Substitutions of Asn-726 in the Active Site of Yeast DNA Topoisomerase I Define Novel Mechanisms of Stabilizing the Covalent Enzyme-DNA Intermediate*

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Eukaryotic DNA topoisomerase I (Top1p) catalyzes changes in DNA topology and is the cellular target of camptothecin. Recent reports of enzyme structure highlight the importance of conserved amino acids N-terminal to the active site tyrosine and the involvement of Asn-726 in mediating Top1p sensitivity to camptothecin. To investigate the contribution of this residue to enzyme catalysis, we evaluated the effect of substituting His, Asp, or Ser for Asn-726 on yeast Top1p. Top1N726S and Top1N726D mutant proteins were resistant to camptothecin, although the Ser mutant was distinguished by a lack of detectable changes in activity. Thus, a basic residue immediately N-terminal to the active site tyrosine is required for camptothecin cytotoxicity. However, replacing Asn-726 with Asp or His interfered with distinct aspects of the catalytic cycle, resulting in cell lethality. In contrast to camptothecin, which inhibits enzyme-catalyzed religation of DNA, the His substituent enhanced the rate of DNA scission, whereas the Asp mutation diminished the enzyme binding of DNA. Yet, these effects on enzyme catalysis were not mutually exclusive as the His mutant was hypersensitive to camptothecin. These results suggest distinct mechanisms of poisoning DNA topoisomerase I may be explored in the development of antitumor agents capable of targeting different aspects of the Top1p catalytic cycle.

Biochemical and crystallographic data suggest that the free DNA end rotates around the intact complementary strand, affecting alterations in the linkage of the two DNA strands (1, 6–9).

Camptothecin specifically interferes with the catalytic cycle of DNA topoisomerase I by reversibly stabilizing the covalent intermediate or cleavable complex (3, 4, 10, 11). During S-phase, this ternary drug-enzyme-DNA complex produces an irreversible inhibition of DNA synthesis, double-stranded DNA breaks, cell cycle arrest in G2 phase, and cell death via a mechanism of apoptosis. The cytotoxic action of the drug has been attributed to replication fork stalling or breakage when the advancing polymerase complex collides with the drug-stabilized enzyme-DNA intermediate (12–16). This model is supported by the fact that inhibitors of DNA synthesis abrogate camptothecin-induced lethality. Moreover, rad52 Δ yeast cells, defective in the recombinational repair of double-stranded DNA breaks and collapsed replication forks, are hypersensitive to the drug (17–19).

Significant advances have been made in defining the structural features of camptothecin important for the productive interaction of this agent with DNA topoisomerase I and DNA (3, 20–22). Recent crystallographic data on the structures of reconstituted and truncated versions of human DNA topoisomerase I in complex with DNA provide further insights into mechanistic aspects of enzyme function (6, 7, 23). Most of the mutations known to affect enzyme sensitivity to the drug are clustered along one face of the DNA in the Top1p-DNA co-crystal structure, close to the active site tyrosine (2, 3, 6). However, despite extensive investigation of camptothecin-resistant yeast and mammalian enzymes, the specific interactions required for the effective binding of the drug to the covalent intermediate remain largely unknown.

The residues preceding the active site tyrosine are highly conserved among the C-terminal domains of eukaryotic cellular enzymes (Fig. 1) (24, 25). A conservation of function is also supported by several studies involving specific amino acid substitutions. For example, mutation of these residues in either yeast or human Top1 to match the Ser-Lys-Arg-Ala-Tyr sequence found at the corresponding position in the camptothecin-resistant vaccinia virus enzyme rendered the mutant Top1vac enzymes resistant to the drug (26, 27). Conversely, substitution of Ala for Thr-722 enhanced the stability of the covalent enzyme-DNA complex in the absence of camptothecin, which proved lethal when top1T722A was expressed in yeast or mammalian cells (28–30). Similar effects on human Top1p function have recently been reported for the analogous replace-
The structures of human Top1p in covalent and noncovalent complexes with DNA demonstrate remarkably few contacts between the DNA substrate and the C-terminal domain (6, 23). Among residues near the active site tyrosine, only those immediately N-terminal to Tyr (Thr, Lys, and Asn in Fig. 1) interact with phosphate groups near the site of DNA scission. Mutation of this Asn to Leu in yeast Top1N726Ip diminished the catalytic activity and camptothecin sensitivity of the enzyme (26). Substitution of Ser for the same Asn residue in human DNA topoisomerase I also conferred camptothecin resistance, without a concomitant decrease in activity (32).

To directly address the contribution of this residue to DNA topoisomerase I catalysis, we investigated the consequences of substituting His, Ser, and Asp for Asn-726 in yeast Top1p. Here we report that these mutations had dramatically different effects on enzyme binding to DNA, cleavage of DNA, and camptothecin sensitivity. Our studies indicate a basic residue immediately conserved domainal interactions (29, 31).

Yeast Cell Sensitivity to Camptothecin—The camptothecin sensitivity of yeast cells transformed with various Top1p constructs was determined as described (2, 15, 27). Individual transformants were grown in selective medium plus dextrose and serially 10-fold diluted, and 5-ml volumes were spotted onto selective plates supplemented with 25 mM HEPES, pH 7.2, 2% dextrose (induced) or 10 mM dextrose (noninduced) and 100 ng/ml camptothecin. Compounds were spotted in a final 0.25% MεSO. No drug control plates contained 0.25% MεSO. Cell viability was scored following incubation at 30 °C.

DNA Topoisomerase I Activity Assays and DNA Cleavage Reactions—DNA topoisomerase I purified from EK3 cells transformed with various pGAlI-Top1p constructs as described (26, 27, 33). Briefly, extracted fractions from 1 liter of galactose-induced cultures were fractionated by sucrose gradient and 30 and 75% saturation ammonium sulfate precipitations and phosphocellulose chromatography. Proteins bound to the phosphocellulose column were eluted with successive 2-column volumes of TEEG buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10% glycerol) containing 0.2, 0.4, 0.6, 0.8, and 1 mg/ml KCl. Top1 proteins in the 0.6 mg/ml KCl fractions, were adjusted to a final 50% glycerol and stored at −80 °C. Protein concentrations were determined with the Bio-Rad protein assay. Top1 protein integrity was assessed in immunoblots probed with rabbit antibodies specific for yeast DNA topoisomerase I and stained with alkaline-phosphatase-coupled secondary antibodies (26, 27).

DNA topoisomerase I activity was assayed by the relaxation of supercoiled plasmid DNA, as described (26–28, 33). Briefly, serial 10-fold dilution of proteins corrected for concentration were incubated in 20-μl reaction volumes with 0.3 μg of negatively supercoiled pH624 DNA in 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 10 mM MgCl₂, 50 μg/ml gelatin, and 150 mM KCl. Where indicated, the final KCl concentration was adjusted from 50 to 200 mM, in 25 mM increments. Following incubation at 30 °C, the reactions were terminated by the addition of 1% SDS, and the extent of plasmid DNA relaxation was assessed following electrophoresis in agarose gels. Topoisomerase bands were stained with ethidium bromide and photographed.

Enzyme sensitivity to camptothecin was assayed in DNA cleavage reactions (26–28). A single 3′-end-labeled DNA substrate was prepared by cleaving plasmid pHCAK3–1 with BglII, repairing the ends with [α-32P]dATP, dNTPs, and Sequenase, and restricting with BamHI. Following purification in a 5% polyacrylamide gel, ~7.5 mg (8000 cpm) of the 480-base pair DNA fragment was incubated with equal concentrations of Top1p in 50-μl reaction volumes containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.1 mM EDTA, 50 mM KCl, 50 μg/ml gelatin, and a final 50 μM camptothecin. After 30 min at 30 °C, the covalent complexes were trapped with 1% SDS at 75 °C for 10 min. The samples were digested with 0.2 μg/ml proteinase K, ethanol precipitated, and electrophoresed in an 8% polyacrylamide, 7 mA urea gel in 0.1 M Tris-Borate buffer at 20 V/cm. The cleavage products were visualized by autoradiography.

Oligonucleotide-based DNA Cleavage Assays—Single-stranded oligonucleotides containing a modified high affinity Tetrahymena rDNA cleavage site sequence (5′-GG↓AT-3′) were synthesized with [γ-32P]ATP, dNTPs, and Sequenase, and restricted with BamHI. Following purification in a 5% polyacrylamide gel, ~7.5 mg (~8000 cpm) of the 480-base pair DNA fragment encoding the active tyrosine region of yeast Top1p was cloned into M13mp19 RF DNA. The single-stranded DNA fragment, was then annealed to equimolar amounts of unlabelled complementary DNA(s) in annealing buffer (10 mM Tris-HCl (pH 7.8), 100 μM NaCl, 1 mM EDTA) by heating to 95 °C for 5 min, followed by slow cooling over 3 h. Suicide DNA substrates were similarly prepared by annealing a 3′-end-labeled sciissile strand oligonucleotide (5′-GATCTAAAAAGATTGGAA*A-3′) to an unlabeled complementary nonscissile strand oligonucleotide (5′-GATCTTTTTTTTTTAAATTTC-AAGTCTTTTAGATC-3′). Cleavage reactions were performed at room temperature by incubating equal concentrations of enzyme with 20 nM DNA in 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.1 mM EDTA, 50 mM KCl, and 50 μg/ml gelatin. Reactions were terminated with SDS (0.5% final) followed by the addition of 3.3 volumes of loading buffer (1 mg/ml bromphenol blue, 1 mg/ml xylene cyanol in either 95% formamide or 80% formamide, 10 μg/ml NaOH). Samples were loaded on 16% polyacrylamide, 7 μA gels and electrophoresed at 40 mA at 50 °C.

DNA Topoisomerase I Activity in vivo—JCW28 cells were co-transformed with plasmid YEpTopA-PGPD, which constitutively expresses bacterial DNA topoisomerase I (28, 38), and the yeast YCpGAlI-top1p-L constructs. Cells were grown at the permissive temperature (25 °C) in C.-caryophyltan, -leucine dextrose medium, diluted into selective medium containing raffinose, and induced with 2% galactose at A595 = 1.3–1.4. After 5 h, half of each culture was shifted to the nonpermissive

1 The abbreviations used are: S.C., synthetic complete; Cpt, camptothecin.
temperature (35 °C) for an additional 3 h. To prevent changes in plasmid DNA topology during cell lysis, the cultures were mixed with an equal volume of buffered ethanol/toluene (20 mM Tris, pH 8.0, 95% ethanol, 3% toluene, and 10 mM EDTA) prechilled to −20 °C (28).

Following cell lysis, plasmid topoisomers were resolved by two-dimensional agarose gel electrophoresis, where the 0.6 mg/ml chloroquine of the first dimension was increased to 3 ug/ml in the second dimension (26, 28). Plasmid topoisomers were visualized with a random-primed 32P-labeled probe containing the 2-μm plasmid origin of replication followed by autoradiography.

RESULTS

Mutation of Asn-726 in Yeast DNA Topoisomerase I Affects Cell Viability and Sensitivity to Camptothecin—The residues preceding the active site Tyr-727 in yeast DNA topoisomerase I (Fig. 1) are highly conserved among eukaryotic cellular enzymes (24, 25). Mutation of these residues in yeast and human Top1p has previously been reported to effect changes in enzyme activity or camptothecin sensitivity (15, 25–32). For instance, in either enzyme, substitution of Ala for the conserved Thr, five residues N-terminal to the active site Tyr, enhanced the stability of the covalent intermediate, which proved lethal in yeast and mammalian cells (28–31). However, mutations involving the Asn residue adjacent to the active site Tyr (yeast and mammalian Top1p) are in bold. Oligonucleotide-directed mutagenesis was used to substitute the indicated amino acid residue for Asn-726 in yeast Top1p. The active site tyrosine of the enzyme is numbered (Tyr-727), whereas the mutant allele designations are at the left.

To assess the phenotypic consequences of these mutations, the top1 alleles were expressed from the galactose-inducible GAL1 promoter on a single copy ARS/CEN plasmid in a yeast strain deleted for TOP1 (top1Δ). As shown in Fig. 2A, yeast cells expressing either top1N726H or top1N726D exhibited at least a 3-log drop in cell viability in comparison to cells expressing wild-type TOP1. Whereas galactose-induced expression of top1N726S had no demonstrable effect on cell viability, the cells were resistant to camptothecin (Fig. 2B). This was in contrast to the dramatic decrease in viability induced by camptothecin in cells expressing comparable levels of wild-type Top1p.

The Top1N726S Protein Is Catalytically Active in Yeast—Because resistance to camptothecin may be a consequence of diminished catalytic activity (e.g., Top1N726Lp (26)), the ability of Top1N726Sp to catalyze the relaxation of plasmid DNA was evaluated in vivo. As reported by Giaever and Wang (38), transcription from divergent plasmid promoters generates local domains of negatively supercoiled DNA behind the transcription complexes and positively supercoiled DNA ahead. In a top1Δ, top2ts yeast strain, constitutively expressing the bacterial topA gene and shifted to the nonpermissive temperature, the only DNA topoisomerase activity detected is that of the bacterial enzyme. Unlike eukaryotic Top1p and Top2p, the bacterial enzyme will only catalyze the relaxation of negatively supercoiled DNA, resulting in the net accumulation of positively supercoiled plasmid DNA (indicated by (−) in Fig. 3). However, as previously reported (26, 28), when wild-type TOP1 is reintroduced on a plasmid and expressed at the nonpermissive temperature, TOP1 activity produces a quantitative shift in the topoisomer distribution from positive to negative (indicated as (−) in Fig. 3). The Top1N726S mutant protein was also active in vivo, producing a similar redistribution of plasmid DNA topoisomers (Fig. 3). Thus, unlike Leu (26), substitution of Ser for Asn-726 did not produce an appreciable decrease in catalytic activity.

Camptothecin sensitivity is greatly enhanced in yeast cells deficient in recombinational repair because of the deletion of the RAD52 gene (17–19). Yet, galactose-induced expression of the camptothecin-resistant mutants, top1N726L and top1vac,
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in cells lacking functional Rad52p, is lethal in the absence of camptothecin (26). These results indicated that the alterations in catalytic activity necessary for drug resistance induce sufficient DNA damage to cause cell death in the absence of Rad52-mediated repair. As both top1N726L and top1Nvac involve Asn-726 substituents, we asked if the camptothecin-resistant phenotype of the Ser for Asn-726 mutant would also correlate with adverse affects on rad52 cell viability.

As shown in Fig. 4, repair-proficient RAD52 cells expressing wild-type TOP1 were sensitive to camptothecin, whereas cells expressing top1N726L or top1N726S were resistant. Consistent with previous findings (26), galactose-induced top1N726L expression was lethal in a repair-deficient rad52Δ strain in the absence of camptothecin. Expression of TOP1 and top1N726S slightly diminished cell growth relative to the top1Δ controls. However, viable cells expressing top1N726S were evident even at high camptothecin concentrations. Thus, in contrast to other Asn-726 substitutions, replacement of this residue with Ser conferred a high degree of drug resistance, even in the absence of recombinational repair.

Substitution of Asn-726 Produces Differential Stability of the Covalent Enzyme-DNA Complex and Altered Enzyme Sensitivity Toward Camptothecin—To further investigate the effects of replacing Asn-726 with Ser, Asp, or His on enzyme mechanism and camptothecin sensitivity, the wild-type and mutant proteins were purified from galactose-induced top1Δ cells expressing plasmid borne pGAL1-top1 constructs. The proteins were corrected for concentration, serially diluted, and assayed in plasmid DNA relaxation assays. At the salt concentrations optimal for wild-type Top1p (150 mM) the specific activity of Top1N726Sp and Top1N726Hp was comparable to that of wild-type Top1p (Fig. 5.). Thus, consistent with in vivo data (Fig. 3), the camptothecin resistance of Top1N726Sp-expressing cells could not be attributed to a loss in enzyme function. However, under the same conditions, Top1N726Dp-catalyzed relaxation of plasmid DNA was reduced by ~20-fold. Repeated assays indicated a consistent 20–50-fold reduction in specific activity. The catalytically inactive Top1Y727F mutant (25, 29, 30) served as a negative control.

The effects of the Asn-726 substituents on covalent enzyme-DNA complex stability were assessed in DNA cleavage assays in the presence and absence of camptothecin. As described (27, 28, 30), enzyme preparations were incubated with a single 3’-end-labeled DNA fragment containing a high affinity binding site (marked with an asterisk). Covalent complexes were irreversibly trapped with SDS, and the extent of DNA cleavage was determined following electrophoresis in DNA sequencing gels.

As shown in Fig. 6, replacement of Asn-726 with Ser in Top1N726Sp abrogated the ability of camptothecin to stabilize the covalent complex. The low level of DNA cleavage by Top1N726Sp was unaffected by drug addition. In contrast, camptothecin induced a dramatic increase in cleavable complexes formed with equivalent amounts of wild-type Top1p, at the high affinity site as well as other sites.

Of the lethal Asn-726 substitutions, only His enhanced the stability of the covalent complex in the absence of camptothecin (Fig. 6). Whereas cleavage was most pronounced at the high affinity site, stable complexes were also formed at other sequences. Relative to wild-type Top1p, the His mutant was hypersensitive to camptothecin as evidenced by the increase in the intensity and distribution of DNA cleavages in the presence of the drug. Although similar results were obtained when Thr-722 was mutated to Ala in Top1T722Ap (28), the pattern of DNA cleavage in the presence of camptothecin reverted to that of wild-type Top1p. In contrast, Top1N726Hp produced a composite of DNA scissions; some were unique to the mutant enzyme, and others were coincident with drug-treated wild-type Top1p. Interestingly, insertion of Asp at residue 726 also conferred camptothecin resistance as cleavage was only slightly

**Fig. 3.** Top1N726Sp is catalytically active in vivo. Yeast strain JC28 (top1Δ, top2Δ), co-transformed with plasmid YEpTopA-PGPD and either YCPGAL1-TOP1-L, YCPGAL1-top1N726-L, or YCPGAL1-L (negative control), was grown in S.C.-tryptophan, -leucine dextrose medium, diluted into medium containing raffinose, and induced with galactose for 5 h at 25°C. Cells were then shifted to the nonpermissive temperature (35°C) for an additional 3 h before harvesting and lysis as described under “Experimental Procedures.” The topological state of the endogenous 2-μm plasmid was evaluated by two-dimensional gel electrophoresis, blotting onto a nylon membrane, and hybridization with a 32P-labeled 2-μm DNA probe. The location of positively supercoiled (+) DNA and negatively supercoiled DNA (−) are indicated.

**Fig. 4.** top1N726S does not induce rad52Δ cell lethality. Exponentially growing EKY3(top1Δ, RAD52) or MBY3 (top1Δ, rad52Δ) cells transformed with the indicated top1 allele were serially 10-fold diluted and spotted onto S.C.-uracil plates containing 2% dextrose or galactose and supplemented with 25 mM HEPES, pH 7.2, and 0.25% Me2SO or 10 μg/ml Cpt in 0.25% Me2SO.

**Fig. 5.** The catalytic activity of purified Top1 proteins. Equal concentrations of wild-type Top1p and the indicated mutant enzymes were serially 10-fold diluted and assayed in a plasmid DNA relaxation assay (as described under “Experimental Procedures”). Reaction products were resolved in an agarose gel and subsequently visualized by ethidium bromide staining. The relative position of the negatively supercoiled DNA substrate is indicated by Sc, whereas the ladder of relaxed DNA topoisomerase bands is labeled R.
enhanced at the high affinity site. Reactions containingTop1Y727Fp, which is able to bind but not cleave DNA, appeared identical to the DNA control. Taken together, these data suggest that efficient stabilization of the covalent complex by camptothecin requires a basic residue, such as Asn or His, immediately N-terminal to the active site Tyr. Replacement of Asn-726 with Ser or Asp abolishes camptothecin sensitivity. Yet, these same substitutions have profoundly different effects on enzyme activity: Ser had no demonstrable effect; His enhanced covalent complex stability; and Asp reduced activity by 20-fold. The increment in covalent complexes formed with the His mutant accounts for the top1N726H lethal phenotype. Less clear is the basis for top1N726D-induced lethality.

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**FIG. 6.** Substitutions of residue Asn-726 affect the camptothecin sensitivity and DNA cleavage activity of DNA topoisomerase I. Equal concentrations of the indicated Top1 proteins were incubated in a DNA cleavage assay with a single end-labeled DNA fragment as described under "Experimental Procedures." As indicated, the reactions contained a final 50 µM camptothecin in 0.4% Me2SO or 0.4% Me2SO alone. Following the addition of 1% SDS and heating to irreversibly trap the covalent enzyme-DNA intermediates, the reaction products were digested with proteinase K, denatured, and resolved in a DNA sequencing gel. C contains DNA substrate alone. The asterisk indicates the position of the high affinity cleavage site derived from *Tetrahymena* rDNA.

**FIG. 7.** Substitution of His for Asn-726 enhances the rate of DNA cleavage. Cleavage of the suicide DNA substrate (shown at the top of the figure) was performed in 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.1 mM EDTA, 50 mM KCl, 50 µg/ml gelatin. Reactions were initiated by the addition of enzyme. At the indicated times, aliquots (10 µl) were withdrawn and immediately quenched with SDS (final concentration of 0.5%). Samples were then electrophoresed in a denaturing 16% polyacrylamide gel and visualized by autoradiography. The position of the 6-mer cleavage product is indicated by the arrow.

G was substituted for A at the +1 position (downstream from the cleavage site) on the scissile strand. The suicide DNA substrate allows for an uncoupling of the DNA cleavage and religation reactions, as the 6-mer (5′-GGAAA*A-3′) liberated upon cleavage readily dissociates from the complex and is unavailable for religation. As shown in Fig. 7, substitution of Ser or Asp for Asn-726 had little effect on the rate of enzyme-mediated DNA cleavage. Similar results were obtained with the lethal Top1T722Ap mutant.2 In contrast, the rate of DNA cleavage was stimulated by the His mutation. Thus, the increased steady-state levels of covalent complexes obtained with Top1N726Hp in Fig. 6 could be attributed to enhanced DNA cleavage. This represents a novel mechanism of Top1 poisoning that contrasts with the inhibition of DNA religation ascribed to camptothecin (39).

Noncovalent binding of DNA topoisomerase I to DNA involves bipartite interaction of the enzyme with the DNA phosphodiester backbone upstream and downstream of the cleavage site. To assess the contribution of specific enzyme-DNA contacts that enhance or suppress the cleavage reaction, nicks were introduced at different sites along the nonscissile strand. As diagrammed in Fig. 8, the resulting double-stranded DNA substrates consist of a 3′-end-labeled 37-mer scissile strand (containing the same cleavage site in the suicide substrate) paired to complementary nonscissile strands. This substrate contains the high affinity site described above, except

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2 J. R. Vance and M.-A. Bjornsti, unpublished results.
of the incubation period to reverse covalent complex formation (lane 2), as well as in the presence of camptothecin (lane 3) with salt reversal (lanes 4). A truncated version of human DNA topoisomerase I (hTop1) was also included.

As a basis for comparison, enzyme-mediated cleavage was first evaluated on the fully double-stranded DNA substrate without nicks. In Fig. 8, the behavior of wild-type Top1p, Top1N726Sp, Top1N726Hp, and Top1Y727Fp mirrored that observed with longer DNA substrates (compare Figs. 6 and 8). Further, all covalent complexes, whether drug- or mutation-induced, were salt reversible. However, the levels of DNA cleavage by Top1N726Dp in the absence of camptothecin exceeded that obtained at the high affinity site in the longer DNA substrate. This may reflect the difference in base composition at +1 (CTT ▼>GGG versus CTT ▼>AGG). Nevertheless, covalent complex formation remained unaffected by the addition of camptothecin.

As was previously reported for human Top1 (37), the introduction of a nick between −4 and −3 suppressed DNA cleavage by yeast Top1p, Top1N726Sp, Top1N726Hp, and Top1N726Dp. This is consistent with a requirement for a phosphate at this position for efficient binding of the yeast enzyme to DNA. Further, with the exception of the catalytically inactive Top1Y727Fp, a nick at the −2/−1 position produced a 2-base pair shift upstream in the site of cleavage resulting in the liberation of a 25-mer product (indicated by the open arrow).

Shifting the nick between −1 and +1 directly opposite the site of cleavage strongly enhanced cleavage even in the absence of camptothecin. Cleavage opposite the nick would produce a blunt-ended double strand break. Unless the untethered duplex DNA was tightly held by the protein, the noncovalently bound DNA ends could dissociate from the complex, effectively acting as a suicide substrate. Indeed, the lack of salt reversal attests to the formation of such abortive double-stranded breaks. Cleavage was most pronounced with the His mutant, followed by wild-type Top1p, and then the Ser and Asp mutants. Camptothecin did augment complex formation with wild-type Top1p, but not Top1N726Sp or Top1N726Dp, suggesting some reversal of the cleavage reaction that was drug inhibitable.

The effect of moving the nick to position +2/+3 differed significantly for Top1N726Dp compared the other enzymes tested, as the covalent Top1N726Dp-DNA complexes alone were not reversed upon salt addition. Moreover, relative to other DNA substrates examined, covalent complex stability was enhanced. These findings suggest that the Asp mutant enzyme may be defective in binding DNA or may alter DNA structure when bound. However, once the covalent intermediate was formed, any reduction in DNA binding would be localized to noncovalent interactions downstream from the cleavage site. The selective loss of the noncovalently bound DNA from the reaction intermediate would preclude efficient religation of the nicked DNA.

Because noncovalent binding of DNA topoisomerase I to DNA is dependent on the ionic strength of the reaction, enzyme catalytic activity was re-examined over a range of salt concentrations. As previously reported (26, 29), wild-type Top1p

Fig. 8. DNA substrates containing nicks in the nonscissile strand distinguish differences in mutant enzyme binding and cleavage of DNA. Top1 proteins were reacted with double-stranded DNA substrates containing nicks in the lower strand as indicated above each panel. Reactions were terminated by the addition of SDS to a final concentration of 0.5% (lanes 1 and 3) or NaCl (final concentration 0.5 M) for 30 min. followed by SDS (lanes 2 and 4). Lanes 3 and 4 also contained 10 μM camptothecin. C contains DNA substrate alone. Arrows and numbers correspond to the position and size of the cleavage products, respectively.
achieved maximal activity at -150 mM KCl and retained activity at concentrations as high as 175 mM (Fig. 9). In stark contrast, Top1N726Dp catalytic activity was optimal in 75-100 mM KCl and rapidly decreased at salt concentrations in excess of 150 mM. Moreover, even at lower salt concentrations (<100 mM), Top1N726Dp exhibited a distributive mode of plasmid DNA relaxation, as evidenced by a continuum of bands extending from the negatively supercoiled DNA substrates to R, relaxed DNA topoisomers.

**DISCUSSION**

**DNA Topoisomerase I Sensitivity to Camptothecin**—A shift in equilibrium between the concerted DNA cleavage and religation reactions catalyzed by DNA topoisomerase I can have dire consequences on cell viability. Indeed, numerous studies attribute the cytotoxic action of camptothecin to its ability to reversibly inhibit the religation reaction (Fig. 10, step III (reviewed in Refs. 2–4)). The resultant stabilization of the covalent enzyme-DNA complexes increases the probability of catastrophic collisions with advancing replication forks (step V) producing the DNA lesions that trigger cell cycle arrest and cell death. Previous studies have shown that substitution of Ala for the conserved Thr, five residues N-terminal to the active site tyrosine of yeast or human Top1p, also enhances the stability of the covalent complex and causes drug-independent cytotoxicity in yeast and mammalian cells (28–31). Although the step(s) in the catalytic cycle altered by the Thr to Ala replacement have in yeast and mammalian cells (28–31). Although the step(s) in the catalytic cycle altered by the Thr to Ala replacement have in yeast and human Top1p, Top1N726Dp, and Top1N726Hp were incubated with supercoiled plasmid pH624 DNA at 30 °C for 30 min followed by termination with SDS. For each enzyme the concentration of KCl in the reaction mix varied from 50–200 mM as indicated. Reaction products were electrophoresed and visualized as described in Fig. 5. Sc, negatively supercoiled DNA substrate; R, relaxed DNA topoisomers.

![Fig. 9. DNA topoisomerase I mutants exhibit different salt optima in plasmid DNA relaxation assays. Equal concentrations of Top1p, Top1N726Dp, and Top1N726Hp were incubated with supercoiled plasmid pH624 DNA at 30 °C for 30 min followed by termination with SDS. For each enzyme the concentration of KCl in the reaction mix varied from 50–200 mM as indicated. Reaction products were electrophoresed and visualized as described in Fig. 5. Sc, negatively supercoiled DNA substrate; R, relaxed DNA topoisomers.

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**FIG. 10. The effects of Cpt and Asn-726 substitutions on the catalytic cycle of DNA topoisomerase I.** The proposed conformational changes necessary to effect DNA binding by the Top1 protein clamp (steps I and IV) are based on recent structures of the human enzyme (23). An alternative possibly is that DNA binding to a constitutively open structure drives the conformational changes that accompany clamp closure.

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To more directly assess the function of Asn-726 in enzyme catalysis and drug sensitivity, we analyzed the effects of several substituents on yeast Top1 function. Our studies indicated that a basic residue, such as Arg or His, was essential for the Cpt sensitivity of the enzyme. The replacement of this residue with Ser (as previously shown for human Top1p (32)) or Asp abolished the ability of Cpt to stabilize the covalent complex. However, the Ser mutant was distinguished from all other Asn mutants studied by the lack of detectable alterations in catalytic activity. Unlike the previously reported yeast top1vac and Top1N726L mutants, placement of Ser immediately N-terminal to the active site tyrosine was not accompanied by an increase in DNA damage and rad52Δ cell death in the absence of camptothecin. What contribution the side chain -OH may play in enzyme catalysis has yet to be defined.

Two models were recently proposed to describe camptothecin bound to the covalent intermediate formed by human Top1 and DNA (6, 40). Both models are consistent with the hypothesis that Cpt is stacked against the base 3′ to the DNA cleavage site (the +1 position) (41). However, the orientation of the drug is essentially flipped in these models, such that the proposed stacking interactions of Asn with camptothecin is a key feature of the Redinbo et al. (6, 7) model, whereas a hydrogen bond between the oxygen of the Asn side chain and the 20-OH of Cpt is a prominent feature in the model by Fan et al. (40) model (3). Clearly, the data presented here regarding the alterations in drug sensitivity because of mutation of the Asn residue will allow for a refinement of these models.

**Enzyme-catalyzed DNA Cleavage and Religation**—The Asn-726 substitutions had even more dramatic effects on the DNA cleavage and religation reactions catalyzed by Top1p. The His and Asp mutants were lethal even in the absence of Cpt. The results of DNA cleavage assays using end-labeled DNA fragments, nicked DNA molecules, or suicide substrates indicate that replacing Asn-726 with His (N726H) enhances the rate of DNA scission catalyzed by the enzyme (Fig. 10, step II). The net effect is the relative accumulation of covalent intermediates,
albeit via a mechanism distinct from that ascribed to camptothecin. Nevertheless, this would also increase the probability of replication-induced DNA lesions as described for ternary camptothecin-enzyme-DNA complexes (12, 14).

The increase in salt concentration necessary for optimal relaxation of supercoiled plasmid DNA (Fig. 9) is consistent with a higher affinity of the Top1N726H protein for DNA. However, the mechanistic basis for elevated rates of DNA scission remain unclear. This may reflect an increase in nucleophility of the active site tyrosine induced by the close proximity of the His residue. In this case, His may act as a base to accept the proton from the attacking hydroxyl of the tyrosine. Indeed, available crystallographic data have failed to identify an amino acid residue in close enough proximity to act as a general base in the cleavage reaction. With wild-type Top1p, it has been proposed that a water molecule may act in this capacity. In Top1N726Hp, the presence of His may usurp this function, facilitating DNA scission. Although, the clarification of this liability was observed in an

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