Werner Protein Stimulates Topoisomerase I DNA Relaxation Activity

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ABSTRACT
Werner syndrome (WS) is a human premature aging disorder characterized by the early onset of age-related clinical features and an elevated incidence of cancer. The Werner protein (WRN) belongs to the RecQ family of DNA helicases and is required for the maintenance of genomic stability in human cells. Potential cooperation between RecQ helicases and topoisomerases in many aspects of DNA metabolism, such as the progression of replication forks, transcription, recombination, and repair, has been reported. Here, we show a physical and functional interaction between WRN and topoisomerase I (topo I). WRN colocalizes and interacts directly with topo I. WRN stimulates the ability of topo I to relax negatively supercoiled DNA and specifically stimulates the religation step of the relaxation reaction. Moreover, cell extracts from WS fibroblasts exhibit a decrease in the relaxation activity of negatively supercoiled DNA. We have identified two regions of WRN that mediate functional interaction with topo I, and they are located at the NH2- and COOH-termini of the WRN protein. In a reciprocal functional interaction, topo I inhibits the ATPase activity of WRN. Our data provide new insight into the relationship between RecQ helicases and topoisomerases in the maintenance of genomic integrity and prevention of tumorigenesis.

INTRODUCTION
WS is an autosomal recessive disorder characterized by the early onset of age-related symptoms including atherosclerosis, osteoporosis, cataracts, diabetes, and a high incidence of malignant neoplasms, especially those of mesenchymal origins (1–3). The gene defective in WS, designated WRN, encodes a nuclear 1432-amino acid protein with seven conserved motifs found in the RecQ family of DNA helicases (4, 5). In addition to WRN, there are at least four other human RecQ homologs. Defects in two of these, BLM and RECQL4, are also associated with human diseases and an increased incidence of cancer (6). RecQ helicases are required for preserving the genomic integrity and thus have been classified as caretakers of the genome (7). Similar to other RecQ helicases, the Werner protein (WRN) is a DNA-dependent ATPase and a 3'–5' helicase (2). However, WRN is unique among the human RecQ helicases in that it also contains a region of conserved exonuclease motifs and possesses a 3'–5' exonuclease activity (8). Cells from WS patients show elevated levels of DNA deletions, translocations and chromosomal breaks (9), and display replicative defects including an extended S-phase and premature senescence (10, 11). WS cells are also sensitive to certain DNA-damaging agents including 4-nitroquinoline-1-oxide (12) and drugs such as the topo I inhibitor CPT (13). All together, these data support a role for WRN in replication and/or anti-recombination pathways (2, 14).

Recent data indicate cooperation between helicases and topoisomerases in DNA metabolism during progression of the replication fork, segregation of newly replicated chromosomes, disruption of nucleosomal structure, DNA supercoiling, recombination, and DNA repair (15, 16). DNA topoisomerases solve the topological problems associated with DNA manipulation by introducing a transient break in the DNA and resolving the DNA structure (16). Type I topoisomerases are monomeric, cleave one strand of duplex DNA, and require no energy cofactor. In contrast, the type II enzymes function as dimers, cleave both strands of the DNA, and pass an intact DNA duplex through the transient double-stranded break in an ATP-dependent manner (17).

There is increasing evidence to suggest that RecQ helicases may act in concert with topoisomerases to preserve genomic integrity. Previous studies in bacteria and yeast have identified an interaction between topoisomerases and members of the RecQ helicase family. In Escherichia coli, RecQ and topo III together catalyze the linking and unlinking of covalently closed circular DNA molecules (18). The yeast homologue of RecQ helicases, SGS1, interacts both physically and genetically with topo III, a type I topo. Mutations in SGS1 partially suppress the pleiotropic effects of TOP3 mutations, suggesting that Sgs1p and Top3p act in the same pathway (19, 20). Wu et al. (21) have shown that the Bloom protein (BLM) also interacts with human topo III and have reported recently that BLM stimulates the ability of topo III to relax supercoiled DNA (22). Furthermore, a previous study found that topo I was coimmunoprecipitated with an antibody against WRN from human colon carcinoma SW480 cell extracts (23). In addition, the inhibition of topo I activity by CPT results in a higher induction of chromosomal damage and apoptosis in WS cells, compared with normal cell lines, in the S and G2 phases of the cell cycle (13, 24, 25). Topo I is a molecular target of CPT, which interferes with the topo I mediated DNA breakage-reunion reaction by stabilizing the enzyme bound to the DNA single-strand break intermediate and consequently inhibiting the DNA rejoicing activity of topo I (26). Furthermore, recent work in yeast showed that expression of an sgs1 mutant lacking DNA helicase activity was sufficient to rescue the hypersensitivity of the sgs1 mutant strain to topo inhibitors but failed to rescue the premature aging phenotype (27).

In this study, we report that there is a physical and two-way functional interaction between WRN and topo I. Topo I inhibits the WRN ATPase and helicase activities, whereas WRN stimulates the topo I relaxation activity and stimulates the religation step of the topo I relaxation cycle. WS fibroblast cells are sensitive to CPT, and WS cell extracts show a decrease in DNA relaxation activity, compared with controls. These data suggest that WRN and topo I function together in a common pathway in the maintenance of genomic stability.

MATERIALS AND METHODS

Proteins. Human topo I purified from freshly extracted human placenta was purchased from TopoGEN, Inc. (Columbus, Ohio). Recombinant WRN protein was purified using a baculovirus/insect cell expression system as described previously (28). The COOH-terminal fragment of WRN, designated as C-WRN, corresponding to residues 940-1432 of the WRN sequence and

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overexpressed in E. coli, was purified as described previously (29). The N-terminal fragment of WRN, designated N-WRN, corresponding to residues 1–368 of the WRN sequence, was overexpressed in E. coli and purified as described previously (28). Recombinant GST-WRN fusion proteins, overexpressed in E. coli, were purified as described previously (30). Recombinant human DNA polymerase β was a generous gift from Dr. Samuel H. Wilson (National Institute of Environmental Health Sciences, Triangle Park, NC). Recombinant human AP endonuclease I was obtained from Dr. David M. Wilson (National Institute on Aging, Bethesda, MD). Recombinant human FEN-1 was a kind gift from Dr. Grigory Dianov (Medical Research Council, Harwell, United Kingdom).

Characterization of WRN-Topo I Interaction by ELISA. ELISA was performed as described previously (30), but with some modifications. The blocking and binding steps were performed in the same buffer (2% BSA, 0.1% Tween 20 in PBS). Wells were coated with 75 ng of purified topo I diluted in carbonate buffer (50 mM) or with BSA as a background control. After blocking, various amounts of purified WRN (0, 75, or 150 ng) were added in the binding step (50 µl), and ethidium bromide (10 µg/ml) was included in a control reaction. As a positive control and to estimate the amount of WRN protein bound to topo I, wells were coated with various amounts of WRN (0–300 ng) and were incubated with blocking buffer during the binding step. Following washes, primary antibody (1:1,500, anti-rabbit IgG against WRN; Novus, Littleton, CO) was added and incubated for 1 h at 37°C. Wells were washed, and secondary antibody (1:10,000, anti-rabbit IgG; Horsedradish peroxidase; Vector, Burlingame, CA) was added and incubated for 1 h at 37°C. Bound WRN was detected with o-phenylenediamine dihydrochloride followed by termination with 3 m H2SO4. Absorbance was read at 490 nm, and values were corrected for background observed in BSA only wells. To estimate the amount of WRN protein bound to topo I, the absorbance values for wells coated with WRN only were plotted against WRN protein amounts. Linear regression analysis was performed on the linear portion of the curve and used to calculate the amount of WRN protein bound to topo I-coated wells. Only those absorbance readings in the linear range were used. Values represent the means of four experiments, and error bars represent the SD.

Cell Lines and Culture. h-TERT AG03141 (normal) and h-TERT AG03141 (transgenic) cell lines were a generous gift from Jerry W. Shay. The h-TERT cell lines, SV40-transformed VA13 (normal) and AG11395 (WS), were cultured in DMEM (Life Technologies, Inc.) supplemented with penicillin/streptomycin (Life Technologies) and 10% fetal bovine serum. Normal (W138) and WS AG03141C (primary fibroblasts) were cultured in MEM (Life Technologies) with 15% fetal bovine serum, essential and non-essential amino acids, vitamins, 2 mM l-glutamine, and penicillin/streptomycin. Cultures were incubated at 37°C in a 5% CO2 atmosphere.

Immunofluorescence. Exponentially growing normal cells (h-TERT GM01604) (normal) and h-TERT AG03141 (transgenic) cell lines were a generous gift from Jerry W. Shay. The h-TERT cell lines, SV40-transformed VA13 (normal) and AG11395 (WS), were cultured in DMEM (Life Technologies, Inc.) supplemented with penicillin/streptomycin (Life Technologies) and 10% fetal bovine serum. Normal (W138) and WS (AG03141C) primary fibroblasts were cultured in MEM (Life Technologies) with 15% fetal bovine serum, essential and non-essential amino acids, vitamins, 2 mM l-glutamine, and penicillin/streptomycin. Cultures were incubated at 37°C in a 5% CO2 atmosphere.

Helicase Assay. Reactions (20 µl) were performed as described previously (32) in standard reaction buffer [40 mM Tris-HCl (pH 8), 4 mM MgCl2, 5 mM DTT, and 0.1 mg/ml BSA] with 2 nM ATP. The 22-mer duplex (0.5 mM) was 5’-end-labeled and prepared as described previously (32). Protein concentrations were as indicated in the figure legend. Reactions were initiated by the addition of WRN and were incubated at 37°C for 15 min. Reactions were terminated by the addition of 3X stop dye (50 mM EDTA, 40% glycerol, 0.9% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol) to a 1X final concentration. Products were run on 12% native polyacrylamide gels, visualized using a PhosphorImager, and quantitated using ImageQuant software (Molecular Dynamics, Inc.). The percentage of displaced products was quantitated using the following formula: percent displacement = 100 × P/(total DNA), where P is the amount of displaced strand product. Background correction was carried out using control reactions that excluded the enzyme and included either native or heat-denatured substrate.

Electrophoretic Mobility Shift Assay. Binding (20 µl) was conducted in standard reaction buffer. Protein and DNA substrate concentrations were as indicated in figure legends. Reactions were incubated for 20 min at 4°C and stopped by the addition of 3X dye (0.125% bromophenol blue and 40% glycerol) to a 1X final concentration. Samples were loaded on 4% polyacrylamide (29:1) gels and electrophoresed overnight at 30 V, 4°C in 1X TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8). Products were visualized using a PhosphorImager.

ATPase Assay. Reactions (10 µl) contained 150 ng of DNA cofactor, 1 µCi of [γ-32P]ATP (3,000 Ci/mmol) in standard reaction buffer, with protein amounts indicated in the figure legend. Reactions were conducted at 37°C for 30 min and then stopped by adding 5 µl of 0.5 mM EDTA. Samples (2 µl) were analyzed on a polyethylenimine-cellulose TLC and developed in 0.75 M KH2PO4. ATP hydrolysis was visualized by PhosphorImager and quantitated by ImageQuant. Results from at least three independent experiments are reported as mean ± SD.

Topo I DNA Relaxation Assay. Topo I activity was measured by the relaxation of supercoiled plasmid pBluescript SK II DNA. The 20-µl assay mixture contained 40 µM Tris-HCl (pH 8), 4 mM MgCl2, 5 mM DTT, 7.5 µg/ml pBluescript SK II, and 0.1 mg/ml BSA. 2 mM ATP was added only where indicated. The reaction was initiated by the addition of 1 unit of topo I and allowed to proceed at 37°C for 30 min. Full-length WRN or WRN fragments were added prior to the addition of topo I at concentrations described in the figure legends. Products were run on a 1% agarose gel at 75 V for 30 min in TBE buffer. The gels were stained with ethidium bromide (0.5 µg/ml) for 30 min. The bands were visualized by illumination from below with short-wave UV light and photographed. Experiments using WCEs were conducted with the same buffer and under the same conditions. Amounts of total proteins are specified in the figure legends. Agarose gels were scanned using FluorImager SI software and quantitated using ImageQuant software (Molecular Dynamics, Inc.). The percentage of relaxation activity was calculated using the following formula: relaxed/(relaxed + supercoiled) × 100. The values for the percentage of relaxation activity were corrected for background in the no-enzyme control experiment. Data were analyzed by simple t test.

WCE Preparation. Cell pellets (100-µl packed cell volume) were resuspended in an equal volume of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40, 2 mM NaVO4, 10 mM NaF, and protease inhibitors (1 µg/ml chymotrypsin, pepstatin, aprotinin, leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Cell lysates were centrifuged at 4°C, 15,000 × g for 20 min, and the supernatants were aliquoted. WCE concentrations were determined by the Bio-Rad assay using BSA as a standard (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis. Equal amounts of WCE were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated in blocking buffer (5% nonfat milk, 0.5% Tween 20 in 1X PBS) for 1.5 h at room temp. The membranes were then incubated with rabbit anti-topo I (1:2,500; Topogen) and goat anti-lamin B (1:1,000; Santa Cruz Biotechnology; NM marker) polyclonal antibodies overnight at 4°C. After washing twice (10 min each) with blocking buffer, the membranes were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (1:10,000; Vector Laboratories) for 1 h at room temperature. After three washes (15 min each), enhanced chemiluminescence was performed (Amersham Biociences), following the manufacturer’s instructions.

Detection of Cell Death. Cells were grown on coverslips and exposed for 1 h to 15 µM CPT (stocks were dissolved in DMSO; Sigma; Ref. 13), washed...
twice, and recovered in drug-free medium for 14 h. Samples were subsequently analyzed for cell death. The final DMSO concentration did not exceed 1% in CPT-treated cultures. PI (0.5 μg/ml; Sigma) dye was used to distinguish the dead cells from viable cells. After treatment, the coverslips were washed once in 1× PBS, followed by incubation with PI for 20 min at room temperature. After three washes in 1× PBS, the coverslips were mounted on Vectashield containing DAPI stain and viewed under a fluorescence microscopy (Zeiss). Reported values represent the means of two independent experiments in which at least 1,000 cells were scored for each.

**Topo I DNA Strand Transfer/Religation Assay.** Reactions were conducted as described previously (33). Briefly, an 18-mer DNA oligonucleotide (5′-CGTTGTCGCCCTATTCCC) was 5′-end labeled in the presence of [γ-32P]ATP and T4 polynucleotide kinase and then hybridized to a complementary 30-mer strand (5′-TGTTAGCTACTCGGAATAAGGCCAACC) to form the 18-mer/30-mer cleavage substrate. Covalent topo I-DNA complexes were formed in the identical buffer as in the relaxation assay, containing (per 20 μl) 10 fmol of 18-mer/30-mer DNA and 150 fmol of topo I. The mixture was incubated for 5 min at 37°C. The strand transfer reaction was initiated by the addition of 0.5 pmol of an 18-mer acceptor strand, 5′-ATTCCCATAGTGACTACA (a 50-fold molar excess over the input DNA substrate), while simultaneously adjusting the reactions to 0.3 M NaCl. Addition of NaCl during the reaction promotes dissociation of the topo I after strand ligation and prevents recleavage of the strand transfer product. The reactions were terminated by the addition of SDS and formamide to 0.2 and 50%, respectively. The samples were heat-denatured and then electrophoresed through a 14% polyacrylamide gel containing 7 M urea in TBE. The extent of the cleaved 12-mer/topo I complex was retarded in the gel (data not shown).

**RESULTS**

**WRN and Topo I Interact Directly.** To test for a WRN and topo I physical interaction, we preformed ELISA assays with purified recombinant proteins. The results demonstrate that purified WRN (9 and 18 nm) binds directly to purified topo I (16 nm; Fig. 1A, columns 3 and 4). This interaction is specific, because BSA fails to show an interaction with WRN under the same conditions (Fig. 1A, column 1). The WRN/topo I interaction was not mediated by DNA because the binding was unaffected by the presence of ethidium bromide (data not shown). To estimate the percent of WRN that bound to topo I, we coated wells with WRN protein alone, performed ELISA, and measured the absorbance for each WRN amount. Absorbance values were plotted against WRN protein amounts (Fig. 1B), and the linear portion of the curve was analyzed by linear regression (see “Materials and Methods”). On the basis of this analysis, for the binding reaction in which 75 ng of WRN was added to the topo I-coated wells, ~26% of the total WRN remained bound to topo I (Fig. 1A, column 4). The absorbance values for the binding reaction containing 150 ng of WRN were outside the linear range. These experiments indicate that WRN and topo I bind to each other in vitro.

**WRN and Topo I Colocalize.** Indirect immunofluorescence of methanol-fixed GM01604 cells indicate that WRN and topo I are present throughout the nucleus in a punctate pattern but colocalize primarily in nucleolar foci (Fig. 2A, yellow). After cells were treated with CPT, both WRN and topo I were no longer present in the nucleolus (Fig. 2B). The distribution of topo I was similar in the WS cell line AGO3141 (data not shown). In GM01604 cells stained for WRN (green) and topo I (red), 60% of the cells examined showed colocalization of both proteins in the nucleolus (Fig. 2A, yellow merge). In contrast, after treatment with CPT, only 6% of the cells showed nucleolar colocalization, and both WRN and topo I were mostly absent from the nucleolus (Fig. 2B). However, in 13% of the cells treated with CPT, a discrete non-nucleolar WRN/topo I colocalization can be discerned, indicating the presence of WRN/topo I protein complexes in the nucleoplasm and peripheral to the nucleolus (Fig. 2B, yellow stain in enlarged panels). We further examined the
colocalization of these proteins in a volumetric reconstruction of a CPT-treated cell (Table 1). We found that 58% of the WRN spots localized with 5% of the topo I spots. Another abundant nuclear protein that is associated with WRN in the nucleolus, the AAA ATPase VCP, did not localize with topo I (data not shown), thus supporting the specificity of the WRN/topo I colocalization.

**Topo I Inhibits the Helicase and ATPase Activity of WRN.** To determine whether topo I and WRN interact functionally, we tested the ability of topo I to modulate WRN helicase activity on a 22-bp forked duplex. Suboptimal amounts of WRN were tested so that either stimulation or inhibition of unwinding could be detected. WRN alone (12 fmol) unwound 33% of the forked duplex (Fig. 3A, Lane 2). The incubation of WRN (12 fmol), together with increasing amounts of topo I (12–96 fmol), resulted in an inhibition of WRN helicase activity (Fig. 3A, Lanes 5–8). The addition of the highest amount of topo I tested (96 fmol) inhibited the WRN unwinding activity 3.6-fold (Fig. 3A, compare Lanes 2 to 8). This inhibition was not attributable to components in the topo I storage buffer (Fig. 3A, compare Lanes 2 to 3). The ability of topo I to inhibit WRN helicase may be attributable, in part, to topo I binding to the DNA substrate. Standard gel-shift analysis indicates that incubation of topo I with the helicase substrate led to a shift in the migration of the substrate corresponding to a topo I-DNA complex (Fig. 3B, compare Lanes 1 and 3). Moreover, WRN did not stably bind the substrate (Lane 2), which is in accordance with our previous observations (28). Next, we investigated the effect of topo I on the ATPase activity of WRN. WRN (75 fmol) was incubated alone or with topo I (300 fmol) in the presence of either the M13 ssDNA or supercoiled DNA as effectors. The addition of topo I to the reaction led to a substantial inhibition (75%) of the WRN ATPase activity with the M13 ssDNA, from 10.2 ± 0.2% (WRN alone) to 2.4 ± 0.4% (WRN and topo I; Fig. 3C, compare Lanes 1 and 2). Topo I does not bind ssDNA with high efficiency compared to dsDNA, suggesting that the inhibition observed using the M13 ssDNA as DNA effector may be attributable to the direct interaction between topo I and WRN. A similar inhibition of the WRN ATPase activity (78%), from 6.3 ± 0.6% (WRN alone) to 1.4 ± 0.5% (WRN and topo I), was observed using the supercoiled DNA as the DNA effector, a substrate that topo I can act upon (Fig. 3C, compare columns 3 and 4). These

![Fig. 2. CPT treatment changes the pattern of WRN and topo I nuclear expression and colocalization. A, untreated h-TERT GM01604 cells. B, h-TERT GM01604 cells treated with 15 nM CPT for 1 h. Cells were processed for indirect immunofluorescence as described in “Materials and Methods” and stained with anti-WRNp (1:200, green), anti-topo I mAb (1:100, red), and the DNA stain DAPI (blue). Z-sections (0.1 μm) were obtained on a Zeiss Axioplan 2i microscope and deconvolved. Merge is the merged image of the three fluorescence channels of the same deconvolved Z-section. A nucleolar area (dashed square in B) of each cell presented was enlarged (×6), and the colocalization (yellow) can clearly be seen. Images are of two representative cells, and Cell B in B shows two different optical slices that are 2.0 μm apart. All images, ×630. Bar, 10 μm.](image-url)
results indicate that topo I inhibits the WRN ATPase activity. Because WRN is an ATP-dependent helicase, topo I inhibition of the WRN helicase may be attributable, at least partially, to its ability to inhibit the WRN ATPase.

**WRN Stimulates Topo I DNA Relaxation Activity.** To test whether the WRN interaction with topo I modulates topo I activity, DNA plasmid relaxation assays were carried out as described in “Materials and Methods.” We purified pBluescript SK II DNA plasmid from *E. coli* to obtain a negatively supercoiled DNA substrate. After incubation of topo I (0.15 pmol) with the DNA substrate, 42% of the negatively supercoiled DNA was converted to relaxed topoisomerase (Fig. 4A, Lane 2). As observed in Fig. 4A, topo I relaxation activity was stimulated by the addition of an increasing amount of WRN (compare Lanes 2 to Lanes 3–6). Titration of WRN (0.3, 0.6, 1.2, to 2.4 pmol) led to an increase in topo I activity up to 64–6%, whereas the highest amount of WRN alone did not affect the substrate (data not shown). Because these reactions were conducted in the absence of ATP, the stimulation of topo I by WRN does not require the catalytic activity of the WRN ATPase-dependent helicase. Addition of ATP did not alter the level of stimulation (data not shown).

**Mapping of the Functional Interaction Between WRN and Topo I.** We next investigated which domain of the WRN protein was responsible for the stimulation of topo I DNA relaxation. For this study, we used several truncated WRN proteins corresponding to different catalytically active domains of the protein (Fig. 4B). N-WRN, corresponding to the exonuclease-proficient NH2 terminus of WRN (Fig. 4C, Lanes 4 and 5), and C-WRN, corresponding to the COOH terminus (Fig. 4C, Lanes 8 and 9), stimulated topo I activity similarly to the full-length WRN. The topo I stimulation by C-WRN indicates that neither the catalytic helicase nor exonuclease activities of WRN are required for stimulation. No stimulation was observed with the truncated WRN protein corresponding to the helicase domain of WRN (Hel-WRN; Fig. 4C, Lanes 6 and 7). These data suggest the existence of two separate topo I-interactive sites in the WRN protein, one present in the NH2 terminus and another in the COOH terminus. In an effort to further map the topo I-interactive sites, we tested a battery of smaller GST-WRN protein fragments (Fig. 4D) for their ability to stimulate the topo I relaxation activity. Each fragment (0.6 pmol) was incubated with 0.15 pmol of topo I. Only the GST-WRN fragment 2 (corresponding to the first 51 amino acids) was able to stimulate topo I, narrowing the first topo I-interactive site in the NH2 terminus to the first 51 amino acids of WRN (Fig. 4E, Lane 4). GST alone and GST-WRN fragments 3 (WRN949–1092), 4 (WRN949–1236), and 5 (WRN1072–1236) corresponding to regions in the C-WRN (amino acids 949–1432) all failed to stimulate topo I activity (Fig. 4E, Lane 10, and Fig. 4E, Lanes 5–7). Incubation of either GST (Fig. 4C, Lane 11) alone or the different GST-WRN fragments (data not shown)
with the DNA substrate alone did not affect the substrate. These data suggest that the WRN region corresponding to amino acids 1236–1432 (because that region was not present in the GST subfragments) is necessary for the stimulation of topo I observed with C-WRN. This region represents part of the second topo I-interactive site but is not necessarily sufficient for the stimulation of topo I by the COOH terminus of WRN. Regions encompassed in the COOH-terminal GST-WRN fragments (3–5) were not sufficient for topo I stimulation but could still contribute to the stimulation observed with full-length C-WRN. Taken together, these data indicate that the WRN N-terminal amino acids 1–51 and COOH-terminal amino acids 949–1432 functionally interact with topo I (Fig. 4G).

To test the specificity of the functional interaction between WRN and topo I, i.e., the ability of WRN to stimulate the topo I relaxation activity, we assayed various proteins known to be implicated in DNA replication, repair, and recombination processes and that bind a variety of DNA structures. APE-1, Pol β, and FEN-1 were incubated (Fig. 4F, Lanes 3 and 4; Lanes 5 and 6; and 7, respectively) with topo I. No stimulation was detected upon addition of these proteins, supporting the specificity of the WRN protein to stimulate topo I activity.

WS Cell Extracts Exhibit a Decrease in DNA Relaxation Activity.

The observation that WRN stimulates topo I DNA relaxation activity in vitro predicts that WS cells should exhibit a decreased relaxation activity of negatively supercoiled DNA, compared with WRN-proficient cell lines. To test this hypothesis, we prepared WCEs from various WS and normal cell lines and compared levels of DNA relaxation activity using a negatively supercoiled plasmid. The assay conditions and substrate were chosen to specifically favor the detection of DNA relaxation activity attributable to topo I (see “Discussion”). DNA relaxation assays with equal amounts of WCE were carried out under the same conditions as the experiments with purified protein. We first tested fibroblast cell lines that were immortalized from various WS and normal cell lines and compared levels of DNA relaxation activity attributable to topo I in vitro. The h-TERT WS (AG03141) cell line exhibited significantly less relaxation activity compared with the h-TERT normal (GM01604) cell line (46 ± 6% and 75 ± 5% activity, respectively; P < 0.001; Fig. 5A). A similar deficiency was observed when comparing SV40-transformed WS (AG11395) and normal (VA13) cell lines, 60 ± 3% and 80 ± 11% respectively (P < 0.001; Fig. 5B), and WS (AG03141 C) and normal (W138) primary fibroblasts, 27 ± 5% and 44 ± 6%, respectively (P < 0.001; Fig. 5C). To determine whether the decrease in relaxation activity is specific to the WRN-stimulated topo I relaxation activity, the reactions contained topo I alone (0.15 pmol; Lane 2) or topo I together with 0.6 or 1.2 pmol of the various GST-WRN recombinant fragments described in D, F, specificity of the WRN-stimulated topo I relaxation activity. The reactions contained topo I alone (0.15 pmol; Lane 2) or topo I together with either 0.15 or 1.2 pmol of APE-1 (Lanes 3 and 4) or Polβ (Lanes 5 and 6) or 1.2 pmol of FEN-1 (Lane 7). G, map of the topo I interactive sites to WRN.

Fig. 4. WRN stimulates topo I DNA relaxation activity. B and D, structural map of WRN protein motifs and recombinant proteins used in this study. B, conserved RecQ ATPase/helicase and exonuclease motifs, RecQ COOH-terminal region (RQC), helicase-related domain (HRDC), and nuclear localization sequence (NLS) are designated. WRN recombinant proteins N-WRN, Hel-WRN, and C-WRN correspond to truncated forms of the WRN proteins. The brackets represent the length of the different fragments. D, GST-WRN recombinant fragments. Numbers on top of each fragment indicate the WRN amino acid sequences. A, C, E, and F, relaxation activity of topo I in the presence of the various WRN fragments. All of the reactions contained 0.15 pmol of topo I and 150 ng of supercoiled plasmid. Products were separated on 1% agarose gels and stained with ethidium bromide.

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The observation that WRN stimulates topo I DNA relaxation activity in vitro predicts that WS cells should exhibit a decreased relaxation activity of negatively supercoiled DNA, compared with WRN-proficient cell lines. To test this hypothesis, we prepared WCEs from various WS and normal cell lines and compared levels of DNA relaxation activity using a negatively supercoiled plasmid. The assay conditions and substrate were chosen to specifically favor the detection of DNA relaxation activity attributable to topo I (see “Discussion”). DNA relaxation assays with equal amounts of WCE were carried out under the same conditions as the experiments with purified protein. We first tested fibroblast cell lines that were immortalized from various WS and normal cell lines and compared levels of DNA relaxation activity attributable to topo I in vitro. The h-TERT WS (AG03141) cell line exhibited significantly less relaxation activity compared with the h-TERT normal (GM01604) cell line (46 ± 6% and 75 ± 5% activity, respectively; P < 0.001; Fig. 5A). A similar deficiency was observed when comparing SV40-transformed WS (AG11395) and normal (VA13) cell lines, 60 ± 3% and 80 ± 11% respectively (P < 0.001; Fig. 5B), and WS (AG03141 C) and normal (W138) primary fibroblasts, 27 ± 5% and 44 ± 6%, respectively (P < 0.001; Fig. 5C). To determine whether the decrease in relaxation activity is specific to the WRN-stimulated topo I relaxation activity, the reactions contained topo I alone (0.15 pmol; Lane 2) or topo I together with 0.6 or 1.2 pmol of the various GST-WRN recombinant fragments described in D, F, specificity of the WRN-stimulated topo I relaxation activity. The reactions contained topo I alone (0.15 pmol; Lane 2) or topo I together with either 0.15 or 1.2 pmol of APE-1 (Lanes 3 and 4) or Polβ (Lanes 5 and 6) or 1.2 pmol of FEN-1 (Lane 7). G, map of the topo I interactive sites to WRN.

WS Cell Extracts Exhibit a Decrease in DNA Relaxation Activity.

The observation that WRN stimulates topo I DNA relaxation activity in vitro predicts that WS cells should exhibit a decreased relaxation activity of negatively supercoiled DNA, compared with WRN-proficient cell lines. To test this hypothesis, we prepared WCEs from various WS and normal cell lines and compared levels of DNA relaxation activity using a negatively supercoiled plasmid. The assay conditions and substrate were chosen to specifically favor the detection of DNA relaxation activity attributable to topo I (see “Discussion”). DNA relaxation assays with equal amounts of WCE were carried out under the same conditions as the experiments with purified protein. We first tested fibroblast cell lines that were immortalized from various WS and normal cell lines and compared levels of DNA relaxation activity attributable to topo I in vitro. The h-TERT WS (AG03141) cell line exhibited significantly less relaxation activity compared with the h-TERT normal (GM01604) cell line (46 ± 6% and 75 ± 5% activity, respectively; P < 0.001; Fig. 5A). A similar deficiency was observed when comparing SV40-transformed WS (AG11395) and normal (VA13) cell lines, 60 ± 3% and 80 ± 11% respectively (P < 0.001; Fig. 5B), and WS (AG03141 C) and normal (W138) primary fibroblasts, 27 ± 5% and 44 ± 6%, respectively (P < 0.001; Fig. 5C). To determine whether the decrease in relaxation activity is specific to the WRN-stimulated topo I relaxation activity, the reactions contained topo I alone (0.15 pmol; Lane 2) or topo I together with 0.6 or 1.2 pmol of the various GST-WRN recombinant fragments described in D, F, specificity of the WRN-stimulated topo I relaxation activity. The reactions contained topo I alone (0.15 pmol; Lane 2) or topo I together with either 0.15 or 1.2 pmol of APE-1 (Lanes 3 and 4) or Polβ (Lanes 5 and 6) or 1.2 pmol of FEN-1 (Lane 7). G, map of the topo I interactive sites to WRN.
activity exhibited by the WS cell lines was attributable to decreased levels of topo I. Western blot analyses were carried out. Equal amounts of WCE were separated by PAGE, transferred, and blotted with an antibody against human topo I, along with an antibody against lamin B to control for loading. WCE, from h-TERT (Fig. 5D), SV40 transformed (Fig. 5E), and primary fibroblast (Fig. 5F) cells displayed a similar level of topo I, suggesting that the decrease in the topo relaxation activity observed in the WS cell lines was not attributable to decreased expression levels of topo I.

**WS Fibroblasts Are Hypersensitive to CPT.** Previous studies have shown that WS EBV-transformed lymphoblast cell lines display hypersensitivity to CPT-induced chromosomal damage compared with normal cell lines (13, 25). Pichierri et al. (13) have also reported an increase in the apoptotic induction in WS lymphoblasts treated with CPT compared with normal cells. To determine whether the WS h-TERT fibroblast cells are also hypersensitive to CPT, an assay for cell death and apoptosis was conducted using h-TERT normal (GM01604) and h-TERT WS (AG03141) fibroblast cell lines. The different cell lines were treated with 15 μM CPT and allowed to recover for 14 h in drug-free media as described previously (13). The control and CPT-treated cells were stained with PI to identify dead cells. Staining of the DNA with PI (red staining) requires the loss of cytoplasm and nuclear membrane integrity. Approximately 1,000 cells were scored for each cell line. A very low percentage of dead PI-stained cells was observed among the untreated normal cells (Fig. 6A, c), treated normal cells (Fig. 6A, g), and untreated WS cells (Fig. 6B, c), approximately 2.5, 5.5, and 5%, respectively. In contrast, 17% of treated WS cell lines exhibited a staining pattern consistent with cell death (Fig. 6B, g). These results are consistent with previous reports using lymphoblast cell lines and demonstrate that the WS fibroblast cell line used in this study also exhibits an increase of cell death after treatment with CPT.

**WRN Stimulates the Religation Step of Topo I DNA Relaxation Activity.** The topo I catalytic cycle can be divided into four steps: binding of the enzyme to the DNA, cleavage of the DNA, single strand passage, and DNA religation. The lack of religation defines the topo I “cleavable complex” as a covalent DNA-topo I complex intermediate. CPT specifically stabilizes these cleavable complexes which, if left unrepaired, can lead to genomic instability. We hypothesized that WRN might shorten the duration of this cleavable complex in the cell by stimulating topo I relaxation activity and more specifically by stimulating the religation step of the topo I relaxation cycle (reviewed in Ref. 16). To form the cleavable complex consisting of topo I covalently bound to the DNA donor complex, topo I (0.15 pmol) was preincubated with 10 fmol of a DNA complex composed of a 5′-32P-labeled 18-mer annealed to a 30-mer strand (Fig. 7A). Site-specific cleavage of the DNA substrate by topo I triggered the release of a six-nucleotide oligomer from the 3′ extremity of the 18-mer top strand. The resulting DNA cleavable complex consisted of a 12-mer strand annealed to a 30-mer strand, complementary to the 5′ single-stranded tail of the DNA cleavable complex, together with increasing amounts of WRN (0.15, and 0.3 pmol). These reactions were simultaneously adjusted to 0.3 M NaCl to promote topo I dissociation after ligation and to prevent recleavage of the strand transfer product (see “Materials and Methods”). Ligation of the 18-mer strand to the 12/30-mer complex leads to the formation of a 30-mer top

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Fig. 5. WS cell lines exhibit a decrease in relaxation activity of negatively supercoiled DNA plasmid. A, relaxation activity of cell extract from h-TERT normal cell lines (GM01604) and WS cell lines (AG03141). B, relaxation activity of cell extracts from SV40-transformed normal (VA13) and WS (AG11385) cell lines. C, relaxation activity of cell extracts from normal (WI38) and WS (AG03141C) primary fibroblasts. Relaxation assays (20 μl) were conducted in the presence of 150 ng of pBluescript SK II DNA plasmid and equal amounts of WCEs (43 ng). Oc, open circle; R, relaxed topoisomerases; Sc, supercoiled; (−), no extract was added. *a value significantly different from wild-type cell lines (P < 0.001, see “Materials and Methods.”) The percentage of relaxation activity for each extract was calculated as described in “Materials and Methods.” [a] wild-type cell lines. [b] WS cell lines. Values represent the means from six independent experiments; bars, SD. D–F, detection of the amount of topo I in each cell extract. WCEs were tested by Western blot analysis using a rabbit polyclonal antibody against human topo I. WCEs from cell lines used in A–C were loaded. The control for equal loading is indicated by blotting with an antibody against lamin B.
strand product (Fig. 7A). After 1-h incubation at 37°C, 8.2 ± 3.8% of the substrates were converted to the expected 30-mer strand by topo I alone (Fig. 7B, Lane 3, and 7C, column 1), whereas 9.4 ± 1.5% and 18.2 ± 4.2% were converted upon addition of the WRN protein (0.15 and 0.3 pmol, respectively; Fig. 7B, Lanes 4 and 5, and 7C, columns 2 and 3). This 2.2-fold increase in strand transfer upon addition of the WRN protein indicates that the WRN protein stimulates the religation step of the topo I catalytic cycle.

**DISCUSSION**

Human topo I is a prime target for anticancer drugs because of its pivotal role in relaxing supercoiled DNA for replication and transcription. The DNA relaxation activity of topo I involves recombination events that can lead to DNA double-strand breaks or illegitimate recombination events if unregulated. In this study, we observed that WRN physically and functionally interacts with topo I in vitro. WRN and topo I have been shown to coimmunoprecipitate with a replication complex (23), but whether this interaction was direct or mediated by another protein was unknown. Using ELISA, we showed that topo I binds directly to WRN, and that the interaction is not mediated by DNA. We demonstrate that in the majority of untreated GM01604 cells, WRN and topo I colocalize in the nucleoli of untreated GM01604 cells and that CPT treatment relocates both WRN and topo I to the nucleoplasm. We have also detected WRN/topo I complexes in the nucleolar periphery and in the nucleoplasm of some of the CPT-treated cells, indicating that the interaction between a subset of these proteins may continue in the nucleoplasm. We next showed that topo I inhibited the helicase activity of WRN. Topo I was shown to bind the substrate, and this interaction with the substrate may, in part, inhibit the unwinding activity of WRN. Additional experiments on the ATPase activity of WRN revealed a direct functional inhibition of the ATPase activity of WRN by topo I, which may also contribute to the helicase inhibition.

In the reciprocal interaction, we showed that WRN stimulated the ability of topo I to relax negatively supercoiled DNA and that this stimulation was not dependent on WRN catalytic activities. Moreover, we found that the topo I functionally interactive sites of WRN are located in the NH2 terminus (1–51 amino acids) and in the COOH terminus (949–1432) of WRN. The WRN extreme COOH terminus (1236–1432 amino acids) is necessary for topo I stimulation via interaction with the WRN COOH terminus. Our results are consistent with previous studies that found yeast Top3p interacts with the NH2 terminus and COOH terminus of Sgs1p (20), and human topo III interacts with the extreme NH2 terminus (1–212 amino acids) and the COOH terminus (1266–1417) of the BLM protein (21). A study in *Saccharomyces cerevisiae* demonstrated that a helicase-deficient mutant of the yeast RecQ homologue, sgs1, failed to rescue the premature aging phenotype of the SGS1 mutant strain but was still able to rescue...
the hypersensitivity of the SGS1 mutant to topo inhibitors (27). Another study suggests a bipartite structure of sgs1p and concluded that the NH2 terminus of Sgs1p has an essential role, distinct from its DNA helicase activity (34). Furthermore, BLM stimulates the DNA relaxation activity of human topo III (22), as reported here for WRN and topo I. However, a truncated BLM mutant that retains helicase activity, but lacks the NH2 and COOH termini, failed to stimulate topo III (22, 35). Similarly, we found that a WRN truncated mutant that also retains helicase activity but lacks the NH2 and COOH termini failed to stimulate topo I. Together these data suggest that modes of interaction between RecQ helicases and their topo partners may be conserved. However, it is important to note that topo I and topo III belong to different type I subfamilies, differ mechanistically and structurally, and have distinct cellular roles (17). Although WRN and BLM belong to the same RecQ helicase family, defects in these proteins give rise to distinctly different syndromes. Differences in protein partners, including associated topoisomerases, may reflect differences in WRN and BLM involvement in DNA metabolic pathways.

GFP-topo I has been shown to shuttle dynamically between the nucleoplasm and nucleolus (36). After treatment with the CPT derivative, topotecan, GFP-topo I is rapidly sumoylated (37). Sumoylation of the NH2-terminal region of topo I can prevent topo I binding to nucleolar sites, and CPT treatment increases sumoylation (38). Our finding that WRN domains interact with topo I can provide the means by which this rapidly cycling protein is recruited to WRN-associated repair complexes. CPT treatment relocates both WRN and topo I to the nucleoplasm, where we observed several foci containing both WRN and topo I, indicating that a subset of both proteins are present in the same protein complex. However, there may be additional proteins in the WRN/topo I complex that may be essential for topo I function. Topo I is present in the nucleoli of WRN-null cells such as AGO3141, arguing that WRN does not have a direct role in sequestering topo I to the nucleolus or retaining it there. Further research is required to understand the dynamics of these multifunctional proteins.

Studies have shown a hypersensitivity of WS cell lines to CPT (13), and one characteristic of this sensitivity is an increase in apoptotic induction after treatment with a sufficient dose of CPT. Here, we report that h-TERT WS fibroblast cell lines are also sensitive to CPT. Interestingly, cell extracts from h-TERT, SV40, and primary WS fibroblast cell lines exhibited a decrease in the overall relaxation activity on a supercoiled DNA plasmid, compared with the control cell lines. The reaction conditions were chosen to enrich specifically for topo I activity. ATP was omitted from the relaxation buffer to prevent potential relaxation activity from topo II, which is ATP dependent (39). Moreover, topo III-catalyzed relaxation of negatively supercoiled DNA is more efficient at a higher temperature (52°C; Ref. 40). Furthermore, E. coli and eukaryotic topo III characterized to date require a hypernegatively supercoiled plasmid DNA substrate for efficient relaxation (41–43). The above evidence suggests that the
relaxation activity observed with the various cell extracts was primarily attributable to topo I. Our evidence is consistent with the decrease in the relaxation activity exhibited by the WS cell extracts as a likely consequence of the lack of WRN protein, and in turn, a lack of stimulation of topo I activity.

In this study, we show that WS cells display sensitivity to CPT without a significant increase in the level of topo I (Fig. 6). CPT interferes with the DNA breakage-ligation reaction by stabilizing the intermediate DNA-topo I complex, consequently inhibiting the relaxation activity of topo I. We suggest that WRN might shorten the duration of this cleavable complex in the cell by stimulating topo I relaxation activity and, more specifically, the relaxation step of the topo I relaxation cycle (Fig. 7). Fig. 8 shows a model for how WRN and topo I might act together in vivo. An intermediate step in the relaxation cycle of topo I is the formation of the cleavable complex. If the relaxation cycle goes to completion, the DNA is relaxed. Control of the superhelical state of the DNA and its relaxation are important during replication, transcription, and the segregation of newly replicated chromosomes. Accumulation of negatively (−) or positively (+) supercoiled regions behind or ahead of the replication and transcription machineries can lead to genomic instability by blocking these DNA metabolic processes and by inducing illegitimate recombination events (44, 45).

The presence and duration of these topo I-DNA cleavable complexes in the cell is an important element because religation may occur with a heterogeneous DNA molecule, resulting in recombinant DNA (35). Covalent DNA-topo I complexes are also formed after UV-irradiation and may participate in the recognition of DNA damage (46). On the other hand, these complexes are potentially dangerous because of their ability to create single-strand breaks and to mediate illegitimate recombination leading to genomic instability. Consequently, the availability and duration of these complexes must be regulated. WRN is known to be an anti-recombination protein, and our data suggest that one way WRN prevents genomic instability is by regulating the duration of the topo I relaxation activity. By stimulating the religation step, WRN may prevent the cell from accumulating these DNA-topo I complexes. CPT is an important topo I inhibitor and is currently used for treatment of colon and ovarian carcinoma. WRN and other RecQ helicases members are now defined as caretakers of the genome (7). They do not directly regulate tumorigenesis but instead influence genomic stability and, therefore, the rate of accumulation of the necessary genetic alterations that are required for tumorigenesis. The WRN/topo I interaction offers further insight into how WRN protects the cell from events leading to genomic instability and cancer.

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PHYSICAL AND FUNCTIONAL INTERACTION BETWEEN WRN AND TOPO I

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