

Alterations in Linker Flexibility Suppress DNA Topoisomerase I Mutant-induced Cell Lethality*

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Eukaryotic DNA topoisomerase I (Top1p) catalyzes changes in DNA topology via the formation of a covalent enzyme-DNA intermediate, which is reversibly stabilized by the anticancer agent camptothecin (CPT). Crystallographic studies of the 70-kDa C terminus of human Top1p bound to duplex DNA describe a monomeric protein clamp circumscribing the DNA helix. The structures, which lack the N-terminal domain, comprise the conserved clamp, an extended linker domain, and the conserved C-terminal active site Tyr domain. CPT bound to the covalent Top1p-DNA complex limits linker flexibility, allowing structural determination of this domain. We previously reported that mutation of Ala⁶⁵³ to Pro in the linker increases the rate of enzyme-catalyzed DNA religation, thereby rendering Top1A653Pp resistant to CPT (Fiorani, P., Bruselles, A., Falconi, M., Chillemi, G., Desideri, A., and Benedetti P. (2003) *J. Biol. Chem.* 278, 43268–43275). Molecular dynamics studies suggested mutation-induced increases in linker flexibility alter Top1p catalyzed DNA religation. To address the functional consequences of linker flexibility on enzyme catalysis and drug sensitivity, we investigated the interactions of the A653P linker mutation with a self-poisoning T718A mutation within the active site of Top1p. The A653P mutation suppressed the lethal phenotype of Top1T718Ap in yeast, yet did not restore enzyme sensitivity to CPT. However, the specific activity of the double mutant was decreased *in vivo* and *in vitro*, consistent with a decrease in DNA binding. These findings support a model where changes in the flexibility or orientation of the linker alter the geometry of the active site and thereby the kinetics of DNA cleavage/religation catalyzed by Top1p.

Human DNA topoisomerase I (Top1p)³ plays a critical role in processes such as replication, recombination, and transcrip-

tion (1–4). This 91-kDa enzyme catalyzes the relaxation of supercoiled DNA through the transient cleavage of one strand of the DNA duplex. The Top1p catalytic cycle comprises five sequential steps: DNA binding, cleavage, strand rotation, religation, and release (1–4). During enzyme catalysis, Top1p undergoes large conformational changes, from an “open” structure that allows DNA binding, to the “closed clamp” conformation observed in co-crystal structures, in which the enzyme completely embraces the duplex DNA (5). DNA relaxation appears to proceed via a mechanism of DNA strand rotation, where the covalent attachment of Top1p to the 3'-phosphoryl end of the nicked DNA strand liberates the 5'-OH end to rotate around the intact non-scissile DNA strand (6). Remarkably, the dynamic changes in protein clamp conformation and linkage of DNA strands occur in the absence of any energy cofactors, such as ATP.

Eukaryotic DNA topoisomerase I is the cellular target of the antitumor drug camptothecin (CPT) (7–9). CPT specifically and reversibly binds the covalent Top1p-DNA complex to inhibit DNA religation and induce S-phase-dependent cell lethality. In the yeast *Saccharomyces cerevisiae*, single amino acid substitutions have been defined in Top1p that mimic the cytotoxic activity of CPT by inducing a terminal G₂-phase phenotype and increased rates of recombination (10–14). For example, substitution of Ala for residue Thr⁷²² (within the active site Tyr⁷²⁷ domain) induces a dramatic reduction in cell viability when expressed in yeast or human cells (10, 15). Similar to the action of CPT, this mutation enhances the stability of covalent enzyme-DNA complexes by a reduction in the rate of DNA religation (14). Substitution of Ala for the corresponding residue Thr⁷¹⁸ of human Top1p (top1T718A) induces a similar phenotype and alterations in enzyme catalysis (12). Thus, in yeast and human Top1p, mutation of this conserved Thr residue (located just five residues N-terminal to the active site Tyr) induces cell lethality as a consequence of an increase in the half-life of the covalent enzyme-DNA intermediate (14, 16). Recent structures of the C-terminal 70-kDa fragment of human Top1p (Topo70) with DNA and the ternary topotecan Topo70-DNA complex indicate the formation of a hydrogen bond between the hydroxyl of Thr⁷¹⁸ and the nonbridging oxygen of the +1 phosphodiester of the DNA (17). These data suggest that substitution of Ala in this position may alter the orientation of the 5'-OH nucleophile to alter the rate of DNA religation.

Structural and biochemical data indicate DNA topoisomerase I contains multiple domains that form a protein clamp around duplex DNA (18) (see Fig. 1). Based on limited proteol-

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³ The abbreviations used are: Top1p, DNA topoisomerase I; SUMO, small ubiquitin-related modifier; CPT, camptothecin; BSA, bovine serum albumin; nt, nucleotide; Me₂SO, dimethyl sulfoxide; WT, wild type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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ysis studies and crystal structures (18, 19) the protein can be divided into four major domains: a highly charged N-terminal region, the conserved core domain that forms the protein clamp, a pair of extended α -helices that form a positively charged linker domain and the highly conserved C-terminal domain containing the catalytic Tyr residue. The core domain contains all of the catalytic residues except the active site Tyr. The linker domain assumes a protruding coiled-coil conformation that is stabilized by hydrophobic side chain interactions between conserved heptads repeating residues. The linker connects the core with the C-terminal domain to position the active site Tyr within the catalytic pocket of the core. Evidence that the linker domain contacts DNA in solution is supported by proteolysis studies, which show that the linker is 10-fold more resistant to limited proteolysis when the enzyme is bound non-covalently to duplex DNA (20). It has been shown that breaking the physical linkage between the linker and core reduces enzyme binding of DNA and shifts the DNA cleavage-religation equilibrium toward religation. Moreover, molecular dynamic simulations suggest that the CPT resistance induced by a single Ala⁶⁵³ to Pro mutation within the linker domain (Fig. 1) results from an increase in linker flexibility, which enhances the rate of DNA religation (21). Taken together, these results suggest that linker flexibility may regulate the resolution of the Top1p-DNA covalent complex.

However, despite a wealth of biochemical, structural and modeling data on Top1p structure and activity, there is little direct evidence of linker domain flexibility influencing the geometry of the active site. To address this question we asked if the enhanced rate of DNA religation, imparted by the A653P linker mutation, would suppress the DNA religation defect induced by the T718A active site mutation. Indeed, here we describe that the combination of the two mutations (in Top1A653P/T718A), abolished the lethal phenotype of yeast cells expressing the single T718A mutant. The double mutant enzyme was catalytically active *in vitro* and *in vivo*, yet was resistant to CPT. Taken together these data indicate long range communications between the flexible linker domain and the active site of the enzyme.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Camptothecin (Sigma Aldrich) was dissolved in Me₂SO at a final concentration of 4 mg/ml and stored at -20°C . Anti-FLAG M₂ affinity gel, FLAG peptide and M₂ monoclonal antibodies were from Sigma Aldrich. *S. cerevisiae* strains EKY3 (*MAT α* , *ura3-52*, *his3 Δ 200*, *leu2 Δ 1*, *trp Δ 63*, *top1 Δ ::TRP1*), JCW28 (*MAT α* , *ura3-52*, *his3 Δ 200*, *leu2 Δ 1*, *trp Δ 63*, *top1 Δ* , *top2-4*), and RRY82 (*MAT α* , *ura3-52*, *his3 Δ 200*, *leu2 Δ 1*, *trp Δ 63*, *top1 Δ ::TRP1*, *ubc9-10*) were described previously (10, 22, 23). YE_pGAL1-hTOP1, YC_pGAL1-hTOP1, and YE_pGPD-topA have been described (24, 25).

The top1A653P/T718A mutant was obtained by ligating a 0.9-kb SphI-NheI fragment from YC_pGAL1-hTop1A653P (21), a 1.4-kb NheI-Clal fragment from YC_pGAL1-hTop1T718A plasmid (6), and a 9.4-kb SphI-Clal fragment from YC_p-GAL1-heTop1. The YC_pGAL1-heTOP1 contains the N-terminal FLAG epitope DYKDDDY recognized by the M2 monoclonal

antibody (12). For ease of presentation, the prefix has been dropped in the following discussions.

Drug Sensitivity and Cell Viability Assays—Yeast *top1 Δ* strains were transformed with vector YC_p50, YC_pGAL1-TOP1, YC_pGAL1-top1A653P, YC_pGAL1-top1T718A, or YC_pGAL1-top1A653P/T718A by LiOAc treatment (26) and selected on synthetic complete (SC)-uracil medium supplemented with 2% dextrose. Individual transformants were grown to an $A_{595} = 0.5$, and 5- μl aliquots of serial 10-fold dilutions were spotted onto SC-uracil plates plus 2% dextrose or 2% galactose, with or without the indicated concentrations of CPT. Cell viability was assessed following incubation at 30°C . For comparisons of EKY3 (*UBC9*) and RRY82 (*ubc9-10*) strains, cell viability was assayed at 36°C .

Purification of DNA Topoisomerase I—Epitope-tagged Top1 proteins were partially purified from galactose-induced EKY3 cells transformed with YC_pGAL1-TOP1 or YC_pGAL1-top1A653P/T718A by phosphocellulose chromatography as described (27, 28). To obtain homogeneous preparations, Top1 proteins eluted in TEEG (50 mM Tris (pH 7.4) 1 mM EDTA, 1 mM EGTA, 10% glycerol) plus 0.8 M KCl were diluted to a final 150 mM KCl and applied to an anti-FLAG M₂ affinity gel column. The column was washed with TBS (50 mM Tris (pH 7.4), 150 mM KCl), and the eTop1 proteins eluted with a solution of 100 $\mu\text{g/ml}$ FLAG peptide in TBS. The fractions were adjusted to a final 40% glycerol and stored at -20°C . Top1 protein levels and integrity were assessed in immunoblots, with the M₂ antibody.

Top1p Activity in Vitro—Top1 activity was assayed in a DNA relaxation assay (24, 27). 120 pmol of Top1 proteins were incubated in 30- μl reactions containing 0.5 μg of negatively supercoiled pHC624 plasmid DNA in 20 mM Tris, pH 7.5, 0.1 mM Na₂EDTA, 10 mM MgCl₂, 50 $\mu\text{g/ml}$ acetylated BSA and the indicated concentration of KCl. Reactions were incubated at 37°C and terminated at the times indicated with a final 0.5% SDS. Reaction products were resolved in 1% agarose gels, stained with ethidium bromide and visualized with a Bio-Rad Gel doc system. To quantitate relative levels of supercoiled and relaxed DNA topoisomers, the stained gels were first exposed to UV light to induce photo-nicking of the DNA, then re-stained with ethidium bromide. These steps obviated any contribution of DNA topology to ethidium bromide binding, and consequently, relative band intensity.

Top1p Activity in Vivo—JCW28 (*top1 Δ* , *top2-4*) cells, co-transformed with YE_pGPD-topA and either YC_pGAL1, YC_pGAL1-TOP1, YC_pGAL1-top1A653P, or YC_pGAL1-top1A653P/T718A were cultured in selective medium plus dextrose at 26°C , then diluted into selective medium supplemented with 2% raffinose. At $A_{595} = 1.0$, TOP1 expression was induced with a final 2% galactose. After 5 h, the culture was shifted to 36°C , the nonpermissive temperature for *top2-4*, for an additional 3 h. The cells were fixed with pre-chilled toluene/ethanol and the plasmid DNA purified as described (10, 29). The distribution of 2- μm DNA topoisomers was assessed by two-dimensional gel electrophoresis and Southern blotting as described (10, 27, 29).

DNA Cleavage Assays—CPT-induced stabilization of Top1-DNA covalent complexes was assessed in DNA cleavage assays

as described (10, 27, 29). Briefly, equal concentrations of purified Top1 proteins were incubated in 50- μ l reactions with a single 3' 32 P-end-labeled, 900-bp DNA fragment, which contains the exadecameric high affinity Top1p cleavage site (30) and was excised from plasmid pBlueAK3-1 (28). Where indicated, reactions were supplemented with 50 μ M CPT. Me₂SO was added to the no drug controls. Following incubation at 37 °C for 30 min, reactions were terminated by the addition of 1% SDS, and the cleavage products were resolved in a 7 M urea/8% polyacrylamide gel and visualized by Phosphorimager analysis (31).

Suicide Cleavage Assays—A 52-mer oligonucleotide (5'-GCTATACGAATTCGCTATAATTCATATGATAGCGGATCCAAAAAAGACTTAG-3'), which contains a Top1p high affinity cleavage site, was 5' end-labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. A 100-nt (3'-CGATATGCTTAAGCGATATTAAGTATACTATCGCCTAGGTTTTTTCTGATCTTTTTTTTTTCGAATTCGTTGTATACCATAGCAGCCTTAAGTTACTC-5') noncissile DNA strand and a 50-nt (5'-AGAAAAAAAAAAGCTTAAGCAACATATGGTATCGTCGGAATTCATGAG-3') oligonucleotide were 5'-phosphorylated with unlabeled ATP. The three strands (kind gift from Birgitta Knudsen, Aarhus University) were annealed at a molar ratio of 2:3:1 of 100 nt: 52 nt: 50 nt in 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol by heating to 95 °C for 5 min, followed by slow cooling to room temperature. The resulting DNA substrate (20 nM) was incubated with equal concentrations of Top1 proteins in 20 mM Tris, pH 7.5, 0.1 mM EDTA, 10 mM MgCl₂, 50 μ g/ml acetylated BSA, and 50 mM KCl at 25 °C. At the times indicated, 10- μ l aliquots were quenched with 0.5% SDS and heated to 75 °C. Half of these mixtures were ethanol-precipitated and then resolved in 7 M urea/12% acrylamide gels (Fig. 8B). The second half of the reaction was added to 2.5 ml of protein sample loading buffer (156 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, 12.5% mercaptoethanol, 0.0025% bromophenol blue), boiled for 5 min, and 10 ml of each sample were analyzed by 10% Tricine, SDS-PAGE (Fig. 8C). The percentage of cleavage was determined by PhosphorImager and ImageQuant software and normalized on the total amount of radioactivity in each lane.

Religation Assays—To assess enzyme-catalyzed DNA cleavage and religation, the truncated suicide oligonucleotide substrate described above was used. The 50-nt (5'-AGAAAAAAAAAAGCTTAAGCAACATATGGTATCGTCGGAATTCATGAG-3') religation oligonucleotide was not 5'-phosphorylated to allow for enzyme-catalyzed religation. The resulting DNA substrate (20 nM) was incubated with equal concentrations of Top1 proteins in 20 mM Tris, pH 7.5, 0.1 mM EDTA, 10 mM MgCl₂, 50 μ g/ml acetylated BSA, and 50 mM KCl at 25 °C. At the times indicated, 5- μ l aliquots were quenched with 0.5% SDS and heated to 75 °C. The reaction products were resolved in 7 M urea/16% acrylamide gels and visualized with a PhosphorImager (Fig. 9B).

RESULTS

The CPT-resistant A653P Mutation Suppresses Top1T718A-induced Lethality—In a yeast genetic screen for catalytically active, CPT-resistant human *top1* mutants, we previously

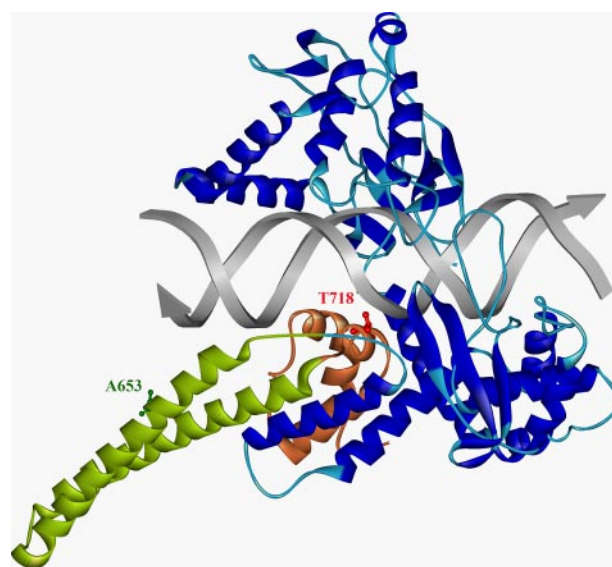


FIGURE 1. Structure of the 70-kDa C terminus of human DNA topoisomerase I noncovalently bound to duplex DNA. Residues comprising the core protein clamp are in blue, the coiled-coil linker domain is green, and the active site tyrosine domain is in brown. Ball and stick representations of linker residue Ala⁶⁵³ and active site domain Thr⁷¹⁸ are shown.

reported an Ala⁶⁵³ to Pro substitution within the linker domain (Fig. 1) that sufficed to confer drug resistance (21). Subsequent biochemical studies and molecular dynamic simulations suggested a model whereby a mutation-induced increase in linker flexibility enhanced the rate of Top1p-catalyzed DNA religation, which in turn decreased the ability of CPT to stabilize the covalent enzyme-DNA intermediate (21). These findings further suggested that the mobility of the linker domain directly impacts the geometry and activity of the active site. If this interpretation were correct, we reasoned that the increased rate of DNA religation imparted by the A653P linker mutation would suppress the decreased rate of DNA religation, and consequently the lethality, of the active site T718A mutant (see Fig. 1). Indeed, as seen in Fig. 2, this prediction was borne out in *top1* Δ yeast cells expressing the human Top1 mutants.

As previously reported (12), expression of the top1T718A mutant from the galactose-inducible pGAL1 promoter, produced a dramatic reduction in yeast cell viability (Fig. 2). In contrast, cells expressing either the single top1A653P or the double top1A653P/T718A mutant were viable. Although pGAL1-promoted expression of top1T718A is cytotoxic, biochemical studies with purified protein demonstrated that the enzyme is sensitive to CPT (12). To assess the CPT sensitivity of the double *top1A653P/T718A* mutant, yeast cells transformed with pGAL-promoted TOP1, top1A653P, top1T718A, and top1A653P/T718A were assessed for growth on selective medium containing CPT. As shown in Fig. 2, the double mutant top1A653P/T718A conferred a CPT-resistant phenotype comparable to that of the single top1A653P mutant.

To ensure that the CPT-resistant phenotype did not result from the loss of Top1A653P/T718A catalytic activity, the *in vivo* activity of the wild-type and mutant enzymes was assessed. As previously reported (10, 27, 29), Top1-catalyzed relaxation of local domains of positively supercoiled DNA can be readily addressed in JCW28 (*top1* Δ ,*top2ts*) cells that constitutively

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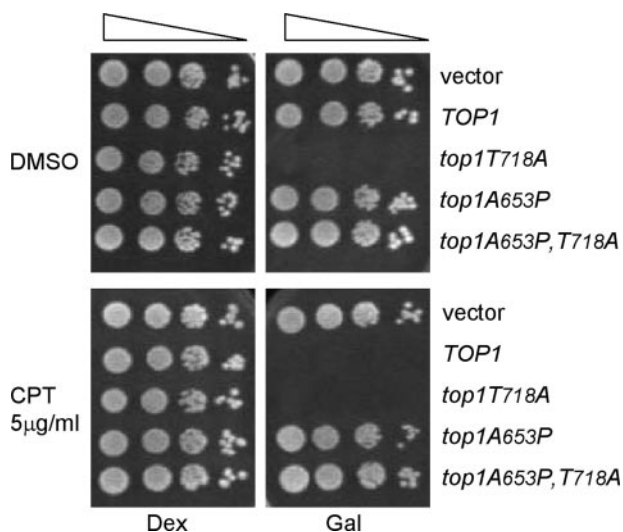


FIGURE 2. The A653P mutation suppresses the lethal phenotype induced by expression of top1T718A. Exponential cultures of EYK3 (*top1Δ*) cells transformed with vector control, YCpGAL1-*TOP1*, YCpGAL1-*top1A653P*, YCpGAL1-*top1T718A*, or YCpGAL1-*top1A653P/T718A* were serially 10-fold diluted and spotted onto SC-uracil plates supplemented with dextrose (*Dex*) or galactose (*Gal*) and, as indicated, CPT. Cell viability was assessed following incubation at 30 °C; *DMSO*, Me₂SO.

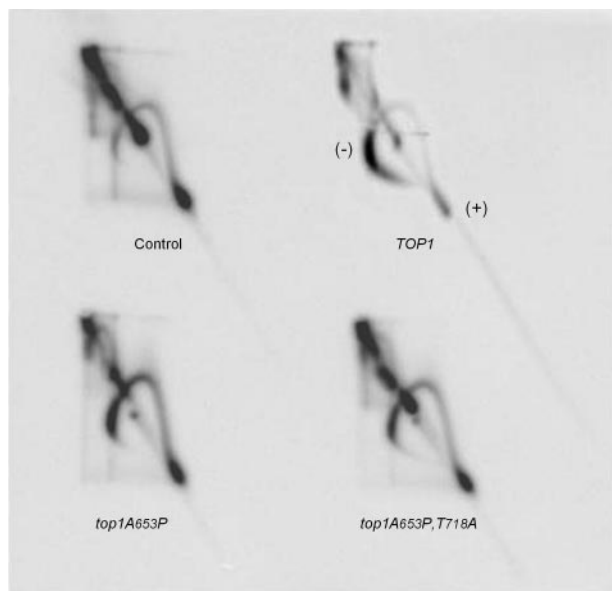


FIGURE 3. *In vivo* activity of Top1A653Pp and Top1A653P/T718Ap. JCW28 (*top1Δ, top2^{ts}*) cells, co-transformed with YEpGPD-*topA* and either vector control, YCpGAL1-*TOP1* (*TOP1*), YCpGAL1-*top1A653P* (*top1A653P*), or YCpGAL1-*top1A653P/T718A* (*top1A653P/T718A*) and cultured at 26 °C in selective media containing raffinose, were shifted to 36 °C and induced with galactose. Plasmid DNA topoisomers were purified from toluene-arrested cells, resolved by two-dimensional gel electrophoresis and visualized in Southern blots. The relative positions of positively supercoiled (+) and negatively supercoiled (–) plasmid DNAs are shown in the *TOP1* arc of topoisomers.

express the bacterial *topA* gene. Transcription from divergent promoters in the 2- μ m plasmid induces local domains of positive and negative supercoils (25). At the nonpermissive temperature, cells lack endogenous yeast Top1 and Top2 activity. However, bacterial TopA preferentially catalyzes the relaxation of negatively supercoiled DNA, resulting in the accumulation of positively supercoiled plasmids (see control in Fig. 3). In contrast, when a catalytically active human Top1 is also expressed

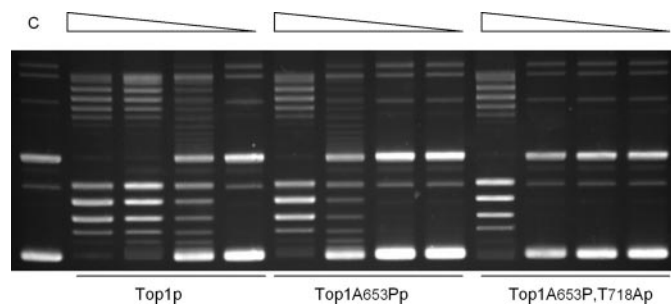


FIGURE 4. *In vitro* catalytic activity of wild-type and mutant Top1p. 120 pmol of purified Top1p, Top1A653Pp, and hTop1A653P/T718Ap were serially 10-fold diluted and incubated in DNA relaxation assays with negatively supercoiled plasmid DNA. Following incubation at 37 °C, the reaction products were resolved in agarose gels and visualized after staining with ethidium bromide. C indicates no enzyme control.

in these cells, Top1 catalyzes the relaxation of both positively and negatively supercoiled DNA. This produces a shift in plasmid topoisomer distribution from that of positively supercoiled (+) to the slightly negatively supercoiled distribution of plasmid DNA assembled with nucleosomes (–) (*TOP1 panel* in Fig. 3). The single mutant Top1A653Pp and double mutant Top1A653P/T718Ap were also catalytically active (Fig. 3), albeit at a slightly lower level than that seen for wild-type Top1p or reported for Top1T718Ap (12). Nevertheless, the CPT resistance of yeast cells expressing the double mutant could not be attributed to the loss of catalytic activity *in vivo*.

Top1A653P/T718Ap Catalytic Activity *In Vitro*—To assess whether the combination of the two mutations alters the catalytic cycle of Top1p, the proteins were purified to homogeneity from galactose-induced yeast *top1Δ* cells, as detailed under “Experimental Procedures.” The specific activity of Top1p, Top1A653P, and Top1A653P/T718Ap was determined in plasmid DNA relaxation assays. Serial dilutions of equal protein concentrations were incubated with negatively supercoiled plasmid DNA, and the reaction products resolved in agarose gels, as described (24, 27). As illustrated in Fig. 4, the specific activity of both mutants were reduced relative to that of the wild-type enzyme. Top1A653Pp activity was ~10-fold lower than Top1p (as reported in Ref. 21), whereas Top1A653P/T718Ap was reduced by 10–20-fold. Thus, specific activity of the double mutant was about 2–3-fold lower than that of the single mutant Top1A653Pp, consistent with the slight decrease in enzyme activity reported in our earlier studies of Top1T718Ap (12). The observed reduction in catalytic activity of the mutant Top1A653P/T718A enzyme could result from several alterations in enzyme catalytic cycle, including reduced DNA substrate binding or decreased rates of DNA cleavage/religation.

To discriminate between these possibilities, we first assessed the effects of ionic strength on mutant enzyme catalysis. Top1 activity exhibits a salt optimum around 150 mM (32). Deviations from this value have previously been shown to correspond with increased or decreased DNA binding, for salt optimum > or <150 mM, respectively. Limiting amounts of Top1p or Top1A653P/T718Ap were incubated in plasmid relaxation assays containing increasing concentrations of KCl (Fig. 5A). The percentage of relaxed plasmid DNA topoisomers produced ((relaxed DNA topoisomers)/(relaxed + supercoiled DNA

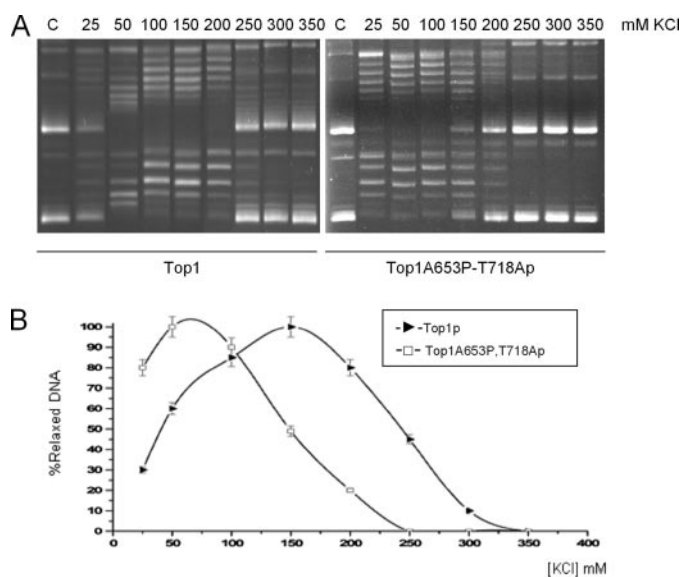


FIGURE 5. The Top1A653P/T718A mutant enzyme exhibits a lower salt optimum than wild-type Top1p in plasmid DNA relaxation assays. *A*, limiting amounts of purified Top1p or Top1A653P/T718Ap were incubated with plasmid DNA at 37 °C, in the presence of the indicated concentration of KCl. The reaction products were resolved in agarose gels as described in the legend to Fig. 4. *B*, the % of relaxed plasmid DNA topoisomers obtained with Top1p (▲) and Top1A653P/T718Ap (□) at the indicated concentration of KCl were determined in a minimum of three independent assays. Error bars indicate S.D.

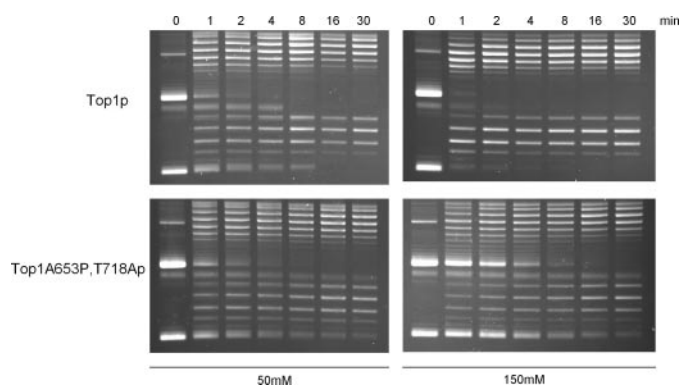


FIGURE 6. Time course of plasmid DNA relaxation catalyzed by Top1p and Top1A653P/T718Ap. Equal concentrations of Top1p or Top1A653P/T718Ap were incubated with supercoiled plasmid DNA in the presence of 50 or 150 mM KCl. At the times indicated, aliquots were treated with 0.5% SDS, and the reaction products were resolved in agarose gels as in Fig. 4. In each case, the $t = 0$ sample was resolved in the same gel as the corresponding reactions.

topoisomers)) following incubation with the enzymes was quantified and plotted as a function of salt concentration (Fig. 5*B*). The double mutant shows a significantly lower salt optimum (50 mM KCl) than the WT enzyme and was reduced at 150 mM KCl. Moreover, the kinetics of double mutant-catalyzed DNA relaxation was enhanced at lower salt, whereas exactly the opposite was observed with wild-type Top1p (Fig. 6). These results are consistent with a lower affinity of Top1A653P/T718Ap for DNA than wild-type Top1p. Thus, consistent with the increase in enzyme catalysis at low salt, alterations in the linker domain and the active site of Top1p appears to increase the dissociation rate of the enzyme from DNA.

Top1A653P/T718Ap Sensitivity to CPT—To assess the mechanism of Top1A653P/T718Ap resistance to CPT, the abil-

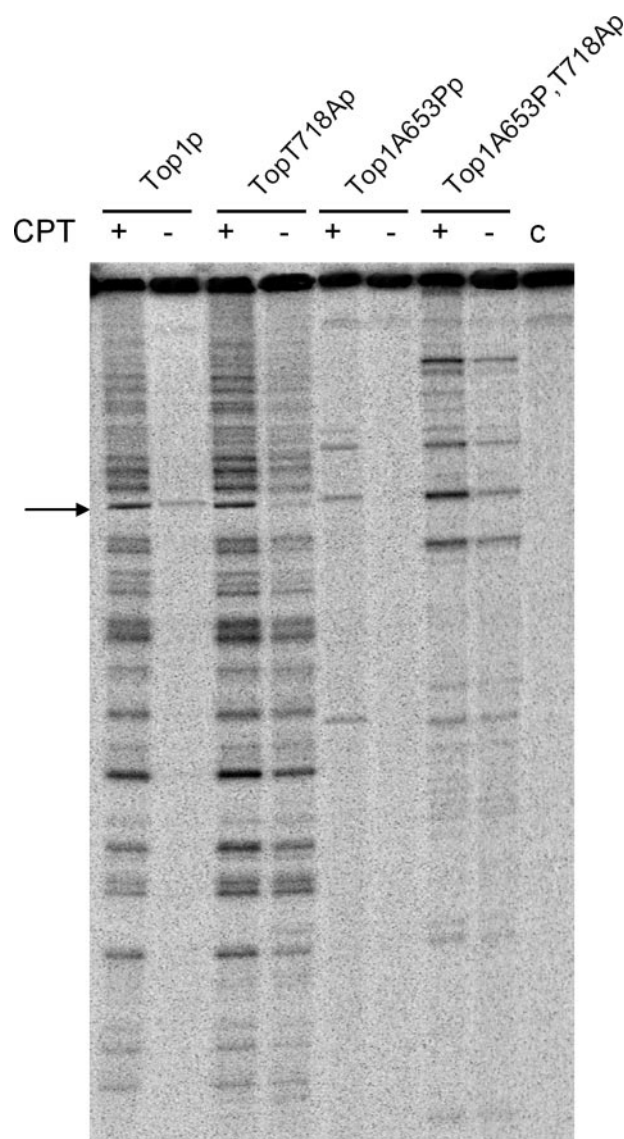


FIGURE 7. Top1A653P/T718Ap exhibits altered patterns of CPT sensitivity *in vitro*. Equal concentrations of Top1p, Top1A653P/T718Ap, Top1A653Pp, and Top1T718Ap were incubated with a single ^{32}P end-labeled DNA fragment in absence or presence of CPT as described under "Experimental Procedures." After 30 min, the reactions were stopped with 0.5% SDS, and the cleavage products resolved by urea/PAGE. C indicates no enzyme control. The arrow indicates the position of a high affinity cleavage site.

ity of CPT to stabilize the covalent DNA-enzyme complex was assessed in a DNA cleavage assay (31). Equal protein concentrations were incubated with a 900-bp DNA fragment uniquely labeled at one 3'-end, in the presence of 50 mM KCl and, where indicated, CPT. The reactions were terminated with SDS, and the products resolved in sequencing gels. Under these conditions, the relative intensity and distribution of cleaved DNA fragments can be used to assess mutation and/or CPT-induced alterations in the steady state levels of covalent enzyme-DNA intermediates. As can be seen in Fig. 7, little DNA cleavage by wild-type Top1p was detected in the absence of drug, whereas CPT induced a dramatic increase in cleaved DNA.

With the double Top1A653P/T718Ap mutant, a low, but reproducible level of DNA cleavage was observed in the absence of CPT (Fig. 7). The mutant enzyme also exhibited

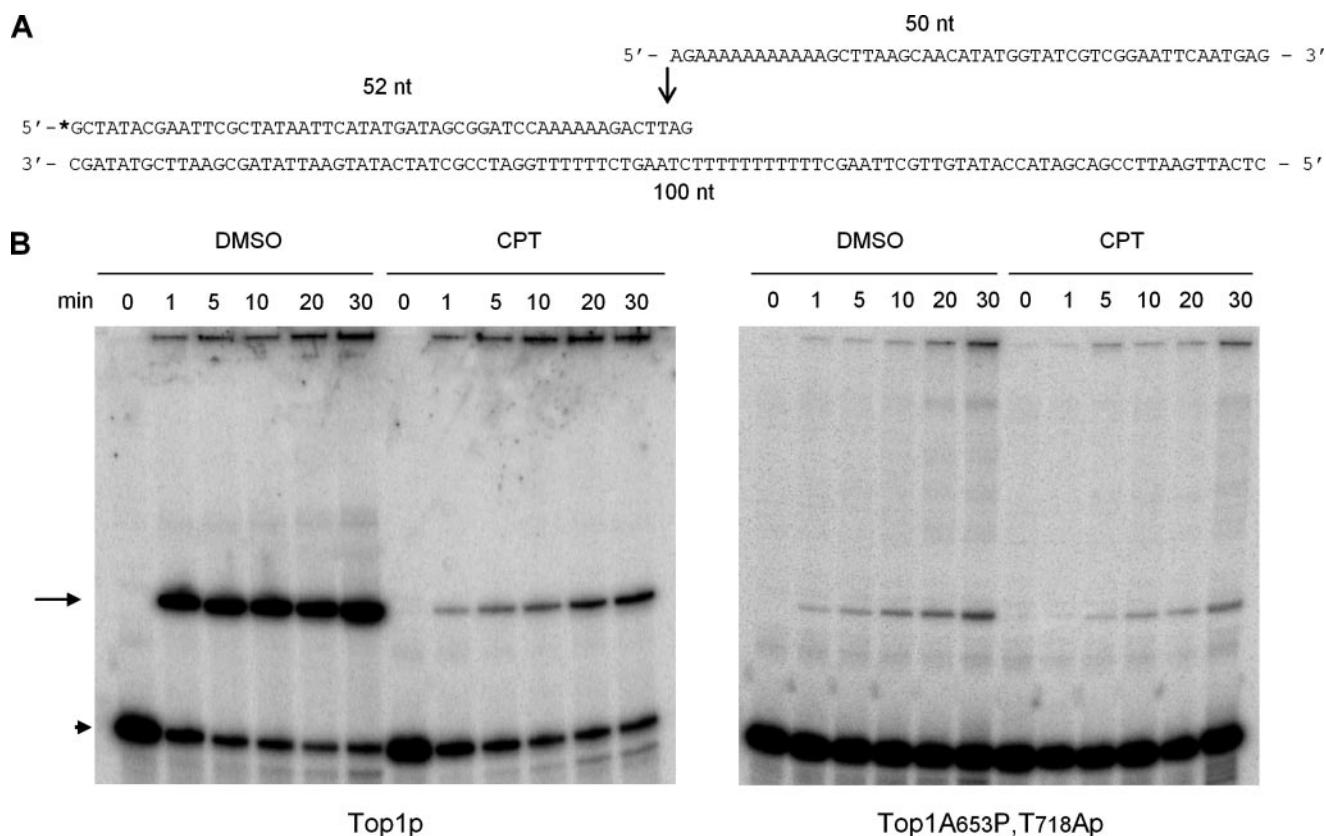


FIGURE 9. DNA ligation by Top1A653P/T718Ap is not sensitive to CPT. *A*, as in Fig. 8*A*, cleavage of the 5'-radiolabeled scissile strand (52 nt, arrowhead) liberates a AG dinucleotide to trap the covalent DNA-Top1p complex. However, ligation of the cleaved DNA strand to the unphosphorylated 50-nt oligonucleotide generates a radiolabeled 100-nt fragment (arrow). *B*, equal concentrations of Top1p and Top1A653P/T718Ap were incubated in the cleavage/religation assay depicted in *A*, in the absence or presence of CPT. At the indicated time points, following the addition of the religation oligonucleotide, aliquots were treated with 0.5% SDS, and the reaction products resolved by urea/PAGE and visualized by PhosphorImaging. DMSO, Me₂SO.

In contrast, the unphosphorylated 50-nt strand, diagrammed in Fig. 9*A*, may participate in the religation of the Top1p-cleaved DNA, such that mutation-induced effects on DNA religation can also be assessed. Typically, shorter DNA suicide substrates are used, and the religation DNA strand is added after covalent complexes are trapped by cleavage of the suicide substrate alone (14, 16). However, consistent with the cleavage pattern observed in Fig. 7, the double Top1A653P/T718Ap mutant was unable to cleave DNA fragments smaller than 100 bp. Moreover, we were unable to detect any cleavage of suicide DNA substrates in the absence of an annealed religation strand (data not shown).

As seen in Fig. 8*B*, both wild-type Top1p and Top1A653P/T718Ap cleaved the 52-nt oligonucleotide at the preferred cleavage site producing a covalent complex that did not enter the gel. To quantitate the amount of cleavable complex obtained, the same samples were analyzed by SDS-PAGE (Fig. 8*C*). For both enzymes, the formation of covalent complexes reached a plateau after 5-min incubation. However, the extent of DNA cleavage by the double mutant was reduced ~60% at any given time (Fig. 8*D*). Taken together, these data suggest the alterations in DNA binding induced by the linker A653P mutation are localized to linker-DNA substrate interactions 3' to site of DNA scission on the scissile DNA strand, *i.e.* to the left of the active site in the view shown in Fig. 1.

In the presence of Top1p, religated DNA molecules accumulate very rapidly in the absence of CPT, whereas the rate of DNA religation is dramatically reduced in the presence of CPT (Fig. 9*B*). These data are consistent with DNA cleavage being the rate-limiting step in enzyme catalysis (33) and the ability of CPT to selectively inhibit the resolution of the covalent Top1p-DNA intermediate (34). In contrast, the rate of DNA cleavage by Top1A653P/T718Ap was substantially reduced in the absence of CPT, with CPT inducing only marginal effects on the rate of DNA religation. These data are consistent with a decrease in mutant enzyme binding of DNA. Yet, the alteration in the apparent rate of DNA religation of the double mutant is quite interesting. In yeast, the rate of DNA religation catalyzed by Top1T722Ap (analogous to human Top1T718A) is reduced (14), whereas the A653P mutation in human Top1 has been shown to enhance the rate of DNA religation (21). A comparison of the relative amounts of DNA substrate and ligated DNA product indicate wild-type Top1p and Top1A653P/T718Ap exhibit comparable rates of DNA religation in the absence of CPT. Thus, the A653P mutation suppressed the religation defect imparted by the T718A mutation.

Defects in SUMOylation Enhance the CPT Sensitivity of Cells Expressing Top1A653Pp or Top1 A653P/T718Ap—As suggested by molecular dynamic simulations, the substitution of Ala⁶⁵³ with a Pro residue could substantially impact the coiled-

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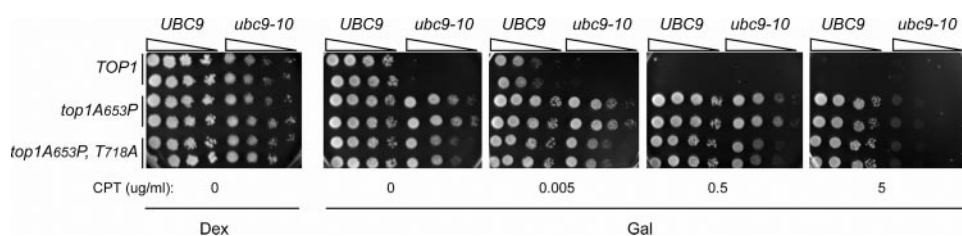


FIGURE 10. Defects in SUMOylation enhance the CPT sensitivity of cells expressing Top1A653Pp or Top1 A653P/T718Ap. Exponential cultures of isogenic wild-type *UBC9* and *ubc9-10* mutant strains (deleted for *TOP1*), transformed with YCpGAL1-*TOP1*, YCpGAL1-*top1A653P*, or YCpGAL1-*top1A653P/T718A*, were serially 10-fold diluted and spotted onto selective medium supplemented with dextrose (*Dex*) or galactose (*Gal*) and the indicated concentration of CPT. Cell viability was assessed following incubation at 36 °C.

coil structure and flexibility of the linker domain (21). However, the ⁶⁵³AKKE⁶⁵⁶ residues also match the consensus site (Ψ KX(E/D)) for the covalent modification of Lys⁶⁵⁴ with SUMO (for small ubiquitin-related modifier) (35). SUMOylation of Top1p has been reported and suggested to play a role in modulating cell sensitivity to CPT (36–38). We recently described a temperature sensitive mutation in the SUMO-conjugating enzyme, yeast Ubc9, which enhances cell sensitivity to a wide range of DNA damaging agents (23, 40). However, mutation of consensus SUMO sites in yeast Top1 lead us to conclude that the defect in mutant *ubc9-10* cell sensitivity to CPT could not be ascribed to alterations in Top1 SUMOylation (23). Nevertheless, as one of these sites corresponds to the ⁶⁵³AKKE⁶⁵⁶ sequences in human Top1p, we asked if alterations in SUMO conjugation might affect the CPT sensitivity of these mutant enzymes *in vivo*.

In contrast to isogenic wild-type *UBC9* cells, galactose-promoted expression of wild-type human Top1p proved lethal to *ubc9-10* mutant cells at the nonpermissive temperature, even in the absence of CPT (Fig. 10). However, at higher drug concentrations, expression of either Top1A653Pp or Top1A653P/T718Ap restored the CPT sensitivity of cells defective for global Ubc9-catalyzed SUMO conjugation. These data indicate that while the *in vivo* CPT sensitivities of both mutant enzymes is less than wild-type Top1p, they are, nevertheless, comparable and regulated by alterations in global SUMOylation.

DISCUSSION

Several lines of investigation with specific Top1p linker defects support a critical role for this domain in modulating enzyme dynamics. For example, biochemical assays of a reconstituted human enzyme (topo58/6.3) comprising the clamp core and conserved C-terminal domain, but lacking the linker (19), or Topo58/12, in which the linker domain is not covalently attached to the core domain (41), or a topo70 660–688 deletion mutant containing a truncated linker domain (42) all exhibit a reduction in specific enzyme activity, a reduction in DNA binding and a more distributive mode of DNA relaxation. As with the single A653P mutation in the linker domain (21), these defects in linker architecture also result in reduced enzyme sensitivity to CPT, corresponding with a shift in the cleavage-religation equilibrium toward religation.

A dynamic coupling between the linker domain flexibility and the active site of Top1p may also be inferred from x-ray structures of Topo70 in the presence and the absence of the

CPT analog Topotecan (17). In the Topo70-DNA covalent complex, the flexibility of the linker domain prevents its structural determination. However, in the ternary Topo70-DNA-Topotecan complex, the linker domain is oriented at an oblique angle to the DNA helix (17).

Specific contacts with the DNA also involve other Top1p domains. Residues of the opposable “lip” domains contact the phosphodiester backbone of the DNA and

form a salt bridge to close the Top1p clamp around duplex DNA. Using molecular modeling to design a reversible disulfide bond across the opposable “lip” domains, we demonstrated that DNA rotation is inhibited within the locked Top1p-clamp-DNA complex (5). Thus, some flexibility of Top1p protein domains is required for enzyme binding of DNA, and the rotation of DNA strands is necessary to effect changes in DNA topology.

More recently, the real time analysis of DNA strand rotation within individual Top1p-DNA complexes was assessed using magnetic tweezers in a single molecule analysis (43). The data suggest the enzyme imposes considerable friction to impede DNA rotation, consistent with the interaction of several Top1p protein domains with the DNA. Based on these considerations, we considered that interactions of the flexible linker domain with the DNA might create some of the friction that restricts strand rotation, while also modulating the geometry of the active site in conjunction with DNA rotation.

In co-crystal structures of Topo70 and DNA, the side chain of Thr⁷¹⁸ is hydrogen-bonded with the phosphate group of the G + 2 base of the scissile strand (17). Staker *et al.* posited that this interaction might position the 5'-OH end of the cleaved DNA for nucleophilic attack of the 3'-phosphotyrosyl bond to effect DNA religation (17); thereby providing an explanation for the lethal phenotype of the T718A mutant (10, 12, 15). Indeed, recent molecular dynamic simulations of the Topo70-DNA complex further support the formation of a hydrogen bond between Thr⁷¹⁸ and the G + 2 phosphate group, consistent with a model whereby enzyme controls the relative position of the +1 base with regard to the active site, by means of hydrogen bonds with the +2 G phosphate group (44). Taken together, these observations indicate that the two mutations A653P and T718A induce opposite effects on enzyme catalysis: A653P enhances (21), whereas T718A decreases DNA religation (14, 16).

In this article, we examined the combined effects of these mutations to assess the relative contributions of the linker domain and active site residues on Top1p catalysis. Indeed, these studies indicate that the flexibility of the linker domain affects the geometry of the active site, to influence the DNA cleavage/religation equilibrium of the enzyme through long-range conformational contacts. Our results show that the A653P mutation in the linker domain suppressed the lethal phenotype of the T718A mutant. Moreover, the A653P-in-

duced alterations in linker domain function also abolished the enhanced CPT sensitivity of Top1T718Ap.

Although the double mutant Top1A653P/T718Ap was active *in vivo*, the specific activity of the mutant enzyme *in vitro* was about 20-fold lower than wild-type Top1p. The more distributive mode of plasmid DNA relaxation and the reduced salt optimum of the mutant enzyme relative to wild-type Top1p were consistent with decreased mutant enzyme affinity for DNA. In fact, the salt optimum of 50 mM KCl exhibited by Top1A653P/T718Ap in plasmid DNA relaxation assays, compared with 150 mM KCl for wild-type Top1p, indicates a similar defect in DNA binding exhibited by a panel of intragenic suppressors of yeast Top1T722A-induced lethality (39). The decreased rate of DNA cleavage seen with the double mutant enzyme in the suicide cleavage assay depicted in Fig. 8 further implicates the concerted action of the active site and linker domain mutations in the effecting alterations in DNA binding and cleavage. However, as the steady state levels of mutant enzyme-DNA complexes stabilized by CPT increase to those seen with wild-type Top1p as the length of the DNA substrate exceeds 250 bp (see Fig. 7), these data suggest Top1A653P/T718Ap binding to DNA may also be affected by alterations in DNA structure, such as DNA ends. As the pattern of CPT-stabilized complexes also differs from that observed for wild-type Top1, these data further suggest DNA sequence may also influence Top1A653P/T718Ap DNA binding/cleavage. Moreover, Top1A653P/T718Ap was only able to cleave the suicide substrate diagrammed in Fig. 8A when the religation strand was annealed, but not in its absence. These data suggest the mutant enzyme requires duplex DNA 3' to the site of DNA scission to effectively bind and cleave a DNA substrate. This defect in DNA binding was not observed with either single mutant.

The basis for the reduced sensitivity of the double mutant to CPT must therefore be found in some step beyond the binding of the drug to the enzyme-DNA covalent complex. In this context it is interesting to analyze in more detail the functional properties of the mutant in comparison to the wild-type enzyme. Cleavage-religation assay indicates a defect in DNA cleavage by the double mutant. However, there is no predictive value in assessing the rate of religation because of the apparent defect in DNA binding and cleavage using a short DNA substrate. This is also apparent in the DNA cleavage assay, where CPT efficiently stabilizes complexes where cleavage occurs at a site >250 bp from the 3'-end, but is progressively less effective in stabilizing complexes formed near the end. All of this can be interpreted in terms of a defect in DNA binding. However, the fact that CPT has little effect on religation using a short DNA substrate further suggests that the alterations in protein-DNA interactions also affect drug binding to the covalent complex. In other words, CPT binding requires a specific geometry of the enzyme-DNA complex.

The results obtained with the SUMOylation defective *ubc9-10* mutant (23, 40) suggest the DNA damage induced by the top1A653P/T718A double mutant or the single top1A653P mutant in the presence of CPT is comparable. Presumably, issues of DNA substrate length *in vivo* are not at issue. Nevertheless, the double mutant also exhibits a slow growth pheno-

type in the absence of drug, consistent with the low level of covalent complexes detected *in vitro* in the absence of CPT.

The interesting finding here is that the alterations in enzyme catalysis and, by extension, DNA binding, are induced by a mutation in the linker, which has been postulated to increase the flexibility of the coiled-coil structure of this domain. These findings support a model whereby changes in the flexibility or orientation of the linker, relative to the catalytic pocket of the enzyme, alters the kinetics of the DNA cleavage/religation reactions catalyzed by Top1p.

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Alterations in Linker Flexibility Suppress DNA Topoisomerase I Mutant-induced Cell Lethality

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