Synergic and Opposing Activities of Thermophilic RecQ-like Helicase and Topoisomerase 3 Proteins in Holliday Junction Processing and Replication Fork Stabilization

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Background: RecQ helicases and topoisomerase 3 enzymes play essential functions in all DNA activities, and their malfunctioning is associated with genomic instability.

Results: A RecQ-like helicase and topoisomerase 3 from thermophilic archaea interact physically and functionally.

Conclusion: The thermophilic enzymes show synergic and antagonistic activities on different DNA substrates.

Significance: The results suggest a novel mechanism of modulation of RecQ-like helicases by topoisomerase 3 enzymes.

RecQ family helicases and topoisomerase 3 enzymes form evolutionary conserved complexes that play essential functions in DNA replication, recombination, and repair, and in vitro, show coordinate activities on model recombination and replication intermediates. Malfunctioning of these complexes in humans is associated with genomic instability and cancer-prone syndromes. Although both RecQ-like and topoisomerase 3 enzymes are present in archaea, only a few of them have been studied, and no information about their functional interaction is available. We tested the combined activities of the RecQ-like helicase, Hel112, and the topoisomerase 3, SsTop3, from the thermophilic archaean Sulfolobus solfataricus. Hel112 showed coordinate DNA unwinding and annealing activities, a feature shared by eukaryotic RecQ homologs, which resulted in processing of synthetic Holliday junctions and stabilization of model replication forks. SsTop3 catalyzed DNA relaxation and annealing. When assayed in combination, SsTop3 inhibited the Hel112 helicase activity on Holliday junctions and stimulated formation and stabilization of such structures. In contrast, Hel112 did not affect the SsTop3 DNA relaxation activity. RecQ-topoisomerase 3 complexes show structural similarity with the thermophile-specific enzyme reverse gyrase, which catalyzes positive supercoiling of DNA and was suggested to play a role in genome stability at high temperature. Despite such similarity and the high temperature of reaction, the SsTop3-Hel112 complex does not induce positive supercoiling and is thus likely to play different roles. We propose that the interplay between Hel112 and SsTop3 might regulate the equilibrium between recombination and anti-recombination activities at replication forks.

Helicases and topoisomerases are essential enzymes playing fundamental roles in DNA activities. Genetic and biochemical studies demonstrated functional, and in some cases also physical, interaction between Type IA topoisomerase 3 (Top3) enzymes and helicases of the RecQ family, including human and Drosophila BLM-TopoIIIα, yeast Sgs1-Top3, and Escherichia coli RecQ-Top3. These enzymes are involved in DNA replication, recombination, repair, and chromosome segregation, and their malfunctioning may cause genome instability, premature aging, infertility, and cancer (for recent reviews, see Refs. 1–5). At the biochemical level, RecQ-Top3 enzymes induce coordinate DNA topoisomerase, ATPase, DNA unwinding, and DNA annealing and act on particular DNA structures to modify their topological linkages. Depending on the substrate, these combined activities may result in DNA catenation/decatenation, dissolution of double Holliday junctions, resolution of replication and recombination intermediates, restart of stalled replication forks, DNA damage repair, and solution of telomere replication problems (6–14). A few helicases with RecQ-like activity have been characterized from several archaean strains (15–18). Type IA topoisomerases are highly conserved in organisms from the three domains of life and relax negative torsional stress (19–21).

RecQ-Top3 complexes show structural and functional similarity with reverse gyrase, a peculiar enzyme specific to thermophilic archaea and bacteria, consisting of fused SF2 helicase-like and topoisomerase IA domains, which induces positive supercoiling and plays a role in genome stability in organisms living at high temperature (22–26). The analogies between RecQ-Top3 complexes and reverse gyrase led Plank and Hsieh (27) to propose the term “helicase-appended topoisomerases.” Several models have been proposed to explain the complex mechanisms utilized by these molecular machines, all including the switch between DNA unwinding and rewinding steps. In the first step, double-strand (ds) DNA is unwound by the helicase action, and single-strand (ss) DNA regions are exposed, facili-
Cooperation of Thermophilic Helicase and Topoisomerase

EXPERIMENTAL PROCEDURES

Cloning of E. coli recQ and S. solfataricus topA —The E. coli recQ gene was amplified from E. coli K12 genomic DNA using the oligonucleotides R-5’ and R-3’ (see supplemental Table 1). The oligonucleotides match the 5’- and the 3’-terminal ends of the coding sequence with the addition of a BamHI site at the 5’-end and an XhoI site at the 3’-end. The BamHI/XhoI fragment was cloned in PET29a in-frame with a sequence coding for a histidine tag at its C terminus, producing Pet-29a-RecQ. The S. solfataricus topA gene (coding for SsTop3) was cloned without any tag by using the procedure described previously for the two reverse gyrase (30).

Proteins — All chromatographic separations were performed on AKTA FPLC systems (GE Healthcare, Buckinghamshire, UK); protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad), and purity was assessed by SDS-PAGE. All proteins were diluted in RG buffer (20 mM phosphate buffer, pH 6.5, 150 mM NaCl, 20% glycerol). Before purification, all cell extracts were extensively digested with DNase, and the absence of contaminant nucleic acids in purified protein preparations was always assessed by ethidium bromide-stained agarose gel electrophoresis. Recombinant His-tagged S. solfataricus Hel112 was purified as described previously (18), using only the monomeric fraction, endowed with both helicase and annealing activity. Recombinant S. solfataricus SSB was purified from E. coli transformed with plasmid pET28c-SSB (provided by M. F. White, University of St. Andrews, Fife, Scotland, UK) using the procedure described previously (31). Recombinant S. solfataricus SsTop3 was expressed and purified as described previously for the two reverse gyrase (30). Recombinant His-tagged E. coli topoisomerase 3 was purified as described previously (26). The RecQ protein was overexpressed in E. coli BL21-AI strain from Pet-29a-RecQ. Cultures were grown at 37 °C in 3 liters of Luria-Bertani (LB) medium supplemented with 50 mg/ml kanamycin until A600 was at 0.5, induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and 0.2% arabinose, and incubated overnight at 37 °C. Cells were harvested by centrifugation and resuspended in 30 ml of resuspension buffer (20 mM Tris-HCl, pH 7.5, 1.2 M NaCl, 1 mM EDTA, 1 mM DTT, 10 mM β-mercaptoethanol), incubated at 37 °C for 30 min with lysozyme (1 mg/g of wet cells), broken by freeze-thawing and sonication, and incubated with Benzonase (25 units/g of wet cells) in the presence of PMSF (1 mM). The lysate was clarified, and streptomycin was added to a final concentration of 3%, centrifuged, and applied to a phenyl-Sepharose column (GE Healthcare) after the addition of 0.8 M NH4SO4. Fractions were analyzed by SDS-PAGE and Western blotting with the anti-His antibody (GE Healthcare); positive fractions were pooled, loaded on a nickel-Sepharose column (GE Healthcare, HisTrap, 1 ml), and eluted with a linear gradient (0–1 M) of imidazole. Positive fractions analyzed by SDS-PAGE were pooled and stored at 20 °C with the addition of 20% glycerol. Activity was checked by helicase assay.

Pulldown Assay — His-tagged Hel112 (5 μg) was incubated at 4 °C for 1 h without or with SsTop3 (2 μg) in a buffer containing 10 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazole. The mixture was then combined with 50 μl of nickel Ni-NTA-agarose slurry (pre-equilibrated in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM imidazole, and 5 μg of pancreatic ribonuclease to saturate nonspecific binding). As a negative control, SsTop3 was incubated under the same conditions with His-tagged E. coli glucosamine kinase. After 3 h, the agarose beads were collected by centrifugation at 500 × g for 1 min and washed five times at room temperature in 500 μl of wash buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 5 mM imidazole). Finally, 50 μl of SDS-PAGE loading buffer was added to the samples, which were then boiled at 100 °C for 5 min and separated on an 8% SDS-polyacrylamide gel. The gel was silver-stained, and the protein band at the SsTop3 molecular weight was excised from the gel and identified by electrospray ionization mass spectrometry.

Far Western Analysis — Appropriate amounts of SsTop3 were subjected to SDS-PAGE and transferred to Hybond-P PVDF membrane (GE Healthcare). All subsequent steps were performed at 4 °C. The membrane was incubated twice in denaturation buffer (6 mM guanidine HCl in phosphate-buffered saline (PBS)) for 10 min, followed by six 10-min incubations in serial dilutions (1:1) of denaturation buffer supplemented with 1 mM DTT. The membranes were blocked in PBS containing 5% powdered milk, 0.1% Tween 20 for 1 h before being incubated for 2 h with either recombinant Hel112 (0.8 mg/ml) or BSA, for controls, in PBS supplemented with 0.25% powdered milk, 0.1% Tween 20, 1 mM DTT. Membranes were washed four times for 10 min in PBS containing 0.1% Tween 20, 0.25% powdered milk. The second wash contained 0.0001% glutaraldehyde. Western analysis was then performed to detect the presence of Hel112 using the anti-His as primary antibody and the ECL Plus system (GE Healthcare).

DNA Substrates — Oligonucleotides, either unmodified or with Cy5 and Cy3 modifications, were purchased from PRIMM (Milan, Italy) and listed in supplemental Table 1. The oligonu-
FIGURE 1. A, sequence comparison of selected Type IA topoisomerases. Schematic diagrams derived from protein BLAST sequence alignments are reported. Conserved motives are indicated by white boxes. TOPRIM superfamily, topoisomerase-primase domain; TopIA superfamily, DNA topoisomerase IA DNA-binding, ATP-binding, and catalytic domain. Hatched boxes indicate putative nucleotide binding sites. The triangle indicates the catalytic site of SsTop3. BLAST alignment scores are indicated by gray-scale code as depicted at the bottom; numbers in parentheses indicate the query coverage percentage. S. cerevisiae, Saccharomyces cerevisiae; H. sapiens, Homo sapiens; RG-Cter, C-terminal domain of reverse gyrase. B, DNA relaxation assay. pQE31 plasmid (final concentration 0.3 nM) was incubated for the indicated time spans at 75 °C with SsTop3 (10 nM) without (upper panel) or with S. solfataricus SSB (100 nM, lower panel). The right panel shows the plasmid incubated for 50 min under the same conditions with no protein or with SSB (100 nM). In all samples, total protein concentration was adjusted adding the required amounts of BSA. Samples were analyzed by mono-dimensional agarose gel electrophoresis. The gels are representative of at least three independent experiments. C, DNA binding property of SsTop3. Increasing amounts of protein were used in the presence of the indicated DNA molecules (40 nM). Plots of the shifted DNA versus the protein concentration are shown; values are mean ± S.E. of three independent experiments. D, SsTop3 does not process HJs. Synthetic HJ (40 nM) was incubated at 55 °C for 30 min with the following proteins: lanes 1 and 3, SsTop3 (150 nM); lanes 2 and 4, SsTop3 (300 nM); lane 5, SSB (150 nM); lane 6, SsTop3 (300 nM) and SSB, (150 nM). 5 mM ATP was added to lanes 3 and 4. E, SsTop3 annealing activity. Oligonucleotides A1, A2, A3, and A4 (40 nM each) were incubated at 55 °C for 30 min without (lane 1) or with increasing concentrations of SsTop3 (lane 2, 150 nM; lane 3, 300 nM; lane 4, 600 nM). F, quantification of the annealing activity. Averaged results (± S.E.) from three independent experiments are shown. G, the same substrates reported in E were incubated without (lane 1) or with EcTop3 (300 nM). Scissors indicate the EcTop3 cleavage products.
ucleotide A1, labeled with the Cy5 fluorophore at its 5’-end, was used as single-strand DNA substrate in DNA binding assay. HJ and Y-shaped fork were prepared by annealing oligonucleotides in the appropriate combinations: HJ, A1-A2-A3-A4; Y-shaped fork, A1-A2 as described previously (26). The fork regression substrate contained a 21-base oligonucleotide as the leading daughter strand and was prepared as described (32), with the difference that two oligonucleotides were labeled with different fluorophores (i.e. 70 lead-Cy5 labeled and 30 lag-Cy3 labeled), whereas the 70 lag and the 21 lead were unlabeled.

**DNA Topoisomerase Assays**—Assays were performed and analyzed by either one-dimensional or two-dimensional agarose gel electrophoresis as reported (33), using PQE31 plasmid (Qiagen) as substrate.

**DNA Helicase Assay**—DNA substrates (40 nM) were incubated in HJ buffer (2.5 mM Tris-HCl, pH 8.0, 0.25 mM β-mercaptoethanol, 5 mM NaAc, 0.5 mM MgCl₂) at the indicated temperatures for 30 min without or with different amounts of the indicated proteins. ATP (5 mM) was added where indicated. To minimize variations among samples within each experiment, a single mix with all common components was set up; in both negative controls and samples, total buffer concentration was kept constant by correcting with the appropriate amount of RG buffer. Reactions were terminated by adding 5× stop solution (0.5% SDS, 40 mM EDTA, 0.5 mg/ml proteinase K, 20% glycerol), immediately loaded on 8% polyacrylamide gel containing 0.1% SDS and run in 0.5× Tris-borate-EDTA buffer at 150 V/cm. Reaction substrates and products were analyzed as described previously (26) by gel imaging on a VersaDoc 4000™ (Bio-Rad) using the preset laser excitation and emission setting for the Cy5 fluorophore. The relative percentage of each DNA species in each reaction was determined by Quantity One software and used to calculate the corresponding absolute amount (expressed in nanomoles).

**Strand-pairing Assay**—Reaction mixtures (10 μl) contained HJ buffer and complementary synthetic oligonucleotides (A1-A2-A3-A4) at a concentration of 40 nM each. Proteins were added to the mixture, and samples were incubated for 30 min at 55 °C in a heated-top PCR machine to prevent evaporation. Reactions were stopped by the addition of 5× stop solution. Samples were subjected to gel electrophoresis as described for the DNA helicase assay. Reaction substrates and products were analyzed by gel imaging as described above.

**Strand Exchange Assay**—Reaction mixtures (10 μl) contained HJ buffer, synthetic HJ (40 nM), and single-strand synthetic oligonucleotide (A2-mod; 40 nM) labeled with the Cy3 fluorophore at its 3’ terminus. Proteins were added to the mixture, and the samples were incubated for 30 min at 55 °C. Reactions were stopped by the addition of 5× stop solution. Samples were subjected to gel electrophoresis as described for the DNA helicase assay. Reaction substrates and products were analyzed by gel imaging on a VersaDoc 4000™ (Bio-Rad) using the preset laser excitation and emission setting for Cy5 and Cy3 fluorophores as described previously (26).

**Fork Regression Assay**—The substrate (20 nM) was preincubated in HJ buffer (supplemented with 5 mM ATP if needed) for
5 min at 4 °C without or with the indicated enzymes and then transferred to 55 °C for the indicated time spans. Reactions were stopped by the addition of 500 μl stop solution, and the samples were subjected to gel electrophoresis as described for the DNA helicase assay. Reaction substrates and products were analyzed and quantified by gel imaging on a VersaDoc 4000™ (Bio-Rad) as described before.

ATPase Assay—ATPase assay reaction mixture (10 μl) contained HJ buffer, 20 μM [γ-32P]ATP (0.5–1 μCi), 20 nM HJ, and the indicated amounts of enzymes. Total protein concentration was adjusted with BSA. Incubations were performed for 30 min at the indicated temperatures in a heated-up PCR machine to prevent evaporation and stopped on ice. 1 μl aliquots of each mixture were spotted on a polyethyleneimine-cellulose thin layer plate (Macherey-Nagel, Duren, Germany) and developed in 0.5 M LiCl and 1 M formic acid. The amounts of [γ-32P]ATP hydrolyzed to [32P]orthophosphate were quantified using a PhosphorImager (Amersham Biosciences).

FIGURE 2. A, sequence comparison of selected helicases. Schematic diagrams derived from protein BLAST sequence alignments are reported. Conserved motives are indicated by white boxes. DEXDc, DEAD-like helicase superfamily domain containing the ATP-binding region; HELICc, helicase superfamily C-terminal domain; DEAD-assoc sf, DEAD-associated superfamily domain, often found with the other conserved helicase domains; S. cerevisiae, Saccharomyces cerevisiae; H. sapiens, Homo sapiens; Nter, N-terminal domain. BLAST alignment scores are indicated by gray-scale code as depicted at the bottom; numbers in parentheses indicate the query coverage percentage. B, DNA binding property of Hel112. Increasing amounts of Hel112 were used in the presence of the indicated DNA substrates. Plots of the shifted DNA versus the protein concentration are shown; values are the mean ± S.E. of three independent experiments. C, HJ processing by Hel112. HJ (40 nM) was incubated at 55 °C for 30 min without (lanes 5 and 6) or with different concentrations of Hel112 (lane 1, 35 nM; lane 2, 75 nM; lanes 3 and 7, 150 nM; lanes 4 and 8, 300 nM); 5 mM ATP was added to lanes 1–5. D, quantification of the Hel112 unwinding activity on HJ. Values are the mean ± S.E. of three independent experiments. E, effect of S. solfataricus SSB on HJ unwinding by E. coli RecQ. HJ (40 nM) was incubated at 55 °C for 30 min in the presence of 5 mM ATP without (lane 4) or with 150 nM RecQ (lanes 1–3). Increasing concentrations of SSB were added as indicated (lane 2, 75 nM; lane 3, 150 nM). F, effect of SSB on HJ processing by Hel112. HJ (40 nM) was incubated at 55 °C for 30 min in the presence of 5 mM ATP without (lane 7) or with 300 nM Hel112 (lanes 1–4). Increasing concentrations of SSB were added as indicated (lanes 2 and 5, 75 nM; lanes 3 and 6, 150 nM; lanes 4 and 7, 300 nM).
RESULTS

*SsTop3 DNA Relaxation and Annealing Activity*—Besides the C-terminal domain of reverse gyrase (34), *S. solfataricus* encodes only one Type IA topoisomerase, namely *SsTop3*, showing high sequence similarity with mesophilic members of this family, including those from humans, yeast, and *E. coli* (Fig. 1A). This protein induces relaxation of negative supercoils (28), but also ssDNA annealing (29), and inactivation of the corresponding gene in the related *Sulfolobus islandicus* strain induced chromosome instability and defects in the coordination between replication and segregation (35). No data on functional or physical interactors of *SsTop3* are available. Recombinant *SsTop3* purified from *E. coli* overexpressing strains (supplemental Fig. S1A) showed ATP-independent relaxation of negatively supercoiled DNA at 75 °C (Fig. 1B, upper panel), a reaction typically performed by mesophilic topoisomerase 3 members at 37–55 °C. These latter are known to be stimulated by ssDNA-binding proteins, which stabilize ssDNA, facilitating topoisomerase binding and preventing reannealing of denatured products (36); however, similar data are lacking for *SsTop3*. We thus tested the effect of the *S. solfataricus* ssDNA-binding protein, SSB, (31) on *SsTop3* activity. As shown in Fig. 1B (lower panel), SSB added up to 10-fold molar excess did not modify significantly the efficiency or the kinetics of the *SsTop3*
DNA relaxation activity. Similar results were obtained when samples were analyzed by two-dimensional agarose gel electrophoresis (supplemental Fig. S3). Like mesophilic Type IA topoisomerases, SsTop3 binds ssDNA with higher affinity as compared with dsDNA (29) (Fig. 1C). Thus, the lack of stimulation by SSB suggests that ssDNA regions required for SsTop3 binding are stably exposed in the negatively supercoiled substrate at the high reaction temperature, even in the absence of SSB.

We extended the analysis of binding efficiency to mixed and structured substrates, such as Y-shaped forks and synthetic HJs, and found that the enzyme binds both, albeit less efficiently as compared with ssDNA (Fig. 1C). In functional assays, the enzyme showed no activity on the HJ at 55 °C (Fig. 1D), and starting from a mixture of complementary ss oligonucleotides and forks, the enzyme induced efficient formation of HJ (Fig. 1, E and F). To test whether production of HJ by oligonucleotide pairing is a general property of topoisomerase 3 enzymes, we tested the E. coli Top3 protein, which is proficient in DNA relaxation at 55 °C (26). Assayed under the same conditions as SsTop3, this enzyme did not induce ss oligonucleotide annealing (Fig. 1G), suggesting that this is a peculiarity of the thermophilic topoisomerase.

Hel112 Activity on Model Recombination and Replication Intermediates—Bona fide homologs of RecQ helicases are absent from archaeal genomes, although ORFs potentially coding for SF2 helicases, showing sequence motifs shared by the RecQ family, are commonly found. In S. solfataricus, three such
proteins have been characterized, namely the reverse gyrase N-terminal domain, Hel308/Hjm, and Hel112 (Fig. 2A and supplemental Fig. S2). Although the N-terminal domain is an ATPase, but not a processive helicase (34), both Hel112 and Hel308/Hjm were reported to induce duplex unwinding (15–18), but also ssDNA annealing (17, 18), a feature shared by several eu-karyotic RecQ homologs (2, 5).

Most RecQ helicases are known to bind and process model replication and recombination intermediates, such as synthetic forks, flaps, and HJ. Previous data showed that preferred Hel112 binding substrates are ssDNA and forks, whereas dsDNA is bound with much lower affinity; the enzyme unwinds forked and 3’-ss tailed DNA structures, but not blunt-ended duplexes or bubble-containing molecules (18). We found Hel112 to bind the synthetic HJ with efficiency comparable with its preferred substrates, fork and ssDNA (Fig. 2B), suggesting that, despite the obvious absence of ssDNA, at least at the temperatures used for binding (25–37 °C), some structural feature of the HJ makes it a good substrate for Hel112 binding. At 55 °C, the protein was able to process the HJ in an ATP-dependent reaction (Fig. 2, C and D); the main products of the Hel112 reaction on the HJ were Y-shaped forks, whereas only a limited amount of ssDNA was occasionally observed. Several mesophilic RecQ family helicases show the ability to unwind synthetic HJs, but in most cases, their reactions proceed up to production of ssDNA and are stimulated by ssDNA-binding proteins (37–40). We reasoned that this difference might be due to spontaneous reannealing of the ssDNA products at 55 °C. We could test directly this hypothesis using the E. coli RecQ protein, which we found to be relatively stable at 55 °C, as testified by its ATPase and helicase activities (supplemental Fig. S4). When assayed under the same conditions used for Hel112, RecQ was able to unwind the HJ up to ssDNA, and the addition of S. solfataricus SSB or E. coli SSB greatly stimulated the reaction (Fig. 2E and data not shown, respectively); thus, ssDNA is stable under the reaction conditions used. In contrast, SSB did not stimulate the Hel112-catalyzed processing of HJ, nor induce accumulation of ssDNA (Fig. 2F). We conclude that in the Hel112 reaction, the final reaction products are Y-shaped forks, whereas ssDNA is never produced; alternatively, the protein might stimulate rapid annealing of ssDNA formed, preventing SSB from binding. Indeed, pairing of oligonucleotides forming dsDNA, forks, or bubbles has been shown for Hel112 at 75 °C (18), and Fig. 3 (A and B) shows that at 55 °C, the helicase also induced efficient ATP-independent annealing of oligonucleotides forming the HJ. Interestingly, in the presence of ATP, Hel112 induced unwinding of a Y-shaped fork at lower protein concentrations, whereas higher concentrations switched the reaction from unwinding to annealing (Fig. 3, C and D). Taken together, these results indicate that in the processing of synthetic HJ, Hel112 uses its unwinding and annealing activities to produce Y-shaped forks.

Although the E. coli RecQ protein did not promote ssDNA annealing at either 37 °C or 55 °C (data not shown), some eu-karyotic RecQ proteins show both unwinding and annealing activities and use them to perform a number of in vitro reactions, including strand exchange (41, 42). Although De Felice et al. (18) showed that Hel112 is unable to perform strand exchange through three-way junction intermediates, we decided to test this activity, adapting the experimental design used for BLM and WRN (42) (Fig. 4A). Hel112 was incubated in the presence of ATP with the Cy5-labeled HJ and different amounts of one of the HJ arms labeled with the Cy3 fluorophore, allowing us to follow its fate during the reaction. As shown in Figs. 4B and 7D, in the absence of any protein, a small amount of the added Cy3-labeled oligonucleotide was swapped with the unlabeled arm in the fork; in the presence of Hel112, the strand exchange was very efficient as significant amounts of Cy3-containing Y-shaped forks were produced. Similar experiments led to the suggestion that BLM and WRN catalyze strand annealing in concert with unwinding, resulting in coordinated strand exchange (42). Analogously, our data suggest that that Hel112 might unwind the fork, whereas it simultaneously anneals the complementary Cy3-labeled strand. However, we cannot rule out the possibility that the observed strand exchange is due to either Hel112 activity, i.e. unwinding (providing greater levels of the complementary ss oligonucleotides), or annealing of the available ss oligonucleotides. In any case, the strand exchange reaction of Hel112 is highly efficient as a 0.5:1 donor: acceptor strand ratio was sufficient to observe the reaction.

FIGURE 4. Strand exchange activity of Hel112. A, scheme of the experiment. B, HJ (40 nM) was incubated at 55 °C for 30 min without (lanes 5–8) or with 300 nM Hel112 (lanes 1 and 4). Increasing concentrations of the A2-mod oligonucleotide (Cy3Oligo) were added where indicated (lanes 2 and 6, 5 nM; lanes 3 and 7, 10 nM; lanes 4 and 8, 20 nM). The same gel was scanned under different excitation/emission conditions to visualize the Cy5 (upper panel) and Cy3 (lower panel) fluorophores, respectively, as described under “Experimental Procedures.” For quantification, see Fig. 7D. Cy3Oligo, Cy3-labeled oligonucleotide.
Coordinate unwinding and annealing activities are exploited by helicases to catalyze regression of replication forks. We tested this hypothesis for Hel112 using a model replication fork substrate that is entirely double-strand, apart from an ssDNA region of 11 bases on the leading arm and a 5-bp bubble (31) (Fig. 5A); thus, it is not the substrate for Hel112-catalyzed unwinding because the protein needs a 3’-ss tail for this reaction. On this substrate, fork regression by the WRN and BLM proteins induced the formation of the two parental and daughter duplexes. In contrast, the E. coli RecQ protein did not regress the fork; rather, it catalyzed forward unwinding to generate partial duplexes and ss products (31). The behavior of Hel112 was radically different from both. Although in the absence of any protein, the substrate incubated at 55 °C was spontaneously processed to ds and ss molecules, the addition of Hel112 had a strong stabilization effect of the ds fork, blocking its spontaneous regression (Fig. 5B and C). This reaction was ATP-independent, and the addition of 5 mM ATP had no effect (supplemental Fig. S5). This experiment suggests that Hel112 blocks the substrate in its fork configuration, preventing its conversion to HJ; indeed, if this latter structure would form, it could be processed by the helicase. It is important to observe that although the Hel112 activities on ds forks and HJs are opposite (stabilization versus unwinding), the main products of both reactions are forks (either Y-shaped or double-strand). Collectively, the results reported in this paragraph show that Hel112 has three different activities: ATP-dependent processing of HJ, ATP-independent annealing of ssDNA, and protection of ds forks. All these activities converge to stabilization of structures resembling replication forks.

**Physical and Functional Interaction of SsTop3 and Hel112**—Considering existing data and results shown so far, we reasoned that SsTop3 and Hel112 might be good RecQ-like and Top3 candidates to test their interaction. Because some, although not all, RecQ and Top3 enzymes interact physically, we first tested the possible physical interaction of purified untagged SsTop3 and His-tagged Hel112 in pulldown analysis. Fig. 6 (left panel) shows that indeed a protein band of the expected molecular mass was specifically pulled down when purified SsTop3 was passed over nickel-column bound Hel112, but not unrelated
His-tagged protein; mass spectrometry analysis confirmed that the pulled protein band contained SsTop3. To confirm the interaction by an independent technique, we performed a far Western experiment in which filter-blotted SsTop3 indeed interacted specifically with Hel112 (Fig. 6, right panel). Although these experiments are not conclusive about the possibility that SsTop3 and Hel112 form a complex in vivo, they indicate that the two proteins can interact, at least in vitro.

In functional assays, SsTop3 strongly inhibited the ATP-dependent HJ unwinding by the helicase (Fig. 7, A and B); inhibition depended on the topoisomerase concentration, and the addition of SSB failed to restore Hel112 unwinding activity if SsTop3 was also present (Fig. 7C). SsTop3 also prevented the Hel112 unwinding activity on the Y-shaped fork (not shown). Moreover, SsTop3 abolished the Hel112-stimulated strand exchange reaction (Fig. 7D), thus confirming that the SsTop3 ssDNA annealing activity counteracts the Hel112-catalyzed DNA unwinding.

Previously, human topoisomerase IIα and topoisomerase I were reported to inhibit the BLM and WRN helicases, respectively, through inhibition of their ATPase activity (43, 44); in contrast, human TopoIIIα had no effect on BLM ATPase activity (9). We found that the Hel112 DNA-independent ATPase activity was not affected by 2-fold molar excess of SsTop3 (Fig. 8A), suggesting that SsTop3 inhibits specifically the Hel112 unwinding activity, but not its ability to hydrolyze ATP, ruling out the possibility of unspecific inactivation of Hel112 by either the topoisomerase or some other reaction component. In addition, we have shown that Hel112 binds Y-shaped forks and HJ with higher affinity as compared with SsTop3, whereas both enzymes show comparable affinity for ssDNA (Figs. 1C and 2B), thus making the possibility that inhibition is due to competition for the substrate unlikely. The most probable explanation of our data is that inhibition of the Hel112 unwinding activity may be due, at least in part, to the peculiar SsTop3 annealing activity that, in addition to that shown by Hel112, drives the reaction equilibrium toward HJ stabilization.

When assayed on the ds fork, SsTop3 did not induce stabilization of the substrate and slightly reduced the amount of ssDNA, likely inducing its annealing to other species; when the two proteins were combined, SsTop3 did not affect significantly the Hel112 stabilizing activity (Fig. 8, B and C). This result suggests that the ds fork stabilization is a specific activity of Hel112, whereas SsTop3 fails to show such activity, and the net result of the concomitant presence of the two proteins is ds fork stabilization against heat-induced denaturation.

Lastly, we tested the functional interaction of the two proteins in DNA topology modification. In such assays, co-incubation of E. coli RecQ and Top3 catalyzes the relaxation of negatively supercoiled DNA molecules (6). In contrast, the S. solfataricus reverse gyrase TopR1, as well as the combination of its isolated helicase-like domain (N-terminal) and topoisomerase domain (C-terminal), induce positive supercoiling (34). Because this latter reaction requires high temperature, in...
principle, it is possible that combination of the thermophilic helicase-topoisomerase enzymes might catalyze a reverse gyrase-like reaction. Both SsTop3 and Hel112 showed similar affinity for a negatively supercoiled plasmid DNA (data not shown). At 75 °C, Hel112 (in the presence of ATP) did not induce any topological modification of a negative supercoiled plasmid (Fig. 9A, right panel). The addition of equimolar amounts (Fig. 9A, left panel) or 4-fold molar excess (not shown) of Hel112 had no significant effect on the efficiency or the kinetics of the relaxation activity of SsTop3, and similar results were obtained when samples were analyzed by two-dimensional agarose gel electrophoresis with or without SSB (10-fold molar excess; Fig. 9, B and C). Thus, Hel112 does not affect, either quantitatively or qualitatively, the DNA topology variations catalyzed by SsTop3. In such assays, the thermophilic enzymes behave like their mesophilic homologs and, despite the high temperature of reaction, do not induce positive supercoiling, which remains a peculiar activity of reverse gyrase.

**DISCUSSION**

The availability of thermophilic SsTop3 and Hel112 allowed us to look into a gap toward understanding the effects of helicase-topoisomerase interaction at high temperature to control aspects of replication/recombination, especially allied to DNA repair. We have shown that Hel112 catalyzes three reactions typical of eukaryal RecQ family helicases, namely ATP-dependent processing of a synthetic HJ, ATP-independent annealing of ss oligonucleotides and ds fork stabilization. On the other hand, SsTop3 shows a strong ssDNA annealing activity, resulting in production of HJs. In vitro, the two enzymes make a physical interaction, and SsTop3 inhibits the Hel112 helicase activity. Thus, the two enzymes show synergic ssDNA annealing activity, whereas they are antagonists in HJ formation/processing. Finally, on ds forks, the topoisomerase does not interfere with the Hel112 stabilization activity, and in topology assays, Hel112 does not affect the topoisomerase DNA relaxation activity.

Interestingly, all Hel112 activities converge on fork stabilization, either directly by binding, as in the case of ds model replication forks, or by exploiting its unwinding and annealing activities to resolve HJs, promote strand exchange, and anneal ss oligonucleotides to produce molecules structurally similar to unreplicated forks (Y-shaped forks). The stabilization of replication forks is fundamental for preventing genomic instability in the presence, but even in the absence of genotoxic agents; indeed, genetic evidence suggests that in bacteria replication, fork is often arrested during normal growth, and the PriA helicase plays a role in stalled fork stabilization (45). PriA shows several activities similar to Hel112. It has the same polarity and
specificity for unwinding of unreplicated and lagging strand arms of model forks and stabilizes ds forks in a configuration that prevents the unreplicated arms from being unwound. It is thus possible that Hel112 plays an analogous role in *S. solfataricus*.

HJs may be formed as intermediates in the resolution of stalled replication forks, by unwinding of the template strands, and by winding of the nascent strands; these structures are potentially recombinogenic. Using their combined unwinding/annealing activities, some RecQ helicases, including human BLM, WRN, RECQ1, and yeast Sgs1, catalyze reverse branch migration of HJs, leading to restoration of active replication forks and thus acting as anti-recombinases. However, fork regression is not a general property of RecQ helicases; rather, it is specific to a subset (31). Indeed, the *E. coli* RecQ protein does not detectably regress replication fork substrates, but catalyzes HJ forward unwinding up to ssDNA (31). In contrast, we have shown that fork structures and not ssDNA are the main products of the Hel112 reaction.

The structural and biochemical analogies between RecQ-Top3 complexes and reverse gyrase have been repeatedly pointed out (23, 24, 27), leading to the suggestion that these latter complexes may have reverse gyrase-like positive supercoiling activity (46, 47). However, this is not the case for mesophilic RecQ-Top3 complexes, which catalyze relaxation of torsional stress. We have shown that the thermophilic enzymes behave like their mesophilic homologs and not reverse gyrase; *i.e.* at 80 °C, they induce DNA relaxation and not positive supercoiling. These data strengthen the idea that the positive supercoiling activity of reverse gyrase is a unique attribute of (hyper)thermophiles, specifically evolved to face the problems of genome maintenance at high temperature, and suggest that Hel112 and SsTop3, analogously to their mesophilic homologs, play different cellular roles.

Based on our data *in vitro*, it is tempting to speculate that Hel112 might be involved in protection and stabilization of (stalled) replication forks, preventing them from originating recombinogenic structures. We propose that Hel112 performs this function in three ways. First, it could induce reannealing of denatured strands (for instance after transitory temperature increase or at AT-rich regions). Second, on ds forks, it could prevent temperature-driven transition to HJ and spontaneous fork regression (which should be very efficient *in vivo*, due to the combined action of temperature and positive supercoiling). Lastly, if four-way structures are formed, the action of Hel112 could lead to branch migration, restoring functional forks. However, *in vivo*, it would not be advantageous for Hel112 to bring about the anti-recombination activity at all potential recombination sites.

SsTop3 might modulate this function, promoting HJ formation, and thus recombination, at a subset of sites or under par-
Cooperation of Thermophilic Helicase and Topoisomerase

The inhibition of a RecQ-like helicase by a topoisomerase 3 complex (9), and conversely, BLM (with BLAP75/RM1) stimulates crossing over during homologous recombination.

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