Intragenic Suppressors of Mutant DNA Topoisomerase I-induced Lethality Diminish Enzyme Binding of DNA

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DNA topoisomerases catalyze changes in DNA topology and is the cellular target of the anticancer drug camptothecin (Cpt). Mutation of several conserved residues in yeast top1 mutants is sufficient to induce cell lethality in the absence of camptothecin. Despite tremendous differences in catalytic activity, the mutant proteins Top1T722Ap and Top1R517Gp cause cell death via a mechanism similar to that of Cpt, i.e. stabilization of the covalent enzyme-DNA intermediate. To establish the interdomainal interactions required for the catalytic activity of Top1p and how alterations in enzyme structure contribute to the cytotoxic activity of Cpt or specific DNA topoisomerase I mutants, we initiated a genetic screen for intragenic suppressors of the top1T722A-lethal phenotype. Nine single amino acid substitutions were defined that map to the conserved central and C-terminal domains of Top1p as well as the nonconserved linker domain of the protein. All reduced the catalytic activity of the enzyme over 100-fold. However, detailed biochemical analyses of three suppressors, top1C273Y,T722A, top1G6295V,T722A, and top1G369D,T722A, revealed this was accomplished via a mechanism of reduced affinity for the DNA substrate. The mechanistic implications of these results are discussed in the context of the known structures of yeast and human DNA topoisomerase I.

DNA topoisomerases catalyze changes in the winding or linkage of DNA strands via a conserved mechanism of transient DNA strand cleavage and religation (reviewed in Refs. 1–3). These enzymes play a critical role in such processes as DNA replication, transcription, and recombination. The cellular form of eukaryotic DNA topoisomerase I (Top1p) is a highly conserved enzyme that catalyzes the relaxation of positively and negatively supercoiled DNA. This type IB enzyme cleaves a single strand of a DNA duplex via the nucleophilic attack of the active site tyrosine hydroxyl on a phosphodiester backbone bond of the DNA. This results in the formation of a covalent phospho-tyrosyl linkage with the 3′ end of the cleaved DNA strand. The other end is presumably free to rotate around the noncissile strand, effecting a rewinding or unwinding of the DNA. This transient enzyme-DNA intermediate is resolved by a second transesterification whereby the free 5′-hydroxyl end of the DNA attacks the phospho-tyrosyl bond to reseal the nicked DNA strand.

Camptothecin (Cpt) is a potent antineoplastic agent that targets eukaryotic DNA topoisomerase I by reversibly stabilizing the covalent enzyme-DNA intermediate (reviewed in Refs. 3–6). During S-phase, the collision of the advancing replication fork with these drug-stabilized cleavable complexes is thought to produce the potentially lethal double-stranded DNA breaks that lead to cell cycle arrest in G2 and eventually cell death. In the yeast Saccharomyces cerevisiae, the gene encoding DNA topoisomerase I, TOP1, is nonessential (reviewed in Refs. 2 and 4). top1Δ strains are viable as other proteins such as DNA topoisomerase II or Trf4p can compensate for the loss of Top1p function (7, 8). However, cells devoid of DNA topoisomerase I are resistant to Cpt. The cytotoxic activity of Cpt is restored when plasmid-borne yeast or human TOP1 is expressed in these cells (9, 10). Together, these data establish Top1p as the cellular target of Cpt, which converts the enzyme into a poison by increasing the half-life of the covalent enzyme-DNA intermediate.

In addition to Top1p-targeted drugs, recent studies suggest additional mechanisms of DNA topoisomerase I-mediated cytotoxicity. For example, the introduction of abasic sites or DNA mismatches at preferred sites of DNA cleavage by Top1p dramatically increases the formation of covalent complexes (11, 12), although the cellular consequences of such DNA lesions has yet to be established in vivo. In contrast, single amino acid substitutions have been defined in DNA topoisomerase I that mimic the cytotoxic activity of Cpt, including a terminal G2-arrested phenotype and increased rates of DNA recombination (13, 14). In yeast Top1T722Ap, alanine is substituted for threonine 722, a highly conserved residue just N-terminal to the active site tyrosine 727, whereas in Top1R517Gp the conserved arginine 517 is mutated to glycine (13). Despite tremendous differences in enzyme specific activity and Cpt sensitivity, the cytotoxicity of both mutant proteins could be attributed to an increase in the half-life of the covalent enzyme-DNA intermediate.

Recent structural studies demonstrate a circumferential binding of DNA by DNA topoisomerase I (15–19). The structures of a 26-kDa fragment of yeast Top1p and reconstituted fragments of human DNA topoisomerase I in covalent or noncovalent complexes with DNA illustrate a protein clamp that circumscribes duplex DNA (15, 16, 18). Based on the structures of reconstituted and truncated versions of human Top1p, a “controlled rotation” mechanism has been proposed to explain the changes in DNA strand winding catalyzed by the enzyme (18). In this model, the rotation of the untethered end of the cleaved DNA strand around the uncleaved strand of DNA is
modulated by interactions between the DNA and the charged surfaces of two sets of α-helices projecting outward from the core of the protein clamp. Vaccinia virus Top1p, also a type IB enzyme, forms a C-shaped protein clamp; although distinct differences in structure, DNA sequence specificity and Cpt sensitivity distinguish this enzyme from cellular Top1p (20).

Mutations affecting the Cpt sensitivity of eukaryotic Top1p have been defined (21–29). Although distributed throughout the conserved central and C-terminal portions of the enzyme, they are clustered along one face of the DNA helix in the co-crystal structure of the reconstituted human enzyme with DNA (16). The consequences of other mutations on enzyme function, including the top1R517G mutant, provide insight into residues that constitute the catalytic site of the enzyme (13). However, despite this wealth of biochemical and structural data, a clear picture of the domainal movements and interactions required for DNA binding and the catalytic activity of the intact enzyme remain unclear. To address this, a mutational analysis of yeast Top1T722A was undertaken to define intragenic suppressors that abrogate the cytotoxic activity of the mutant enzyme. All of the top1T722A intragenic suppressors examined abolished enzyme activity. However, the detailed characterization of several mutants suggest this was mediated by a reduced affinity for DNA. The positions of the intragenic suppressors within the structure of human DNA topoisomerase I are discussed in terms of enzyme mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials, Yeast Strains, and Plasmids—**Camptothecin (Sigma) was dissolved in MeSO to a final concentration of 4 mg/ml, and aliquots were stored at −20 °C. Yeast strains EKY3 (MATa, ura3-52, his3D200, leu2Δ1, trplΔ63, top1Δ::TRP1) and JCW28 (MATa, ura3-52, his3D200, leu2Δ1, trplΔ63, top2-4, top1Δ) have been described (30). MB3 is EKY3 deleted for RAP52. To accomplish this, LEU2 sequences were excised with KanI from the rad52Δ::LEU2 disruption construct, pSM20 (obtained from Dr. D. Schild, Berkeley Lawrence Laboratories). BglII linkers were ligated to the repaired DNA ends; the plasmid was cleaved with BglII and ligated to a BamHI fragment containing the HIS3 gene from plasmid YDpH (31). The rad52Δ::HIS3 sequences were excised with EcoRI, ligated for single stranded repair, and used to transform galactose-inducible pGAL1 Top1 promoter vectors that lacked an N-terminal tag were corrected for protein concentration in each DNA sample was used to transform S. cerevisiae strain Abcx74 (provided by Dr. Ed-Doudna, Berkeley, CA) to carry the gene of interest in the galactose-inducible pGAL1-top1T722A-L intragenic suppressors were prepared as described (21). Protein concentrations were determined using the Bio-Rad reagent, and 45 μg of total protein was resolved by SDS-polyacrylamide gel electrophoresis. Top1p levels and integrity were assayed in immunoblots with a polyclonal antibody specific for yeast Top1p (22).

Untagged and epitope-tagged Top1 proteins were partially purified from galactose-induced EKY3 cells as described (22). Top1 proteins labeled with an N-terminal tag were recovered for single stranded repair in immunoblots as above. eTop1 protein levels were determined in immunoblots probed with an epitope-specific M2 monoclonal antibody (IBI) and stained with alkaline phosphatase-conjugated secondary antibody. Top1 protein concentrations were determined using the Bio-Rad reagent, and 45 μg of total protein was resolved by SDS-polyacrylamide gel electrophoresis. Top1p levels and integrity were assayed in immunoblots with a polyclonal antibody specific for yeast Top1p (22).

**DNA Topoisomerase I Activity in Vivo—**JCW28 (top1Δ, top2Δ) cells were cotransformed with YEpV2 and a plasmid constitutively expressing bacterial DNA topoisomerase I, and one of the following plasmids: YCpGAL1-eTop1p, YCPGAL1-eTop1p-L, YCpGAL1-eTop1p-L, or YCpGAL1-eTop1p-L. Individual transformants grown in S. cerevisiae strain AB1157 were able to grow on uracil-containing media, indicating that the plasmid DNA Topoisomerase I activity in vivo is dependent on the presence of eTop1p.

**DNA Binding Assay—**A 32P-labeled, 17-base pair DNA fragment containing a high affinity DNA topoisomerase I binding site was amplified from plasmid pBlueAK3-1 (23) using Amplitaq polymerase (Perkin-Elmer), the SK and KS primers (Strategene), and [α-32P]ATP. The epitope-tagged wild-type and mutant Top1 proteins (0–50 pmol) were incubated in a final 140 μl with 5 μg of biotinylated M2 antibody (Kodak, IBI) and 25 μl of streptavidin-coated cellulose beads (Pierce) in 140 μl with 5 μg of biotinylated M2 antibody.
20 mM phosphate (pH 7.0), 150 mM NaCl, for 1 h on ice. After sequential washes with 250 µl of 20 mM phosphate (pH 7.0), 1 M NaCl and 2× 250-µl volumes of binding buffer (20 mM Tris (pH 7.5), 10 mM MgCl₂, 0.1 mM EDTA, 50 mM KCl, 50 µg/ml gelatin, and 1 mM β-mercaptoethanol), the bead-bound enzymes were incubated on ice with 12,000 cpm (50–100 fmol) of the ³²P-labeled DNA substrate in 250 µl of binding buffer in cell culture acetyl microcentrifuge spin columns (Micron Separations Inc.). After 10 min, the unbound DNA was eluted by centrifugation at 6000 rpm (2800 × g) and a subsequent wash with 250 µl of binding buffer. The label in the combined eluent fractions and that retained on the beads was counted in a Beckman LS801 liquid scintillation counter. The percentage of bound DNA was calculated as (bead-bound cpm)/(bead-bound cpm + (combined eluent cpm)). In side by side reactions, the concentration of bead-bound protein was determined in immunoblots with the M2 antibody, in comparison with known concentrations of homogeneous eTop1p. Column fractions prepared from top1Δ strains were included as DNA-binding controls.

RESULTS

The Cytotoxic Mutation in Top1R517G Is an Intragenic Suppressor of Top1T722A-induced Lethality—Cpt converts DNA topoisomerase I into an S-phase poison by reversibly stabilizing the covalent Top1p-DNA intermediate. Specific mutations in DNA topoisomerase I have also been shown to increase the formation of covalent complexes with similar effects on cell viability (13, 14). Expression of yeast mutants, top1T722A or top1R517G, from the galactose-inducible pGAL1 promoter, produced a dramatic reduction in cell viability and a terminal G₂-arrested phenotype indistinguishable from that obtained following Cpt treatment of cells expressing wild-type TOP1 (13, 30). Further biochemical analyses demonstrated an increase in the stability of the covalent enzyme-DNA intermediate, albeit via distinct alterations in catalytic activity (13).

In the structure of reconstituted human Top1p, the corresponding residues, Thr-718 and Arg-590, lie in close proximity to the active site tyrosine Tyr-723 (16, 18). Indeed, Arg-590 (residue 517 in yeast Top1p) appears to be one of four residues that form the catalytic site of the enzyme (18). Along with Arg-488 and His-632, Arg-590 may coordinate the scissile phosphate during the cleavage reaction. In contrast, the mechanism of Top1T722Ap-enhanced DNA cleavage remains unclear. As a prelude to establishing the molecular interactions necessary for DNA topoisomerase I catalysis, we first addressed this question by asking what effect the Arg-517 to Gly substitution would have on Top1T722Ap function.

As shown in Fig. 1A, galactose-induced expression of either single mutant in a top1Δ strain produced at least a 3-log drop in the number of viable cells. In contrast, expression of the double mutant top1R517G,T722A had little effect on yeast cell viability. This correlated with a lack of detectable enzymatic activity in crude extracts (Fig. 1B). As previously reported (13), the specific activity of Top1T722Ap was ~1/3 that of wild-type Top1p when assayed in plasmid DNA relaxation assays, whereas Top1R517Gp activity was not detectable. Similarly, the introduction of R517G into the Top1T722A mutant enzyme abolished the activity of the double mutant. However, in contrast with Top1R517Gp, the double mutant was no longer cytotoxic suggesting the lack of covalent complex formation by Top1R517G,T722Ap. This was confirmed in the DNA cleavage assay shown in Fig. 1C, where the introduction of the R517G mutation suppressed the enhanced stability of the covalent DNA-Top1T722A complex at a high affinity cleavage site. This intragenic suppressor of top1T722A-induced lethality further diminished the camptothecin hypersensitivity of Top1T722A. These data, in turn, imply a functional interaction between residues Arg-517 and Thr-722 in wild-type Top1p.

Intragenic Suppressors of Top1T722A-induced Lethality Map throughout Conserved Domains of the Enzyme—Intragenic suppression and complementation are widely used genetic approaches to define specific interactions between protein domains and/or residues (37, 38). Such functional assignments are especially critical in defining enzyme mechanism once the structure of the protein is known. To clarify such interactions in the various aspects of Top1p catalysis, a genetic screen for intragenic suppressors of the top1T722A lethal phenotype was undertaken.

As described in Fig. 2, plasmid YCpGAL1-top1T722A was mutagenized with hydroxyurea. After establishing relative mutation rates of the plasmid-borne URA3 marker in E. coli
MC1066 cells, a pool of mutagenized plasmids was amplified. The mutated top1T722A alleles, designated top1T722Am, were excised and subcloned into a fresh plasmid backbone. This step eliminated mutations affecting pGAL1 function or plasmid maintenance by restricting the selected mutations to the coding region of top1T722A. The resultant top1T722Am pool was transformed into EKY3 (top1D) cells and plated on selective media containing galactose. To ensure plasmid integrity and that the mutations conferring viability were plasmid-borne, individual plasmids were purified, analyzed by restriction enzyme digestion, and transformed into top1D cells to screen for viability on galactose. Of these, 92 individual top1T722Am mutants were further analyzed.

Exponentially growing cultures of top1T722Am transformants were induced with galactose. After 8 h, cell extracts were prepared; equal loadings of total protein were resolved by SDS-polyacrylamide gel electrophoresis, and Top1p expression was assessed in immunoblots with polyclonal antibodies specific for yeast Top1p. Of the 92 top1T722Am mutants examined, 15 encoded full-length proteins with steady state levels comparable to wild-type TOP1 and top1T722A-expressing controls (data not shown).

To identify the specific lesions that suppress top1T722A-induced lethality, three overlapping ~1-kbp restriction fragments (see Fig. 3) were subcloned into the same sites of an unmutagenized top1T722A vector. Each construct was re-screened in EKY3 cells to define the DNA sequences containing the intragenic suppressor, which were then sequenced. Asterisks indicate the number of times a specific mutation was identified. One of the three indicated for Arg-517 is the R517G substitution in Fig. 1. Substitution of Phe for the active site tyrosine, Tyr-727, was previously shown to suppress top1T722A-induced lethality (13). The DNA relaxation activity of each double mutant was determined in crude cell extracts as described in the legend to Fig. 5. The number of + signs indicates specific enzyme activity, relative to wild-type Top1p.
that abolished enzyme activity. This class of mutants was not pursued.

As seen in Fig. 3, the intragenic suppressors were distributed throughout the gene, with the exception of the N-terminal portion of the protein. This domain has previously been shown to be dispensable for the catalytic activity of the yeast and human enzymes (36, 39). The suppressors that mapped to Cys-273, Gly-295, His-299, Gly-303, Gly-369, Arg-517, and Pro-730 all involve highly conserved residues (16, 40). Gly-295 corresponds to human residue Gly-363, which when mutated to cysteine renders the human enzyme resistant to Cpt (26). Gly-295, His-299, and Gly-303 correspond to human residues Gly-363, His-367, and Gly-371, respectively, which lie within one of the “lip” domains that close the protein clamp around the DNA (16). As with R517G in Fig. 1, substitution of Arg-517 with His also suppressed top1T722A-induced lethality. Residues Leu-598 and Pro-603 lie within a nonconserved linker region. Regardless of their distribution, all nine mutations severely affected enzyme activity in plasmid relaxation assays (Fig. 3, data not shown). In cell extracts assayed in plasmid relaxation assays, only the double mutants Top1C273Y,T722Ap and Top1G369D,T722Ap exhibited any appreciable activity. However, this activity was ~100-fold less than that detected in Top1T722Ap extracts (Fig. 3).

Although pGAL1-promoted expression of top1T722A is cytoxic, biochemical studies with purified protein demonstrated that Top1T722Ap is sensitive to Cpt (13). To facilitate the isolation of top1T722Am mutants that suppress the lethal phenotype by restoring wild-type Top1p function, over 1,700 top1T722Am transformants surviving on galactose were individually scored for Cpt sensitivity on plates containing the drug. Only three transformants were drug-sensitive. However, as no plasmids could be retrieved from these cells, the restoration of drug sensitivity appeared to result from some genomic alteration, possibly involving integration of the plasmid sequences via homologous recombination with the 3’ end of the top1Δ sequences to regenerate wild-type TOP1. Remarkably, no catalytically active or Cpt-sensitive intragenic suppressors were obtained from this screen, suggesting that the alteration in Top1T722Ap function cannot be complemented by a second mutation in Top1p. Rather, a reduction in catalytic activity constituted the preferred mechanism of top1T722A suppression.

**Suppression of Top1T722A-induced Lethality Involves a Mechanism of Decreased DNA Binding by DNA Topoisomerase I**—The observed reduction in catalytic activity of the top1T722Am mutant enzymes could result from several alterations in enzyme activity, including a reduction in DNA substrate binding or DNA cleavage. Indeed, substitution of phenylalanine for the active site tyrosine was previously shown to abolish the catalytic activity and cytotoxicity of Top1T722Ap (13). However, mutation of the active site tyrosine in and of itself is insufficient to abolish DNA binding (41). To define mechanisms involved, we asked if the second mutation in the Top1T722Am enzymes affected DNA binding. To assess this, a bead-binding protocol previously developed to assay yeast DNA topoisomerase I activity in mammalian nuclear extracts was adapted (32). The introduction of an N-terminal epitope tag in yeast Top1p (Fig. 6A) had no discernible effects on enzyme activity or Cpt sensitivity (32). Moreover, tethering of the tagged enzyme to beads via an M2 monoclonal antibody specific for the epitope did not alter enzyme activity. The bead-bound form of wild-type Top1p or Top1T722Ap exhibited similar levels of catalytic activity, Cpt sensitivity, and DNA cleavage as proteins free in solution (32). The N-terminal position of the tag further ensured that only intact proteins were assayed, as any proteolytic processing of Top1p in yeast preferentially liberates the N terminus (data not shown). Modifying this assay to include binding of radiolabeled DNA (see Fig. 6B), the effects of specific suppressor mutations on full-length Top1p and Top1T722Ap binding to DNA could be assessed.

The three mutants selected for further analysis included the two with marginal catalytic activity, top1C273Y,T722A and top1G369D,T722A, and the mutant top1G295V,T722A. In the structure of the reconstituted human enzyme, residues corresponding to Cys-273 and Gly-369 (Cys-341 and Gly-437, respectively) lie in close proximity to the putative hinge region of the enzyme, on the opposite side of the protein clamp from the active site tyrosine and lip domains (16). The clamp must open and close around the DNA helix to effectively bind DNA, so
mutations close to the suspected hinge may affect the requisite conformational changes. On the other hand, Gly-295 corresponds to human Gly-363, which is important for the Cpt sensitivity of the enzyme (26), and lies within one of the lip domains involved in the closure of the protein clamp around the DNA (16). Substitutions of this residue may affect the interactions of adjacent residue side chains with the DNA and/or alter the structure of the lip domain and preclude DNA binding.

The introduction of the N-terminal epitope tag (indicated by an e prefix) did not affect eTop1p sensitivity to Cpt, eTop1T722Ap-induced lethality, or suppression of top1T722A cytotoxicity evident in eTop1C273Y,T722Ap-, eTop1G295V,T722Ap-, or eTop1G369D,T722Ap-expressing cells (data not shown). To assess the relative levels of mutant enzyme activity in vivo, pGAL1 expression vectors containing the indicated etop1 allele were co-transformed into top1Δ, top2ts yeast strain JCW28 along with a vector that constitutively expresses bacterial DNA topoisomerase I. As described by Giaever and Wang (42), transcription from divergent promoters bisect a plasmid DNA into local domains of positively and negatively supercoiled DNA. At the nonpermissive temperature, JCW28 cells lack DNA topoisomerase I and II. However, the bacterial enzyme preferentially relaxes the negatively supercoiled DNA resulting in the accumulation of positively supercoiled plasmid topoisomers (marked (+) in the pGAL1 control shown in Fig. 4). In contrast, when wild-type DNA topoisomerase I is also expressed in these cells, Top1p catalyzes the relaxation of the positively supercoiled DNAs (13, 22). In Fig. 4 (eTOP1), this was evident in a quantitative shift of the positively supercoiled plasmid topoisomers to the position of slightly negatively supercoiled DNAs (marked (+)). Similar results were obtained with catalytically active eTop1T722Ap (Fig. 4). As in vitro, the double mutant eTop1C273Y,T722Ap was partially active as evident in a slight but significant decrease in the relative intensity of the (+) topoisomers. This activity was distinct from that observed with eTop1G295V,T722Ap (Fig. 4), where an even distribution of topoisomers spanned the arc from positively to negatively supercoiled, suggesting a more distributive mode of activity, consistent with a lower affinity for DNA. eTop1G295V,T722Ap appeared less active as the topoisomer distribution resembled that of the vector control (compare pGAL1 and eTop1G295V,T722A).

The epitope-tagged double and single mutant proteins were partially purified as described (22, 30). The specific activity of the purified proteins was determined in plasmid DNA relaxation assays at varying salt concentrations (Fig. 5). As reported (22, 23), wild-type Top1p activity was maximal at 150 mM KCl and exhibited a 5-fold decrease at 50 mM KCl. The specific activity of Top1T722Ap was indistinguishable at high and low salt concentrations. The diminished activity of the double mutants at 150 mM KCl mirrored the in vivo data; eTop1G295V,T722Ap was inactive, and the limited activity of

**FIG. 5. In vitro activity of top1T722A suppressors.** Equal concentrations of the purified proteins were serially 10-fold diluted in reaction buffer (20 mM Tris (pH 7.5), 10 mM MgCl₂, 0.1 mM EDTA, 50 μg/ml gelatin, 1 mM β-mercaptoethanol, and KCl at a final 50 or 150 mM, as indicated). In a final 20 μl, 2 μl of the proteins were incubated with 0.3 μg of negatively supercoiled plasmid pBlueAK3 DNA in the same buffer for 60 min at 30 °C. Reactions were terminated by the addition of SDS and the products resolved in agarose gels, followed by ethidium bromide staining. C contains DNA alone. The relative positions of negatively supercoiled (−) and relaxed DNA topoisomers are as described in the legend to Fig. 1.

**FIG. 6. In vitro Top1p DNA binding assay.** A, the sequence of the N-terminal epitope tag in eTop1p is shown. The underlined residues are recognized by the monoclonal M2 antibody (Kodak, IBI). B, a schematic presentation of the DNA binding assay. As detailed under “Experimental Procedures,” the percent radiolabeled DNA bound to eTop1 proteins tethered to streptavidin-coated beads via the biotinylated, epitope-specific M2 antibody was calculated as bound-bound cpm/(bead-bound cpm) + (combined eluent cpm). C, percent DNA bound by increasing amounts of partially purified eTop1p, eTop1T722Ap, and eTop1Y727Fp. Fractions were corrected for eTop1p concentration in immunoblots probed with the epitope-specific M2 monoclonal antibody.
eTop1C273Y,T722Ap appeared processive, whereas that of eTop1G369D,T722Ap was more distributive. In the context of the wild-type Thr-722 residue, the specific activity of each single mutant (Top1C273Yp, Top1G295Vp, and Top1G369Dp) was also decreased about 100-fold. Surprisingly, the single mutants and the partially active double mutants eTop1C273Y,T722Ap and eTop1G369D,T722Ap exhibited an ~10-fold increase in activity at lower salt. This indicates that the single suppressor mutations (C273Y, G295V, and G369D) diminish the ability of the enzyme to bind DNA, rather than abrogate catalytic activity. When conditions favor tighter DNA binding, the specific activity of the mutant enzymes was enhanced. The exception was eTop1G295V,T722Ap, where the combination of the two mutations abolished enzyme activity at all salt concentrations. This suggests a more direct interaction of these mutated residues in Top1p catalysis.

To assess the relative affinities of wild-type and mutant proteins for DNA, increasing concentrations of eTop1 proteins were analyzed in the DNA binding assay (Fig. 6B). The eTop1 proteins were first bound to streptavidin-coated cellulose beads via the biotinylated M2 antibody, extensively washed to remove unbound protein, and then incubated with a $^{32}$P-labeled DNA fragment containing a high affinity Top1p-binding site (23, 43). The bead-bound protein-DNA complexes and unbound DNA fractions were recovered in spin columns, and the percent DNA bound was calculated. In controls with wild-type eTop1p, eTop1T722Ap, and the catalytically inactive eTop1Y727Fp, the relative affinities of the three proteins for DNA were identical (Fig. 6C). Similar results were obtained in nitrocellulose filter binding assays with homogeneous proteins. Since mutation of the active site tyrosine in eTop1Y727Fp precludes the formation of covalent intermediates, noncovalent DNA binding was measured under these conditions.

As shown in Fig. 7, the percent DNA bound by the single or double mutant proteins was significantly reduced relative to eTop1p and eTop1T722Ap and approached background levels defined with preparations from top1A cells. Consistent with the reduced catalytic activity observed in vitro, the DNA binding profiles obtained with the single mutants eTop1C273Yp and eTop1G369Dp overlapped those of the corresponding double mutants (Fig. 7, A and C). Extrapolation of these data suggests that the dissociation constants of the single mutants and the partially active double mutants, eTop1C273Y,T722Ap and eTop1G369D,T722Ap, were equivalent and were 5-fold higher than that of wild-type eTop1p or eTop1T722Ap (compare binding curves in Fig. 7, A–C). In contrast, the affinity of the inactive eTop1G295V,T722Ap for DNA was reduced about 10-fold (Fig. 7B). Although the single suppressors diminished DNA binding by Top1p, the combination of G295V with T722A further decreases enzyme affinity for DNA. This supports a direct interaction of the wild-type residues, Gly-295 and Thr-722, in the efficient binding of DNA.

This interpretation predicts that the double mutants, although less active, still exhibit an enhanced stability of the covalent enzyme-DNA intermediate, albeit at levels too low to detect in vitro. The enhanced Cpt sensitivity of yeast cells defective in the repair of double-stranded DNA breaks, due to deletion of RAD52 (9, 10), suggests that low levels of covalent enzyme-DNA complex may be detected in rad52D strains. This prediction was borne out in Fig. 8, where expression of the double mutants Top1C273Y,T722A, Top1G295V,T722A, or Top1G369D,T722A from pGAL1 was sufficient to induce top1A, rad52A cell death. In contrast, expression of the single mutants had little effect on cell viability. Thus, alterations in Top1p catalytic activity imparted by the T722A mutation were required for rad52A cell lethality. In repair-proficient strains, the diminished DNA binding induced by the suppressors reduces the formation of covalent enzyme-DNA complexes to sublethal levels.

**DISCUSSION**

Mutation of the conserved residue Thr-722 in yeast Top1T722Ap was previously shown to increase the stability of the covalent intermediate. The top1T722A mutant is lethal when overexpressed in yeast (13) and mammalian cells (32) and appears to mimic the cytotoxic activity of Cpt. Similar effects on enzyme function, yeast cell cycle progression, and cell viability were observed when the same substitution was made.

2 J. Fertala and M.-A. Bjornsti, unpublished results.

![Fig. 7. Intragenic suppressors of top1T722A reduce DNA binding.](Image 314 to 548)
at the corresponding position (Thr-718) in human DNA topoisomerase I. This underscores the conservation in DNA topoisomerase I function evident in the high degree of sequence and structural similarities between yeast and human Top1p.

Recent biochemical and structural studies of Top1p illustrate the formation of a protein clamp that circumscribes the duplex DNA substrate (15, 16, 18–20). However, the role that specific residues play in mediating Top1p binding to DNA and the conformational changes that must accompany protein clamp opening and closing during the catalytic cycle are mostly inferred from the crystal structures of the DNA-bound forms of the protein and await functional studies.

To define residues critical for the interdomainal interactions necessary for enzyme catalysis, we initiated a yeast genetic screen to identify intragenic suppressors of the lethal top1T722A mutant. This approach was supported by the demonstration that top1T722A-induced lethality was suppressed by a second lethal mutation, R517G. The combination of the two mutations abolished the catalytic and cytotoxic activity of the enzyme. As the mutated residues lie in separate conserved domains of the protein (13), their combined effect suggested that alterations in interdomainal interactions could greatly affect DNA topoisomerase I function. We reasoned that random mutagenesis would provide an unbiased approach to define second site mutations that affect various aspects of Top1T722Ap catalysis, including DNA binding, cleavage, and religation. Moreover, since alterations in the catalytic activity of Top1T722Ap mimics the action of Cpt, the results of these analyses would address the cytotoxic mechanism of Top1p poisoning.

Surprisingly, an exhaustive mutant hunt failed to identify second site mutations that restore wild-type Top1p function, as evidenced by Cpt-dependent cell death. Although the spectrum of hydroxylamine mutations is limited to GC → AT transitions, these results suggest that single amino acid substitutions are unable to counteract the alterations in enzyme activity that stabilize the covalent intermediate and re-establish the rapid turnover of the covalent Top1p-DNA complex. In the case of Cpt, the drug inhibits the religation reaction (44). With Top1T722Ap, studies with suicide substrates suggest an increase in the rate of DNA cleavage. The common feature in both is an increase in the levels of covalent enzyme-DNA intermediates. Although numerous mutations affecting enzyme sensitivity to Cpt have been reported (21–29), the exact mechanism of resistance has not been established. That is, drug resistance may be manifest as an inability of the drug to bind to the enzyme-DNA complex. Alternatively, the drug, although bound, may not affect the rate of DNA religation. The fact that no mutations in our screen restored the normal equilibrium of DNA cleavage and religation catalyzed by Top1T722Ap might argue a defect in drug binding is more likely.

In contrast, single amino acid substitutions were identified that suppress the cytotoxic activity of Top1T722Ap by reducing enzyme-specific activity over 100-fold. These mutations map throughout the conserved central and C-terminal domains of the enzyme, as well as the nonconserved linker region. Detailed biochemical characterization of three of these mutants, including assays of specific activity at varying salt concentrations and DNA binding by bead-bound enzymes, defined a mechanism.

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3 P. Fiorani, J. F. Amatruda, A. Silvestri, G. Francese, M.-A. Bjornst, and P. Benedetti, unpublished results.
whereby the affinity of the mutant enzymes for DNA was dramatically reduced. This constitutes the first demonstration that single amino acid substitutions in diverse structural domains of cellular DNA topoisomerase I can have such dramatic effects on DNA binding. Although single residue changes in vaccinia DNA topoisomerase I abolish DNA binding, considerable differences in structure, drug sensitivity, and DNA sequence specificity distinguish this enzyme from cellular Top1p (20, 45–47). In particular, the vaccinia enzyme lacks the lip domain necessary for protein clamp closure around the DNA helix and appears to contain a different “hinge” region than that defined in the cellular enzyme (20, 46). Given the recently reported structures of human DNA topoisomerase I, testable predictions may be made with regard to the yeast Top1T22A suppressors to explain the observed changes in the first critical step of the catalytic cycle, i.e. DNA binding.

The suppressor mutants, Top1C273Y, Top1G295V, and Top1G369D, involved substitutions of conserved residues Cys-273, Gly-295, and Gly-369, corresponding to human Top1p residues Cys-341, Gly-363, and Gly-437, respectively (16, 40). The double mutants exhibited a drastic reduction in catalytic activity, in vitro and in vivo. In DNA binding assays using intact enzymes tethered to beads via an N-terminal tag, the single mutant proteins and Top1C273Y, T722A and Top1G369D, T722A proteins exhibited a similar reduction in DNA binding. These effects were exacerbated in Top1G295V, T722A. Here, the combination of the two mutations produced a more severe reduction in enzyme activity and DNA binding than the single G295V mutation, suggesting a more direct interaction of these residues in the efficient binding of the enzyme to DNA.

In the human Top1p structure, residues corresponding to yeast Cys-273 and Gly-295 (Cys-341 and Gly-363, respectively) lie in core subdomain I, whereas the conserved residue Gly-369 (Gly-437 in human Top1p) lies at the beginning of a long a-helix in subdomain III that connects to subdomain I (16). Presumably, this helix forms part of the flexible hinge necessary to effect the conformational changes associated with protein clamp opening and closing. Cys-341 is in a β-strand structure in close proximity to this a-helix (16). Since mutation of these residues (Cys to Tyr and Gly to Asp) diminished DNA binding, it is tempting to speculate that such drastic changes in amino acid composition may affect the flexibility of the hinge region, with adverse affects on DNA binding and processive enzyme activity.

In the human enzyme, Gly-363 (Gly-295 in yeast Top1p) lies across the protein clamp from the hinge and resides in one of two lip structures that interact with each other and the bound DNA in the closed clamp structure (16). This corresponds to the Cpt loop defined in the structure of the 26-kDa fragment of yeast Top1p, based on the observation that substitution Gly-363 in human Top1p with Cys rendered the enzyme resistant to Cpt (26). Substitutions of adjacent residues also affect enzyme activity and Cpt sensitivity (48). Within this lip, Arg-364 makes minor groove contacts with bases adjacent to the cleavage site in the covalent and noncovalent enzyme-DNA complexes, and Lys-369 forms the single salt bridge with the other lip through residue Ghu-497 (16). Thus, it is likely that a substitution of the branched valine for the smaller, more flexible glycine at position 363 would alter the structural features of this critical domain. Similar arguments can be made for the H299Y and G303D suppressors identified in this screen.

The top1T722A suppressors L598F and P603S lie in the nonconserved linker region. Yet limited sequence similarity suggests these residues lie close to the junction of the two α-helices projecting out from the protein clamp (18, 40). Changes here may affect the extended helical structure upon which the controlled rotation model is based (18). Whether this affects DNA binding per se or the relative movement of DNA strands following DNA cleavage has yet to be determined. In the latter case, DNA cleavage-religation would occur without a concomitant change in DNA linking number. Studies are under way to examine these possibilities.

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