Domain Interactions Affecting Human DNA Topoisomerase I Catalysis and Camptothecin Sensitivity

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ABSTRACT

DNA topoisomerase I (Top1p) relaxes supercoiled DNA by the formation of a covalent intermediate in which the active site tyrosine is transiently bound to the severed DNA strand. The antineoplastic agent camptothecin (Cpt) specifically targets Top1p and several mutations have been isolated that render the enzyme Cpt resistant. The mutated residues, although located in different regions of the enzyme, may constitute part of the Cpt binding site. To begin identifying the structural features of DNA Top1p important for Cpt-induced cytotoxicity, we developed a novel yeast genetic screen to isolate catalytically active, yet Cpt-resistant enzymes from a pool of human *top1* mutants. Among the mutations isolated were substitutions of Ser or Val for Gly363, which like the Gly363 to Cys mutation previously reported by us, suppressed the Cpt sensitivity of Top1p. In contrast, each amino-acid substitution differed in its ability to suppress the lethal phenotype and catalytic activity of a human *top1* mutant top1T718A that resembles Cpt by stabilizing the covalent intermediate. Biochemical analyses and molecular modeling support a model where interactions between two conserved domains, a central "lip" region containing residue Gly363 and the residues around the active site tyrosine (Tyr723), directly affect the formation of the Cpt-binding site and enzyme catalysis.

DNA topoisomerase I (Top1p) catalyzes the relaxation of supercoiled DNA through the transient cleavage of one strand of a DNA duplex and is fundamental to processes such as replication, recombination, and transcription (Hsiang et al., 1985; Chen and Liu, 1994; Wang, 1996; Nitiss, 1998). Eukaryotic Top1p is the cellular target of the antitumor drug camptothecin (Cpt), which reversibly stabilizes the cleavable complex, an intermediate in the enzyme's catalytic cycle (Hsiang et al., 1985; Chen and Liu, 1994; Pommier et al., 1998). Genetic and biochemical studies have led to the identification of amino-acid substitutions in Top1p that render the enzyme resistant to Cpt. Mutations that yield catalytically active, yet drug-resistant enzymes are clustered in highly conserved regions, which are themselves widely separated along the linear amino-acid sequence of the protein. One region surrounds the active site tyrosine, where several

such mutations have been identified (Knab et al., 1993, 1995; Fujimori et al., 1995; Wang, 1997). Another is located around residue 363 (Gly363) of the human enzyme, where two mutations have been independently mapped (Benedetti et al., 1993; Rubin et al., 1994). Collectively, these data led to the working hypothesis that the Cpt-binding site is formed by a cleft that contains DNA and residues responsible for Cpt resistance.

Recently, the three-dimensional structures of a 26-kDa fragment of yeast DNA Top1p (yTop1p) (Lue et al., 1995) and of reconstituted and N-terminal truncated versions of human DNA Top1p (hTop1p) in complexes with a 22-base pair DNA molecule (Redinbo et al., 1998; Stewart et al., 1998) have been solved. The enzyme is organized in multiple domains that "clamp" around the DNA molecule. DNA relaxation has been proposed to proceed via "controlled rotation" in which the covalently bound enzyme holds one end of the DNA duplex, and allows the end downstream of the cleavage site to rotate around the remaining phosphodiester bond (Redinbo et al., 1998). The residues mutated in the Cpt-resistant mutants are positioned in regions of the enzyme engaged in DNA-protein interactions and are clustered along one face of the DNA molecule. In hTop1p, Gly363 lies within a loop structure that constitutes one of two "lips" that interact with

ABBREVIATIONS: Top1p, DNA topoisomerase I; yTop1p, yeast DNA topoisomerase I protein; hTop1p, human DNA topoisomerase I protein; Cpt, camptothecin; DMSO, dimethyl sulfoxide.

the DNA and each other in the closed DNA bound protein clamp (Redinbo et al., 1998). The corresponding structure of the 26-kDa yTop1p fragment, called the Cpt loop, also contains this residue (Gly295) (Lue et al., 1995). Lue et al. (1995) suggested that the Cpt loop was opposite the active site tyrosine domain of the protein and formed a catalytic cleft. Indeed, the close proximity of these domains is borne out in the cocrystals of the reconstituted central and C-terminal fragments of hTop1p with DNA (Redinbo et al., 1998).

We describe a novel genetic screen to identify additional mutations that suppress the Cpt sensitivity of hTop1p without abolishing catalytic activity. Among the mutants isolated were additional substitutions of Gly363. Substitution of Ser or Val for Gly363, in mutants htop1G363S and htop1G363V, respectively, abolished the Cpt sensitivity of these mutant proteins with only marginal effects on enzyme activity. This was in contrast to the more than 100-fold reduction in specific activity seen in the corresponding yeast top1 mutant Top1G295Vp (Hann et al., 1998b). To address this apparent discrepancy in the activities of identical amino-acid substituents in hTop1p and yTop1p, despite the conservation of amino-acid sequence and structure in this domain, we further investigated the effects of these Gly363 substitutions in suppressing the lethal phenotype of a Top1 mutant that mimics Cpt. Herein, we show that substitution of Thr718 to Ala in hTop1p mirrors the alterations in catalytic activity observed with the same substitution in yTop1T722Ap, both in vitro and in vivo (Megonigal et al., 1997; Hann et al., 1998a,b) Moreover, we show that the Cpt-resistant mutations at Gly363 have widely varied affects in suppressing the lethal phenotype and catalytic activity of the hTop1T718A mutant enzyme. Furthermore, molecular models were created for the mutants, based on the three-dimensional structure of the human enzyme. Along with biochemical analyses, these data suggest that distinct interactions between the two conserved lip domains mediate the Cpt sensitivity and catalytic activity of wild-type DNA Top1p. The implications of this intramolecular suppression are discussed in the context of current structural models for eukaryotic DNA Top1p.

Experimental Procedures

Materials, Yeast Strains, and Plasmids. Cpt (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at 4 mg/ml. *Saccharomyces cerevisiae* strains EKY2 (*MATa*, *ura3-52*, *his3△200*, *leu2△1*, *trp1△63*, *top1::HIS3*) and JN2-134 (*MATa*, *rad52::LEU2*, *trp1*, *ade2-1*, *his7*, *ura3-52*, *ise1*, *top-1*, *leu2*) were described previously (Bjornsti et al., 1989; Kauh and Bjornsti, 1995). Strain JCW28 (*Mata*, *ura3-52*, *his3△200*, *leu2△1*, *trp1△63*, *top2-4*, *top1△*) was kindly provided by Dr. J. C. Wang, Harvard University. YCp*GAL1*-hTOP1 and ptac-h*TOP1* have been described (Bjornsti and Wang, 1987; Bjornsti et al., 1989). YEptopA-pGPD was reported previously (Giaever and Wang, 1988). pBlueAK3-1 DNA contains a high-affinity DNA Top1p cleavage site (Knab et al., 1995).

htop1T718A was generated by oligonucleotide-directed mutagenesis of the h*TOP1* gene *(hTOP1)* cloned into the M13 mp19 vector (Amersham Corp., Arlington Heights, IL) with the oligo: 5′-GATT-GCCCTGGGAGCCTCCAAACTCATTATCT-3′. The mutated h*TOP1* gene was then cloned into *Bam*HI-*Sal*I-cut pBM125 to yield YCp-GAL1-htop1T718A. The mutant h*top1G363C* has been described (Benedetti et al., 1993). The mutants h*top1G363V* and h*top1T718A, Y723F* were generated by oligonucleotide-directed mutagenesis of plasmid ptac-hTOP1 (Bjornsti et al., 1989) with the transformer system from Clontech (Palo Alto, CA) and the oligonucleotides 5′-

GACTTTTCCGTGTCCGCGGCAAC-3′ and 5′-CTGGGAGCCTC-CAAACTCAATTTTCTGGAC-3′, respectively. Randomly mutagenized h*top1* pools were produced as described (Benedetti et al., 1993). To express h*top1* mutants from the p*GAL1* promoter, fragments bearing the mutations were excised from bacterial expression vectors and exchanged for the wild-type sequences in YCpGAL1-hTOP1 to yield plasmids YCpGAL1-htop1G363S, YCpGAL1-htop1G363V, and YCpGAL1-htop1T718AY723F. Plasmids YCp-GAL1-htop1G363S, T718A, YCpGAL1-htop1G363C, T718A, and YCpGAL1-htop1G363V, T718A were made by replacing wild-type sequences in YCpGAL1-htop1T718A with fragments containing the Gly363Ser, Gly363Cys, or Gly363Val mutations, respectively.

To construct YCp-SchTOP1*,* in which hTop1p is under the constitutive yTop1p promoter, the 4.4-kb *Apa*I/*Sal*I fragment of plasmid YEp-SchTOP1 (Bjornsti and Wang, 1987) was joined to the 3.8-kb *Apa*I/*Sal*I fragment of pRS416 (Sikorski and Hieter, 1989). YCpSc-hTOP1 was then digested with *Sph*I/ClaI and the 2.4-kb *Sph*I/ClaI fragment of YCpGAL1-htop1T718A inserted to create vector YCpSc-htop1T718A.

Cell Viability Assays*. Top1△* yeast strains were transformed with the YCpGAL1-hTOP1 constructs by LiOAc treatment and selected on synthetic complete (SC)-uracil plus 2% dextrose. Transformants were grown to an $A_{595} = 0.5$ and 5-μl aliquots of serial 10-fold dilutions were spotted onto SC-uracil plates plus 2% dextrose or 2% galactose. Alternatively, cultures were diluted 1:100 into SC-uracil media containing 2% raffinose and, at an $A_{595} = 0.3$, induced with a final 2% galactose. At various time points, aliquots were serially diluted and plated onto SC-uracil, 2%dextrose. The number of colonies was counted following incubation at 30°C.

DNA Top1p Activity In Vivo. Yeast strain JCW28 (top1△, top2-4ts) was cotransformed with plasmid YEptopA-pGPD, which constitutively expresses the bacterial *topA* gene (Giaever and Wang, 1988), and either YCpGAL1, YCpGAL1-hTOP1, or YCpGAL1-htop1T718A. Transformants were grown at 25°C, a permissive temperature for the top2-4ts mutant, in SC-uracil-leucine plus dextrose. At $A_{595} = 1.0$, Top1p expression was induced with 2% galactose. After 5 h, half of the culture was shifted to 37°C for 3 h to inactivate DNA Top II. The cells were harvested and disrupted as described (Worland and Wang, 1989; Gartenberg and Wang, 1992) and the linking number distributions of purified plasmid DNAs were determined by two-dimensional gel electrophoresis (Benedetti et al., 1993; Megonigal et al., 1997). DNA was transferred to a nylon membrane (Amersham Corp.) and probed for 2-μm plasmid sequences with a ^{32}P-labeled fragment prepared by random priming (Pharmacia, Piscataway, NJ). Phosphoimager analysis was used to establish the ratio between positive supercoils and relaxed DNA in each sample.

Purification of Mutant hTop1p. Partial purification of DNA Top1ps from galactose-induced EKY2 cells was achieved by phosphocellulose column chromatography as described (Benedetti et al., 1993; Knab et al., 1995). Coomassie-stained SDS-polyacrylimide gel electrophoresis gels indicate the purity of these enzymes to be ~50 to 60%. Alternatively, a one-step purification was achieved with the FLAG system (Kodak) as follows: sequences encoding the epitope DYKDDDY were inserted at the 5′ end of h*TOP1* by polymerase chain reaction. The tagged he*TOP1* gene was subcloned into YCp-GAL1 to produce YCpGAL1-heTOP1. Western blot and immunfluorescence analyses verified that full-length heTop1p was recognized by the epitope-specific monoclonal antibody M2 (data not shown). To purify heTop1p, strain EKY2 was transformed with YCPGAL1-he-TOP1 or YCpGAL1-hetop1T718A, grown in SC-uracil plus dextrose and diluted 1:100 in SC-uracil plus raffinose. At an $A_{595} = 1.0$, the cells were induced with 2% galactose for 6 h. Cells were harvested by centrifugation, washed with TEEG (TRIS, EDTA, EGTA, glycerol) buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 10% (v/v) glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 800 μg/ml NaHSO$_3$, 20 μg/ml benzamidine, 2 μg/ml pepstatin A, 2 μg/ml leupeptin) and resuspended in TEEG buffer at 2 ml buffer/g cells (wet weight). Then

0.5 volumes of 0.5-mm glass beads were added, and the cells were disrupted with ten 30-s cycles of vortexing alternating with 30 s on ice. The lysate was clarified by centrifugation and incubated for 1 h at 4°C with the M2 antibody covalently coupled to an insoluble resin (Kodak). The resin was washed three times for 10 min with 10 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and used directly in activity or cleavage assays.

DNA Top1p Activity In Vitro. DNA Top1p activity was assayed in DNA relaxation reactions (Bjornsti and Wang, 1987; Bjornsti et al., 1989). Briefly, DNA Top1p preparations were incubated in 30-μl reaction volumes containing 0.5 μg of negatively supercoiled plasmid pHC624 DNA and reaction buffer [20 mM TRIS (pH 7.5), 0.1 mM Na$_2$EDTA, 10 mM MgCl$_2$, 50 μg/ml acetylated BSA, and 100 mM KCl]. After 1 h at 37°C, the reaction products were analyzed in 1% agarose gels. One unit of activity was the amount of enzyme needed to completely relax 0.5 μg of negatively supercoiled plasmid under standard reaction conditions. To assess the effects of Cpt on enzyme activity, 50 μM Cpt was added to the reactions and the extent of plasmid DNA relaxation at 0.5, 1, 5, 15, and 30 min was determined with a Bio-Rad GS 700 imaging densitometer with Multi Analyst software.

DNA Cleavage Assays. As described (Benedetti et al., 1993; Knab et al., 1995; Megonigal et al., 1997), cleavage of DNA by DNA Top1p was assayed by incubating enzyme preparations or immuno-tag-purified enzymes with a double-stranded DNA fragment, radiolabeled at one unique 3′ end, in the presence or absence of camptothecin. A 900-base pair DNA fragment containing a high affinity DNA Top1p cleavage site (Bonven et al., 1985) was single ^{32}P-end labeled and purified from plasmid pBlueAK3–1 as described (Knab et al., 1995). Enzyme preparations were incubated in 50-μl reactions with DNA, reaction buffer and, where indicated, 100 μM camptothecin. DMSO was added to the no-drug controls. Following incubation at 37°C for 1, 5, or 30 min, reactions were terminated by the addition of 1% SDS, heated to 75°C for 15 min, and treated with 0.2 μg/ml proteinase K. The cleaved DNA fragments were resolved in 8 M urea/8% polyacrylamide gels and visualized by autoradiography (Benedetti et al., 1993; Knab et al., 1995)

Fluorescence Microscopy EKY2 cells, transformed with YCp-GAL1-htop1T718A and induced with galactose for 6 h, were fixed by the addition of a final 3.7% formaldehyde. After 30 min, cells were pelleted and resuspended in 100 mM KPO$_4$, pH 7.0, and 3.7% formaldehyde. After 30 min, the fixed cells were washed three times with 100 mM KPO$_4$, sonicated, and stored at 4°C. To visualize nuclei, cells were resuspended in mounting medium (10 mM NaPO$_4$ pH 7, 150 mM NaCl, 90% glycerol, 1 μg/ml p-phenylenediamine) plus 1.0 μg/ml 4,6-diamidino-2-phenylindole and viewed in a Leitz Axioplan microscope equipped with a UV filter set. Large-budded cells (defined as cells in which the bud was at least one-half as large as the mother cell) were scored for the presence of a single nucleus, two nuclei, or one nucleus in the bud neck. One hundred cells were scored for each

of five experiments, by an observer blinded to the identity of the samples.

Molecular Simulations. Models of enzyme mutants were constructed based on the coordinates of hTop1p (70 kDa) in noncovalent complex with a 22-base pair DNA duplex (Redinbo et al., 1998; Stewart et al., 1998). Hydrogens were added and the whole structure energy-minimized. Appropriate amino-acid substitutions were made and each structure was energy-minimized with only residues within a 20Å-radius from each mutation free to move. The programs Insight II, Biopolymer, and Discover (Molecular Simulations, Inc., Sunnyvale, CA) were used for viewing and creating the models. Minimizations were performed with the AMBER all-atom forcefield (Weiner, 1981; Weiner et al., 1984), a distance-dependent dielectric constant (4xR) and the conjugate gradients algorithm to final maximum derivative of 0.01 kcal/A.

Results

Yeast Genetic Screen for Catalytically Active, Cpt-Resistant hTop1 Mutants. Previously, we examined DNA Top1 mutants in yeast (for review, see Bjornsti et al., 1994; Benedetti et al., 1998; Reid et al., 1998) and showed that cytotoxicity requires the presence of an active enzyme because *top1△* yeast lacking DNA Top1 are Cpt resistant. A large percentage of Cpt-resistant *top1△* yeast cells transformed with a mutagenized pool of h*TOP1* will express enzymes with reduced catalytic activity. To avoid brute-force screening of the subset of catalytically active, Cpt-resistant htop1 mutants, a genetic screen was devised that took advantage of the fact that yeast strains deficient in DNA repair (due to deletion of the *RAD52* gene) tolerate only limited amounts of h*TOP1*. JN2–134 (*rad52△*) cells tolerate p*GAL1*-promoted h*TOP1* expression from a single copy vector, but exhibit decreased viability when the enzyme is expressed from p*GAL1* on a multicopy vector (Bjornsti et al., 1989). Yet, leaky expression from this same multicopy YEpGAL1-hTOP1 vector is sufficient to render JN2–134 cells sensitive to 5 to 10 μg/ml Cpt on dextrose plates. Thus, catalytically active, Cpt-resistant h*top1* mutants may be selected by replica plating Cpt-resistant colonies onto galactose plates and identifying the inviable cells (Fig. 1).

h*TOP1* sequences, randomly mutagenized as described (Benedetti et al., 1993) were subcloned into the multicopy YEpGAL1-hTOP1 under the p*GAL1* promoter. Following transformation into JN2–134 (*rad52△*) cells, colonies grown on dextrose Cpt plates were replica plated onto galactose plates to score catalytically active, Cpt-resistant h*top1* mu-
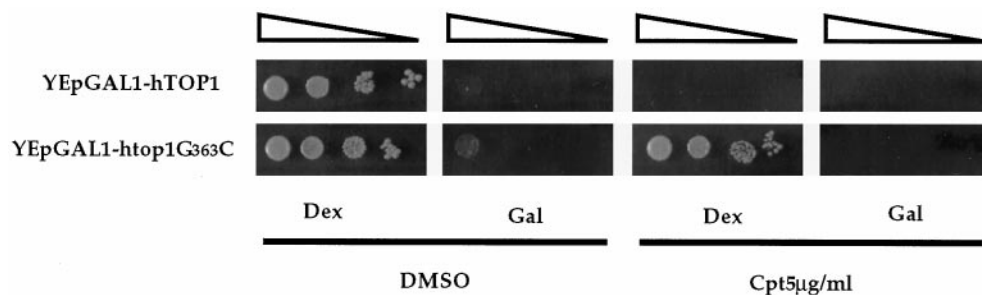
Fig. 1. Isolation of Cpt resistant h*top1* mutants. With the catalytically active Cpt^r mutant h*top1G363C* as an example, a yeast screen was developed to isolate catalytically active, Cpt-resistant hTop1p mutants. Exponentially growing cells in dextrose, transformed with YEpGAL1-hTOP1 or YEpGAL1-htop1G363C, were serially 10-fold diluted starting from A_{595} = 0.5 and 5 μl was spotted on selective media under repressed (Dex) or induced (Gal) conditions, in the presence or absence of 5 μg/ml Cpt. The figure shows that it is possible to score for resistance (YEpGAL1-htop1G363C in dextrose with Cpt) or sensitivity (YEpGAL1-hTOP1, under the same conditions).

tants. Selected colonies were assesed for hTop1p catalytic activity plasmid DNA relaxation assays. YEpGAL1-htop1 plasmid DNA was recovered (Robzyk and Kassir, 1992) and rescreened in yeast JN2–134, to establish that the mutations were plasmid borne. The relevant amino-acid substitutions were determined by DNA sequencing. To ensure that the substitutions identified were responsible for the Cpt-resistant phenotype, the single mutations also were generated by site-specific mutagenesis. Amino-acid substitutions at position Gly-363 (in h*top1G363S* and h*top1G363V*) and Ala-653 (where Ala is mutated to Pro) were sufficient to confer Cpt resistance.

hTop1G363Sp and hTop1G363Vp Are Cpt Resistant. h*top1G363S* and h*top1G363V* were cloned into the single copy vector YCpGAL1-hTOP1 to assess Cpt sensitivity in repair proficient EKY2 cells. As reported for h*top1G363C*, where Gly363 is mutated to Cys (Benedetti et al., 1993), cells expressing h*top1G363S* and h*top1G363V* were resistant to high doses of Cpt (5 μg/ml) (Fig. 2A).
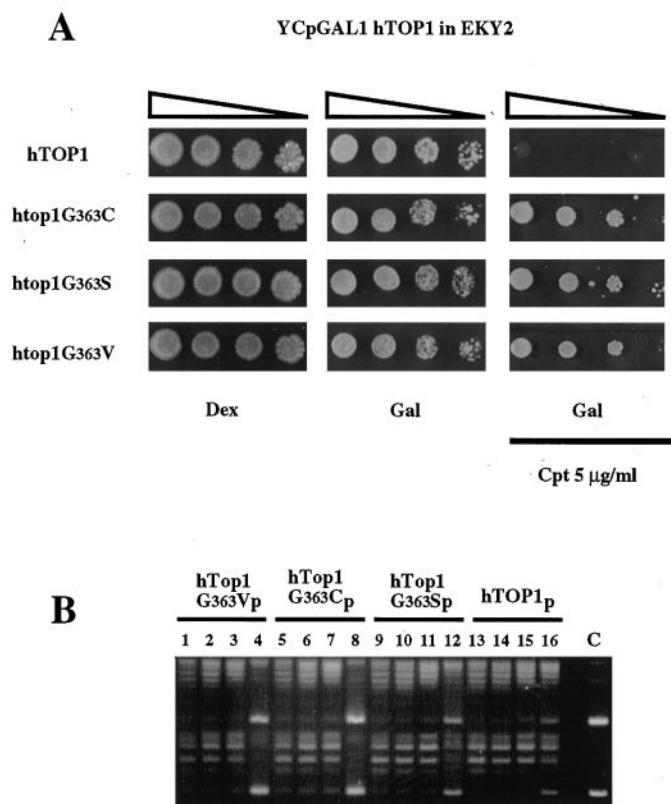
To establish the mechanism of drug resistance, the mutant proteins were partially purified (Benedetti et al., 1993; Knab et al., 1995), and assayed for activity. In Fig. 2B, serially diluted

Fig. 2. h*top1G363S* and h*top1G363V* are resistant to Cpt. A, 5 μl of 10-fold serial dilutions starting from A_{595} 0.5 of EKY2 (*top1△*) cells transformed with YCpGAL1-hTOP1, YcpGAL1G363S, or YcpGAL1G363V were spotted onto SC-uracil plates containing 2% dextrose or 2% galactose in the presence or absence of 5 μM Cpt, and incubated at 30°C. B, to assess enzyme activity in DNA relaxation assays, partially purified hTop1p and Cpt-resistant mutant proteins were corrected for total protein concentration. Equal Top1p concentrations were confirmed in immunoblots. One microliter of serial 10-fold dilutions of hTop1G363Vp (lanes 1–4), hTop1G363Cp (lanes 5–8), hTop1G363Sp (lanes 9–12), and hTop1p (lanes13–16) were incubated with 0.5 μg of negatively supercoiled plasmid DNA (pHC624) as described in *Experimental Procedures*. C, no enzyme added.

aliquots of hTop1p, hTop1G363Sp, and hTop1G363Vp were incubated with supercoiled plasmid DNA and the extent of plasmid DNA relaxation assessed in agarose gels. As with hTop1G363Cp, the specific activities of the mutant proteins were comparable to that of wild-type hTop1p. Enzyme activity was assayed at 100 mM KCl because our previous studies of the Gly363 to Cys mutant established enzyme-specific activity was maximal at 100 mM KCl in the presence of Mg^{2+} (Benedetti et al., 1993). Substitution of Ser or Val for Gly363 had similar effects on enzyme activity.

The mutant enzymes were resistant to Cpt in vitro. hTop1p, hTop1G363Sp, and hTop1G363Vp were incubated with a 3′ single end-labeled DNA fragment, in the presence or absence of 100 μM Cpt. Following incubation at 37°C for 1, 5, or 30 min, the cleavable complexes were trapped by SDS/proteinase K treatment and the cleaved DNA fragments resolved in a denaturing polyacrylamide gel, followed by autoradiography. As with hTop1G363Cp (Benedetti et al., 1993), Cpt did not stabilize the cleavable complexes formed by catalytically active hTop1G363Sp and hTop1G363Vp (data not shown).

The results obtained with the Gly363 to Val substitution in hTop1p differed from those obtained with the same substitution at the corresponding position in yTop1p. Substitution of Val for Gly295 in yTop1G295Vp reduced enzyme activity by 100-fold, as a result of diminished enzyme binding of DNA (Hann et al., 1998b). Although the increased salt sensitivity of the analogous hTop1G363Vp mutant suggests an alteration in DNA binding, the overall effect of this mutation on hTop1p activity is minimal. Given the structural conservation of this domain and its proximity to the active-site tyrosine domain, this apparent discrepancy in human and yeast enzyme function was investigated by examining the ability of Gly363 substitutions to suppress the lethal phenotype of a Thr718 to Ala mutation. This mutation, five residues N-terminal to the active site tyrosine, was chosen because the same change in yeast Top1p (y*top1T722A*) renders the enzyme lethal when overexpressed. yTop1T722Ap causes cell death via a mechanism that mimics Cpt, i.e., by stabilizing the covalent enzyme-DNA intermediate (Megonigal et al., 1997). We found that the $T_{718}A$ mutation in hTop1p mirrored the alterations in catalytic activity reported for yTop1T722Ap. Given the close proximity of Thr718 to the active-site tyrosine, we then asked if the Gly363 substitutions suppressed h*top1T718A*-induced cytotoxicity. Unlike the similar effects on Cpt sensitivity, distinct phenotypes resulted from the combination of each Gly363 substitution with the T718A mutation, suggesting specific interactions between the Cpt loop and the C-terminal domain of the enzyme.
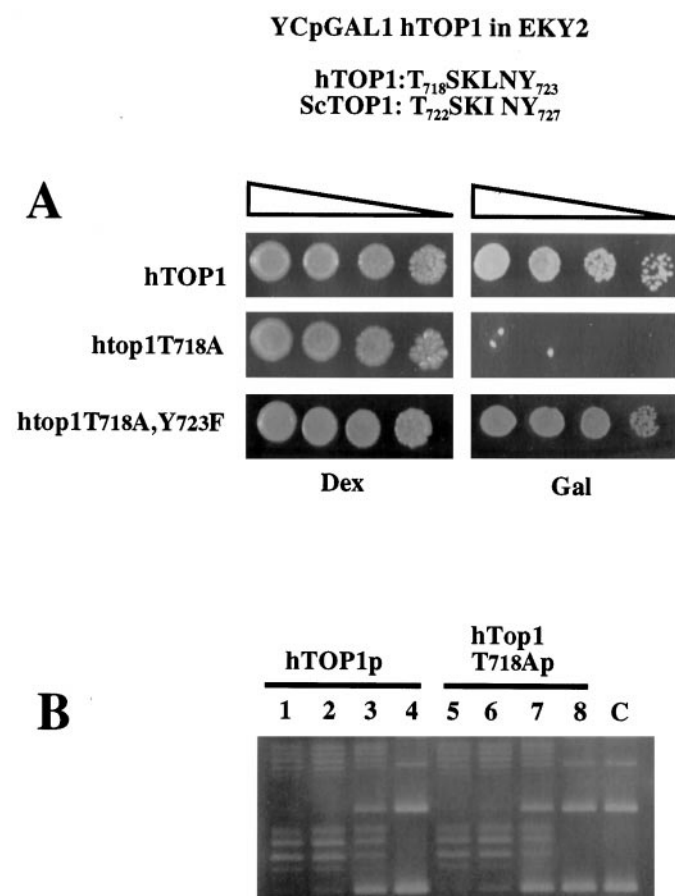
hTop1T718A Is Lethal When Overexpressed in Yeast. The h*top1T718A* mutant, generated by site-directed mutagenesis, was cloned into plasmid YCpGAL1-htop1T718A and transformed into EKY2 (*top1△*) cells. When cultured in galactose, cells expressing h*top1T718A* exhibited a dramatic reduction in cell viability (Fig. 3A). The cytotoxic activity of hTop1T718Ap required catalytic activity, as mutation of the active site Tyr723 to Phe abrogated h*top1T718A*-induced lethality. Moreover, low constitutive levels of h*top1T718A* expression from the yeast *TOP1* promoter in YCpSc-htop1T718A was easily tolerated in EKY2 cells. In contrast, although JN2–134 cells transformed with YCpSc-hTOP1 were viable, no transformants were obtained with YCpSc-htop1T718A. Low levels of hTop1T718Ap were cytotoxic in

rad52△, repair deficient strains (data not shown). Thus, hTop1T718Ap-induced lethality was dependent upon cellular levels of mutant protein and the resultant DNA damage.

hTop1T718Ap Is Catalytically Active In Vitro and In Vivo. As shown in Fig. 3B, the specific activity of hTop1T718Ap was indistinguishable from that of wild-type hTop1p. The in vivo activity of hTop1p and hTop1T718Ap was assessed by examining the relaxation of the positive supercoils generated by transcription of the endogenous yeast 2-μm plasmid as described in Benedetti et al. (1993), Knab et al. (1993), and Megonigal et al. (1997). JCW28 (*top1△, top2-4ts*) cells, deleted for *TOP1* and bearing a temperature-sensitive mutation in DNA topoisomerase II, were cotransformed with YEptopA-pGPD and either YCpGAL1, YCpGAL1-hTOP1, or YCpGAL1-htop1T718A. YEptopA-pGPD constitutively expresses bacterial DNA Top1p. At 37°C, DNA topoisomerase II is inactivated and the negative supercoils that accumulate during transcription are selectively relaxed by bacterial DNA Top1p (Giaever and Wang, 1988). As seen in Fig. 4A, JCW28 cells transformed with YEptopA-pGPD and a control plasmid expressing no DNA Top1p accumulate positively supercoiled (PS) 2-μm plasmid

YCpGAL1 hTOP1 in EKY2

hTOP1: T₇₁₈SKLNY₇₂₃
ScTOP1: T₇₂₂SKI NY₇₂₇

Fig. 3. Overexpression of *htop1-T718A* is lethal in yeast. A, 5 μl of 10-fold serial dilutions starting from A_{595} 0.5 of EKY2 (*top1△*) cells transformed with YC*pGAL1*-hTOP1, YC*pGAL1*-htop1-T718A, or YC*pGAL1*-htop1T718A, Y723F were spotted onto SC-uracil plates containing 2% dextrose or 2% galactose, and incubated at 30°C. A comparison of human and yeast sequences around the active site tyrosine is shown. B, partially purified hTop1p (lanes 1–4) and hTop1 T718Ap (lanes 5–8) were serially 10-fold diluted in reaction buffer and 1 μl was assayed for their ability to relax 0.5 μg of supercoiled plasmid DNA as described in *Experimental Procedures*.

DNA upon shift to the nonpermissive temperature (37°C). When JCW28 was transformed with YCpGAL1-htop1T718A or YCpGAL1-h*TOP1* and shifted to nonpermissive temperature, the mutant or wild-type enzyme catalyzed the relaxation of the positive supercoils (Fig. 4, B and C). Thus, hTop1T718Ap is catalytically active in vivo.

Substitution of Ala for Thr718 in hTop1p Enhances Stability of Covalent Enzyme-DNA Intermediate. To assess the phenotypic consequences of h*top1T718A* expression, the stability of the covalent complex formed by the mutant enzyme was assessed. Equal concentrations of heTop1 and heTop1T718A proteins, epitope tagged at the N terminus, were bound to beads via the epitope specific M2 monoclonal antibody as described in Hann et al. (1998). The inclusion of the epitope had no effect on mutant or wild-type enzyme activity, either in solution or in the bead bound form (data not shown). The tethered proteins were incubated with a 3′ end-labeled DNA fragment, in the presence or absence of 100 μM Cpt. Following incubation at 37°C for 1, 5, or 30 min, the cleavable complexes were trapped with SDS/proteinase K treatment and the cleaved DNA fragments resolved in a denaturing polyacrylamide gel. When wild-type heTop1p is incubated without Cpt, very little cleavage of the labeled DNA strand was detected (Fig. 5). However, when heTop1p is exposed to Cpt, a dramatic increase in cleaved DNA fragments was observed. In contrast, heTop1T718Ap-DNA complexes were stabilized even in the absence of Cpt, as evidenced by the increased intensity of cleaved fragments relative to heTop1p (Fig. 5). Yet, the apparent differences in band intensities produced in the presence and absence of Cpt (compare heTop1T718Ap lanes with Cpt and DMSO) indicate that heTop1T718Ap remains sensitive to Cpt. Moreover, because the pattern of bands was indistinguishable between heTop1T718Ap and heTop1p in the presence of Cpt, the preferred sites of cleavage by heTop1T718Ap are altered by the drug (see arrow in Fig. 5, A and C). Band (B) corresponds
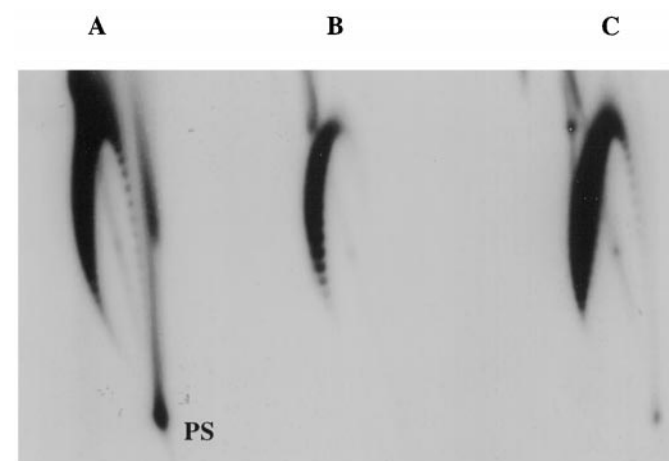

Fig. 4. hTop1T718A is active in vivo. Relaxation of positively supercoiled yeast 2-μm plasmid DNA by hTop1p and htop1T718Ap were assayed in vivo. Strain JCW28 (*top1△, top2-4*) constitutively expressing a plasmid-borne *Escherichia coli topA* gene encoding bacterial DNA topoisomerase I, was transformed with a control plasmid YCpGAL1 (A), YCpGAL1-htop1T718A (B), or YCpGAL1-hTOP1 (C). Cultures were incubated at 25°C, a permissive temperature for *JCW28* strain, and induced with galactose. Cultures were then shifted to 37°C to inactivate yeast DNA topoisomerase II. The 2-μm plasmid DNA was recovered from each culture after the temperature shift and analyzed in southern blots of two-dimensional agarose gels electrophoresis. PS, positive supercoils.

to the preferred cleavage site derived from rDNA (Bonven et al., 1985).

The action of Cpt is S-phase specific and results in G2 arrest (Tobey, 1972; Holm et al., 1989; D'Arpa et al., 1990; Kauh and Bjornsti, 1995). A similar terminal phenotype has been described for yeast cells expressing y*top1T722A* (Megonigal et al., 1997). Therefore, the pattern of nuclear segregation in *top1△* strains expressing h*TOP1* or h*Top1T718A* was examined 6 h after galactose induction (Fig. 6). There was a striking difference in the distribution of nuclear DNA in the large-budded, G2/M phase cells. In cells expressing h*TOP1*, >70% of the large-budded cells had segregated the replicated nuclei into mother and daughter cells and had two distinct nuclear masses. In contrast, cells expressing h*Top1T718A* exhibited a marked derangement in nuclear segregation; 65% of the large-budded cells contain a single nucleus, either in the mother cell or in the bud neck between mother and daughter cells.

Gly363 Substitutions Are Distinguished in Their Ability to Suppress Cytotoxic Activity of hTop1T718A. As detailed in Hann et al. (1998), random mutagenesis identified a Gly295 to Val substitution as an intragenic suppressor of y*top1T722A*-induced lethality. This resulted from a dramatic decrease in DNA binding and, therefore, the catalytic activity of the double mutant. In contrast, our results demonstrate that the same substitution (Gly363 to Val) in hTop1p had only a minimal effect on enzyme activity, yet suppressed the Cpt sensitivity of the human enzyme. To investigate whether the interactions between the "lip" domain and the active-site tyrosine residues differ in the yeast and human enzymes, the ability of all three Gly363 substituents to suppress h*top1T718A*-induced lethality was assessed.
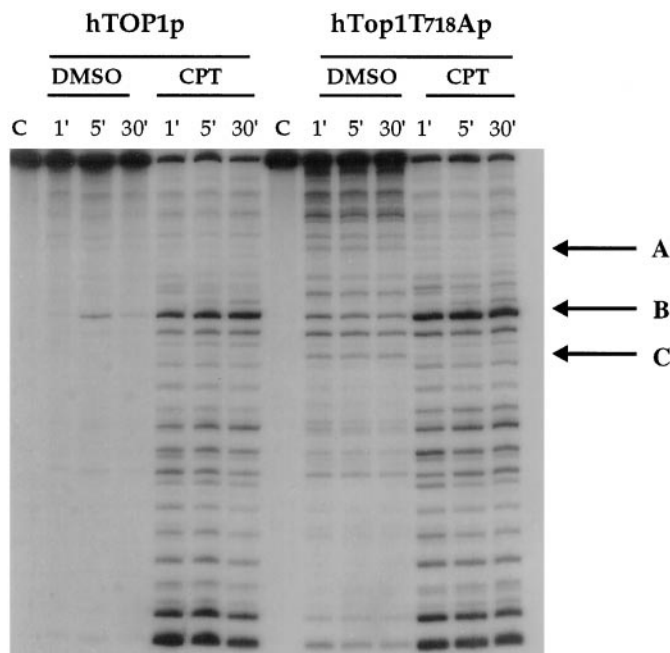

Fig. 5. hTop1T718Ap increases the stability of the covalent enzyme-DNA intermediate in the absence of Cpt. In vitro DNA cleavage activity of bead-bound epitope-tagged ehTop1p and ehTop1T718Ap was assayed. A 3′ ^{32}P end-labeled DNA fragment was incubated with immunoprecipitated ehTop1p or ehTop1T718Ap for the times indicated, in the presence of 4% DMSO or 100 μM Cpt. Control, no enzyme added (C). Total protein (110 ng), with comparable specific activity, was used in each assay. Arrows A and C indicate cleavage sites mostly evident in the hTop1T718Ap in the absence of Cpt and arrow B indicates the preferred cleavage site derived from *Tetrahymena* rDNA.

As shown in Fig. 7A, only the Gly363-to-Val mutation suppressed the h*top1T718A* lethal phenotype. Cells expressing the double mutant h*top1T718A, G363V* were viable in the presence or absence of Cpt. In contrast, cells expressing the double mutant h*top1T718A, G363S* were inviable on galactose, whereas only a modest increase in viability was evident in cells expressing the double mutant h*top1T718A, G363C*. In both cases, the cytotoxicity of the double mutants also was observed in the presence of Cpt.

A second-site mutation could suppress the lethality of hTop1T718Ap by restoring normal catalytic activity, decreasing enzyme activity, destabilizing the protein, or interfering with its entry into the nucleus. To address the mechanism of T718A suppression, the mutants were transformed into the *top1△, rad52△* strain, JN2–134. Galactose-induced mutant hTop1p expression was cytotoxic to this repair-deficient strain, suggesting a critical level of DNA damage was induced by each double mutant.

The specific activities of the mutant proteins also were determined in plasmid DNA relaxation assays. As shown in Fig. 7B, the ability of the various Gly363 substituents to suppress the lethal activity of hTop1T718Ap correlated with a reduction in catalytic activity. The strongest suppressor, hTop1T718A, G363Vp exhibited the least catalytic activity. The activity retained by hTop1T718A, G363Sp and hTop1T718A, G363Cp was sufficient to render the enzyme sensitive to Cpt in vitro. Assays of specific activity with increasing salt further demonstrate that hTop1T718A, G363V was more sensitive to alterations in ionic strength than hTop1p, hTop1T718A, G363Sp, or hTop1T718A, G363Cp because its activity was impaired at salt concentrations in excess of 100 mM (Fig. 7C). These data suggest that the combination of Gly363 to Val with Thr718 to Ala compromises the catalytic activity of the enzyme, possibly through a reduction in DNA binding.

Time course assays (Hertzberg et al., 1989; Crow and
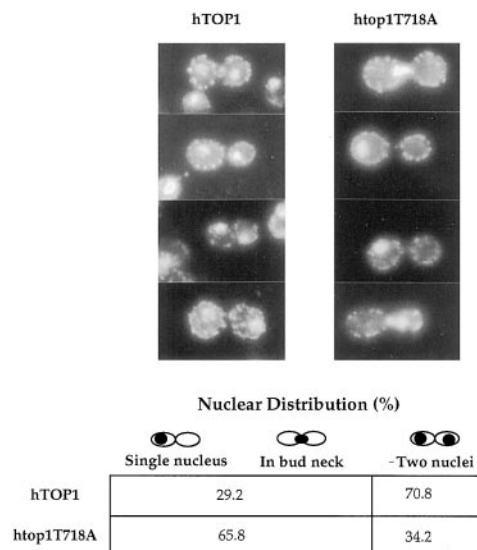
Fig. 6. Nuclear segregation is defective in strains overexpressing h*top1T718A*. EKY2 (*top1△*) cells were transformed with YCpGAL1-hTOP1 or YcpGAL1-htop1-T718A, and hTop1p expression was galactose-induced for 6 h before cells were processed for microscopy. The table shows the percentage of large-budded cells displaying a single or double nucleus. Scoring was done by an observer blinded to the identity of the samples.

Crothers, 1992) also were used to examine the ability of Cpt to inhibit the relaxation activity of hTop1p, hTop1T718Ap, hTop1G363Vp, and hTop1G363V, T718Ap. As shown in Fig. 8A, the presence of 50 μM Cpt decreased the initial velocity of hTop1p and mutant hTop1T718Ap catalyzed DNA relaxation significantly, but had no effect on the activity of mutant hTop1G363Vp or hTop1G363V, T718Ap (Fig. 8B).

These results are in agreement with the findings of Hann et al. (1998). However, although all of the Gly363 substitutions abrogated the Cpt sensitivity of hTop1p, only the Gly363-to-Val substitution abolished the catalytic activity of hTop1T718Ap. This distinguishes the relative contributions of Gly363 substituents to the cytotoxic action of Cpt and enzyme catalysis.

Molecular Modeling Analysis of Mutations. Molecular simulations were based on the coordinates of the three-dimensional structure of hTop1p (70 kDa) in noncovalent complex with a 22-base pair DNA duplex (Stewart et al., 1998).
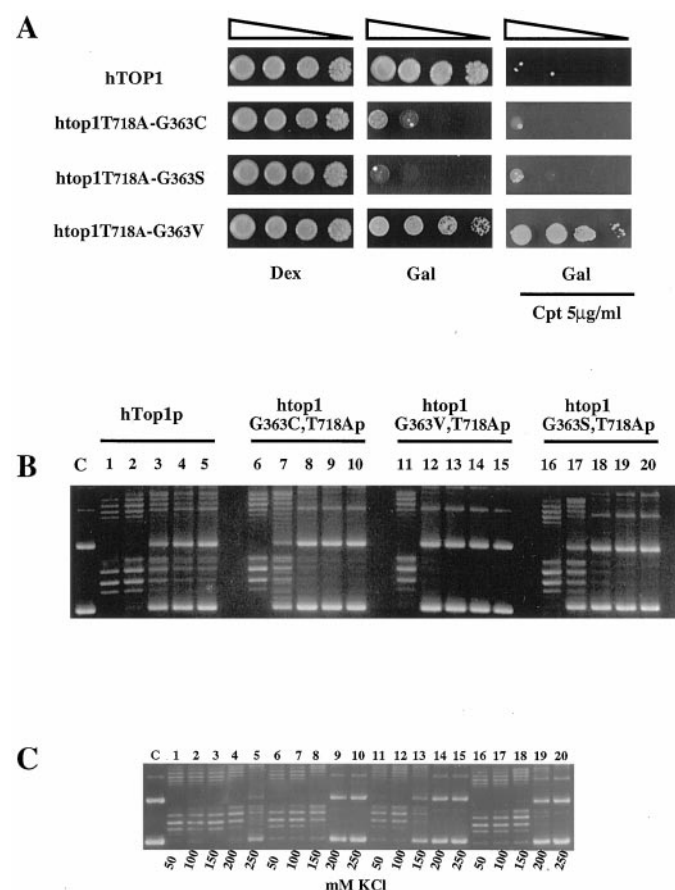
Fig. 7. Mutations at position 363 can suppress the lethality of h*top1T718A*. A, viability of EKY2 (*top1△*) cells transformed with the indicated YCpGAL1-hTOP1 construct. Five microliters of serial 10-fold dilutions of cultures starting from $A_{595} = 0.5$, were spotted onto SC-uracil plates containing 2% dextrose or 2% galactose and 5 μg/ml Cpt. B, 1 μl of serial 5-fold dilutions of partially purified wild-type or mutant hTop1p were assayed for their ability to relax 0.5-μg supercoiled plasmid DNA. Lanes 1–5, hTop1; lanes 6–10, hTop1G363C, T718Ap; lanes 11–15, hTop1G363V, T718Ap; lanes 16–20, hTop1G363S, T718Ap. Control (C), no protein added. Equivalent protein concentrations were verified by western blotting with an antibody specific for hTop1p. C, 1 μl of partially purified wild-type hTop1 (1–5) hTop1G363C, T718Ap (6–10) hTop1G363V, T718Ap (11–15), or hTop1G363S, T718Ap (16–20) were assayed for their ability to relax 0.5-μg supercoiled plasmid DNA in the presence of the KCl concentrations.

The complete structure was energy-minimized, after addition of the hydrogens, with the conjugate gradients algorithm and AMBER forcefield. Individual substitutions, T718A, G363C, G363S, and G363V, and double combinations were introduced and the structures were again minimized for all residues within a 20-Å radius from each mutation.

Surprisingly, substitution of Ala for Thr718 did not alter enzyme structure to any significant extent. Fig. 9A shows the structure of the minimized mutant enzyme in the region of Thr718. Wild-type and mutant structures are superimposed and there was only a minor disturbance at position 718 due to the smaller dimensions of alanine.

The effect of Gly363 substitutions was very different. Figure 9B shows the superimposed structures of hTop1p (Gly363) and hTop1pG363Vp around residue 363. Many lateral groups of the Cpt loop are altered and the backbone itself is distorted. The displacement of Arg364, which is proposed to directly interact with Cpt (Redinbo et al., 1998), is greatest with substitution of Cys for Gly363. The major effect in this loop structure is the distortion in residues that contact the second "lip" region from residue 489 to 501, as shown, for example, by the position of Pro368. The least active mutant, hTop1G363Vp, shows the greatest backbone distortion in the lip region, whereas hTop1G363Sp is the most similar to the wild type.

These models suggest the correct positioning of the lip regions is essential for enzyme activity and Cpt sensitivity. The highly conserved Cpt loop could serve as a DNA "sensor", via Arg364, Arg362, and other residues that face the DNA duplex. The Cpt loop forms the sole salt bridge between the lips close the protein clamp around the DNA duplex. Thus, the interaction of these lip domains may be critical to the proper positioning of the active site tyrosine domain in the catalytic pocket.

Discussion

Genetic, biochemical, and crystallographic studies contribute to our current understanding of eukaryotic DNA Top1p structure and mechanism (Lue et al., 1995; Wang, 1996; Berger, 1998; Pommier et al., 1998; Redinbo et al., 1998; Stewart et al., 1998). Recent crystallographic data (Redinbo et al., 1998; Stewart et al., 1998) reveal that the enzyme forms a protein clamp around the DNA with basic residues in contact with the phosphate backbone and the active site tyrosine poised to attack the phosphodiester linkage. Stewart et al. (1998) proposed that the enzyme catalyzes the relaxation of supercoiled DNA via a mechanism of "controlled rotation" in which the protein clamp binds the DNA helix to create the single-stranded break. The DNA molecule 3′ to the cleavage site rotates about the phosphodiester bond opposite the nick, with the positively charged surfaces of the "linker region" (a coiled-coil structure that connects the central globular core of the enzyme with the active site-tyrosine domain) and a set of cone helices extending from the top of the clamp, acting as a brake to slow the rotation of the negatively charged DNA. Specific contacts with the DNA involve several Top1p domains. The residues of the Cpt loop/lip region of core subdomain I also are involved in interdomainal interactions with the lip region of core subdomain III and in directly binding the DNA helix to form the closed clamp structure.

Among the Cpt-resistant mutants isolated in this screen,

substitution of Ala653 with Pro is interesting given its location in the coiled-coil region of the linker domain. This mutant has reduced catalytic activity (data not shown) and is particularly sensitive to proteolysis. Substitution of Pro for Ala could disrupt the alpha helix of the coiled-coil, thereby affecting the interaction of the enzyme with DNA. Although the linker domain is dispensable for relaxation activity (Stewart et al., 1997), the presence of a modified structure could alter the controlled rotation of DNA and influence the relaxation activity of the enzyme.

Cpt resistance was previously ascribed to mutations in the Cpt loop/lip region of core subdomain I (Benedetti et al., 1993; Rubin et al., 1994; Lue et al., 1995; Li et al., 1997). Herein, we report that substitution of Val or Ser for Gly363 had little effect on catalytic activity, yet decreased the Cpt sensitivity of the enzyme to levels similar to those observed with the Cpt-resistant htop1G363C mutant. Subtle differences highlight the strict structural context required for Cpt sensitivity and presumably drug binding. Our molecular models suggest that substitutions in this lip region could affect enzyme activity and sensitivity to Cpt by altering the conformation of the loop. Arg364, which makes direct contact with the minor groove of the DNA opposite the site cleaved by the active site tyrosine (Redinbo et al., 1998) is slightly distorted in the minimized structures, with the G363C mutation being most pronounced. This distortion may influence the hydrogen bonding with Cpt as proposed by Redinbo et al. (1998) or might affect the overall structure of the loop impor-

tant for Cpt binding. In addition, as a single salt bridge exists between the two lip domains, any alteration in the correct positioning of these two regions might adversely affect the interdomainal interactions needed to effect clamp closure. The opening and closing of the enzyme clamp around the DNA also could influence the Cpt binding-equilibrium. If hTop1p mutations alter the efficiency of clamp closure, the equilibrium would shift toward the unbound state.

Fan et al. (1998) recently proposed an alternate model for Cpt binding, based on molecular-modeling studies of different Cpt analogs and DNA. Substitution of Ala for Thr718 produces different patterns of DNA cleavage in the presence or absence of Cpt. In the presence of Cpt, however, the pattern was indistinguishable between hTop1T718Ap and hTop1p. Similar results were obtained with yTop1T722Ap (Megonigal et al., 1997), suggesting that Cpt binding to the mutant enzyme-DNA complex restores the microenvironment around the active site. This is in agreement with the Fan et al. (1998) model.

Mutation of Gly363 to Val provided the strongest suppression of h*top1T718A* lethality. The molecular models suggest the introduction of the branched side chain of Val, as opposed to the smaller, linear side chains of Cys or Ser, produces a more severe alteration in the Cpt loop. This was reflected in a pronounced decrease in catalytic activity, but only in the context of the Thr718-to-Ala substitution. Thus, the flexibility imparted to the Cpt loop by Gly363 may be more critical in maintaining the altered catalytic activity of *hTop1T718Ap*
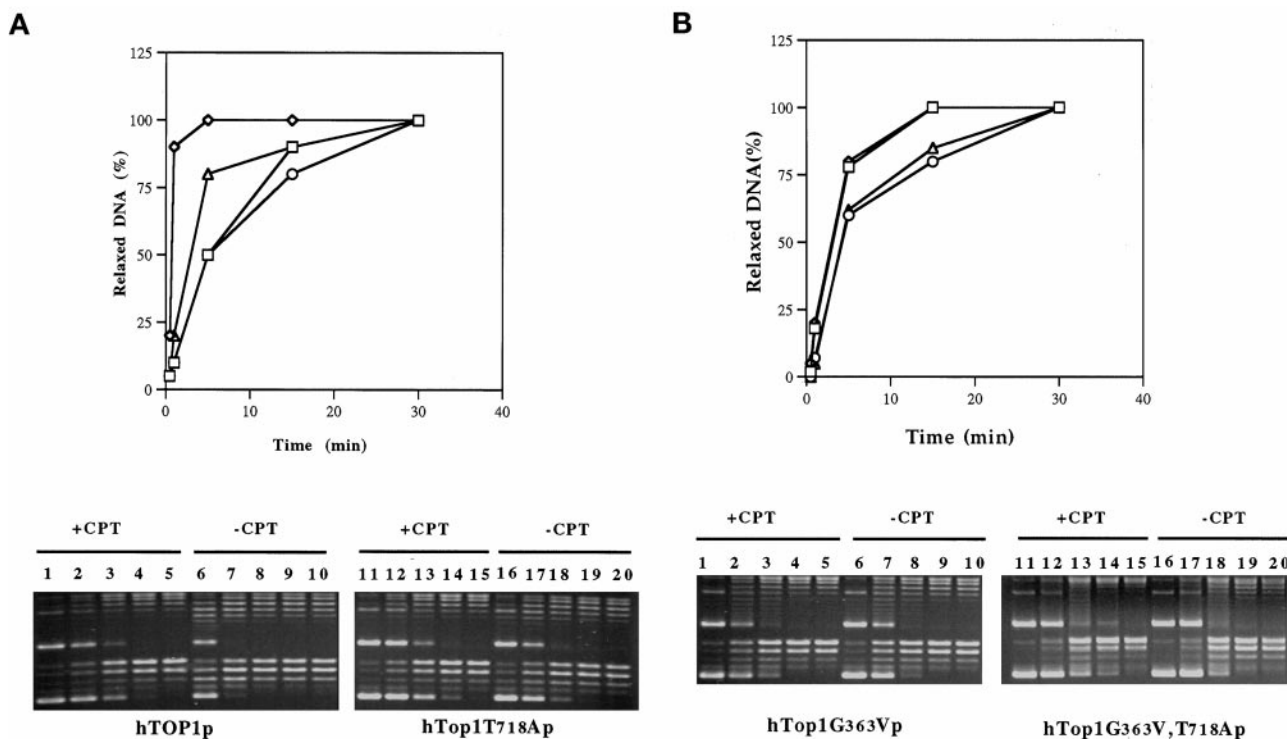
Fig. 8. Time course of Cpt inhibition of hTop1p, hTop1T718Ap, hTop1G363Vp, and hTop1G363V, T718Ap relaxation activity. A, 1 μl of partially purified hTop1p or hTop1T718Ap was used in each assay in the presence or absence of 50 μM Cpt. At the following times, the reactions were terminated with 0.5% SDS: 0.5 (lanes 1, 6, 11, 16), 1 (lanes 2, 7, 12, 17), 5 (lanes 3, 8, 13, 18), 15 (lanes 4, 9, 14, 19), or 30 min (lanes 5, 10, 15, 20). Reaction products were resolved in agarose gels and visualized by staining with ethidium bromide. The percentage of relaxed DNA, quantitated with the Bio-Rad imaging densitometer and Multi Analyst software, is presented in graphic form. □, hTOP1p (50 μM CPT); ◇, hTOP1p (no drug); ○, htop1T718Ap (50 μM CPT); △, htop1T718Ap (no drug). B, as described in (A), 1 μl of partially purified hTop1G363Vp or hTop1G363V, T718Ap was assayed in the presence or absence of 50 μM Cpt. Reactions were terminated with 0.5% SDS at 0.5 (lanes 1, 6, 11, 16) 1 (lanes 2, 7, 12, 17), 5 (lanes 3, 8, 13, 18), 15 (lanes 4, 9, 14, 19), or 30 min (lanes 5, 10, 15, 20). The percentage of relaxed DNA is presented in graphic form. □, hTop1G363Vp (50 μM CPT); ◇, hTop1G363Vp (no drug); ○, hTop1G363Vp (50 μM CPT); △, hTop1G363Vp (no drug).
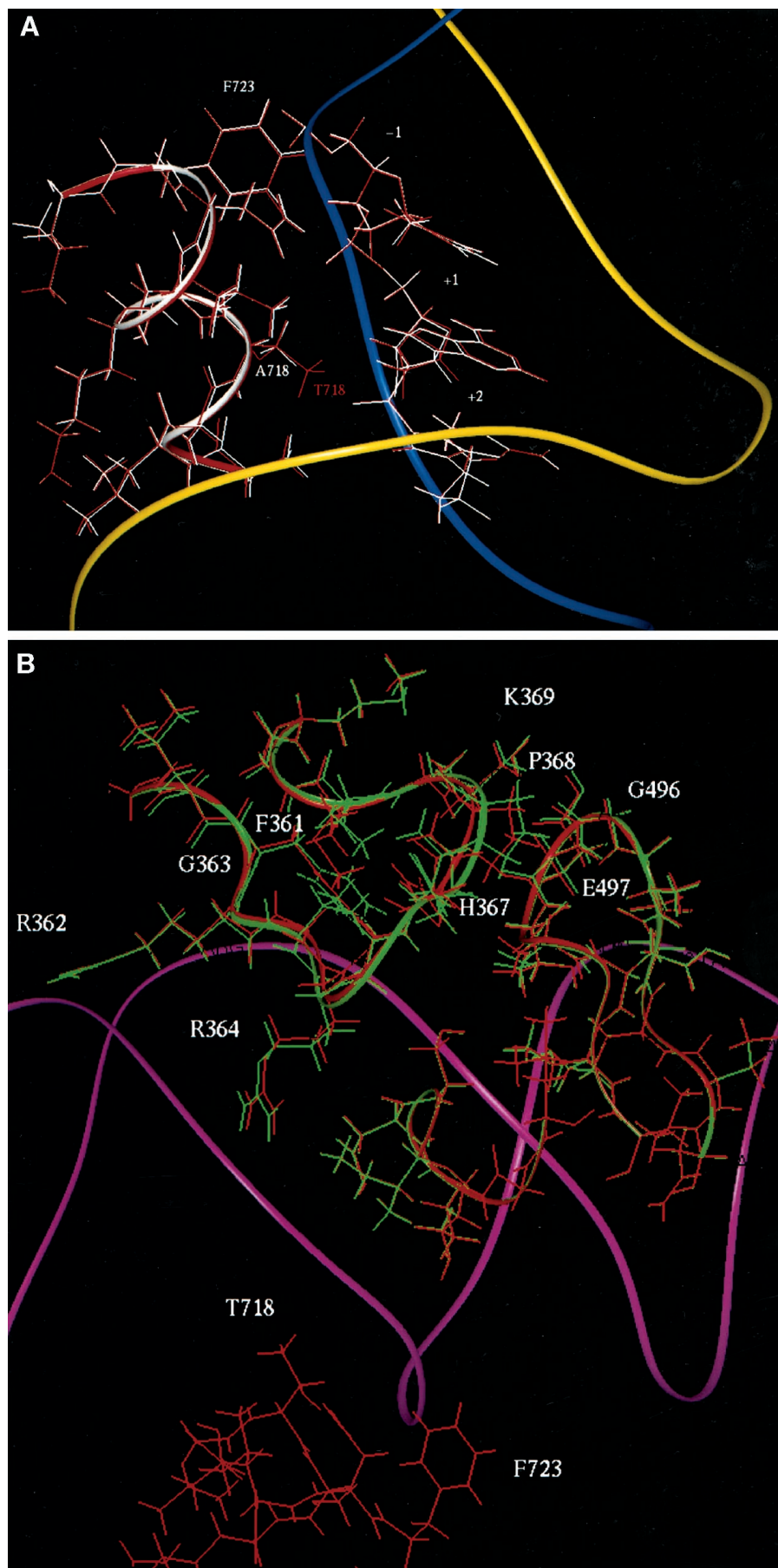
Fig. 9. A, minimized structures in the region of the Thr718 and the active site tyrosine. Superimposition of wild-type hTop1p (red) and hTop1T718Ap (Ala substituted for Thr-718) (white) structures after minimization. The yellow DNA ribbon indicates the nonscissile strand. B, minimized structures in the region of the Cpt-loop. Superimposition of the wild-type hTop1p (red) and Val substituted for Gly-363 (green).

than wild-type hTop1p. Indeed, substitution of Val, Ser, or Cys for Gly363 had comparable effects on wild-type enzyme activity and Cpt sensitivity: a modest decrease in activity at higher salt and resistance to Cpt.

The results of Gly363 substitutions in hTop1p differed from that obtained following the substitution of Val for the corresponding residue (Gly295) in yTop1p, where a 100-fold decrease in yTop1G295Vp was attributed to a reduction in DNA binding (Hann et al., 1998b). Yet, consistent with the results presented herein, this effect was exacerbated in the context of the lethal Thr to Ala substitution. Thus, interactions of the Cpt loop/lip region with residues near the active site tyrosine affect the catalytic activity of both yTop1p and hTop1p.

Alterations in the catalytic activity of hTop1p imparted by the Thr718 to Ala mutation mirror those reported for yTop1T722Ap (Megonigal et al., 1997). The DNA relaxation activity of hTop1T718Ap was essentially identical with that of the wild-type enzyme. Furthermore, hTop1T718Ap mimics the cytotoxic effects of Cpt. Wild-type and mutant enzyme sensitivity to Cpt are comparable with regards to the degree and site specificity of DNA cleavage. Yet, in the absence of Cpt, hTop1T718Ap formed elevated levels of cleavable complexes. Therefore, either treatment with Cpt or mutation of Thr718 to Ala enhances the stability of the covalent enzyme-DNA intermediate. It is possible that different steps in the catalytic cycle are affected, which would not be distinguished by the assays used. Indeed, differences in the DNA cleavage patterns created by hTop1T718Ap in the presence or absence of Cpt support the hypothesis that the Thr718-to-Ala mutation and Cpt treatment interfere with distinct aspects of the catalytic cycle.

Yeast and hTop1p share a high degree of similarity in terms of sequence, structure, and enzyme mechanism. Moreover, the human enzyme functions in yeast (Bjornsti et al., 1989) and the yeast enzyme functions in mammalian cells (Hann et al., 1998). Yet, despite the overwhelming similarities in enzyme structure and function, significant differences have been reported (Merino et al., 1993; Knab et al., 1995; Reid et al., 1997). For example, yTop1p could not substitute for hTop1p in suppressing the basal level of transcription in vitro. Because hTop1p catalytic activity was not required, this implied a critical difference in structure between enzymes. In these studies, mutation of most conserved residues elicited similar alterations in enzyme activity, yet significant differences were apparent. As numerous Cpt analogs and other Top1p inhibitors are being developed as chemotherapeutic agents, deciphering the structural features of Top1p critical for Cpt sensitivity is important.

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