of either Top3-Sgs1(Δ1–106) or



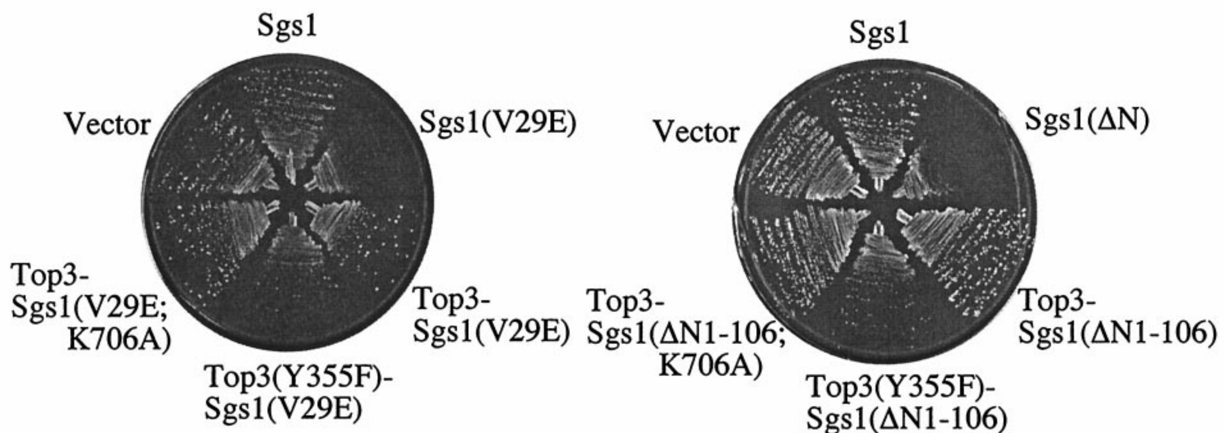


Fig. 4. Complementation of *sgs1* and *sgs1 top1* strains by the expression of Top3-Sgs1 fusion proteins. The *sgs1* (RB103) and *sgs1 top1* (AMR58) strains were transformed with plasmids expressing the indicated Top3-Sgs1 fusion proteins. Colonies were picked and restreaked and grown at 30°C for 2–3 days. (*A*) Strain *sgs1 top1*. (*B*) Strain *sgs1*.

Top3-Sgs1(V29E) showed no deleterious effect on growth (Fig. 4*B*). Here, the inactivation of the helicase activity of the fusion protein by introducing the K706A mutation had little effect, but the topoisomerase activity of the fusion proteins again was found to be critical. Thus, the growth of *sgs1* cells was severely hindered by the expression of Top3(Y355F)-Sgs1(Δ1–106) or Top3(Y355F)-Sgs1(V29E) (Fig. 4*B*).

Discussion

A major conclusion of the present work is that the Sgs1 domain comprising amino acid residues 1–106 is functionally important because of its interaction with Top3. The functional importance of the N-terminal region of Sgs1 has been well recognized (21, 27, 32, 33). The hypersensitivity to MMS or HU of *sgs1* cells, for example, is complemented by the expression of full-length Sgs1 but not by the expression of Sgs1(Δ1–106) (this work), or other N-terminal truncations (32). Nonetheless, this hypersensitivity is complemented fully by the expression of Top3-Sgs1(Δ1–106), a fusion protein in which Top3 replaces residues 1–106 of Sgs1 (see Fig. 2*A*). These results indicate that when the Top3 and Sgs1 polypeptides are joined in tandem to facilitate complex formation between the two, residues 1–106 of Sgs1 are dispensable. Therefore, in the native Sgs1 protein, residues 1–106 are unlikely to have any function other than the binding of Top3. This inference is strengthened further by similar results from experiments with the point mutant Sgs1(V29E), which is apparently incapable of interacting with Top3 without physically linking the two polypeptides.

A corollary of the above conclusion is that the hypermorphic phenotype of *sgs1* or *sgs1 top1* cells expressing Sgs1 within the N-terminal 106 residues is the result of expressing an Sgs1 protein unable to interact with Top3. In the present work, expression of Sgs1(Δ1–106) or Sgs1(V29E) in *sgs1* or *sgs1 top1* cells was found to impede growth, which is in agreement with previous findings that the growth of *sgs1* or *sgs1 top1* cells is retarded by the expression of Sgs1 lacking the N-terminal 158 amino acid residues (32) or a point mutant Sgs1(L9F) that does not properly interact with Top3 (27). When the Top3 and Sgs1 polypeptides are fused in tandem, however, neither the deletion

of the N-terminal 106 residues of Sgs1 nor the introduction of the V29E mutation into Sgs1 results in growth retardation of *sgs1* or *sgs1 top1* cells. Together with the MMS- and HU-hypersensitivity results discussed earlier, the results summarized here strongly suggest that growth inhibition caused by the deletion of the N-terminal segments of Sgs1 is mediated through the association between Sgs1 and Top3.

A key issue in the study of Sgs1 and Top3 is the molecular characteristics of their intracellular substrates. The results reported in this and other articles (10, 21, 27, 33) strongly suggest that the two proteins mainly function as a complex. We speculate that the Sgs1 component of the complex has a key role in directing the complex to its proper cellular substrates. In contrast to *E. coli* DNA topoisomerase I, which binds preferentially to junctions of double- and single-stranded DNA (43, 44), yeast Top3 by itself is highly specific for single-stranded DNA but shows little additional substrate specificity (40). This difference in substrate specificity between the two type IA DNA topoisomerases can be attributed to several DNA-binding domains that are absent from the yeast enzyme but are present in the C-terminal region of the bacterial enzyme (45). Sgs1, on the other hand, has been shown to bind preferentially to junctions of double- and single-stranded DNA, double-stranded DNA with very short single-stranded gaps, three-way and four-way DNA junctions (46), as well as G–G paired DNA (47). Thus, the association of Top3 with Sgs1 may direct the complex to various intracellular substrates, such as junctions of double- and single-stranded DNA, junctions of multiple duplex DNA, and perhaps, as well, damaged duplex DNA that Top3 by itself cannot readily access. It is of interest that the N terminus of human Blm protein seems to be required for targeting Top3α to distinct nuclear locations (48).

The postulated substrate specificity of these proteins is consistent with the observation that the toxicity of Sgs1 lacking the N-terminal Top3-interacting domain is manifested in *sgs1* or *sgs1 top1* cells but not in $SGS1^+$ or $SGS1^+$ *top1* cells. Presumably, so long as the Top3/Sgs1 complex is present, it can act efficiently at the appropriate intracellular substrates and thus prevent their access by Sgs1 helicase not associated with Top3. With the construction of physiologically functional Top3-Sgs1 fusion proteins, the postulated substrate specificity of the Top3/Sgs1 complex should be more easily testable.

Our experiments with the *top3*(Y355F) mutation have provided evidence that the DNA breakage and rejoining activity of Top3, or at least the DNA-breakage activity, is essential for the function of the Top3/Sgs1 complex. Replacement of the active-site tyrosine by phenylalanine, either in a Top3-Sgs1 fusion protein or in Top3 itself, inactivates the protein. The molecular role of Top3 is often thought of in terms of a decatenase that allows the untwining of duplex DNA, but the enzyme might act also in the rewinding of DNA strands separated by the helicase associated with it. Topoisomerase-mediated rewinding of separated, complementary DNA strands can be viewed as a special case of topoisomerase-mediated relaxation of negatively supercoiled DNA.

Finally, we believe that it is significant that both the helicase and topoisomerase activities of the Top3-Sgs1 fusion proteins are needed to complement the MMS and HU hypersensitivity of *sgs1* cells and the slow growth of *sgs1 top1* cells. In wild-type yeast cells, the Top3/Sgs1 complex probably is involved in the repair of DNA damage caused by these and other agents. Whereas a number of phenotypes of *top3* nulls are suppressed by *sgs1* nulls, hypersensitivity of *top3* nulls to MMS and HU is not suppressed by mutating *SGS1* (K. Kwan, R.J.B., and J.C.W., unpublished results; see also figure 1 in ref. 33).

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