Mutation of Gly^{721} Alters DNA Topoisomerase I Active Site Architecture and Sensitivity to Camptothecin

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DNA topoisomerase I (Top1p) catalyzes the relaxation of supercoiled DNA via a concerted mechanism of DNA strand cleavage and religation. Top1p is the cellular target of the anticancer drug camptothecin (CPT), which reversibly stabilizes a covalent enzyme-DNA intermediate. Top1p clamps around duplex DNA, wherein the core and C-terminal domains are connected by extended α-helices (linker domain), which position the active site Tyr of the C-terminal domain within the catalytic pocket. The physical connection of the linker with the Top1p clamp as well as linker flexibility affect enzyme sensitivity to CPT. Crystallographic data reveal that a conserved Gly residue (located at the juncture between the linker and C-terminal domains) is at one end of a short α-helix, which extends to the active site Tyr covalently linked to the DNA. In the presence of drug, the linker is rigid and this α-helix extends to include Gly and the preceding Leu. We report that mutation of this conserved Gly in yeast Top1p alters enzyme sensitivity to CPT. Mutating Gly to Asp, Glu, Asn, Gln, Leu, or Ala enhanced enzyme CPT sensitivity, with the acidic residues inducing the greatest increase in drug sensitivity in vivo and in vitro. By contrast, Val or Phe substituents rendered the enzyme CPT-resistant. Mutation-induced alterations in enzyme architecture preceding the active site Tyr suggest these structural transitionsmodulate enzyme sensitivity to CPT, while enhancing the rate of DNA cleavage. We postulate that this conserved Gly residue provides a flexible hinge within the Top1p catalytic pocket to facilitate linker dynamics and the structural alterations that accompany drug binding of the covalent enzyme-DNA intermediate.

Eukaryotic DNA topoisomerase 1 (Top1p)^2 alters DNA topology by introducing a transient break in a single strand of the DNA duplex, thereby allowing strand rotation at the site of DNA scission to relieve superhelical tension (1–3). The enzyme plays critical roles in processes such as replication, transcription, recombination, and chromosomal condensation, with a reaction mechanism highly conserved from yeast to human. The active site tyrosine (Tyr^{727} in yeast) acts as a nucleophile to cleave a single DNA strand, forming a covalent phosphotyrosyl linkage between the enzyme and the 3’-end of the DNA. The free 5’-OH end of the scissile strand then rotates about the uncleaved DNA strand in a manner dictated by torsional strain in the DNA. In a second transesterification reaction, the nucleophilic attack by the 5’-OH resolves the covalent Top1p-DNA intermediate, and the enzyme is liberated from the religated DNA.

DNA cleavage by Top1p is, at least in part, rate-limiting, thus ensuring low steady state-levels of the covalent enzyme-DNA complex (4). However, increased concentrations of covalent complexes, either as a consequence of drug action, Top1p mutation, or the formation of DNA adducts, converts Top1p into a cellular poison (5–16). The camptothecin (CPT) class of chemotherapeutics reversibly stabilizes the covalent intermediate by intercalating into the protein-linked DNA nick and displacing the 5’-OH end of the DNA to prevent religation (5, 10, 12). Moreover, recent studies demonstrate that CPT binding preferentially impedes Top1p-catalyzed uncoiling of positively supercoiled DNA (17). Thus, during S-phase, either the ternary CPT-Top1p-DNA complex itself, or the positive supercoils that accumulate between the replication machinery and the ternary complex, present obstacles to advancing replication forks and trigger the irreversible DNA lesions that induce cell death. Mutation of conserved residues within the enzyme active site may also alter the DNA cleavage/religation equilibrium catalyzed by Top1p, leading to increased covalent complex formation and cell death (7, 8, 11). For example, as with CPT treatment of wild-type Top1p, substitution of Ala for Thr^{722} reduces the rate of enzyme-catalyzed religation, while mutation of Asn^{726} to His enhances the rate of DNA cleavage (6). The same substitutions of the corresponding residues in human Top1p also produce “self-poisoning” enzymes with similar effects on enzyme catalysis (8, 18).

Co-crystal structures of a 70-kDa C-terminal fragment of human Top1p (Topo70) in noncovalent or covalent complexes with DNA revealed the unusual architecture of the monomeric enzyme (19–21). Top1p forms a protein clamp around duplex DNA, where interaction of opposable “Lip” domains completes the circumscission of the tightly packed DNA by the protein core. We used molecular modeling to design a reversible disulfide bond between the lip domains, and demonstrated that locking the clamp prevents DNA rotation within the covalent Top1p-DNA complex (22). Moreover, expression of a catalytically inactive Top1Y^{722}P-p-clamp proved toxic in the absence of DNA cleavage. The Top1p clamp consists of a conserved cen-
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tral core that includes all of the active site residues necessary for enzyme catalysis, except the active site Tyr\textsuperscript{727} (19, 21, 23). A flexible linker physically connects the protein core with the conserved C-terminal active site tyrosine domain. The linker consists of a pair of \( \alpha \)-helices, arranged in an antiparallel orientation, which extends out from the Top1p clamp and positions the C-terminal domain for enzyme catalysis. The poorly conserved N-terminal domain is missing in the Topo70 structures. Although these sequences have been implicated in DNA binding and in mediating protein–protein interactions, the N terminus is dispensable for catalytic activity (1, 24–26).

Mutation of conserved residues in catalytically active yeast and human Top1 mutants has been reported to confer resistance to CPT (reviewed in Refs. 12, 27, 28). The majority of the mutated residues cluster along the Top1p-DNA interface or serve to alter the conformation of the protein clamp. Quantitative assays of drug binding are currently lacking; nevertheless, co-crystal structures of the CPT analog topotecan (TPT) bound to wild-type and mutant Topo70-DNA complexes suggest mutation-induced alterations in drug binding (20, 27).

In the covalent Topo70-DNA structures, the linker domain extends ~56 Å from the enzyme active site at an oblique angle to the DNA when TPT is bound (see Fig. 1C). In the case of yeast Top1p, residues corresponding to the linker domain are predicted to form a similar structure that would extend beyond 110 Å. In the absence of drug, the structure of these \( \alpha \)-helices in the covalent human Topo70-DNA complex was not resolved (Fig. 1A). Although present in Topo70, biochemical, genetic and molecular dynamic simulation data suggest that the flexibility of the linker in the absence of TPT would preclude structural determination (20, 29–31). Indeed, the physical connection and/or flexibility of the linker contribute to the CPT sensitivity of Top1p. For instance, when Topo70 is reconstituted from separate polypeptides comprising the Top1p core and the linker/C-terminal domains, the enzyme is catalytically active yet exhibits reduced sensitivity to CPT (26). Within the N-terminal \( \alpha \)-helix of the linker, molecular dynamic simulations suggest mutation of Ala\textsuperscript{653} to Pro (A\textsuperscript{653}P) increases the flexibility of this coiled-coil structure, to enhance the rate of DNA religation and Top1p resistance to CPT (30).

Studies suggest that Gly\textsuperscript{721} in yeast Top1p constitutes a flexible hinge between the linker domain and a short \( \alpha \)-helix (delineated at the C terminus by the active site Tyr) that modulates the geometry of the active site during enzyme catalysis, which directly impacts Top1p sensitivity to CPT.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Yeast Strains, and Plasmids**—Camptothecin purchased from Sigma was dissolved in Me\textsubscript{2}SO and stored at ~20 °C.

*Saccharomyces cerevisiae* strains EKY3 (MATa, ura3–52, his3DA200, leu2Δ1, trplΔ63, top1Δ:TRPI) and MMY3 (EKY3, rad9Δ:hisG) were described previously (11, 33). In plasmid YcpGal1–eTOP1, wild-type Top1p containing an N-terminal FLAG tag was expressed from the galactose-inducible GAL1 promoter on an ARS/CEN, URA3 vector (11). Mutants top1G721D, top1G721F, or top1G721V, where residue Gly\textsuperscript{721} was mutated to Asp, Phe, or Val, respectively, and top1L720Q, where Leu\textsuperscript{720} was mutated to Gin, were isolated from a pool of top1 mutants generated by degenerate oligonucleotide-directed mutagenesis of residues flanking the active site Tyr\textsuperscript{727}, as described in Ref. 11. Individual mutant sequences, excised in a Bsu36I-Xba1 DNA fragment from YcpGal1–top1 vectors, were ligated into YcpGal1–eTOP1 to generate YcpGal1–etop1G721D, YcpGal1–etop1G721F, YcpGal1–etop1G721V, and YcpGal1–etop1L720Q. In plasmid pTP1–4, Gly\textsuperscript{721} was mutated to Leu, Asn, Glu, or Ala (top1G721L, top1G721N, top1G721E, top1G721Q, or top1G721A respectively) or Leu\textsuperscript{720} to Glu (top1L720E) using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and pairs of complementary oligonucleotides based on the following sequences: GL (5′-CTCC-CAGGTTTTCAGCTGCTACTCCATGAAAATCCATT-3′); GN (5′-CAGGTTTTCAGCTGCTACTCCATGAAAATCCATT-3′); GE (5′-CAGGTTTTCAGCTGCTACTCCATGAAAATCCATT-3′); GQ (5′-GAAAATCCAGGTTCAGCTGCTACTCCATGAAAATCCATT-3′); and LE (5′-AAATCCAGGTTCAGCTGCTACTCCATGAAAATCCATT-3′).

**Yeast Cell Sensitivity to Camptothecin**—To examine cell sensitivity to CPT, exponential cultures of top1Δ yeast cells transformed with the indicated YcpGal1–top1 vector, were adjusted to an A\textsubscript{955} = 0.3, serially 10-fold diluted, and aliquots were spotted onto SC-uracil agar plates containing 25 mm HEPES pH 7.2, 2% dextrose or galactose and the indicated concentration of CPT in a final 0.125% Me\textsubscript{2}SO. Cell viability was scored following incubation at 30 °C. In more quantitative
assays of cell viability, transformants grown in SC-uracil containing 25 mM HEPES, pH 7.2, (SC-uracil-HEPES) and dextrose, were diluted into SC-uracil-HEPES supplemented with 2% raffinose. At an $A_{595} = 0.3$, galactose (2% final) was added to induce Top1 expression, along with 50 mM CPT in a final 0.43% Me$_2$SO or Me$_2$SO alone. At the times indicated, aliquots were serially diluted and plated onto SC-uracil, dextrose plates, and the number of viable cells forming colonies was determined following incubation at 30 °C.

DNA Topoisomerase I Expression, Purification, and Activity Assays—Extracts of galactose-induced cultures of EKY3 cells expressing plasmid encoded wild-type or mutant Top1 proteins were prepared essentially as described (35) except the glass beads were prechilled to -20 °C. Purified Top1 preparations were obtained by successive ammonium sulfate fractionations followed by phosphocellulose chromatography, as described (35). Top1 protein fractions were adjusted to a final 30% glycerol and stored at -20 °C.

To assess Top1p integrity and relative concentration, crude extracts, corrected for total protein concentration using a Bio-Rad protein assay, or purified proteins were resolved in 4–12% BisTris gels (Invitrogen), transferred onto activated polyvinylidene difluoride membranes (PerkinElmer Life Sciences), immunostained with the M2-FLAG antibody (Sigma) and visualized by chemiluminescence. In the case of crude extracts, immunostaining with tubulin antibodies served as a loading control.

DNA topoisomerase I activity was assayed in plasmid DNA relaxation reaction as described (6, 7). Briefly, serial 10-fold dilutions of Top1 proteins, corrected for concentration, were incubated at 30 °C for 30 min with 0.3 µg of negatively supercoiled plasmid pH624 DNA in 20 mM Tris, pH 7.5, 10 mM MgCl$_2$, 0.1 mM EDTA, 50 µg/ml gelatin, and KCl concentrations ranging from 50 to 200 mM. Reactions products were resolved in agarose gels and visualized by ethidium bromide staining.

DNA Cleavage Assays—Wild-type and mutant enzyme sensitivity to CPT was determined in DNA cleavage assays as described (6, 7). A single 32P-end-labeled DNA substrate was incubated with Top1 proteins in 50 µl reaction volumes containing Cleavage buffer (20 mM Tris, pH 7.5, 10 mM MgCl$_2$, 0.1 mM EDTA, 50 mM KCl, and 50 µg/ml gelatin) and the indicated concentration of CPT in a final 4% (v/v) Me$_2$SO. After 10 min at 30 °C, reactions were terminated with 1% SDS at 75 °C, treated with proteinase K, ethanol-precipitated, and resolved in 8% polyacrylamide/7 M urea gels, and visualized with a PhosphoImager.

DNA Religation Assays—Relative rates of Top1p-catalyzed DNA religation were assessed using oligonucleotide-based religation assays adapted from Colley et al. (6). In the pre-annealed religation substrate reaction, the same 5’-end-labeled DNA suicide substrate described above, which contains the DNA scissile strand (5’-GATCTAAAAGACTT ↓ GG-3’), was incubated with equal concentrations of Top1 proteins in Cleavage buffer at room temperature for 15 min to generate Top1-DNA cleavage complexes. Religation was then initiated (time = 0) by the addition of a 10-fold excess of the religation oligonucleotide (5’-GGAAAAATTTTTTTAAGATC-3’). Alternatively, in reactions containing the pre-annealed religation substrate, the same DNA substrate was incubated with a 10-fold excess of the religation strand for 15 min prior to the addition of Top1 proteins. In both assays, aliquots were processed as described above for the 5’-end-labeled suicide cleavage reactions, except that when indicated, urea was omitted from the trypsin reaction to probe for alterations in active site architecture.

RESULTS

Substitution of Gly721 and Leu720 Alter Top1p Sensitivity to Camptothecin—Within the active site pocket of yeast and human Top1p, residues immediately N-terminal to the active site tyrosine are highly conserved (Fig. 1E). Residues spanning Thr722 to the active site Tyr727 correspond to Thr718 to Tyr723 in human Top1p, which form a short α-helical structure highlighted in yellow in the co-crystal structures of human Topo70 covalently linked to DNA, either in the presence or absence of Topotecan (Fig. 1, A–D) (20, 37). Upon drug binding, however, this helical structure is extended to include conserved residues Gly717 and Leu716 (Gly721 and Leu720 in yeast Top1p). This rather subtle transition in active site structure coincides with a more dramatic restriction in linker domain flexibility, such that the linker is now oriented at an oblique angle to the DNA. Although these changes in structure might reflect differences in crystal packing, additional studies suggest that linker domain

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- [Image 230x26 to 257x38]
flexibility as well as the physical linkage of the linker to both the active site C-terminal domain and the protein clamp to which the C terminus is abutted constitute critical determinants of enzyme sensitivity to CPT (30, 31). Given the flexibility of Gly residues within a polypeptide chain, and the structural transitions apparent in the co-crystal structures with drug bound, we propose that Gly717 (Gly721 in yeast) constitutes a flexible hinge and that alterations in active site architecture at this junction would affect the orientation and flexibility of the linker domain. To address this hypothesis, we examined the effects of single amino acid substitutions of yeast Gly721 and the adjacent residue Leu720. These studies were initiated in yeast for several reasons: 1) the phenotypes of specific top1 mutants could be assessed in the absence of wild-type Top1 (in yeast top1Δ strains) (5); 2) any confounding effects of protein-protein interactions due to the expression of human Top1p in yeast could be avoided and 3) mutant enzyme activity could be assayed in different genetic backgrounds in otherwise isogenic strains.

Individual Gly721 and Leu720 substitutions were isolated from a pool of yeast Top1p active site mutants generated by degenerate oligonucleotide mutagenesis. Two hydrophobic substituents of Gly721, (Val in G721V or Phe in G721F) led us to engineer a Leu mutation (G721L) to expand the analysis of amino acid shape at this position. In contrast, the isolation of a Leu720 to Gln (L720Q) mutation, prompted considerations of charge, so an acidic residue Glu was also engineered at this position (L720E). The effect of these single amino acid substitutions on cell sensitivity to CPT was assessed in top1Δ cells induced to express the indicated mutant allele from the GAL1 promoter on an ARS/CEN vector. As seen in Fig. 2A, substituting a basic or acidic residue for Leu720 abolished the CPT-resistant phenotype of cells expressing Top1L720Qp or Top1L720Ep, respectively. These proteins were expressed at slightly higher levels than wild-type Top1p; nevertheless, the specific activity of the two mutant enzymes was reduced by more than 100-fold (Fig. 2B and C). These data suggest the CPT-resistant phenotype of cells expressing the L720Q or L720E mutant results from a decrease in Top1p activity, rather than specific alterations in enzyme sensitivity to CPT. Indeed, homologous recombination defective rad52Δ, top1Δ cells expressing Top1L720E were sensitive to 10-fold higher CPT concentrations than cells expressing wild-type Top1p (data not shown), indicating the formation of lower levels of CPT-induced Top1L720E-DNA damage.

By contrast, substitution of hydrophobic residues for Gly721 had distinct effects on enzyme activity and cell sensitivity to CPT. The G721L mutation has no obvious affect on CPT-induced toxicity, while cells expressing Top1G721Vp or gly721 as a flexible hinge in Top1p
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FIGURE 2. Mutation of Leu\textsuperscript{720} or Gly\textsuperscript{721} in yeast Top1p induces CPT resistance in vivo. A, exponentially growing cultures of top1Δ cells, transformed with the indicated YCpGAL1-top1 constructs, were adjusted to an A\textsubscript{600} = 0.3, serially 10-fold diluted, and aliquots were spotted onto SC-uracil plates containing 25 mM HEPES pH 7.2, 0.1% Me\textsubscript{2}SO, and either Dex, Gal or galactose plus 5 \mu g/ml CPT. The viability of two individual transformants was assessed following incubation at 30 °C. B, levels of galactose-induced wild-type and mutant Top1p in crude cell extracts were determined in immunoblots with the M2-FLAG antibody. Immunostaining with tubulin antibodies served as a loading control. C, the specific activity of wild-type and mutant Top1 enzymes was determined by incubating serial 10-fold dilutions of crude cell extracts (corrected for protein concentration) in plasmid DNA relaxation assays as described under "Experimental Procedures." After 30 min at 30 °C, the reaction products were resolved in a 1.2% agarose gel and visualized after ethidium bromide staining. The relative positions of relaxed (+) and supercoiled (-) DNA topoisomers are indicated. C is plasmid DNA control.

Top1G\textsuperscript{721}Fp were CPT resistant (Fig. 2A). All three mutant enzymes were expressed at levels comparable to wild-type Top1p (Fig. 2B). The specific activity of Top1G\textsuperscript{721}Lp was comparable to wild-type Top1p, and the G\textsuperscript{721}V and G\textsuperscript{721}F mutants were slightly (~5-fold) less active in plasmid DNA relaxation assays (Fig. 2C). However, expression of these mutants in a Rad9 DNA damage checkpoint defective (rad9\textsuperscript{Δ}) strain revealed that the G\textsuperscript{721}L mutant actually enhanced cell sensitivity to CPT, relative to cells expressing wild-type Top1p or the G\textsuperscript{721}V and G\textsuperscript{721}F mutants (Fig. 3A).

The side chain of Val is branched at the β carbon, while the γ carbon branch of Leu is more distal to the polypeptide backbone. We then made additional substitutions of Gly\textsuperscript{721}, including Asp and Asn (G\textsuperscript{721}D and G\textsuperscript{721}N, respectively), to probe differences in charge independent of side chain geometry, and Glu and Gln (G\textsuperscript{721}E and G\textsuperscript{721}Q, respectively), wherein the acidic and basic side chains are extended by a γ-δ carbon bond. As seen in Figs. 3A and 4, substituting the acidic residues for Gly\textsuperscript{721} increased cell sensitivity to CPT by more than a factor of ten. However, as with the G\textsuperscript{721}L mutant, a significant increase in cell sensitivity to CPT as a result of G\textsuperscript{721}N or G\textsuperscript{721}Q mutant expression was only evident in checkpoint-deficient strains, such as rad9\textsuperscript{Δ} or rad53Δ cells (Fig. 3A and data not shown).

We previously described active site mutations, such as T\textsuperscript{722}A and N\textsuperscript{726}H, which enhance Top1p sensitivity to CPT in vitro (7, 11). However, these mutant enzymes induced cell lethality in the absence of CPT and exhibited other alterations in enzyme catalysis. In contrast, the Gly\textsuperscript{721} mutants that enhanced cell sensitivity to CPT, failed to induce growth defects in the absence of drug. In addition, as shown for Top1G\textsuperscript{721}Lp (Fig. 2, B and C), there were no detectable differences in the levels of wild-type and mutant Top1 proteins in galactose-induced cells, and the specific activities of the purified G\textsuperscript{721}D, G\textsuperscript{721}E, G\textsuperscript{721}N, and G\textsuperscript{721}Q mutant proteins were indistinguishable from that of wild-type Top1p (Fig. 3B). Thus, this enhanced CPT sensitivity constitutes a unique phenotype for a single amino acid substitution in Top1p.

Gly\textsuperscript{721} Mutations Increase Top1p Sensitivity to Camptothecin in Vitro—We next asked if the increased CPT sensitivity of cells expressing the G\textsuperscript{721}D, G\textsuperscript{721}E or G\textsuperscript{721}N mutants was a direct effect of increased CPT-Top1p-DNA covalent complexes. First, the specific activity of these mutant enzymes exhibited the same salt optimum as wild-type Top1p; similar patterns of activity and DNA topoisomerase distributions were observed for G\textsuperscript{721}D, G\textsuperscript{721}E and G\textsuperscript{721}N mutant enzymes (Fig. 5A and data not shown). Equal concentrations of the proteins were then incubated with a \textsuperscript{32}P-single end-labeled DNA and increasing concentrations of CPT, to assess the intrinsic CPT sensitivity of each mutant enzyme relative to wild-type Top1p. In such DNA
cleavage assays (Fig. 5B), all three mutants exhibited higher levels of drug-stabilized Top1-DNA covalent complexes than that observed with wild-type Top1p: the acidic substituent Glu (G721E) conferred high levels of DNA cleavage across a wide range of CPT concentrations, while mutation to Asp (G721D) enhanced DNA cleavage to the greatest extent at higher drug concentrations. Indeed, these findings are consistent with the enhanced CPT sensitivity of cells expressing the G721D and G721E mutants (Figs. 3 and 4). Increased levels of covalent complexes were also evident with the G721N mutant, but not at lower drug concentrations (as with G721E) or to the same extent as G721D at higher concentrations. Together, these data suggest that the increased CPT sensitivity of cells expressing these mutants can be attributed to mutation-induced increases in drug poisoning of Top1p.

Replacing Gly721 with Acidic Residues Affects Top1p Cleavage of DNA and Enzyme Architecture—Top1G721Dp and Top1G721Ep did not exhibit alterations in steady state levels of Top1-DNA covalent complexes in the absence of CPT, or specific enzyme activity in plasmid DNA relaxation assays, yet exhibited increased CPT sensitivity in vivo and in vitro (Figs. 4 and 5). We considered that changes in enzyme architecture induced by these substitutions might enhance CPT binding and coincide with changes in linker domain flexibility. To address such dynamic interactions, we first used a series of oligonucleotide-based DNA substrates to uncouple Top1p catalyzed DNA cleavage from religation.

A common strategy to uncouple DNA cleavage from religation is to use a oligonucleotide-based substrate that contains a truncated scissile strand. For example, as depicted in Fig. 6A, Top1p cleavage of a high affinity site within a suicide DNA substrate liberates a dinucleotide and traps the covalent Top1p-DNA complexes (6, 18, 38). In this case, Top1-DNA covalent complex formation is monitored by the accumulation of a peptide-linked 14-mer, produced by trypsin digestion of the reaction products in the presence of urea (39). These data revealed a decreased rate of DNA cleavage by the G721D mutant, relative to that observed with wild-type Top1p (Fig. 6A). We next asked if the extent of duplex DNA structure 3' to the site of DNA cleavage affected the relative rates of mutant and wild-type Top1 cleavage of DNA. One way to address this question is to use successively longer scissile strands. Such assays did demonstrate a proportional increase in the rate of DNA cleavage by Top1G721Dp (data not shown). However, in this case, the co-migration of the 14-mer + peptide reaction product with the 20-mer substrate required the analysis of a 3' end-labeled substrate. So to avoid such complications, a second suicide substrate depicted in Fig. 6B was employed. In this duplex DNA substrate, a 5'-bridging phosphorothioate at the preferred site of scission produces a 5'-SH in the Top1p-DNA covalent complex, which is ineffective in the transesterification that religates the DNA (6, 36). As the substrate is 5'-end-labeled, the rate of DNA cleavage can be approximated by the accumulation of the same peptide-linked 14-mer as described in Fig. 6A. With this fully duplexed DNA, the G721D mutant exhibited an increased rate of DNA cleavage, relative to that

![Figure 4](image_url)

**FIGURE 4.** Expression of Top1G721Dp and Top1G721Ep increases the cytotoxicity of CPT. At t = 0, exponential cultures were treated with galactose, to induce expression of plasmid-encoded Top1p, Top1G721Dp or Top1G721Ep, 0.43% Me2SO and, as indicated, 50 μM CPT. At the times indicated, aliquots were serially diluted and plated onto SC-uracil media containing dextrose. The number of viable cells forming colonies was assessed after 30 °C for 3 days and plotted relative to the number obtained at t = 0. Results are an average of three independent experiments.

![Figure 5](image_url)

**FIGURE 5.** Top1G721Dp, Top1G721E, and Top1G721Ep exhibit increased levels of covalent complexes in the presence of CPT. A, equal concentrations of Top1p and Top1G721Dp were serially 10-fold diluted and incubated in a plasmid DNA relaxation assay containing the indicated concentration of KCl. After 30 min at 30 °C, the reaction products resolved by agarose gel electrophoresis and visualized with ethidium bromide. The relative positions of relaxed (R) and supercoiled (-) DNA topoisomers are indicated. B, equal concentrations of Top1p and mutant proteins were incubated with a single 32P-end-labeled DNA substrate in a DNA cleavage reaction containing the indicated amount of CPT. After incubation for 10 min at 30 °C, covalent complexes were trapped with SDS at 75 °C and treated with proteinase K. The reaction products were resolved in 8% polyacrylamide/7 M urea gels and visualized using a Phosphomager. Cit is DNA alone, and the asterisk (*) indicates a high affinity Top1p cleavage site.
observed with wild-type Top1p (Fig. 6, B and C). Taken together, these findings indicate that in contrast to wild-type Top1p, the enhanced DNA scission catalyzed by Top1G721Dp required the presence of duplex DNA 3’ to the site of cleavage.

We next considered the effects of Gly721 mutations on the rate of Top1-catalyzed DNA religation, using the strategy depicted in Fig. 7A. Here, the dissociation of the GG dinucleotide facilitates the subsequent annealing of a complementary oligonucleotide, which provides the 5’-OH necessary to resolve the trapped covalent complex and produce a 35-mer. In the post-annealed religation assay, covalent Top1p-DNA complexes were first trapped with the 16-mer suicide substrate, then the complementary religation oligonucleotide is added (time = 0). At the times indicated, the reactions were terminated with SDS at 75 °C, and the relative levels of uncleaved 16-mer, religated 35-mer and covalent Top1-DNA intermediate were assessed in denaturing gels following trypsin digestion. These reactions were initially carried out in the absence of urea, because trypsin digestion of wild-type Top1 (Fig. 7B) efficiently liberated a 7-amino peptide (INY727IDPR) covalently linked to the radiolabeled 14-mer, which migrates slower than the 16-mer suicide oligonucleotide. However, a distinct pattern of bands was obtained in tryptic digests of covalent complexes formed by Top1G721D. As seen in Fig. 7B, two additional bands are observed at zero time, with the slower migrating band (labeled <) being more prominent. Following addition of the religation oligonucleotide, the accumulation of the faster migrating band (labeled >) paralleled that of the religated 35-mer. Similar results were obtained in assays performed at 150 mM KCl (data not shown). These extra bands do not result from illegitimate religation events. Rather, they represent incomplete tryptic digests of the covalent Top1G721D-DNA complexes, as trypsin digestion of these complexes in the presence of urea produced a single 7 residue peptide linked to the radiolabeled 14-mer (Fig. 7C). Moreover, digestion with proteinase K, which cleaves at sites distinct from trypsin, failed to yield multiple bands (data not shown). Nevertheless, if one corrects for differences in the level of covalent complexes formed at t = 0, then there was no difference in the rate of DNA religation (accumulation of 35-mer following the addition of the religation oligonucleotide) catalyzed by Top1 versus Top1G721Dp (Fig. 7C).

To determine if altered tryptic digests coincided with increased enzyme sensitivity to CPT, similar analyses of Top1G721Ep and Top1G721Np were performed. All three mutants produced lower levels of covalent enzyme-DNA intermediates at t = 0, with no obvious defect in DNA religation (Fig. 7, B and D). However, only in the case of the more CPT sensitive Top1G721D and Top1G721E mutant enzymes were the longer peptide-linked oligonucleotides obtained; these bands were not

![FIGURE 6. Top1G721D exhibits altered rates of DNA cleavage. A, as diagrammed, Top1p cleavage (indicated by \( \uparrow \)) of a suicide substrate containing a 5’-radiolabeled oligonucleotide (16-mer) liberates a GG-dinucleotide and traps the covalent [\( \gamma^32P \)]DNA-Top1p complex. B, as diagrammed, Top1p cleavage (indicated by \( \downarrow \)) of a fully duplexed suicide substrate, with a 5’-bridging phosphorothiolate at the site of scission, yields a 5’-SH end and traps the covalent [\( \gamma^32P \)]DNA-Top1p complex. In A and B, intact Top1p is indicated by \( \bullet \). Trypsin digestion of the covalent complexes in the presence of 2 mM urea generates a 7-amino acid peptide covalently linked to a 14-mer. In these assays, equal concentrations of Top1p and Top1G721Dp were incubated with the suicide substrate and at the times indicated (minutes or s for seconds), reaction aliquots were terminated with 0.5% SDS at 75 °C, ethanol-precipitated, and the trypsin products resolved in a denaturing gel and visualized by PhosphorImaging. C, quantitation of the % of DNA cleaved the reactions depicted in B.](image-url)
evident with the less CPT sensitive Top1G721Np (Fig. 7, B and D). Thus, substituting acidic residues for Gly721 appears to enhance CPT poisoning of Top1p and alter the geometry of the active site in the covalent complex.

We next asked if the presence of extended duplex DNA 3′ to the site of DNA cleavage, which affected the rate of DNA cleavage by Top1G721D (Fig. 6B), also impacts enzyme active site architecture and/or rates of DNA religation. To address these questions, we took two approaches. First, as diagrammed for the “pre-annealed religation substrate in Fig. 8A, the 21-mer religation oligonucleotide diagrammed in Fig. 7A was annealed prior to the addition of enzyme. DNA cleavage by Top1p liberates a GG dinucleotide to allow formation of the 35-mer. B, equal concentration of Top1p and Top1G721Dp were added to pre-annealed religation assays at t = 0. At the times indicated (minutes or s for seconds), reaction aliquots were quenched with SDS at 75°C. Covalently linked Top1 proteins were hydrolyzed with trypsin (in the absence of urea), and the reaction products were resolved in 16% polyacrylamide/7 M urea gels. C, trypsin digests of Top1G721Dp, incubated in pre-annealed or post-annealed religation assays, were resolved by 16% polyacrylamide/7 M urea gel electrophoresis. As in Fig. 7, << and < indicate the position of the 14-mer covalently linked to Top1 peptides.

FIGURE 7. Distinct patterns of Top1p-DNA tryptic digests obtained in religation reactions suggest G721D-induced alterations in active site structure. A, in the post-annealed religation assay, the same suicide substrate diagrammed in Fig. 6A, is used to generate covalent [γ-32P]DNA-Top1 complexes. At t = 0, a 21-mer religation oligonucleotide (complementary to the 5′-end of the nonciscle strand) is added. As shown in B, C, and D, the resolution of the covalent Top1-DNA complex to generate a 32P-labeled 35-mer can be followed by the conversion of the trypsin 14-mer + peptide to the 35-mer. B and D, equal concentrations of Top1p, Top1G721Dp, Top1G721Ep, and Top1G721Np were incubated with the suicide substrate for 15 min at room temperature. At t = 0, the 21-mer religation oligonucleotide was added. At the times indicated (minutes or s for seconds), reaction aliquots were terminated with SDS at 75°C, treated with trypsin (in the absence of urea), resolved in 16% polyacrylamide/7 M urea gels visualized using a Phosphohrager. << and < indicate the position of the cleaved 14-mer covalently linked to Top1 peptides longer than 7 residues. C, as for the reactions shown in B, equal concentrations of Top1p and Top1G721Ep were incubated in the post-annealed religation assay. However, the reaction products were digested with trypsin in the presence of 2 M urea prior to 16% polyacrylamide/7 M urea gel electrophoresis.

FIGURE 8. Duplex DNA, 3′ to the site of DNA scission, impacts Top1G721Dp active site architecture and the rate of enzyme-catalyzed DNA religation. A, in the pre-annealed religation assay, the 21-mer religation oligonucleotide diagrammed in Fig. 7A was annealed prior to the addition of enzyme. DNA cleavage by Top1p liberates a GG dinucleotide to allow formation of the 35-mer. B, equal concentration of Top1p and Top1G721Dp were added to pre-annealed religation assays at t = 0. At the times indicated (minutes or s for seconds), reaction aliquots were quenched with SDS at 75°C. Covalently linked Top1 proteins were hydrolyzed with trypsin (in the absence of urea), and the reaction products were resolved in 16% polyacrylamide/7 M urea gels. C, trypsin digests of Top1G721Dp, incubated in pre-annealed or post-annealed religation assays, were resolved by 16% polyacrylamide/7 M urea gel electrophoresis. As in Fig. 7, << and < indicate the position of the 14-mer covalently linked to Top1 peptides.
Top1G721D reactions containing the pre-annealed substrates, relative to that observed with wild-type Top1p (Fig. 8B) or Top1G721D incubated with post-annealed substrates (Fig. 8C). However, because the pre-annealed assay does not uncouple cleavage from religation, it is difficult to determine if the presence of the dinucleotide flap and/or duplex DNA also affects the rate of DNA cleavage.

To address these questions and avoid the potential complications of aberrant DNA structure, we asked if a similar analysis of Top1p-DNA complexes formed with the S’ bridging phosphorothiolate (diagrammed in Fig. 6B) would yield the same pattern of Top1G721D and Top1G721E-derived peptides linked to the 14-mer. Indeed, as shown in Fig. 9, the presence of duplex DNA 3’ to the site of DNA cleavage precluded efficient trypsin digestion of Top1p-DNA complexes formed by the G721D and G721E mutants, but not wild-type Top1. Thus, independent of the presence of a DNA flap or religation, these findings support a model whereby the alterations in trypptic digests of the covalent enzyme-DNA intermediates, induced by the various DNA substrates, coincided with increased mutant enzyme sensitivity to CPT.

Based on these findings, we hypothesize that the increased CPT sensitivity of the Gly721 mutant enzymes results from conformational changes within the Top1p catalytic pocket that limits linker domain flexibility and enhances CPT binding to the covalent enzyme complex. In particular, mutation of the highly flexible Gly might extend the α-helical structure of residues N-terminal to the active site Tyr, as seen in the structures shown in Fig. 1. One prediction of this model is that, independent of side chain charge, an increased tendency for α-helical structure at position 721 would augment CPT poisoning of Top1p. Estimates of the intrinsic helix-forming tendencies of various amino acid residues indicate that while Gly has a low propensity to form α-helices, Ala has the highest (40). Indeed, substituting Ala for Gly721 (in Top1G721Ap) induced a 5-fold increase in cell sensitivity to CPT, relative to cells expressing wild-type Top1p, which was only slightly less than the 10-fold increase induced by the G721D mutation (Fig. 10).

**DISCUSSION**

The architecture of Top1p includes a flexible linker domain, comprised of an extended coiled-coil that positions the C-terminal active site Tyr domain within the catalytic pocket formed in concert with the central protein clamp. Biochemical, x-ray crystallographic and molecular dynamic simulation data suggest that the integrity and flexibility of the linker domain within the Top1p clamp are critical determinants of enzyme sensitivity to the CPT class of chemotherapeutics (20, 29—31, 37). Increasing the flexibility of the human Top1p linker domain, either by mutation (A653P) (30) or by physically uncoupling the linker domain from the Top1p protein clamp (in reconstituted Topo70 preparations) (31), reduces enzyme sensitivity to CPT. In crystallographic structures of human Topo70 and DNA, the presence of CPT analogues appears to restrict the movement of the linker domain thereby enabling the structural determination of this coiled-coil. This contrasts with the mobility of the linker domain within the covalent Topo70-DNA complex in the absence of drug. Close inspection of active site architecture within these structures revealed an extension of a short α-helical structure, spanning Ser719 through the active site Tyr (human Tyr723) in the absence of drug, to include Leu716, Gly717, and Thr718 in the presence of drug (see Fig. 1). These data suggest a dynamic interplay between active site α-helical structure and linker flexibility attends drug binding to the covalent Topo70-DNA complex. Such long range molecular interactions between the Top1 linker and active site in mediating enzyme sensitivity to CPT are also supported by recent studies where the cytotoxicity induced by a defect in human Top1p-catalyzed DNA religation (due to the active site mutation T722A) was suppressed by the increased linker flexibility of the A653P mutation (32). Based on these considerations, we hypothesized that the presence of this conserved Gly residue within the active site of the enzyme (Gly721 in human Top1p; Gly721 in yeast Top1p) functions as a flexible hinge to facilitate the alterations in active site geometry and linker domain flexibility that impact CPT poisoning of Top1p. Indeed, our analyses of Gly721 mutations in yeast Top1p, in vitro and in vivo, support this model and suggest that the charge and geometry of amino acid side chains at this position directly impact active site architecture within the covalent enzyme-DNA complex and the intrinsic sensitivity of Top1p to CPT.

First, our findings indicate that the introduction of a bulky aromatic residue (Phe) or β-branched aliphatic side chain (Val)
decreased the specific activity and CPT sensitivity of Top1p. By contrast, the introduction of an acidic side chain, with carboxyl groups at the β or γ carbon in G721D and G721E, respectively, dramatically enhanced Top1p sensitivity to CPT, without any obvious alterations in specific activity in plasmid DNA relaxation assays. In this case, side chain charge was important, as the increased CPT sensitivity of cells expressing Top1 mutants engineered with the amide forms of these residues (G721N and G721Q) or a α branched aliphatic side chain (G721L) was only evident in the absence of the DNA damage checkpoint. However, the tendency of residues at this position to form α-helices also appears to be a critical determinant of Top1p sensitivity to CPT, as mutating Gly721 to Ala (estimated to have the highest propensity for helix formation (40)) also increased cell sensitivity to CPT. Together, our data support the following rank order of mutant-induced increased in Top1p sensitivity to CPT: (G721E, G721D) > G721A > (G721N, G721Q, G721L) > wild-type > G721V > G721F.

Second, our studies also suggest mutation-induced alterations in enzyme active site architecture coincide with increased CPT sensitivity. The G721D, G721E mutants exhibited the greatest CPT sensitivity in vivo and in vitro, as well as specific alterations in tryptic digests of covalent enzyme-DNA complexes formed with distinct suicide substrates DNA. A comparison of potential trypsin cleavage sites in yeast and human Top1p with the pattern of DNA-bound tryptic fragments obtained with yeast and human Top1p mutants (Figs. 7–9 and data not shown) allows for an accurate determination of the alterations in trypsin digestion. In the residues spanning the active site tyrosine of yeast Top1p, K721-ENQYSGL724TSK725INY727IDPR, trypsin digestion of SDS-denatured wild-type Top1p-DNA covalent complexes typically yields a 7 residue peptide (INY727IDPR) covalently attached to a 5’-end-labeled 14-mer oligonucleotide via a 3’-phosphotyrosyl linkage. Mutation of Gly721 to Asp or Glu (G721D or G721E, respectively) limits trypsin digestion at K724 to yield longer peptides linked to the DNA. One possible explanation is that the G721D and G721E mutations alter the α-helical structure of the active site so as to restrict trypsin digestion of the partially denatured proteins. The presence of an acidic side chain may also preclude efficient trypsin digestion at K724. Nevertheless, the changes in enzyme active site geometry suggested by the altered tryptic digests coincided with increased Top1p sensitivity to CPT. Alterations in trypsin digestion were only observed with the more CPT sensitive G721D or G721E mutants and not with the less CPT sensitive G721N mutant. Moreover, these alterations were observed in the absence of DNA religation or alterations in DNA helical structure (Fig. 9).

The presence of duplex DNA 3’ to the site of DNA scission also altered the rates of Top1G721Dp and Top1G721Ep catalyzed DNA cleavage, as well as the pattern of tryptic digests. These data suggest the enhanced CPT sensitivity of these mutants derives from the increased cleavage of duplex DNA and that downstream protein-DNA contacts impact dynamic interactions between the active site and the linker. Indeed, the linker of human Top1p is 10-fold more resistant to limited proteolysis when the enzyme is noncovalently bound to duplex DNA, consistent with linker-DNA contacts 3’ to the site of DNA scission (23). Recent single molecule analyses of individual human Top1p-DNA complexes using magnetic tweezers also determined that the binding of TPT selectively impede enzyme uncoiling of positively supercoiled DNA (17). While mutation-induced alterations in Top1p structure await x-ray structure determination, our studies implicate the conserved Gly721 residue as a flexible hinge within the active site of Top1p that enable linker domain flexibility and the structural alterations that accompany drug binding of the covalent enzyme-DNA complex.

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